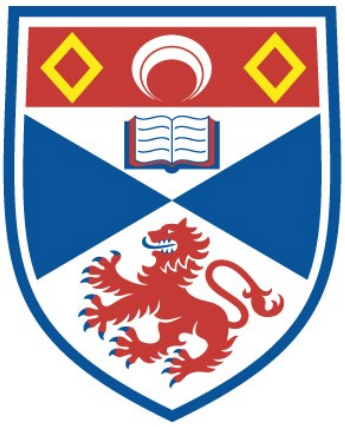
STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE IKBALPHA PROTEIN

Jane F. Wright

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1997

Structural and Functional Analysis of the IκBα protein.

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A thesis submitted in partial fulfilment

of the requirements for

the degree of Doctor of Philosophy

September 1996

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ACKNOWLEDGEMENTS

Thanks to my supervisor Ron Hay for his help during the work, with special thanks going to Sharon Kelly for the CD work, Dr. Kemp for the FPLC work and to Ellis and John for providing me with purified proteins. Thanks also go to the remaining members of the group, recent and departed, for their helpful suggestions and to Bill Blythe for his photographic skills. Finally, I would like to thank Mark, Sarah, Alan, James and my family for their support.

This work was supported by a collaborative grant from the AIDS Directed Programme of the Medical Research Council, U.K. and the Wellcome Foundation.

ABSTRACT

NF-κB/Rel transcription factors and IκB proteins play a central role in the rapid induction of genes transcribed in response to a variety of extracellular stimuli. Signal induction results in the phosphorylation and ubiquitination of IκBα prior to proteasomal degradation and release of NF-κB. The objective of this work was to identify residues within the N- and C-termini of IκBα which were important for the function of the protein, specifically the residues contacting the p65 subunit of NF-κB and those involved in the turnover of IκBα. In addition, the structure of the C-terminal region of IκBα was also examined to gain a better understanding of the functional properties of this domain.

The identification of conserved stretches of acidic residues in the C-terminal region of IκBα prompted the suggestion that perhaps these amino acids were important for interacting with the positively charged nuclear localisation signal of the p65 subunit of NF-κB. Accordingly two regions, residues 284-286 and residues 300-302 (glutamic acid, aspartic acid, glutamic acid in both regions), were targeted for mutation (and referred to as the C-terminal mutants) to examine their role, if any, in IκBα -p65 association.

A second set of mutants were generated following a protease sensitivity study on IκBα which revealed that residues 251 (tyrosine), 258 (tryptophan) and 275 (glutamic acid) were protected from digestion in the presence of p65. These amino acids were located in the low homology sixth ankyrin repeat of IκBα, thought to act as a flexible linker region between the highly conserved central five ankyrin repeats and the C-terminal region of the protein. Consequently, amino acids 258 and 275 were selected for mutation (referred to as the linker mutants).

The *in vitro* characterisation of the C-terminal and linker mutants demonstrated that neither amino acids 258 and 275 nor amino acids 284-286 and 300-302 affected the ability of IκBα to interact with p65 homodimers, even under conditions of varying pH or ionic strength. However, residues 284-286 reduced the inhibitory capacity of IκBα with respect to p65 homodimers. The results indicated that the region of IκBα required for the inhibition of p65 DNA binding activity, possibly located in or around residues 284-286, was separable from the area of the protein responsible for association with p65.

Attempts to phosphorylate the C-terminal and linker mutants *in vitro* using either casein kinase I or casein kinase II demonstrated that the C-terminal mutants were not efficiently phosphorylated by casein kinase II but were phosphorylated by casein kinase I. Both kinases were shown to phosphorylate wild-type IκBα and the linker mutants. Therefore, residues 284-286 and 300-302 were possibly important for the *in vivo* phosphorylation of IκBα through casein kinase II.

The expression of the C-terminal and linker mutants from vectors transiently transfected into 293 cells revealed that residues 284-286 and residues 300-302 were

required for either inducible or constitutive degradation of IκBα, but more likely constitutive turnover. All mutants appeared to undergo signal-induced ubiquitination. Furthermore, the mutants were capable of interacting with p65 and in a different cell line (Cos7 cells) all appeared to allow NF-κB-dependent transcription from a luciferase reporter. The final result was thought to be a consequence of different degradation characteristics existing within Cos7 cells compared to 293 cells. In the light of recent data, it was concluded that the C-terminal residues of IκBα were important for constitutive, rather than inducible turnover of IκBα.

A mutational study carried out in collaboration with colleagues at the Pasteur Institute, France to identify potential lysine residues in the N-terminus of IκBα which may be targeted for ubiquitination revealed that lysine 21 and 22 were the primary targets for ubiquitination, with lysine 38 and 47 acting as secondary targets.

A Circular Dichroism study of proteins corresponding to the C-terminal region of IκBα illustrated that the domain was comprised largely of β-sheet with other secondary structure constituents such as β-turns and possibly areas of random coil contributing to the overall structure. The secondary structure of the C-terminal region may be important for its proposed interaction with the DNA binding domain of p65. The finding that at lower pH's (e.g. pH5.6) the β-sheet content of the C-terminal region was raised suggested that this may mimic the structure adopted by the region when it interacts with the DNA binding domain of p65 (low pH conditions will lead to the protonation of carboxyl groups in the C-terminus, thereby making the groups available for hydrogen bond formation with p65).

In summary, the overall domain structure of IκBα can be seen to comprise an unstructured N-terminal signal response domain essential for inducible phosphorylation and degradation of IκBα, a central α-helical region of five ankyrin repeats responsible for protein-protein interactions, a flexible linker region (the low homology sixth ankyrin repeat) implicated in protein-protein interactions and possibly signal-induced degradation of IκBα and a C-terminal region of high β-sheet content required for constitutive phosphorylation and degradation of IκBα.

LIST OF ABBREVIATIONS

A	adenine
A#	absorbance at # nm
ABC	ATP-binding cassette
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
amp	ampicillin
APS	ammonium persulphate
ATP	adenosine 5'-tris(phosphate)
ß-gal	ß-galactosidase
ß- mer	ß- mercaptoethanol
BSA	Bovine serum albumin
C	cytosine
cDNA	complementary DNA
CMV	Cytomegalovirus
CMV IE	CMV-immediate early promoter/enhancer
ConA	concanavalin A
cpm	counts per minute
C-term.	carboxy terminus
DAPI	4, 6 diamidino-2-phenylindole
dATP..	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEAE	diethylaminoethyl
dGTP..	2'-deoxyguanosine 5'- triphosphate
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNP	dinitrophenol
dNTP(s)	2'-deoxynucleotide 5'-triphosphate(s)
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
ECL	Enhanced Chemiluminesence
E. coli	*Eschericia coli*
EDTA	ethylenediaminetetracetic acid
EGTA	ethyleneglycol-bis(b-aminoethylether)*N,N,N',N'*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	fusion protein
Fab	antigen-binding fragment of antibody
Fc	crystalizable fragment of antibody
FCS	foetal calf serum
G	guanine
GMEM	Glasgow`s modified essential medium
GMEM-10	Glasgow`s modified essential medium plus 10% FCS

hCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HRP	Horseradish Peroxidase
Ig	immunoglobulin
IPTG	isopropyl-ß-*D* -thiogalactopyranoside
KAc	potassium acetate
kb_	kilobases
kbp	kilobase pairs
LB	Luria broth
LPS	lipopolysaccharide
LTAg	Large T Antigen
LTR-	long terminal repeat
mAb	monoclonal antibody
MMTV	murine mammary tumor virus
mRNA	messenger RNA
NaAc	sodium acetate
NP	nucleoprotein
NP40	nonidet P-40
N-term.	Amino terminus
NTP(s)	ribonucleoside triphosphate(s)
OD#	optical density at # nm
OKA	Okadaic acid
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PAGel	polyacrylamide gel
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	pondus hydrogen ($-\log_{10}[H^+]$)
PMSF	phenylmethylsulphonylfluoride
RE	restriction endonuclease
RNA	ribonucleic acid
rpm	revolution per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
SSC	salt-sodium citrate buffer
ssDNA	single-stranded DNA
strep	streptomycin
SV40	Simian Virus 40
T	thymine
T7-RDRP	T7 RNA Dpendent RNA Polymerase

TAE	Tris-acetate-EDTA buffer
TAR	sequence at the 5' end of RNA recognized by tat and cellular proteins
TBE	Tris-borate-EDTA buffer
TCR	T cell receptor
TE	Tris-EDTA buffer
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TNFα	Tumour necrosis factor α
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
TRP	tetracycline responsive promoter
tRNA	transfer RNA
UV	ultra-violet
V_H	variable region of an antibody heavy chain
V_L	variable region of an antibody light chain
v/v	volume per volume ratio
w/v	weight per volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-ß-*D* -galactoside

ABBREVIATIONS FOR AMINO ACIDS

Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
Aspartic acid	asp	D
Cysteine	cys	C
Glutamine	gln	Q
Glutamic acid	glu	E
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	try	W
Tyrosine	tyr	Y

Valine	val	V

GENETIC CODE

TTT	phe	F	TCT	ser	S	TAT	tyr	Y	TGT	cys	C
TTC	phe	F	TCC	ser	S	TAC	tyr	Y	TGC	cys	C
TTA	leu	L	TCA	ser	S	TAA	OCH	Z	TGA	OPA	Z
TTG	leu	L	TCG	ser	S	TAG	AMB	Z	TGG	trp	W
CTT	leu	L	CCT	pro	P	CAT	his	H	CGT	arg	R
CTC	leu	L	CCC	pro	P	CAC	his	H	CGC	arg	R
CTA	leu	L	CCA	pro	P	CAA	gln	Q	CGA	arg	R
CTG	leu	L	CCG	pro	P	CAG	gln	Q	CGG	arg	R
ATT	ile	I	ACT	thr	T	AAT	asn	N	AGT	ser	S
ATC	ile	I	ACC	thr	T	AAC	asn	N	AGC	ser	S
ATA	ile	I	ACA	thr	T	AAA	lys	K	AGA	arg	R
ATG	met	M	ACG	thr	T	AAG	lys	K	AGG	arg	R
GTT	val	V	GCT	ala	A	GAT	asp	D	GGT	gly	G
GTC	val	V	GCC	ala	A	GAC	asp	D	GGC	gly	G
GTA	val	V	GCA	ala	A	GAA	glu	E	GGA	gly	G
GTG	val	V	GCG	ala	A	GAG	glu	E	GGG	gly	G

PHYSICAL UNITS

°C	temperature in degrees Celsius
g	gram
m	metre
mol	mole
s	second
Ci	Curie [radioactivity; 3.7×10^{10} s^{-1} (disintegrations per second)]
Da	Dalton (relative molecular mass)
g	gravitational acceleration (9.81 ms^{-2})
hr(s)	hour(s)
l	litre (volume; $10^{--3}m^3$)
M	molar concentration ($mol.l^{-1}$)
min(s).	minute(s) (time)
S	Svedberg (sedimentation)
U	unit of enzymatic activity
V	volts
A	amperes

ORDER PREFIXES

d	deci	10^{-1}	k	kilo	10^3
c	centi	10^{-2}	M	mega	10^6
m	milli	10^{-3}	G	giga	10^9
µ	micro	10^{-6}	T	tera	10^{12}
n	nano	10^{-9}			

p	pico	10^{-12}
f	femto	10^{-15}
a	atto	10^{-18}

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INTRODUCTION

1.THE ROLE OF TRANSCRIPTION FACTORS IN THE CONTROL OF EUKARYOTIC GENE EXPRESSION.

1.1. Transcription factors and the basal transcription machinery.

The control of gene expression is fundamental to the survival of all living organisms as it provides a route for the selective expression of genes in response to a particular developmental or extracellular signal. Gene expression can be regulated in several ways, for example post-transcriptionally through RNA splicing or stability (Hodges and Bernstein., 1994; Ross *et al*., 1996), at the level of protein translation (Kaufman *et al*., 1994) or via the stability of the mature protein product. The ubiquitin-conjugation system plays an important role in protein stability, essentially marking the protein for proteolytic degradation by the covalent attachment of a small, highly stable protein known as ubiquitin to lysine residues in the target protein (Jennissen, 1995; Jentsch and Schlenker, 1995). This mechanism will be discussed in more detail in section 3 of the introduction.

Although gene expression can be modulated via many routes, the major control point exists at the stage of transcription initiation and in eukaryotes initiation is modulated by sequence-specific DNA-binding proteins referred to as transcription (*trans*-acting) factors. These regulatory proteins can associate with recognition sites in the promoter region (the transcriptional start site) and in additional regulatory (*cis*-regulatory) elements known as enhancers, where they can operate singly or associated with other proteins to either repress or activate transcription initiation.

Typically transcription factors consist of a DNA binding domain which is functionally separable from a transcriptional activation or repression domain. Several different classes of activation domain have been identified including acidic, glutamine-rich and proline-rich. In parallel, specific classes of repression domains have also been discovered, for example alanine-rich and basic regions. The structure and organisation of these domains will be discussed in more detail in section 2 with reference to the ubiquitous mammalian transcription factor, NF-κB.

Transcription factors can act as the rate-limiting step in the assembly of the so-called basal transcription machinery at the core promoter elements (the TATA boxes and initiator elements) by interacting directly or via intermediary proteins with specific components of the basal transcription unit. In the absence of these *trans*-acting factors, *in vitro* studies have shown that only a minimal level of transcription can be supported by the basal transcription machinery (Buratowski, 1994). The basal transcription unit in eukaryotes is comprised of RNA polymerase II (pol II), the enzyme responsible for mRNA synthesis and a plethora of accessory proteins referred to as the basal transcription factors (TFIIA, TFIIB, TFIID (TBP), TFIIE, TFIIF, TFIIH and TFIIJ) (reviewed in Maldonado and Reinberg, 1995). In yeast there are also a number of additional proteins called SRBs (suppressors of RNA polymerase B) which have been shown *in vivo* to participate in transcription initiation (Koleske and Young, 1995). Most of the basal transcription factors are composed of multiple subunits. For example, TFIID is comprised of the TATA box-binding protein (TBP) which can bind the TATA box alone and eight or more TBP-associated factors (TAFs) which can direct promoter selectivity via protein-DNA recognition (Goodrich and Tjian, 1994).

Transcription initiation can be divided into three main stages: preinitiation complex assembly, isomerisation and promoter clearance (Goodrich and Tjan, 1994). The initial step involves the reversible binding of RNA polymerase II and its accessory proteins to the promoter DNA. In the second, almost irreversible step a small region of DNA close to the site of transcription initiation becomes unwound and in the third and final step RNA polymerase leaves the promoter to elongate the primary transcript.

Two models have been proposed for the assembly of the preinitiation complex, the multistep model and the holoenzyme assembly model (Maldonado and Reinberg, 1995). The first model was derived from *in vitro* studies using purified components of the basal transcription machinery. In this multistep model TBP, either alone or in a TFIID multisubunit complex containing TAFs, binds to the TATA element, followed by TFIIB. This intermediatory TFIID/TFIIB complex acts as a bridge for the delivery of RNA polymerase II by TFIIF and is followed by the successive association of the remaining factors, TFIIA, TFIIE, TFIIH and TFIIJ (figure 1).

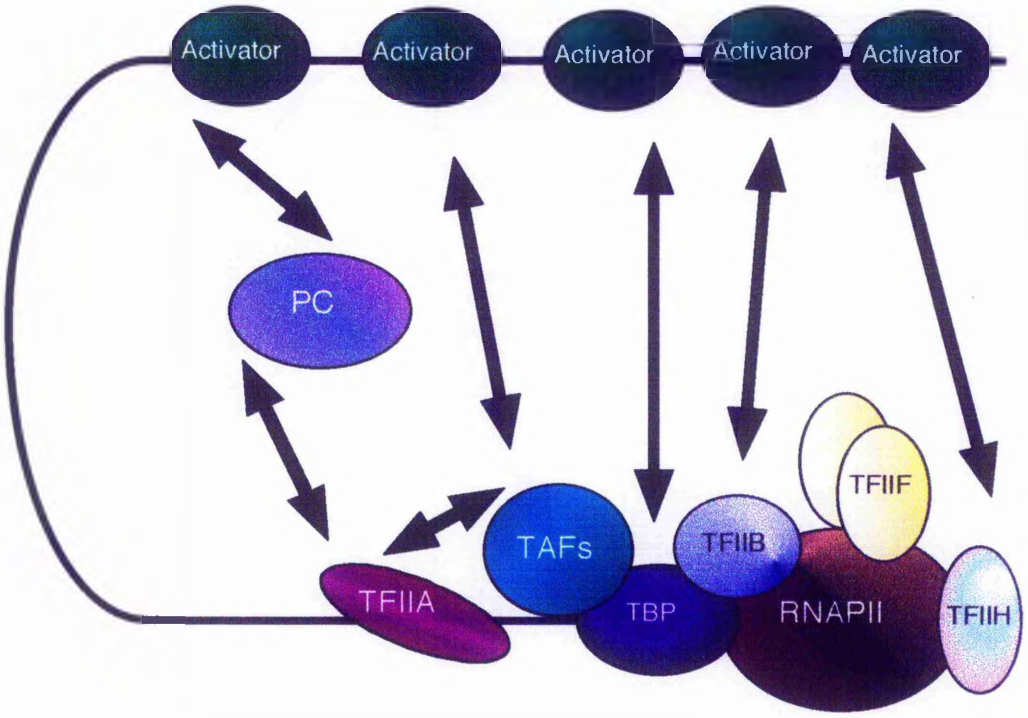


Figure 1: Multistep model for the assembly of the pre-initiation complex.

Multiple cooperative interactions between activators, cofactors and the general or basal transcription factors mediate transcriptional activation. This mechanism is derived mainly from studies performed *in vitro* utilising mammalian systems. TBP, TATA binding protein; TAFs, TBP-associated factors; PC, positive co-activators; RNAPII, RNA polymerase II (adapted from Maldonado and Reinberg, 1995).

The second model for the assembly of the initiator complex was proposed after the discovery of the holoenzyme in yeast (reviewed in Emili and Ingles, 1995) and the identification of SRBs (Koleske and Young, 1994). In this holoenzyme assembly model it is proposed that a pre-formed holoenzyme consisting of RNA polymerase II, TFIIB, TFIIF, TFIIH, the SRBs and other unidentified polypeptides can be recruited to the promoter in one step via interactions with TFIIE and after the binding of TFIID to the TATA box (figure 2). A second form of the yeast RNA polymerase II holoenzyme has also been purified which appears to be a subcomplex of the form represented in figure 2. It is composed of RNA polymerase II, and the so-called "mediator" complex of TFIIF, the SRB proteins, Gal11, Sug1 and other unidentified polypeptides (but lacks TFIIB and TFIIH). Possibly both forms of the holoenzyme exist together *in vivo*. Recently two mammalian forms of the RNA polymerase II holoenzyme have also been identified: One form consists of RNA polymerase II, TFIID, TFIIB, TFIIH, TFIIF and TFIIE (Ossipow *et al.*, 1995) and the other form is comprised of RNA polmerase II, TFIIE, TFIIH and a human homologue of the yeast SRB7 protein (Chao *et al.*, 1996).

One of the major regulatory steps in both of the models for the assembly of the transcription initiation complex is the binding of TFIID to the TATA-box. Indeed, numerous studies have shown that transcriptional regulatory proteins such as the mammalian Sp1 factor can establish specific protein-protein interactions with the polypeptides comprising TFIID, namely TBP (Maldonado and Reinberg, 1995) and the TAFs (Chiang and Roeder, 1995). It has been suggested that regulatory transcription factors may affect either TFIID recruitment to the TATA-box, or induce a conformational change in TFIID once it is bound to the promoter. Efficient activator-dependent transcription initiation cannot be supported by TBP alone but appears to require intact TFIID indicating the importance of the TAF subunits for TFIIDs interaction with *trans*-acting factors. Furthermore, *in vitro* studies using reconstituted TFIID have revealed that distinct transcriptional activators require specific combinations of TAF proteins. For example, the isoleucine-rich activator NTF-1 requires a complex composed of TBP, $TAF_{II}250$ and $TAF_{II}150$ whereas the glutamine-rich activator Sp1 additionally requires $TAF_{II}110$ (Chen *et al.*, 1994).

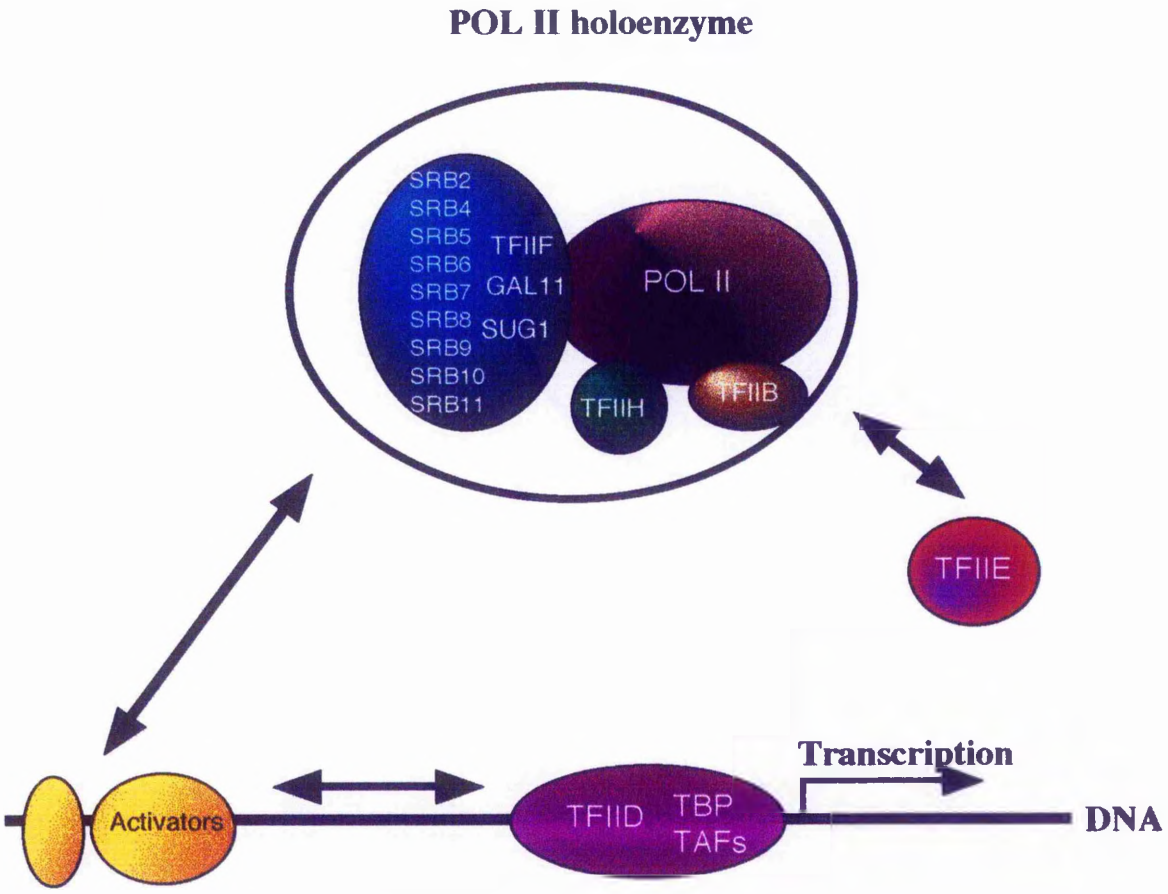


Figure 2: Model for the assembly of the RNA polymerase II (Pol II) holoenzyme into an initiation complex.

Multiple interactions between components of the holoenzyme, activator proteins and transcription factor IID (TFIID) facilitate stable intiation complex formation. TBP, TATA-binding protein; TAFs, TBP-associated factors. Double arrows indicate possible interactions between the proteins of the initiation complex.

Other components of the basal transcription machinery have also been shown to interact with regulatory transcription factors including TFIIA, TFIIB and TFIIH (Maldonado and Reinberg, 1995). TFIIA is required for high level stimulation by transcriptional activators such as herpes simplex virus VP16, Sp1 and NTF-1 *in vitro* (Ozer *et al.*, 1994). Recent crystal structures of TFIIA in complex with TBP and the TATA element have revealed large areas of both TBP and TFIIA that would be accessible to interactions with such activators (in addition to other members of the basal transcription machinery) (Tan *et al.*, 1996; Geiger *et al.*, 1996).

TFIIB has been shown to interact with numerous transcription factors including VP16 (Roberts and Green, 1994) and the ubiquitous human POU domain protein, Oct-1 (Nakshatri *et al.*, 1995). It has been suggested that VP16 may stimulate transcription through TFIIB by inducing a conformational change in TFIIB, thereby exposing the binding sites for TBP, TFIIF and RNA polymerase II and thus driving forward the formation of an preinitiation complex. Experimental evidence also indicates that activators of TFIIB may function by increasing the amount of TFIIB recruited to the preinitiation complex (Roberts *et al.*, 1995). The recent crystal structure of TFIIB in complex with TBP and the TATA binding site has provided a direct view of some of the transcription factor binding surfaces of TFIIB (Nikolov *et al.*, 1995).

TFIIH represents a prime target for regulation by *trans*-acting factors as it has been implicated in other processes besides transcriptional initiation including DNA repair and cell cycle control. Recent studies have shown that the tumour repressor p53 interacts both physically and functionally with TFIIH (Leveillard *et al.*, 1996) and earlier studies demonstrated a direct interaction with VP16 (Xiao *et al.*, 1994).

Besides the components of the basal transcription machinery there exists a group of factors referred to as positive cofactors which are also targets for the regulation of transcription at the stage of initiation. These positive cofactors do not stimulate basal level transcription but rather they enhance the response to a transcriptional activator (Maldonado and Reinberg, 1995). A large amount of information has been collected on the positive coactivator, PC4 which has been shown to interact with free and DNA-bound VP16 activation

domains. Recently it was also demonstrated that PC4 could initiate transcriptional activation during TFIIA-TFIID-promoter complex formation (Kaiser *et al.*, 1995).

In the yeast holoenzyme assembly model, the RNA polymerase II holoenzyme represents a target for regulation by transcription factors and this is believed to be accomplished by a TAF-independent mechanism. As previously described, two different complexes of the yeast holoenzyme have been isolated. Both forms of the holoenzyme are responsive to *trans*-acting factors. The SRB proteins and the Gal11 and Sug1 proteins of the "mediator" are essential for this responsiveness (Koleske and Young, 1995). It has been proposed that *trans*-acting factors may recruit the holoenzyme to the promoter in one step by interacting with these proteins. Recently, the SWI and SNF gene products, formerly identified as global gene regulators whose functions included remodelling of chromatin (see section 1.314), have been found as integral components of the SRB complex of proteins in the yeast holoenzyme (Wilson *et al.*, 1996). The SWI/SNF components possess the ability to disrupt nucleosomal DNA and facilitate the association of the transcription initiation complex with the promoter. However, the first mammalian form of the RNA polymerase II holoenzyme to be isolated from rat liver nuclear extracts (Ossipow *et al.*, 1995) has not been found to be associated with, or stimulated by, the liver-enriched transcriptional activators, HNF1, HNF4 or C/EBPα. Clearly other transcriptional activators will have to be tested before any firm conclusions can be reached on the requirement of regulatory transcription factors by the holoenzyme. In contrast, the second mammalian form of the holoenzyme to be identified was found to be responsive to transcriptional activators (Gal4-VP16) when assayed in the presence of coactivators (PC4) (Chao *et al.*, 1996). Therefore such coactivators may be required to stimulate the activity of the rat liver purified holoenzyme.

1.2. The control of transcription factor activity.

Since transcription factors represent the rate-limiting step in the assembly of the basal transcription machinery their activity will ultimately govern the level of gene expression. Consequently, several mechanisms have evolved to tightly control transcription factor activity. These can be grouped into two basic categories, either those that involve regulating

their synthesis or those that act post-translationally to modify the activity of a pre-existing transcription factor. Control can be exerted by a combination of these mechanisms which serves to create an intricate and finely balanced regulatory program for transcription factor activity. These mechanisms will be discussed under the headings which follow.

1.21. The control of eukaryotic transcription factor synthesis as a mechanism to regulate their activity.

Regulating the synthesis of a transcription factor offers a metabolically inexpensive way of controlling its activity because the protein will only be synthesised when it is required. This mechanism of regulation depends upon generating changes in the actual amount of a transcription factor by modulating the processes which are involved in the proteins synthesis, for example the transcription of the gene which encodes the factor and the splicing and translation of the corresponding mRNA transcript. It can be appreciated that if, for example control is applied at the level of transcription it will be necessary to go through all of the remaining steps in the synthesis pathway before the amount of transcription factor which is produced is accordingly altered. Subsequently, this mechanism of control tends to be adopted in situations where a particular transcription factor activity is maintained for long periods, for example in the expression of cell-type specific or developmentally regulated genes. Alternatively, it can be used to supplement more rapid methods of control for example, the post-translational regulatory mechanisms.

The headings which follow describe some examples of transcription factors which are modulated at three main stages during their synthesis: transcription, RNA splicing or translation of their mRNA.

1.211. Transcriptional regulation.

The transcriptional regulation of a gene encoding a transcription factor usually occurs at the stage of initiation and involves the recruitment of other *trans*-acting factors to either repress or induce gene expression. These additional factors will also be regulated, perhaps transcriptionally by different *trans*-acting factors, leading to complex gene networks and

hierarchies of transcription factors. As previously mentioned, this strategy is exploited by many transcription factors which are involved in cell type-specific and developmental gene expression. For example, the mammalian CCAAT box-binding factor, C/EBPα which directs the expression of several liver and adipose specific genes has been shown to be transcriptionally regulated. Mice deficient in C/EBPα fail to develop normal metabolic functions, such as hepatic glycogen synthesis, in the liver and white and brown adipose tissue (Darlington *et al.*, 1995). Controlling the activity of this transcription factor is therefore essential for the proper functioning of the liver and adipose tissue. The promoter of the gene encoding murine C/EBPα has been shown to be autoregulated: C/EBPα can bind within 200 base pairs of the transcriptional start site to elevate the activity of the C/EBPα gene by approximately three fold. However, the human C/EBPα gene promoter does not contain a binding site for C/EBPα but recent studies have shown that C/EBPα can stimulate transcription from this promoter. This activation has been attributed to a ubiquitously expressed upstream stimulatory factor (USF) which is stimulated by the C/EBPα protein to bind a specific USF element (CACGTG) in the C/EBPα gene promoter (Timchenko *et al.*, 1995).

The expression of two mammalian proto-oncogenes, c-Jun and c-Fos which encode the proteins, Jun and Fos respectively have both been shown to be transcriptionally controlled. Jun and Fos (in addition to other Jun and Fos related proteins) can heterodimerise to form the transcription factor AP-1 which renders genes which contain an AP-1 binding site inducible by treatment with phorbol esters (Angel *et al.*, 1987). Jun can also form homodimers which tend to be less stable than Fos-Jun heterodimers but they are still capable of binding to AP-1 sites (Angel *et al.*, 1991). Both Jun and Fos play a critical role in the induction of phorbol ester- and growth-dependent genes. Transcription of the c-Fos proto-oncogene following treatment with growth factors or mitogens is dependent on the activity of a region located in the c-Fos promoter referred to as the serum response element (SRE). The SRE binds the transcription factor, SRF (serum response factor) and an additional protein, TCF (ternary complex factor) to mediate the induction of c-Fos gene expression (reviewed in Treisman, 1995 and discussed in more detail in section 1.313). Interestingly, the formation and

modification (by phosphorylation-see section 1.313) of the ternary complex at the c-Fos SRE induces DNA bending in the promoter region (Sharrocks and Shore, 1995). This protein-induced DNA bending may allow the ternary complex at the SRE to interact with components of the basal transcription machinery so that transcription can be initiated. Indeed, previous studies have demonstrated that protein-induced DNA bending by the repressor, YY1 at the c-fos promoter act to prevent interactions between the CREB activator protein and members of the basal transcription machinery. YY1 may also affect the binding of the basal transcription factor, TFIID to the TATA box (reviewed in Grosschedl, 1995). The promoter of c-Jun contains binding sites for many transcription factors including Sp1, CTF and AP-1. It has been demonstrated that Jun homodimers and Fos-Jun heterodimers (AP-1) may be partially responsible for activating transcription of the c-Jun gene via two AP-1 binding sites in response to phorbol ester treatment (Unlap *et al.*, 1992). Studies on the serum induction of c-Jun expression in HeLa cells have suggested that serum may regulate expression via a member of the MEF2 family of transcription factors, MEF2D. This protein binds a MEF2 site in the c-Jun promoter and renders the gene responsive to serum. The MEF2 proteins appear to be related to the serum response factor through their DNA binding domains. Both types of transcription factor require this domain to retain their ability to trans-activate. It is possible that the serum induction of c-Fos and c-Jun gene expression may be regulated by common mechanisms (Han and Prywes, 1995).

1.212. Regulation of RNA splicing.

The synthesis of many transcription factors is controlled via the differential splicing of their RNA transcript. This post-transcriptional mechanism of control avoids the need for additional *trans*-acting factors to activate or repress the gene which encodes the transcription factor. However, RNA splicing does require specific splicing factors and their activity will also have to be controlled. To date, little is known about the nature and regulation of the splicing factors involved in the processing of transcription factor RNA transcripts.

The transcript of the mammalian transcription factor, CREB undergoes alternative splicing. CREB is activated in response to cyclic AMP (cAMP) and induces the expression of

specific genes by binding to promoter regions known as cAMP response elements (CRE) (Montminy and Bilezikjian., 1987). Several alternatively spliced forms of CREB have been identified in mice (Ruppert *et al.*, 1993) but the most abundant isoforms with DNA binding activity and transactivation potential appear to be CREBα and CREBΔ (a homologue of the human and rat CREBΔ).The role of many of the other CREB isoforms is unclear but several, including CREBγ and CREBαγ exist at elevated levels in the primary spermatocytes of the testis. It is possible that by generating these isoforms the level of mRNA species encoding active CREBΔ will be lowered, leading to a reduction in CREB-mediated gene expression.

Recently a set of novel β-exon containing CREB splice products have been identified. High levels of these new splice variants heve been correlated with poor responses to cAMP in certain tissues. They possess a transactivation domain but lack a target serine residue which is required for cAMP-dependent phosphorylation (see section 1.312 and activation of CREB). It has been suggested that these variants may assist in reducing CREB activity and hence cAMP responsiveness in some tissues (Ellis *et al.*, 1995). The generation of mice with a mutation in the CREB gene has led to the isolation and characterisation of a further CREB isoform, termed CREBβ (Blendy *et al.*, 1996). In wild-type mice this isoform is expressed at relatively low levels but in the mutant mice it is present in high quantities due to an increase in alternative splicing. This isoform appears to participate in many CREB-related functions suggesting that in instances where CREBΔ and CREBα are absent, CREBβ may partially compensate for this loss.

The primary RNA transcript derived from the gene encoding Oct-2, a member of the POU family of transcription factors that share two common domains (Herr *et al.*, 1988), is alternatively spliced to yield a number of different mRNAs which encode different isoforms of the protein. In addition to activating immunoglobulin gene expression in B cells (Clerc *et al.*, 1987 and Staudt *et al.*, 1988), Oct-2 has also been shown to act as a repressor of transcription in neuronal cells (Dent *et al.*, 1991). Two isoforms of Oct-2 predominate in neuronal cells, Oct-2c and mini Oct-2 (Lillycrop *et al.*, 1992). Oct-2c lacks a C-terminal activation domain whereas mini Oct-2c consists only of the central DNA binding POU

domain. It is thought that these isoforms may be involved in the inhibitory effect of Oct-2 on gene expression in neuronal cells. It has recently been discovered that these isoforms occur in other tissues with the proportion of mini Oct-2 mRNA being much higher in the spleen than the brain. Also, mini Oct-2 mRNA levels are increased in response to differentiation-inducing stimuli and decreased upon exposure to growth factors in neuronal cell lines (Liu *et al.*, 1995). This means that the splicing of the Oct-2 transcript is regulated both in a tissue specific manner and in neuronal cells in response to certain stimuli. As yet the precise mechanism which mediates the regulation of alternative splicing remains to be elucidated.

Several functionally distinct isoforms of the T-cell specific transcription factor, TCF-1 have been recently identified (Vandewetering *et al.*, 1996). TCF-1 is essential for early T-cell differentiation and isoforms of the transcription factor are generated via alternative splicing of the mRNA transcript and also through the existence of two promoters and a second reading frame in the gene which encodes TCF-1. Transfection studies have been performed to determine the ability of the TCF-1 isoforms to transactivate the transcription of a reporter gene via a T-cell receptor α-chain (TCR-α) enhancer (which contains a recognition site for TCF-1) (Vandewetering *et al.*, 1996). It was discovered that the transactivation potential of the TCF-1 isoforms was highly dependent upon their length: the longer isoforms failed to transactivate whereas the shorter isoforms could successfully upregulate gene expression. However, the longer isoforms of TCF-1 were still able to bind DNA. It was therefore proposed that the larger isoforms were sterically hindered in their ability to transactivate the reporter gene possibly through the exclusion of additional proteins which are also required for transactivation (for example the previously described CREB *trans*-acting factor).

1.213. Regulation of translation.

Influencing the translation of transcription factor mRNA in order to regulate synthesis of the protein can be achieved by either altering the levels of the rate-limiting protein factors which participate in translation (generally at the stage of initiation) or by using a more specific mechanism which affects only a single mRNA or class of mRNAs. The synthesis of a yeast transcription factor, GCN4 has been shown to be controlled at the level of

translation initiation. GCN4 is activated under conditions of amino acid starvation to facilitate the expression of genes which are required for amino acid production. This activation occurs when the α-subunit of translation initiation factor 2 (eIF2α, one of three non-identical subunits which comprise eIF2) is phosphorylated (via a protein kinase, GCN2) and inactivated which allows the translation of GCN4 mRNA and an increase in the level of GCN4 protein (reviewed in Hinnebusch , 1994). The increase in GCN4 mRNA translation in response to eIF2α phosphorylation depends on four short upstream open reading frames (uORFs) in the leader GCN4 mRNA. Under starvation conditions the phosphorylation of eIF2α prevents the formation of a ternary complex between eIF2, GTP and initiator transfer RNA charged with methionine (Met-tRNA^{Met}). This is thought to reduce the rate of ribosome binding since under conditions of non-starvation the small ribosomal subunit must associate with the ternary complex before it can successfully bind to the capped 5' end of the mRNA and proceed with translation at the nearest AUG-start codon. However, any ribosomes that do bind to the mRNA will recognise and translate the first uORF (uORF1). Following the translation of uORF1it is believed that ribosomes possessing a bound ternary complex bypass the remaining AUG codons of the uORFs 2,3 and 4 and re-initiate translation further downstream at GCN4. Under conditions of non-starvation where ternary complexes are in abundance and therefore available to bind ribosomes, translation of uORFs 1,2,3 and 4 is favoured. However, unlike ribosomes that translate uORF1, most that translate uORFs 2,3 and 4 dissociate from the mRNA following peptide chain termination and therefore fail to reach the GCN4 start codon.

Several isoforms of the previously mentioned CCAAT enhancer binding transcription factor, C/EBPα and the related factor, C/EBPβ have been detected in mouse liver. Four C/EBPα proteins with molecular masses ranging from 20-42 kDa were found to possess DNA binding activity. Differences existed between the pool levels of each isoform in the nucleus of liver cells. In addition, these levels were altered in response to lipopolysaccharide (LPS) treatment: The levels of the smallest isoform (20kDa) were found to increase whereas the levels of the largest isoforms (42kDa & 30kDa) were shown to decrease. Three C/EBPβ proteins were identified with molecular masses of 35, 20 and 16 kDa and in parallel to the

C/EBPα proteins their nuclear pool levels and response to LPS also varied. It has been proposed that LPS can mediate the differential initiation or inhibition of translation at specific AUG sites within each mRNA. It is believed that the pool levels of each isoform are either regulated by LPS or an upstream-stimulated factor (possibly a translational trans-activator) via the selection of AUG start sites for the activation and repression of translation (An *et al.*, 1996).

The GC-box binding transcription factor, BTEB which can activate HIV type 1 long terminal repeat and cellular gene promoters containing multiple GC-boxes, is partly regulated through its translation. In mammals, BTEB protein expression is confined to the brain, although BTEB mRNA is found in many other mammalian tissues and cell lines. This cell-specific protein expression has been attributed, in part, to an upstream AUG in the 5' untranslated region (5' UTR) of the BTEB mRNA (Imataka *et al.*, 1994). Since the events of translation initiation take place on the 5' UTR of the mRNA both the primary and secondary structures of this region have an important role to play in the efficiency of mRNA translation. Therefore, the presence of additional AUG codon(s) upstream of the initiator AUG reduces the chances of the pre-initiation complex for translation reaching the authentic initiating AUG. It is possible that the activity of the proteins involved in translation initiation (for example, eIF-2α) vary between different cells and tissues. Therefore, the ability of ribosomes to reinitiate translation at the BTEB open reading frame may be more efficient in brain cells compared to other cells.

1.31. Control of eukaryotic transcription factor activity using post-translational mechanisms of regulation.

The modification of a pre-existing transcription factor as a mechanism to control its activity is used in situations where rapid gene induction is required, for example during environmental change or challenge. However, these post-translational mechanisms of control rely on the synthesis of a transcription factor, albeit in an inactive state, even when it is not required and are thus more demanding in terms of energy usage than those mechanisms that only completely synthesise a protein when it is needed.

There are several ways to modify the activity of a transcription factor post-translationally and these include the association of transcription factors with small diffusable ligands, the covalent modification of factors, the recruitment of accessory proteins, the interaction of transcription factors with chromatin or the exclusion of *trans*-acting factors from the nucleus to prevent DNA binding. Each of these methods of control will be discussed under the headings which follow.

1.311. The association of transcription factors with small, diffusable ligands.

In many eukaryotes the ability of a transcription factor to bind its target DNA is controlled via its association with small, diffusable ligands. The activity of the yeast transcription factor, AMT1 is regulated in this way. AMT1 induces the expression of metallothionein genes in response to copper. Activation of AMT1 is achieved by a major conformational change which is induced by the binding of four copper (+) ions and one zinc (2^+) ion to two independent sub-domains located in the N-terminal DNA binding region of the protein. Although the zinc ion does not affect the ability of AMT1 to bind DNA it is thought that it may be critical for the transcriptional activation function of the protein (Thorvaldsen *et al.*, 1994; Farrell *et al.*, 1995).

Members of the nuclear receptor superfamily, which are responsible for the mediation of many extracellular signals into transcriptional responses, can only bind with high affinity to their cognate hormone response elements (HRE) via association (through ligand binding domains (LBD)) with various lipophilic hormonal ligands. Some members of the Orphan receptors (a class of nuclear receptors) appear to lack the requirement for these ligands. However, it is more likely that a group of novel ligands exist for these receptors which, as yet, have not been identified. These ligands include the steroids, retinoids, thyroid hormones and vitamin D_3 (reviewed in Mangelsdorf *et al.*, 1995). They can pass through the lipid bilayer of the cell to interact with their respective receptors where they can act as regulators of development, cell differentiation and organ physiology. The classic model for hormonal induction of receptor DNA binding postulates that hormone binding induces allosteric changes in the receptor which unmasks the DNA binding region of the receptor. This allows

the hormone-receptor complex to associate with specific DNA sequences and modulate transcription.

Studies to date have characterised over 150 members of the nuclear receptor superfamily. The superfamily is often sub-divided into the steroid receptor family and the thyroid/retinoid/vitamin D (or non-steroid) family. In addition, the superfamily can be arranged into four classes based on the ability to dimerise and bind DNA: Class I receptors consist of the steroid hormone receptors which, following ligand induction, bind as homodimers to DNA half-sites organised as inverted repeats; Class II receptors form heterodimers with the retinoid X receptor (RXR) and tend to associate with DNA arranged as direct or symmetrical repeats. This class includes all the other known ligand receptors outwith the steroid hormone receptor class; Class III receptors exist as homodimers and recognise DNA sequences organised as direct repeats; Class IV receptors are monomeric and associate with DNA arranged as an extended core. The Orphan receptors tend to fall into either Class III or IV and it is thought that a set of novel hormonal ligands may exist for these receptors (reviewed in Mangelsdorf and Evans, 1995).

Recently, the structures of unliganded human retinoid X receptor LBD and liganded thyroid receptor α LBD have been resolved using X-ray crystallography (Bourguet *et al.*, 1995; Wagner *et al.*, 1995). The two structures were found to be broadly similar, although RXR exists as a homodimer and the thyroid receptor is monomeric. It has previously been found that the LBD's of all known ligand-activated receptors comprise a carboxy-terminal region of around 225 amino acids. This region is also responsible for dimerisation, hormone-dependent transcriptional activation and, in some instances hormone-reversible transcriptional repression. The most striking feature to emerge from the crystallisation data was the differing arrangement of the receptors respective transcriptional activation domains. The transcriptional activation domain of RXR formed an amphipathic alpha helix (termed AF-2) which extended into the solvent away from the central core of the LBD (containing the putative ligand binding site). In contrast, the the equivalent transactivation region in the thyroid receptor was packed onto the core of the receptor and formed part of the ligand-binding pocket. The ligand itself was buried inside the hydrophobic core of the receptor. Therefore, it appears that ligand

binding probably causes extensive allosteric changes in the receptor which allows the complex to bind specific DNA sequences and regulate transcription.

1.312. The covalent modification of transcription factors.

Several transcription factors are regulated post-translationally by covalent modifications such as phosphorylation or the addition of O-linked monosaccharides. For example, the CREB transcription factor, previously mentioned to undergo regulation through alternative splicing (section 1.212), can also be activated by the phosphorylation of a serine residue at position 133 via protein kinase A (Gonzalez *et al.*, 1989). Protein kinase A is stimulated by cyclic AMP (Nigg *et al.*, 1985) to release its catalytic subunits into the nucleus where they can phosphorylate and activate CREB. The serine residue which is phosphorylated in CREB is located within a region of the protein known as the phosphorylation-box (P-box) or kinase-inducible domain (KID). The P-box is flanked by two transcriptional activation domains, Q1 and Q2, the latter domain appears to be more important for activation (Brindle *et al.*, 1993). Phosphorylation of the serine residue in the P-box stimulates Q1 and predominantly Q2 to activate the transcription of specific genes.

The precise mechanism of the stimulatory action on Q1 and Q2 is not known. However, it has been suggested that like other substrates of protein kinase A, CREB may undergo a conformational change following phosphorylation which allows Q1 and Q2 to interact with the transcription apparatus and activate gene expression (Brindle *et al.*, 1993). Alternatively, it has been shown that phosphorylation of the P-box can enable CREB to interact with a co-activator protein known as CBP (CREB-binding protein) (Chrivia *et al.*, 1993). Transfection experiments have demonstrated the ability of CBP to enhance the activation of CREB-mediated reporter constructs *in vivo* in a manner which is dependent on protein kinase A phosphorylation of CREB (Kwok *et al.*, 1994). Recently, a domain referred to as KIX has been characterised in CBP. KIX was found to bind CREB following phosphorylation of CREB at serine 133 and was able to be recruited to a cAMP response element. It appears that KIX is co-ordinated through a single arginine residue (Arg-600) to the phosphoserine residue in CREB. This association is stabilised by hydrophobic residues in the

KID of CREB which flank serine 133. It has been proposed that novel motifs such as KIX may be responsible for the protein-protein interactions that follow serine/threonine mediated signalling (Parker *et al.*, 1996).

The mammalian Jun transcription factor is activated by the phosphorylation two N-terminal serine residues (Ser 63 and Ser 73) which are located in the transcriptional activation domain of Jun. The Jun N-terminal kinases (JNKs)/stress activated protein kinases (SAPKs), members of the family of mitogen-activated protein kinases (MAPK) are responsible for this N-terminal phosphorylation. *In vitro* studies have shown that the JNK/SAPKs can bind to a so-called "docking site" in the Jun activation domain from residues 30 to 60. Removal of this site from Jun results in a dramatic reduction in the ability of Jun to be phosphorylated *in vivo* in response to a range of JNK/SAPK activators (environmental stress and the proinflammatory cytokines)(reviewed in Hill and Treisman, 1995; Karin and Hunter, 1995).

1.313. The recruitment of accessory proteins by transcription factors.

Many transcription factors require the presence of a co-activator protein to assist in the process of transcriptional regulation. This can be illustrated by the interaction between the transcription factor Oct-2 and the co-activator, OBF-1. As previously described in section 1.212 Oct-2 can upregulate immunoglobulin gene expression in B-cells only by binding to an octamer motif (ATGCAAAT) in the promoter and enhancer regions of most immunoglobulin genes. Substantial evidence exists to suggest that Oct-2 mediates the cell-specific transcriptional regulation of immunoglobulin genes by forming a ternary complex with the B-cell specific factor, OBF-1 on an octamer motif (Gstaiger *et al.*, 1995; Strubin *et al.*, 1995). OBF-1 does not appear to bind the DNA itself but instead associates directly with the POU domain of Oct-2. This domain contains two highly conserved regions which mediate the transactivating potential of Oct-2 and other transcription factors that comprise the POU family (reviewed in Herr *et al.*, 1988). It is thought that OBF-1 may potentiate the activity of Oct-2 by acting as a bridge between a member of the basal transcriptional machinery and the octamer-bound Oct-2.

As outlined in section 1.211 the transcription factor, SRF (serum response factor) requires the coactivator protein, TCF (ternary complex factor) to efficiently induce c-Fos gene expression from the serum response element (SRE) in the c-Fos promoter (reviewed in Treisman *et al.*, 1995; Hill and Treisman, 1995). TCF forms a ternary complex with SRF at the serum response element following treatment with serum growth factors. TCF cannot bind the SRE alone. Various studies have indicated that SRF may occupy the SRE in a ternary complex with Elk-1 even in the absence of serum (Herrera *et al.*, 1989). This indicates that activation of the SRE may involve regulation of transcriptional activation rather than DNA binding.

Several cDNAs that encode proteins with TCF-like activity have been identified. These include the Ets domain proteins, Elk-1, SAP-1a and SAP-2. They are the targets for the mitogen activated protein kinases (MAPK), a family of protein kinases whose members include the mammalian extracellular signal-regulated kinases ERK1 and ERK2. MAP kinases are activated through a signalling cascade which is initiated by the stimulation of receptor tyrosine kinases. This signalling cascade involves the activation of ras kinase followed by the sequential activation of MAPK kinase and ERK-1/ERK-2 (Cobb and Goldsmith, 1995). MAPK phosphorylation sites contain a core sequence motif, S/T-P. Such core motifs have been identified in the C-terminal transcriptional activation domains of the Elk-1, SAP-1a and SAP-2 proteins (Price *et al.*, 1995). These motifs are required for activation of the TCF-like proteins. ERK-2 can phosphorylate each TCF activation domain *in vitro*. Furthermore, ERK activation *in vivo* is capable of inducing transcriptional activation in the presence of each TCF activation domain. Interestingly, SAP-2 cannot bind DNA or form ternary complexes as efficiently as ELK-1 or SAP-1a, suggesting that its binding may be regulated through mechanisms distinct from these two proteins.

A model has been proposed in which SRF is permanently bound to the SRE in a ternary complex with Elk-1. Following exposure to serum MAP kinase phosphorylates Elk-1 leading to the subsequent induction of c-Fos transcription. An alternative signalling pathway for the phosphorylation and subsequent activation of Elk-1has recently been described (Whitmarsh *et al.*, 1995). This pathway has been shown to involve phosphorylation of Elk-1

and a resulting increase in ternary complex formation at the SRE by a group of MAP kinases referred to as the JNKs (see section 1.312). The JNKs are activated through a signal transduction pathway distinct from that of the ERKs. It would therefore appear that the SRE represents an integrative site for two distinct signalling pathways.

1.314. The interaction between transcription factors and chromatin.

In eukaryotes, transcription factors must function in an environment where DNA is compacted into a chromatin structure by repeating units (nucleosomes) of five small, basic proteins (11-16kD) called histones (H2A, H2B, H3, H4 and the linker histone). Beyond the chromatin level of organisation there also exists a higher order structure comprising of chromosomal domains of chromatin fibres (see figures 3 and 4; reviewed in Pruss *et al.*, 1995; Wolffe and Pruss, 1996a). As yet, there is little information on the nature of the contacts which may contribute to the formation of these domains. However, it can be envisaged that large areas of the genome will be rendered inaccessible to transcription factors and also to the basic transcription initiation machinery (RNA polymerase and the basal transcription factors supporting the minimum levels of transcription) which results in the repression of many genes. However, as will be discussed, the effect of chromatin structure on gene transcription is not always one of repression.

Substantial genetic evidence exists to show that the histone proteins can influence the level of transcription. For example, studies were performed on yeast cells where the wild-type histone H4 and H2B genes were replaced with repressed copies which resulted in the normally silent PHO5, CUP1 and HIS3 genes becoming active (Durrin *et al.*, 1992; Kim *et al.*, 1988 and Han *et al.*, 1987). Presumably the repression of the histone genes prevented normal chromatin assembly and thereby permitted the *trans*-acting factors and RNA polymerase to gain access to the promoter and enhancer regions of the PHO5, CUP1 and HIS3 genes so that transcription could proceed.

However, more specific information on the regulatory proteins which interact directly with the histones to control gene expression does exist. This includes the SWI-SNF activator complex in yeast which is composed of eleven proteins, the sucrose non-fermenting (SNF5,

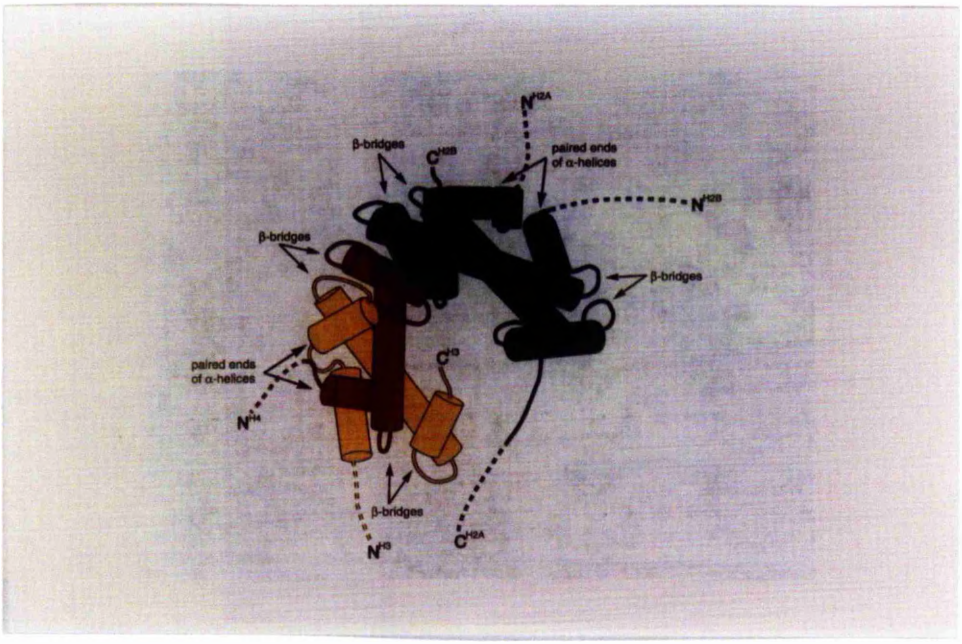


Figure 3: The histone fold and DNA binding motifs.

The relative juxtaposition of the two histone heterodimers as viewed "from the top"(i.e. along the superhelical axis of the DNA). Histones are colour-coded in the following way: H4 (red), H3 (yellow), H2B (blue) and H2A (green). The approximate positions of the flexible histone tails are shown by broken lines. Note the six regularly spaced domains (double arrows) predicted to be involved in DNA binding (taken from Pruss *et al.*, 1995).

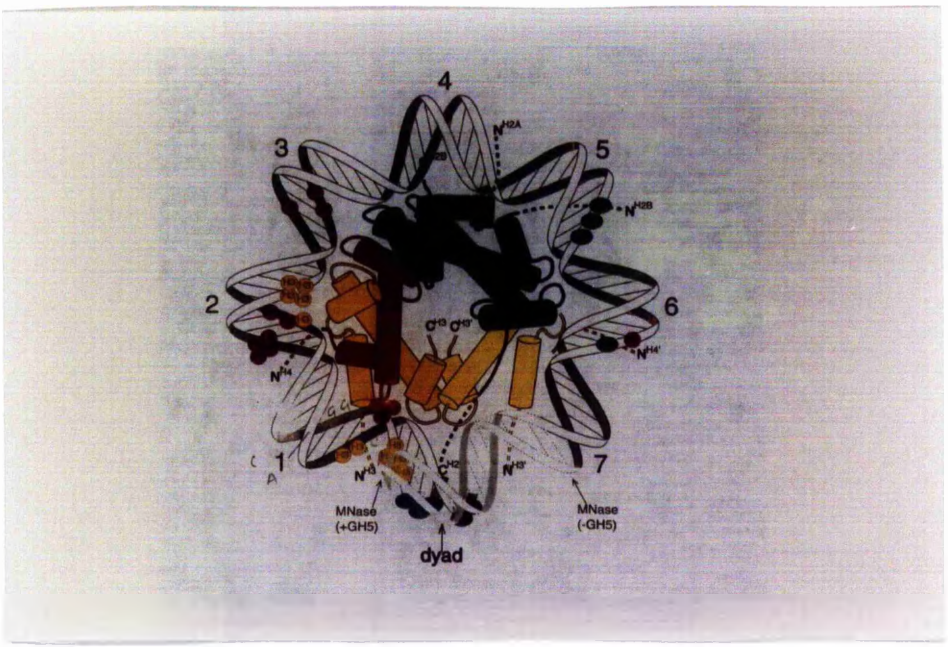


Figure 4. The anatomy of the core particle-a view down the superhelical axis.

The micrococcal nuclease (MNase) digestion boundaries and sites of histone-DNA contact (revealed by crosslinking) (coloured circles) are shown as found on the coding sequence portion of the 5S nucleosome (the numbers of "crosslinking circles" roughly indicate the efficiency of crosslinking). For simplicity, the DNA is shown as a uniform superhelix. The helical turns are numbered relative to the dyad axis (0). GH5, globular region of linker histone H5 (taken from Pruss *et al.*, 1995).

SNF6, SNF11) and switch (SWI1, SWI2, SWI3) proteins in addition to other identified polypeptides, and is required for the expression of many inducible genes (reviewed in Peterson and Tamkun, 1995; Kornberg and Lorch, 1995). SWI-SNF mutations have a profound effect on activator function, for example the activity of the GAL4 transcription factor can be reduced approximately twenty fold in the absence of either SWI1, SNF5 or SNF6. Early clues to the mechanism of transcription factor activation by the SWI-SNF complex arrived when it was demonstrated that yeast strains deficient in SWI gene activity were still capable of inducible gene transcription. This activity was attributed to mutant forms of the sin genes which normally generate proteins which are thought to act like histones (Kruger *et al.*, 1991). Therefore, mutants of these proteins probably failed to compact the DNA so that the promoter and enhancer regions of the genes were exposed allowing transcription initiation. It was thought that the activator complex may similarly affect the integrity of the chromatin structure, unfolding it for transcription initiation.

Biochemical studies using the purified SWI-SNF complex and a derivative of GAL4 have revealed a direct biochemical relationship between the SWI-SNF complex and chromatin (Cote *et al.,* 1994): the SWI-SNF complex does not result in nucleosome displacement although it does directly bind to the nucleosomal DNA, instead it promotes the formation of a ternary complex between the GAL4, its DNA recognition site and the histone proteins. The SWI-SNF complex uses the energy generated from the hydrolysis of ATP to drive this process. The ATPase activity of the complex is thought to reside in the SWI2 component since it possesses ATPase motifs (similar to the DNA stimulated ATPases) and mutations in the protein result in a loss of ATPase activity from the SWI-SNF complex. One current "chromatin remodelling" model for the SWI-SNF complex proposes that the SWI-SNF complex associates with the nucleosomal DNA and then uses the energy produced from ATP hydrolysis to alter DNA-histone interactions thereby promoting the loss of one or both of the histone2A-2B dimers from the GAL4 ternary complex (Cote *et al.*, 1994; Peterson and Tamkun, 1995).

Genetic evidence also exists for the role of the higher order chromatin structure (chromosomal domains) in transcription initiation control. This can be demonstrated by

"position effects" in both the fruit fly, *Drosophila* and the yeast strain, *S. cerevisiae*. Position effects occur when active genes become repressed following their relocation next to inactive highly condensed domains of DNA (heterochromatin) as a result of rearrangement or recombination. This repression is thought to be brought about by the extention of the heterochromatin structure into the previously active gene.

In *Drosophila* and *S. cerevisiae*, the histone proteins (Becker, 1994; Wolffe and Pruss, 1996b) have been shown to play a role in position effects. These transcriptionally repressive effects can be relieved through the hyperacetylation of core histones. This hyperacetylation causes an allosteric change in nucleosome conformation which destabilises the higher-order chromatin structure and allows transcription factors to gain access to the nucleosomal DNA. Recently, a yeast transcriptional activator called GCN5p was shown to function as a histone acetyltransferase suggesting the potential for targeted histone modification (reviewed in Wolffe and Pruss, 1996b). In addition, a specific family of *trans*-acting factors in *Drosophila*, including HP1, a chromodomain associated protein (reviewed in Wolffe, 1994) and a factor encoded by the Brahma gene (brm) in *Drosophila* (Tamkin *et al.*, 1992) can also contribute to position effects. Brm is related to the ATPase component of the SWI-SNF complex, SWI2 and assists DNA binding regulatory proteins to overcome the repressive effects of chromatin on transcription (reviewed in Peterson and Tamkun, 1995). A recent study has discovered that the product of the *Drosophila* snr1 gene (a potential homologue of the SNF5 gene) can form a complex with brm indicating that the yeast SWI-SNF complex may be conserved in higher eukaryotes (Dingwall *et al.*, 1995).

Finally, several examples exist where nucleosome positioning can positively or negatively regulate transcription initiation (reviewed in Grosschedi, 1995). The mouse mammary tumour virus (MMTV) promoter is strongly transcribed in response to steroid hormones but is almost silent in the absence of hormonal stimuli. Full transcriptional activation from the MMTV promoter in the presence of steroid hormones requires the association of the hormone receptors to four hormone-responsive elements (HREs), in addition to the binding of nuclear factor 1 (NF1) and the octamer transcription factor, Oct-1. The low basal activity of the MMTV promoter is believed to result from its organisation in a

positioned nucleosome where it is wrapped around a histone octamer in a precise rotational orientation. This allows access to only two of the HREs by the hormone receptors and completely prevents the binding of NF1 and Oct-1 to their respective sites. Presumably hormone induction remodels the nucleosome allowing the binding of each *trans*-acting factor to its relevant site and transcriptional activation (reviewed in Truss *et al.*, 1995). The *Drosophila hsp26* and alcohol dehydrogenase promoters are arranged into a 3-dimensional structure by nucleosomes (reviewed in Wolffe, 1994). Distant regulatory elements are brought into close proximity by nucleosome positioning and it is thought that this may be required for efficient transcriptional activation.

Many transcription factors can themselves induce bending in the promoter DNA to facilitate the binding of additional transcription factors for transcriptional activation (Grosschedi *et al.*, 1995). For example, the LEF-1 protein (a lymphoid specific member of the high mobility group (HMG) domain family) binds to a specific site in the centre of the T-cell receptor α enhancer (TCRα) and causes the DNA to bend sharply. It is proposed that LEF-1 acts as an architectural element that allows additional transcription factors which are bound to sites flanking the LEF-1 binding region to interact with one another.

1.3.5. Cytoplasmic localisation of transcription factors in an inactive form.

Many transcription factors can exist in the cytoplasm in an inactive form and can be activated by certain stimuli so that they can enter the nucleus to regulate the transcription of specific genes. For example, the interferon stimulated gene factor 3 (ISGF3) is a trimeric transcription factor which resides in the cytosol in an inactive state (reviewed in Hill and Treisman, 1995; Schlinder and Darnell, 1995). It is stimulated in response to treatment of cells with the cytokine, interferon α (IFα) via the JAK-STAT signal transduction pathway. JAK refers to a family of receptor- associated tyrosine kinases and STAT refers to a family of latent cytoplasmic transcription factors, which are activated by tyrosine phosphorylation. ISGF3 is composed of an α-interferon inducible component referred to as ISGF3α and a 48 kDa constitutively active DNA binding component, which can be induced by γ-interferon, known as ISGF3γ (a member of the IRF-1 family of transcription factors). ISGF3α is a dimer

comprised of two STAT (signal transducers and activators of transcription) proteins , STAT2 (113kDa) and either STAT 1α (91kDa) or an 84kDa C-terminally truncated splice product of STAT 1α, referred to as STAT 1β. Following treatment with interferon α the cytoplasmically located subunits of ISGF3α (STAT 2 and either STAT 1α or STAT 1β) are tyrosine phosphorylated on a specific residue towards their C-termini and associate into an active signal transducing factor (STF). The STAT1:STAT2 heterodimer translocates to the nucleus where it forms a complex with ISGF3γ to yield the mature transcription factor, ISGF3. ISGF3 can then bind to a specific sequence which is located in the promoters of interferon responsive genes and is referred to as a type I interferon stimulatable response element (ISRE).

Tyrosine phosphorylation of the STAT1 and STAT2 proteins of ISGF3 is achieved through the activation of the interferon α receptor following stimulation by the binding of IFNα at the extracellular surface. IFNα has been shown to induce rapid phosphorylation of its receptors as well as two receptor associated tyrosine kinases, JAK1 and TYK2, members of the Janus kinase (JAK) family. The JAK kinases appear to be essential for the activation of the STAT proteins. Recent evidence has demonstrated that TYK2 can tyrosine phosphorylate the IFNα receptor subunit 1. This complex can recruit unphosphorylated STAT2 (via the SH2 domain of STAT2) to the receptors phosphorylated residues, essentially acting as a docking site for the protein (Yan *et al.*, 1996). Further studies have shown a dependence of STAT1 phosphorylation on the STAT2 protein but not vice versa (Leung *et al.*, 1995). It has been proposed that STAT1 may be drawn into the receptor complex through the activated STAT2 protein and activated by a JAK1/TYK mediated event. The dissociation of the STAT1:STAT2 complex from the receptor is thought to be controlled by the SH2 domains of these proteins. After initial binding to the receptor tyrosine motif, the SH2 domain is believed to shift its binding specificity to that of the associated tyrosine kinases (JAK1/TYK2) and perhaps to additional stabilising domains. Presumably the subsequent activation of the STATs would than act to destabilise the kinase interaction, promoting the formation of energetically favourable dimers (Gupta *et al.*, 1996).

Cytoplasmically located transcription factors can be held in an inactive form via their association with so-called inhibitory proteins. Various stimuli can release the factor from this protein and permit its translocation to the nucleus where it can participate in DNA binding and hence transcriptional modulation. For example, the previously mentioned steroid hormone receptors (see section 1.311) when not bound to their respective hormone ligands are held in the cytoplasm by a large multiprotein complex of chaperones, including Hsp90 and the immunophilin Hsp56 (reviewed in Pratt., 1993; Beato *et al.*, 1995). The receptors are only released from their protein anchor by the steroid hormones which allows the receptor to dimerise and enter the nucleus where it can bind to specific palindromic DNA sequences and transcriptionally activate genes (the mechanism of activation of the mouse mammary tumour virus long terminal repeat by the glucocorticoid receptor has already been discussed in section 1.314).

Hsp90 interacts with the C-terminal region of the glucocorticoid receptor which also contains the sequences responsible for steroid binding (Pratt, 1995). Immediately adjacent to this region is the DNA binding domain of the receptor and the sequences responsible for receptor dimerisation. It is thought that Hsp90 masks these regions when it is bound to the receptor and only after Hsp90 is displaced by steroids can the receptor dimerise and bind to DNA. It has been demonstrated that disruption of Hsp90 homologues in yeast strains expressing the glucoorticoid receptor does not result in an expected constitutive activation of the receptor. Instead, the ability of the receptor to be stimulated by hormones is dramatically reduced. It has therefore been suggested that the protein chaperones help to maintain the function of the steroid hormone receptors. Other chaperones besides Hsp90 have also been shown to assist in conserving the functional integrity of the steroid hormone receptors. This includes the yeast YDJ1 protein, a member of the DnaJ chaperone family, which can bind to unliganded steroid thyroid hormone receptors (Kimura *et al.*, 1995).

In parallel to the steroid hormones there exists a group of transcription factors known as the Rel/NF-κB family which are also primarily regulated through inhibitory proteins in the cytosol. One of these family members, known as NF-κB has received extensive interest because it plays an important role in several diseases and is also involved in controlling the

expression of many genes including those of the immune and inflammatory responses. NF-κB is a ubiquitous transcription factor present in mammalian cells and is activated by exposure of cells to a range of stimuli including inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), viral infection or the expression of certain viral gene products, B or T-cell activation or UV irradiation (reviewed in Liou *et al*, 1993). Cytosolic activation of NF-κB is modulated through the targeted phosphorylation and subsequent degradation of its inhibitor protein, IκB which releases NF-κB allowing it to translocate to the nucleus and bind specific DNA sequences. There it can regulate the transcription of many viral and cellular genes such as those of the human immunodeficiency virus (reviewed in Roulston *et al*., 1995; Baldwin, 1996). The discussion which follows in sections 2 and 3 will be mainly focused on NF-κB and its inhibitor protein, IκB although some details of the other Rel/NF-κB family members will be given.

2. THE NF-κB AND IκBα PROTEINS.

2.1. Structure and function of NF-κB.

2.11. Identification and purification of NF-κB.

NF-κB was first identified in mature B-cells as a nuclear factor which bound to a specific DNA sequence located in the immunoglobulin κ light chain gene transcriptional enhancer and referred to as the κB motif (5' GGGGACTTTCC 3', the immunoglobulin type of κB motif) (Sen and Baltimore, 1986a). Subsequently, NF-κB DNA binding activity was found to be inducible in pre-B cells following exposure to lipopolysaccharide (LPS) or the active phorbol ester phorbol 12-myristyl 13-acetate (PMA) (Sen and Baltimore, 1986b). In addition, it was discovered that NF-κB DNA binding activity was independent of protein synthesis since protein translation inhibitors such as cycloheximide could activate DNA binding. This indicated that NF-κB may exist in an inactive form prior to stimulation, possibly bound to an inhibitor protein. Another significant discovery in this study was that neither NF-κB nor its potential repressor protein were confined to the B-cell lineage as previously believed: NF-κB could be induced in both T-cell and epithelial cell lines. Subsequent studies identified functional NF-κB recognition sites in the promoters and enhancers of several genes in cells other than those of the B-cell lineage such as the MHC class I and β2 globulin genes (Yano *et al.*, 1987; Baldwin and Sharp, 1988),

The identification of a specific, reversible inhibitor of NF-κB DNA binding activity, named the IκB protein clarified the mechanism of NF-κB activation (Baeuerle and Baltimore, 1988a, b). It was suggested that NF-κB was sequestered in the cytoplasm of cells via its interaction with IκB and that various stimuli could disrupt this association thereby allowing NF-κB to enter the nucleus and transactivate target genes (reviewed in Liou and Baltimore, 1993). A detailed discussion of the relationship between NF-κB and IκB will be given under the sub-section which deals with the mechanisms of NF-κB regulation.

Early efforts to DNA affinity purify NF-κB from human cell lines using double-stranded multimers of the κB recognition site (5' GGGACTTTCC 3') yielded proteins of around 40-60kDa (Kawakami *et al*, 1988; Lenardo *et al*, 1988). More definitive biochemical analyses revealed that the inducible form of NF-κB was comprised of two polypeptides with

apparent molecular sizes of 50kD and 60kD, referred to as p50 and p65 (Baeuerle and Baltimore, 1989).

2.12. Cloning of NF-κB- the emergence of the Rel/NF-κB family of proteins. The first subunit of NF-κB to be cloned was that of p50 and resulted from the independent and simultaneous efforts of several laboratories (Kieran *et al.*, 1990; Ghosh *et al.*, 1990: Bours *et al.*, 1990). The amino acid sequence of p50 was found to be identical to that of the constitutively active nuclear protein, KBF-1 which was previously purified from a mouse T-cell line (Yano *et al.*, 1987). In addition, p50 appeared to be generated from the N-terminal half of a larger precursor protein known as p105 (NF-κB1) which failed to bind DNA. The N-terminal region of p105 was highly homologous to an area in the proto-oncogene product, c-Rel (Wilhelmsen *et al.*, 1984; Brownell *et al.*, 1989) and the *Drosophila* maternal effect morphogen protein dorsal which is involved in the critical developmental pathways that determine the dorsal-ventral patterning of the *Drosophila* embryo (Steward, 1987). Further investigations led to the isolation of cDNAs encoding the p65 (Rel A) subunit of NF-κB (Nolan *et al.*, 1991; Ruben *et al.*, 1991). Like the precursor to p50, the N-terminal region of p65 displayed a high level of sequence similarity to the rel proto-oncogene product. Indeed, p65 appeared to share more amino acid homology with c-Rel than with either p50 or dorsal. Conversely, the amino acid sequence of p50 was more closely related to the *Drosophila* dorsal protein than to either p65 or c-Rel.

The highly conserved N-terminal region present in p50, p65, c-Rel and the *Drosophila* dorsal protein was soon discovered in other proteins. This included the p49 protein (also known as p50B or p52) (Schmid *et al.*, 1991; Bours *et al.*, 1992) which shared extensive amino acid homology with p50 and like p50 was also generated from the N-terminal half of a larger non-DNA binding precursor protein known as p100/p97 (or NF-κB2) via alternative splicing. In addition, the two closely related proteins, murine RelB (Ryseck *et al.,* 1992) and human I-Rel (Ruben *et al.*, 1992a) were cloned and found to possess areas of amino acid homology to the c-Rel protein. Recently, the *Drosophila* immune response protein, Dif which is involved in the regulation of bacteriocidal genes in response to endotoxin was cloned and

its amino acid sequence was found to be highly homologous to the dorsal protein (Ip *et al.*, 1993).

This group of related mammalian proteins have become collectively known as the Rel/NF-κB family (see figure 5) and they are characterised by the presence of a Rel homology domain (RHD), an N-terminal region of around 300 amino acids in length which displays approximately 30 to 61% identity between the various family members. The RHD accomodates the areas of sequence which are involved in protein dimerisation (a prerequisite for DNA binding), DNA binding, nuclear localisation and interactions with various members of the IκB protein family. These functions will be discussed in section 2.14.

The Rel/NF-κB family members can be grouped into two classes on the basis of sequences C-terminal to the RHD. The two members of the first class, p105 (NF-κB1) and p100 (NF-κB2) which are proteolytically cleaved to yield p50 and p49 respectively, contain seven copies of a 33 amino acid repeat called an ankyrin or cell-cycle repeat at their C-termini. These ankyrin repeats are known to mediate protein-protein interactions (reviewed in Michaely and Bennett, 1992) and are present in the IκB inhibitor proteins of the Rel/NF-κB family. The functional significance of these ankyrin repeats in p100 and p105 will be discussed in section 2.23. In addition, the role that proteolytic processing of these proteins plays in the regulation of Rel/NF-κB proteins will be dealt with in section 2.236.

Members of the second class of Rel/NF-κB proteins which includes p65 (RelA), c-Rel, Rel B, Dorsal, Dif and v-Rel (the retroviral counterpart of the cellular c-Rel protein isolated from the avian retrovirus, Rev-T which causes rapid and fatal lymphoma in young birds (Gilmore and Temin, 1986; Herzog and Bose, 1986)), have generally unrelated C-terminal domains that do not contain ankyrin repeats. They are also characterised by the absence of approximately 25 amino acids in the RHD, a length of sequence that is present in the class I Rel/NF-κB proteins.

Recently it has been suggested that the NF-AT family of transcription factors may also be related to the Rel/NF-κB group of proteins. The NF-AT transcription factors are believed to be involved in the inducible transcription of the IL-2 gene and other cytokine genes. Several similarities exist between the NF-AT and Rel/NF-κB groups of proteins

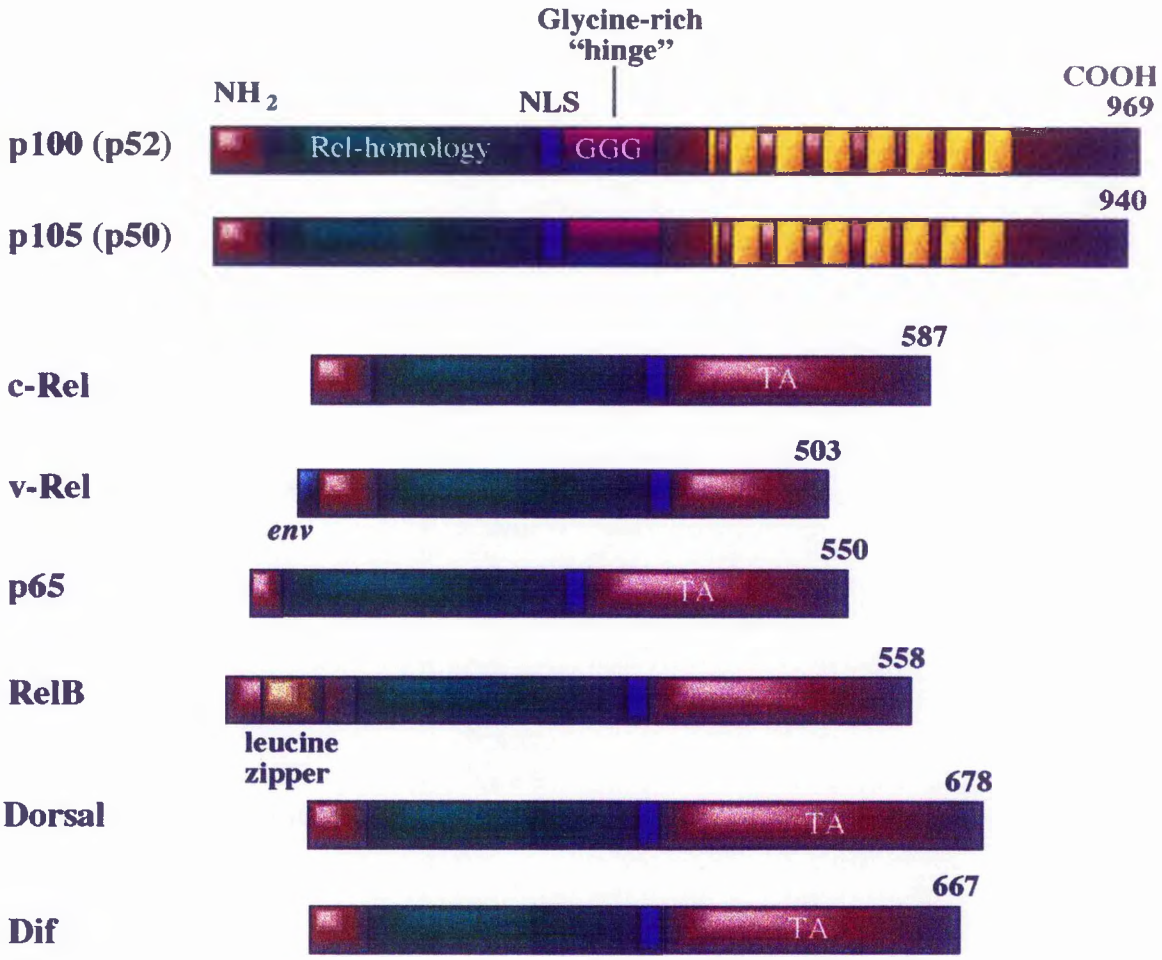


Figure 5: The Rel/NF-κB/Dorsal family.

All the members of this family contain the well-conserved Rel homology domain (green box) which is ~300 amino acids long and is involved in DNA binding and dimerisation. The nuclear localisation signal (NLS) (blue box), a stretch of basic amino acids, is also well coserved. (TA) *Trans*-activation domain present in c-Rel, p65, Dorsal, and Dif. Both p50 and p52 are generated by a proteolytic processing event of the precursor p105 and p100 possibly involving the glycine "hinge" (pink box) region. The carboxy-terminal half of p100 and p105 contains the ankyrin motif (yellow boxes) (adapted from Verma *et al.*, 1995).

including their ability to be induced following the stimulation of T-cells and their use of cytoplasmic/nuclear partitioning as a mechanism of control. In addition, the DNA binding site for NF-AT family members, GGAAAA, resembles one half-site of the κB motif. Most significantly, the recent cloning of members of the NF-AT family has revealed a region similar to the RHD in the Rel/NF-κB proteins (Nolan *et al.*, 1994; Jain *et al.*, 1995). These findings indicate that the Rel/NF-κB proteins may form part of a larger family of transcription factors which appear to regulate important areas of immune function.

2.13. NF-κB: a dimer of distinct subunits.

The active, DNA binding forms of the Rel/NF-κB family are dimeric complexes. Combinatorial mixing of the different family members yields many homo- and heterodimers each with a different affinity for the so-called κB DNA binding site. The general consensus sequence for the DNA binding site is a degenerate decamer (GGGRN/NYYCC, where R is any purine, N is any nucleotide and Y is any pyrimidine) that essentially contains two half-sites. The bi-partite nature of the DNA binding site tends to reflect the type of member that will associate with each half-site.

The most abundant and extensively studied of all the Rel/NF-κB dimeric complexes is NF-κB(p50/ p65(RelA)) which is detectable in most cell types. Other dimers include homodimers of RelA or RelA/Rel heterodimers which usually tend to be detected in very low levels. It appears that certain dimers do not exist, for example RelB will only dimerise with p50 or p52 (Ryseck *et al.*, 1995).

As previously mentioned each dimer type has a preference for a certain κB binding site. For example, classic NF-κB binds to the sequence, 5' GGGRNNYYCC 3' (where R is any purine, N is any nucleotide and Y is any pyrimidine), whereas RelA/c-Rel binds to the sequence, 5' HGGARNYYCC 3' (where H indicates A, C or T; R refers to purine and Y is pyrimidine) (Parry *et al..*, 1994). The two types of subunit comprising NF-κB have been shown to vary in their preference for the two half sites which comprise the prototypical immunoglobulin (Ig) κB site: p50 binds to the 5' half site (5'-GGGAC), while the p65 subunit tends to associate with the 3' half site (3'GGAAA, opposite strand) (Urban & Baeuerle, 1991;

Kunch *et al.*, 1992). Subsequently, homodimers of p50 prefer to interact with palindromic sites consisting of two Ig κB 5' half sites and p65 homodimers bind with higher affinity to duplicated Ig κB 3' half sites. Such selective recognition of κBbinding sites by the various combinations of Rel/NF-κB members increases the number of genes which can be regulated by NF-κB dimers. In addition to differences in binding site preference, different combinations of the Rel/NF-κB family also exhibit distinct transactivation potentials, kinetics of nuclear translocation, interactions with members of the IκB family and activation (reviewed in Siebenlist *et al.*, 1994 and discussed in the sections which follow).

2.14. The protein domains of NF-κB and their functions.

As previously mentioned, the RHD present in all Rel/NF-κB family members is responsible for DNA binding, dimerisation, nuclear localisation and interactions with the various members of the IκB family of proteins. Classical NF-κB (p50/p65) contacts DNA through both subunits as described in section 2.11. The crystal structure of the 50kD NF-κB1 homodimer bound to a κB site (Ghosh *et al.*, 1995; Muller *et al.*, 1995) has revealed that large areas of the RHD constitute the DNA binding surface of the protein and contacts the entire major groove of the bound DNA. In addition, the RHD is arranged into two domains, each of which contribute to DNA binding. The dimerisation surface of the two subunits of NF-κB1 is formed by the side chains from three β-strands in the C-terminal region of each RHD. However, extensive information had been obtained on the mode of NF-κB's DNA binding and the location of the dimerisation domain before the crystal structure of NF-κB1 had been published (reviewed in Grimm and Baeuerle, 1993; Matthews and Hay, 1995). An early study had shown that the dimerisation domain of p50 was contained within the N-terminal region of the RHD, specifically amino acids 201-367 (Logeat *et al.*, 1991). Furthermore, internal deletions within the C-terminal region of the RHD of p50 from amino acids 11-200 prevented DNA binding but did not affect homo- or heterodimer formation. An alternatively spliced transcript of p65 which lacked amino acids 222-231 (equivalent to residues 279-293 in p50) had also been reported and failed to homodimerise or to form heterodimers with p50 (Ruben

et al., 1992b). Indeed, a complementary study of p50 by Bressler *et al* in 1993 reported that the mutation of residues similar to those missing in the p65 transcript impeded dimerisation.

As described above, DNA binding of p50 was prevented by the internal deletion of the N-terminal residues, 22-200 of the RHD (Logeat *et al.*, 1991) which corresponds to the information obtained from the p50 homodimer-κB motif DNA co-crystal structures. Other studies also demonstrated that the N-terminal region of the RHD was critical for p50 DNA binding. This included a study involving the creation of a fusion protein comprised of residues 35-68 of human p50 and residues 46-309 of human p65 (Coleman *et al.*, 1993). The resulting hybrid protein exhibited p50 DNA binding specificity which could be switched to p65 specific DNA recognition by the introduction of a single amino acid change (H67R) to the p50 sequence. Moreover, substituting four residues in the N-terminal region of the p65 sequence for the corresponding p50 residues, generated a mutant with the ability to bind to κB motif and specific p50 DNA sequences but incapable of recognising p65 DNA sites. An additional mutagenesis study found that residues 54-70 of human p50 were important for DNA binding. This finding agreed with the p50 homodimer-κB co-crystal structure since these residues form the start of a loop region in the N-terminal area of the RHD which makes several base and backbone DNA contacts (Toledano *et al.*, 1993).

A small region of amino acids at the beginning of the N-terminal section of the RHD (the R(F/G)(R/K)YXCE motif) was also found to be essential for p50 DNA binding (Toledano *et al.*, 1993) and the p50 homodimer-κB DNA motif co-crystal structure showed that this region made direct contact with the major groove of the DNA recognition sequence. Site directed mutagenesis of the cysteine residue within this motif (conserved amongst all Rel/NF-κB family members) indicated that this amino acid was required for redox regulation of DNA binding activity (C35 of v-Rel, Kumar *et al.*, 1992 and C62 of human p50, Matthews *et al.*, 1992; Toledano *et al.*, 1993). Indeed, prior to this discovery several other groups had suggested that NF-κB DNA binding activity might depend on the redox state of a cysteine residue (Toledano and Leonard, 1991; Matthews *et al.*, 1992 and Hayashi *et al.*, 1993). The modification of cysteine 62 in p50 by treatment with iodoacetate was found to inhibit p50 DNA binding. However, attempts to modify p50 and inhibit its DNA binding activity when

the homodimer was complexed with its specific DNA recognition site failed, indicating that the cysteine residue was probably located near to the DNA (Matthews *et al.*, 1993a). This suggestion was confirmed by the co-crystal structure of p50 homodimers bound to a κB motif which illustrated that cysteine 62 made contact with the DNA backbone (Ghosh *et al.*, 1995; Muller *et al.*, 1995). Photocrosslinking studies implicating residue Y60 of p50 as a determinant of DNA binding specificity (Liu *et al.*, 1994) were also substantiated by the X-ray co-crystal structure of p50 homodimers complexed with the κB motif. The side-chain of Y60 was found to donate a hydrogen bond to a DNA phosphate in the κB motif (Ghosh *et al.*, 1995; Muller *et al.*, 1995).

Partial proteolysis studies have revealed that the binding of p50 to the high affinity H2TF1 type κB motif involves conformational changes in the N-terminal region of the protein (Hay and Nicholson, 1993). More recent studies using circular dichroism (CD) spectroscopy have confirmed that both p50 homodimers and p65 homodimers change conformation upon association with specific or non-specific DNA recognition sites (Matthews *et al.*, 1995). However, corresponding changes in the conformation of the DNA recognition sites which are complexed with either p50 or p65, were limited to only the high affinity type of κB motif. Such protein-induced distortion of the DNA at κB motifs was thought to account for the change in the alkylation pattern observed upon NF-κB binding in early chemical probing studies (Clark *et al.*, 1989, 1990). In contrast, a recent discovery shows that purified p50 homodimers appear to induce minimal DNA bending in a variety of κB sites (Kuprash *et al..*, 1995). This finding was confirmed by the X-ray crystal structures of p50 homodimers complexed with the κB motif (Ghosh *et al.*, 1995 and Muller *et al.*, 1995) which indicated that the DNA recognition sites were only bent slightly upon p50 binding. Taken together with the CD spectroscopy findings (Matthews *et al.*, 1995) the data tends to suggest that a slight unwinding of the κB motif duplex probably occurs upon binding of p50 or p65 homodimers.

The Rel/NF-κB family of proteins all possess a nuclear localisation signal (NLS) which is located towards the end of the RHD's C-terminal region. The NLS consists of a stretch of positively charged amino acids which are required for nuclear uptake of the protein and for interactions with the IκB inhibitor proteins (discussed later) (Blank *et al.*, 1991; Beg

et al., 1992; Ganchi *et al.*, 1992; Henkel *et al.*, 1992; Matthews *et al.*, 1993b and Zabel *et al.*, 1993).

Members of the Rel/NF-κB family of proteins also contain transcriptional activation domains which are found in the carboxyl terminal side of the RHD. The p65 subunit (specifically a stretch of 30 C-terminal amino acids) of NF- κB appears to provide most of the heterodimers transactivation function. Homodimers of p50 fail to activate gene expression whereas p65 homodimers and p50/p65 heterodimers (NF-κB) show strong activation (Schmitz and Baeuerle, 1991). However, there has been some evidence that, in certain circumstances p50 homodimers can activate transcription. For example, when p50 binds to certain types of the κB motif *in vitro*, such as the high affinity H2TF1 site and adopts a chymotrypsin resistant conformation, the protein acts as a strong transcriptional activator (Fujita *et al.*, 1992). Transcriptional activation by p50 is believed to be dependent on the IκB protein, Bcl-3 which is thought to act as a coactivator by forming a ternary complex with p50 homodimers at the promoter region of the gene to be activated (see section 2.233).

Other members of the Rel/NF-κB family including RelB and the closely related I-Rel (thought to be the human homologue of murine RelB) protein are not capable of binding κB sites as homodimers but can act as potent transcriptional activators as heterodimers with, for example p50 (Bours *et al.*, 1994; Ryseck *et al.*, 1992). In contrast, the full length mouse c-Rel protein when fused to GAL4 is a relatively poor transcriptional activator (Bull *et al.*, 1990) and has been shown to interfere with p65 DNA binding resulting in the inhibition of transactivation by p65 homodimers (Doerre *et al.*, 1993). However, fusing the C-terminal residues of c-Rel residues to GAL4 leads to transcriptional activation and it is thought that the N-terminal region of the full length protein may, in some way mask this potential transactivation domain (Bull *et al.*, 1990).

The Rel/NF-κB family of transcription factors like all transcription factors can act as a rate limiting step in the assembly of the basal transcription machinery (see chapter 1). Available evidence has shown that c-Rel, p65 and dorsal can interact with the TATA binding protein (TBP) *in vitro*. It appears that the N-terminal 50 residues of c-Rel can also maintain this interaction *in vivo*. In contrast, v-Rel, p50 and p52 fail to associate with TBP but p50 and

c-Rel can interact with the basal transcription factor, TFIIB *in vitro* (Kerr *et al.*, 1993). More recently, it has been demonstrated that the C-terminal transactivation domain of p65 can interact with TBP, TFIIB and at least one coactivator protein, PC1 *in vitro* (Schimtz *et al.*, 1995). One model has been proposed in which p50/p65 or p50/c-Rel heterodimers once associated with their κB recognition sites, could bind to TBP via p65 or c-Rel and then promote the assembly of the preinitiation complex by interacting with TFIIB via p50 and / or c-Rel.

2.15. Interactions between NF-κB and other cellular transcription factors.

NF-κB is capable of interacting with other transcription factors and there is evidence to suggest that this affects the ability of NF-κB to selectively mediate the transcription of target genes. As detailed in the section above, NF-κB can associate with members of the basal transcription machinery but interactions with transcription factors such as the high mobility group protein HMGI(Y), the CCAAT/enhancer element binding protein (C/EBP) and Sp1 have also been demonstrated, to name but a few.

Both NF-κB and HMG1 are essential for activation of the human interferon β gene (IFNβ). The human IFNβ gene is stimulated by viral infection or by treatment with double-stranded RNA. HMGI(Y) has been shown to stimulate the binding of NF-κB to a κB site in the promoter of the IFNβ gene by associating with the central core of this site and promoting the binding of NF-κB to the outer sequences of the κB site. In addition, it has been demonstrated that HMGI(Y) can interact directly with both the p50 and p65 subunits of NF-κB (reviewed in Baldwin, 1996; Lin *et al*, 1995; Thanos and Maniatis, 1995).

Avian lymphoid cells contain complexes of C/EBP and NF-κB proteins which are capable of binding DNA and a recent study has suggested that such interactions may be important for the regulation of avian leukosis virus LTR (long terminal repeat) driven transcription (avian leukosis virus induces B-cell lymphomas in chickens following integration of the proviral LTR enhancer sequences adjacent to the *c-myc* proto-oncogene) (Bowers *et al.*, 1996). An NF-κB/Rel related factor was found to be a component of the LTR binding complex (which activates transcription) and immunoprecipitation studies on cells

demonstrated that the NF-κB/Rel factor could bind to a C/EBP factor referred to as A1/EBP. In addition, *in vitro* analyses of NF-κB/ C/EBP interactions have indicated that both p50 and p65 can bind to A1/EBP. Furthermore, p65 was able to activate LTR CCAAT/enhancer element transcription.

The transcription factor Sp1, a constitutively expressed protein present in a wide range of cell types which binds to GC-rich sequences in the promoters of many viral and cellular genes, can interact with NF-κB to stimulate HIV-1 transcription. Interestingly the two transcription factors associate with one another through their respective DNA binding domains which allows cooperative DNA binding of each transcription factor to their specific sites in the HIV-1 long terminal repeat. The HIV-1 LTR contains two κB sites in close proximity to three Sp1 sites and it is assumed that this permits NF-κB-Sp1 interaction (Perkins *et al*., 1994).

2.2. The IκB proteins.

2.21. Identification and purification of IκBα.

IκB was first discovered in 1988 by Baeuerle and Baltimore (a,b) as an inhibitor of NF-κB DNA binding activity. It appeared that NF-κB could be prevented from binding target DNA sequences by its association with IκB in the cytoplasm of cells. Furthermore, NF-κB was not associated with IκB in the nucleus indicating that the inhibitory action of IκB on NF-κB DNA binding was exerted in the cytoplasm (Baeuerle and Baltimore, 1988a,b). Therefore, IκB appeared to be able to exert a twofold effect on NF-κB: inhibition of both NF-κB nuclear translocation and DNA binding.

Initial attempts to purify the IκB protein(s) associated with NF-κB (from cytosolic fractions of the human placenta) yielded two chromatographically distinct forms of IκB: A 35-37kDa species, termed IκBα and a minor form (constituting approximately 20-30% of the placental inhibitory activity) named IκBβ of 43kDa (Zabel and Baeuerle, 1990). Both forms of IκB were capable of inhibiting the DNA binding activity of NF-κB but not of p50 homodimers. In addition, both IκBβ and IκBα were shown to dissociate NF-κB from a high affinity DNA binding site.

2.22. Cloning of I κB α and the emergence of a family of I κB proteins.

The cDNA encoding the IκBα protein (or MAD-3; 36-38kDa) was isolated in 1991 from mRNAs which were induced following adherence of human monocytes (Haskill *et al.*, 1991). The IκBα protein could prevent NF-κB and c-Rel DNA binding *in vitro* but could not inhibit DNA binding of p50 homodimers. Furthermore, IκBα was found to contain six copies of the 33 amino acid ankyrin repeat, previously shown to be present at the C-terminal end of the p105 precursor to p50 and to be responsible for its inability to bind DNA (Kieran *et al.*, 1990; Ghosh *et al.*, 1990). This suggested that the ankyrin repeats may play an important role in the functioning of IκB proteins.

The cloning of IκBα was soon followed by the isolation of a cDNA encoding an avian homologue of IκBα , termed pp40 (Davis *et al.*, 1991). Like IκBα, pp40 was able to inhibit the DNA binding activity of p65 or c-Rel containing complexes (NF-κB, p50/c-Rel and c-Rel homodimers). A rat homologue of human IκBα and chicken pp40, termed RL/IF-1 (regenerating liver inhibitory factor), was identified by Tewari *et al* in 1992 and was found to inhibit the DNA binding of NF-κB, c-Rel/p50, RelB/p50 but not homodimers of p50 *in vitro*. Another homologue of IκBα was discovered in pigs and is known as ECI-6 (deMartin *et al.*, 1993). This protein displays both functional and structural similarities to the other IκBα homologues.

Several other functionally related IκB proteins have since been identified which contain a number of copies of the ankyrin repeat region. These include mammalian IκBγ, Bcl-3, IκBR, IκBL and *Drosophila* cactus. IκBγ is a 70kDa protein which is detected in cells of a lymphoid lineage and is the product of an alternatively spliced RNA transcribed from the p105 gene (this gene also generates the p50 protein-see section 2.12). IκBγ corresponds to the C-terminal ankyrin repeat region of the full-length p105 protein. IκBγ was originally shown to be capable of inhibiting the DNA binding of p50 homodimers, NF-κB and c-Rel *in vitro* (Inoue *et al.*, 1992). However, subsequent studies using an independently expressed carboxy-terminal region of p105 (which corresponds to IκBγ) have suggested that IκBγ has an inhibitory specificity for p50 homodimers (Liou *et al.*, 1992).

The Bcl-3 protein, encoded by a proto-oncogene, was originally discovered in 1990 and found to contain seven tandem copies of the ankyrin repeat region (Ohno *et al.*, 1990). Later studies established Bcl-3 as an IκB-like protein through its ability to inhibit the DNA binding of p50 homodimers (*in vitro*) via its ankyrin repeats (Hatada *et al.*, 1992). The Bcl-3 protein has been found to be selective for both p50 homodimers and p52 homodimers (Nolan *et al.*, 1993). In addition, Bcl-3 is thought to act as a coactivator of p50 and p52 homodimers (section 2.233). However, it has been reported that the protein can also inhibit the DNA binding of NF-κB (p50/p65) (Kerr *et al.*, 1992) although titration experiments have shown that this inhibitory capacity is minimal when compared to the inhibition exerted on p50 homodimers by Bcl-3 (Nolan *et al.*, 1993).

The cloning of the *Drosophila cactus* gene and the sequencing of its maternal transcript revealed areas of homology to the mammalian IκB genes (Geisler *et al.*, 1992 ;Kidd *et al* ., 1992). The predicted protein sequence showed a set of seven ankyrin repeats and the bacterially expressed protein was capable of inhibiting the DNA binding activity of the *Drosophila* NF-κB/ Rel related protein, dorsal (Geisler *et al.*, 1992). Indeed, previous studies had demonstrated that cactus activity was required to retain dorsal in the cytoplasm of cells.

A cDNA representing the gene which encodes the IκBR protein (IκB-related) was isolated from human epithelial cells by Ray *et al.*. in 1995. The IκBR protein sequence is highly homologous to other IκB proteins, exhibiting greatest sequence similarity to the *Drosophila* protein, Cactus and containing three carboxy-terminal ankyrin repeats. IκBR was able to inhibit the DNA binding activity of both NF-κB and p50 homodimers *in vitro* but failed to prevent DNA binding of p65 homodimers. In addition, co-transfection of a vector over-expressing the IκBR protein with an NF-κB dependent luciferase expression vector into a murine embryonal carcinoma line (F9), resulted in inhibition of NF-κB-mediated activation of luciferase expression. It is thought that IκBR may regulate the activity of NF-κB and perhaps other unidentified target proteins in epithelial cells.

The cDNA encoding a protein referred to as IκBL also exhibits homology to the other IκB proteins. IκBL possesses two complete ankyrin repeats together with a third incomplete repeat and its gene is located in the major histocompatibility complex (Albertella *et al*, 1994).

The gene encoding the mammalian IκBβ protein was cloned in 1995 (Thompson *et al.*, 1995) and together with IκBα represents one of the most abundant forms of the IκB proteins identified so far in mammalian cells. IκBβ (around 45kDa) was shown to inhibit the DNA binding activity of NF-κB *in vitro* . Furthermore, co-transfection of Cos7 cells with vectors expressing IκBβ and either p50 and p65 or p50 and c-Rel demonstrated the ability of IκBβ to inhibit the DNA binding of both p65 and c-Rel (although the inhibitory effect observed was weaker with respect to c-Rel). Recombinant IκBβ was also shown to interact equally well with p65 and c-Rel but the protein failed to associate with p50.

The proteins described above constitute the IκB family of proteins (figure 6) and include the NF-κB precursor proteins, p105 and p100, previously mentioned in section 2.12. Both p105 and p100 have been found complexed with p50, p65 or c-Rel *in vivo* and can retain these proteins in the cytoplasm of cells (Rice *et al.*, 1992; Mercurio *et al.*, 1993; Rice and Ernst, 1993 and Miyamoto *et al.*, 1994). The IκB proteins are characterised by the presence of several copies of a 30-35 amino acid ankyrin repeat, generally located in the centre of the IκB protein and their ability to interact with members of the NF-κB/Rel family of transcription factors. Ankyrin repeats can also be found in yeast cell-cycle proteins (Breeden and Nasmyth, 1987) and human erythrocyte ankyrin (Lux *et al.*, 1990) although they vary from the distinct sequence of the IκB ankyrin repeats (Nolan and Baltimore, 1992). The ankyrin repeats are required for protein-protein interactions, in effect they enable the individual members of the IκB family to bind to their respective NF-κB/Rel dimeric complexes.

2.23. Regulation of NF-κB by IκBα.

The primary role of IκBα is to regulate the activity of the transcription factor, NF-κB and there are several ways in which IκBα can achieve this control. For example, through the inhibition of both NF-κB DNA binding activity and nuclear translocation, in addition to those mechanisms which mediate the transient loss and resynthesis of IκBα itself. The following headings outline the various control mechanisms exerted on NF-κB via IκBα.

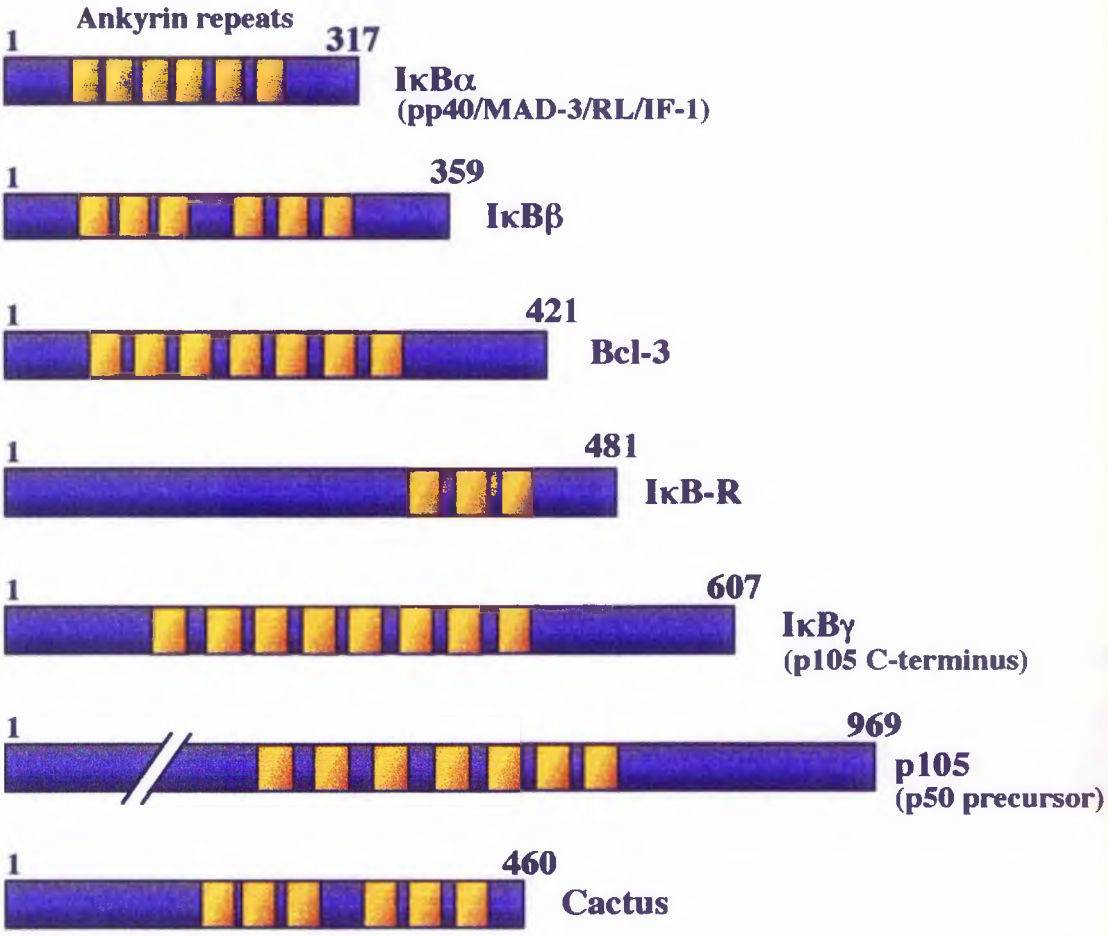


Figure 6: The IκB family.

The IκB proteins are characterised by the presence of ankyrin repeats (yellow boxes) whose numbers vary among the various family members (3 to 7). The ankyrin repeat domains are essential for protein-protein interactions. IκBγ is generated by alternative splicing from p105 mRNA. Cactus is the functional IκB homologue in *Drosophila* (adapted from Verma *et al.*, 1995).

2.231. Interaction of IκBα with NF-κB and the mechanisms of NF-κB cytoplasmic retention.

A combination of mutational analyses and protease sensitivity studies have allowed the identification of regions within IκBα which are important for interacting with NF-κB and for the cytoplasmic retention of NF-κB. Several observations have indicated that IκBα preferentially inhibits the DNA binding activity of the p65 rather than the p50 subunit of NF-κB (reviewed in Grimm and Baeuerle, 1993). For example, the addition of p65 homodimers to an inactive complex of NF-κB bound to IκBα results in the release of active NF-κB. Furthermore, an excess of p65 but not p50 homodimers can block the inhibition of NF-κB DNA binding activity by IκBα (Urban and Baeuerle, 1990). In addition, the inhibitory specificity exhibited by IκBα is for p65 homodimers and p50/p65 (NF-κB) heterodimers but not for p50 homodimers (Baeuerle and Baltimore, 1989; Urban *et al.*, 1991). Therefore, most of the information which follows on the interaction between IκBα and NF-κB concerns the association between the p65 subunit of NF-κB and IκBα.

Structurally, IκBα can be divided into three distinct regions: an N-terminal region of 70 amino acids, an internal domain of 205 amino acids that is comprised of ankyrin repeats, and a C-terminal acidic region of 42 amino acids. Early studies demonstrated that the C-terminal region and the ankyrin repeats of IκBα were required for binding to members of the NF-κB/Rel family. For example, a report on the avian form of IκBα (pp40) and its association with Rel *in vitro* showed that a mutant lacking amino acids 253-317 failed to interact with Rel (Inoue *et al.*, 1992). Furthermore, mutation of individual pp40 ankyrin repeats revealed that all but ankyrin repeat 3 were essential for association with c-Rel. In addition, co-transfection of a vector expressing a pp40 mutant containing an altered fifth ankyrin repeat (which could not bind c-Rel) with an NF-κB-dependent reporter plasmid resulted in a failure of the mutant to inhibit transcription from the reporter (i.e. through the binding of NF-κB in the cytoplasm). In contrast, removal of the N-terminal 51 amino acids did not affect either pp40-c-Rel, pp40-p65 or pp40-p50 interaction. A later study to determine the minimal domains of human IκBα (MAD-3) and p105 required for interaction with members of the NF-κB/Rel family revealed that a deletion of only three amino acids (71-73) at the start of the first ankyrin repeat of

IκBα could markedly reduce its affinity for p65 (Hatada *et al.*, 1993). Also, removal of the C-terminal amino acids, 280-317 and the N-terminal amino acids, 1-70 of IκBα resulted in a loss of interaction with p65 *in vitro*, whereas deletion of only the N-terminal residues from 1-70 had virtually no effect on IκBα-p65 binding. In parallel with IκBα, a deletion mutant of p105 lacking the C-terminal acidic region together with the seventh ankyrin repeat (not present in IκBα) failed to associate with p50. Surprisingly, a p105 deletion mutant lacking the seventh ankyrin repeat exhibited a weak interaction with p50.

In contrast, other more recent mutational studies have found that the acidic C-terminal residues from 279-317 of IκBα are dispensible for association with NF-κB. Ernst *et al*. (1995) generated a C-terminal IκBα mutant lacking amino acids 279-317 which was capable of interacting with p65 both *in vitro* and *in vivo*. Moreover, immunofluorescence analysis of cells cotransfected with vectors expressing p65 and the C-terminal deletion mutant illustrated that p65 could still be retained in the cytoplasm of cells by mutant IκBα. A possible explanation for this discrepancy exists in the nature and origin of the IκBα mutant generated in the earlier study (Hatada *et al.*, 1993). Firstly, Hatada *et al* generated a mutant that lacked the potential stabilising influence of the N-terminal residues (1-70) as well as the C-terminal amino acids, 280-317. Secondly, the mutant was expressed in, and isolated from bacteria, whereas Ernst *et al* performed studies on either *in vitro* translated proteins or proteins expressed in mammalian cells.

Interestingly, it appears that the association between IκBα and v-Rel, the oncogenic viral counterpart of c-Rel, does require the C-terminal region of IκBα as well as the ankyrin repeat region. The C-terminal region also appears to be required for cytoplasmic retention of v-Rel by IκBα. In contrast, the C-terminus of IκBα is dispensible for its association with c-Rel and for the cytoplasmic retention of c-Rel. This was discovered in a recent study using the yeast two hybrid system where a fusion protein of v-Rel and the Gal4 DNA binding domain was analysed for its ability to activate Gal-4 dependent β-galactosidase expression in the absence or presence of either a C-terminal IκBα deletion mutant lacking amino acids 300-317 or 281-317 fused to the Gal4 activation domain. High level β-galactosidase expression was observed in the presence of the C-terminal deletion mutant, 300-317 but expression did not

increase above background levels in the presence of the mutant devoid of residues 281-317 (Rottjakob *et al.*, 1996). In addition, an IκBα Gal4 (the activation domain) fusion mutant containing a deletion of 142 amino acids in the ankyrin did not associate with v-Rel (in fusion with the Gal4 DNA binding domain) in yeast. Immunofluoresence analysis of cells co-transfected with a vector expressing either the C-terminal deletion mutant, 281-317 or an IκBα mutant containing three substituted residues at position 288-290 and a vector expressing v-Rel revealed that neither of the mutants could retain v-Rel in the cytoplasm. This indicated that distinct regions within the C-terminal region of pp40 were required for the cytoplasmic retention of v-Rel.

The above study also revealed that certain mutant forms of both c-Rel and v-Rel (for example a v-Rel mutant containing an amino acid substitution in a consensus protein kinase A site (S275 to A275) and a C-terminally truncated c-Rel protein containing a serine to cysteine substitution at position 26) could associate with pp40 but failed to be retained in the cytoplasm of cells when overexpressed with pp40. This suggested that the association of pp40 with Rel proteins was not sufficient for their cytoplasmic retention and that some other contributory factor was involved, possibly post-translational modifications of either Rel or pp40.

A role for the low homology sixth ankyrin repeat of IκBα in p65-IκBα association has recently been suggested through protease sensitivity studies of IκBα (Jaffray *et al.*, 1995). This report describes the protection of residues 251 (Y), 258 (W) and 275 (E) from proteolytic cleavage in the low homology sixth ankyrin repeat of IκBα when the protein is bound to p65. A more recent study using deletion mutants of IκBα has indicated that the removal of residues 263-317 prevents p65-IκBα interaction *in vivo*. Furthermore, immunofluorescence assays using the overexpressed IκBα deletion mutant (265-317) revealed that it could not localise overexpressed p65 in the cytoplasm of transfected cells. This correlated with the inability of the overexpressed mutant to inhibit NF-κB-dependent transcription from a reporter plasmid in transfected cells (Sun *et al.*, 1996). Similarly, another report demonstrated that the deletion of either amino acids 261-317 or 269-317 in IκBα abolishes binding to p65 *in vivo* (Beauparlant *et al.*, 1996).

The interaction of IκBα with heterodimeric complexes containing either p65 or c-Rel results in the localisation of these complexes in the cytoplasm of cells. It has been shown that both IκBα and the ankyrin repeat region of p105 can interact with, and shield the nuclear localisation signal (NLS) located at the C-terminal end of the RHD of some NF-κB/Rel family members. An early report investigated the effect of removing or substituting the residues corresponding to the NLS's of p50, p65 and c-Rel on their interaction with IκBα. It was discovered that the mutant proteins could not interact with IκBα. In addition, overexpression of the p65 NLS mutant from a transfected vector in cells revealed that the mutant was exclusively cytoplasmic, unlike transfected wild-type p65 which was found in high levels within the nucleus. However, fusion of a second NLS derived from the SV40 large T-antigen to the p65 mutant resulted in its nuclear localisation, indicating that the IκBα could not interact with the new NLS and therefore failed to retain the mutant within the cytoplasm (Beg *et al.*, 1992). Around the same period, a report on the p105 protein emerged which demonstrated that its NLS could not bind an anti-p50 NLS-antibody (Henkel *et al.*, 1992). Indeed earlier experiments using a p105 mutant lacking the C-terminal acidic region (between the sixth and seventh ankyrin repeats) had shown 10% nuclear localisation (whereas wild-type p105 was not found in the nucleus) and it was suggested that this region may interact with the NLS in the full-length p105 protein, preventing nuclear localisation (Blank *et al.*, 1991).

A later study demonstrated that when IκBα was associated with NF-κB, antibodies specific for regions which overlapped the NLS of both p50 and p65 failed to recognise their epitopes on these proteins (Zabel *et al.*, 1993). In contrast, an additional report on IκBα showed that whilst IκBα could block the binding of antibodies to the p65 NLS, an antibody to the p50 NLS was still able to immunoprecipitate p50. However, the p65-NLS antibody could co-immunoprecipitate p50 and p65 following the co-translation of their mRNA's. It was suggested that when p50 and p65 were associated (i.e. as NF-κB) IκBα was able to contact both of their NLS's (Zabel *et al.*, 1993). The discovery that p50 alone (i.e. p50 homodimers) did not interact with IκBα through its NLS was supported by the finding that a complex of p50 and IκBα was accessible to proteolytic cleavage by trypsin, which initially cuts at sequences in or near to the NLS of p50. However, when the C-terminal region of p105

(corresponding to IκBγ) was bound to p50, digestion with trypsin was blocked. Furthermore, p50 failed to be immunoprecipitated with an antibody recognising the NLS in the presence of the p105 C-terminus (Matthews *et al.*, 1993b).

It appears that the NLSs of the c-Rel protein and its oncogenic counterpart, v-Rel are not required for association with pp40. Fusion of c-Rel and v-Rel mutant proteins, containing deleted NLSs (residues 298-303) with the Gal4 DNA binding domain did not affect β-galactosidase expression in yeast cells when tested with a Gal4 activation domain-pp40 fusion protein. However, replacing wild-type pp40 with a C-terminal deletion mutant lacking residues 282-317 reduced β-galactosidase expression in the presence of the v-Rel NLS mutant. This indicates that removal of the NLS from c-Rel renders the protein dependent on the pp40 C-terminal region for c-Rel-pp40 association. Furthermore, it was demonstrated that only a two amino acid difference between c-Rel and v-Rel (amino acids 286 and 302) appeared to be responsible for the pp40 C-terminal requirement in v-Rel-pp40 interactions (Rottjakob *et al.*, 1996).

2.232. Inhibition of NF-κB DNA binding activity and nuclear translocation by IκBα.

The inhibition of NF-κB DNA binding by IκBα has been shown *in vitro* to be controlled by certain regions of IκBα. Numerous mutational studies have demonstrated that, although the ankyrin repeats are required and sufficient for binding IκBα to NF-κB (as discussed in section 2.231) they cannot alone maintain inhibition of NF-κB DNA binding activity. Inhibitory activity appears to additionally require the presence of the acidic C-terminus of IκBα. An early report on the avian form of IκBα, pp40 demonstrated that altering the individual ankyrin repeats of pp40 prevented the protein from inhibiting the DNA binding of NF-κB *in vitro*. However, a pp40 mutant containing the ankyrin repeats only (devoid of both N- and C-termini) and a mutant lacking the C-terminus and a section of the sixth low homology ankyrin repeat (residues 253-317) also blocked the inhibitory capacity of the protein, whereas an N-terminal pp40 deletion mutant was still able to inhibit NF-κB and c-Rel DNA binding (Inoue *et al.*, 1992). Interestingly it appeared that those mutants which were unable to inhibit the DNA binding of c-Rel (the ankyrin repeat only and C-terminal deletion

mutants) also failed to associate with c-Rel. It was suggested that the association of pp40 with p65 and c-Rel may contribute to the inhibition of their DNA binding activity.

Additional experimental evidence for the inhibitory role of the C-terminus of IκBα was presented by Rodriguez *et al* (1995) where the C-terminal residues, 257-317 were deleted resulting in a failure of IκBα to inhibit either p65 or NF-κB DNA binding activity. A similar result was obtained from a study using IκBα mutants lacking either residues 261-317 or 269-317 which also failed to inhibit NF-κB DNA binding activity (Beauparlant *et al.*, 1996). In addition, these mutants were only able to interact weakly with p65. A combined loss of the N- and C-termini of I κBα has also been shown to prevent the inhibition of p65 DNA binding activity and interaction with p65 (Hatada *et al.*, 1993). The possible molecular mechanisms involved in the inhibition NF-κB DNA binding activity by the acidic C-terminus of IκBα will be detailed in the discussion

It is interesting to note that, as well as inhibiting NF-κB DNA binding activity, IκBα can also disrupt preformed NF- κB-DNA complexes (Zabel and Baeuerle, 1990). This finding originally led to the suggestion that IκBα may limit the transcriptional activation by NF-κB through actively removing the complex from its DNA binding site. *In vivo*, this would require the presence of IκBα in the nucleus and indeed many recent studies have indicated that this may be the case. For example, an early investigation on the pp40 protein in REV-T transformed chicken lymphoid cells demonstrated the presence of the protein in complex with $pp59^{v\text{-}Rel}$ within the nucleus (Davis *et al.*, 1990). Further studies on the pp40 protein indicated that it could form complexes with members of the NF-κB/Rel family in both the cytoplasm and nucleus of WEHI-231 murine lymphoma cells (Kerr *et al.*, 1991). Later reports demonstrated the localisation of human IκBα in the nucleus and cytoplasm of a variety of mouse tissues and cell types when the protein was overexpressed from a transfected vector or microinjected into the cytoplasm (Cress and Taub, 1993; Zabel *et al.*, 1993).

In a more recent study it was shown that newly synthesised IκBα (which follows NF-κB-dependent transcription of the IκBα gene-see section 2.237) could accumulate in the nucleus of untransfected HeLa cells following treatment with TNF where it was found in association with NF-κB/Rel complexes. Furthermore, the localisation of IκBα in the nuclear

compartment was accompanied by a reduction in NF-κB DNA binding and NF-κB-dependent transcription. In addition, a massive reduction in the amount of nuclear p50 was observed suggesting that after NF-κB has associated with newly synthesised IκBα the complex is exported out of the nucleus (Arenzana *et al.*, 1995). Interestingly, an investigation using *in vitro* synthesised proteins microinjected into Xenopus oocytes has recently demonstrated that nuclear IκBα can disrupt NF-κB-DNA interaction and then export NF-κB/Rel complexes out of the nucleus. Located in the C-terminus of IκBα is a nuclear export signal (NES-residues 265-277) which appears to mediate the nuclear export of NF-κB/Rel complexes (see figure 7). The fusion of this sequence onto a reporter protein allows the fusion protein to be exported out of the nucleus. Moreover, the NES has been found to be homologous to the NES of protein kinase I and HIV-1 Rev (Arenzana *et al.*, 1996, submitted for publication).

Other IκB proteins have also been found in the nucleus of cells. A species known as p56 which appeared to be identical to the Bcl-3 protein was discovered in both nuclear and cytoplasmic extracts of HeLa cells. A related proteolytically cleaved protein, p38 was also found, but it appeared to be present exclusively in the nucleus (Kerr *et al.*, 1992). Conflicting reports on the localisation of Bcl-3 in the nucleus of cells transfected with a vector expressing the protein have since been published. One report claims that Bcl-3 can localise in the nucleus (Bours *et al.*, 1993) whereas another investigation could only detect Bcl-3 in the cytoplasm (Naumann *et al.*, 1993). A more recent study using transgenic mice constitutively expressing Bcl-3 in thymocytes has demonstrated that Bcl-3 is associated with endogenous p50 and p52 in nuclear extracts from the transgenic mice (Caamo *et al.*, 1996).

2.233. IκBα as a transcriptional activator-co-operation with NF-κB.

It has been demonstrated that the human (MAD-3) and avian (pp40) forms of IκBα in addition to the C-terminal region of p105 (corresponding to IκBγ) and c-Rel can act as transcriptional activators when fused to the Gal4 DNA binding domain and transiently transfected into chicken cells or yeast (Morin and Gilmore, 1992; Morin *et al.*, 1993). It was found that the transcription activation domain of p105 was contained within C-terminal residues, 727-806 (located between the sixth and seventh ankyrin repeat). Interestingly this

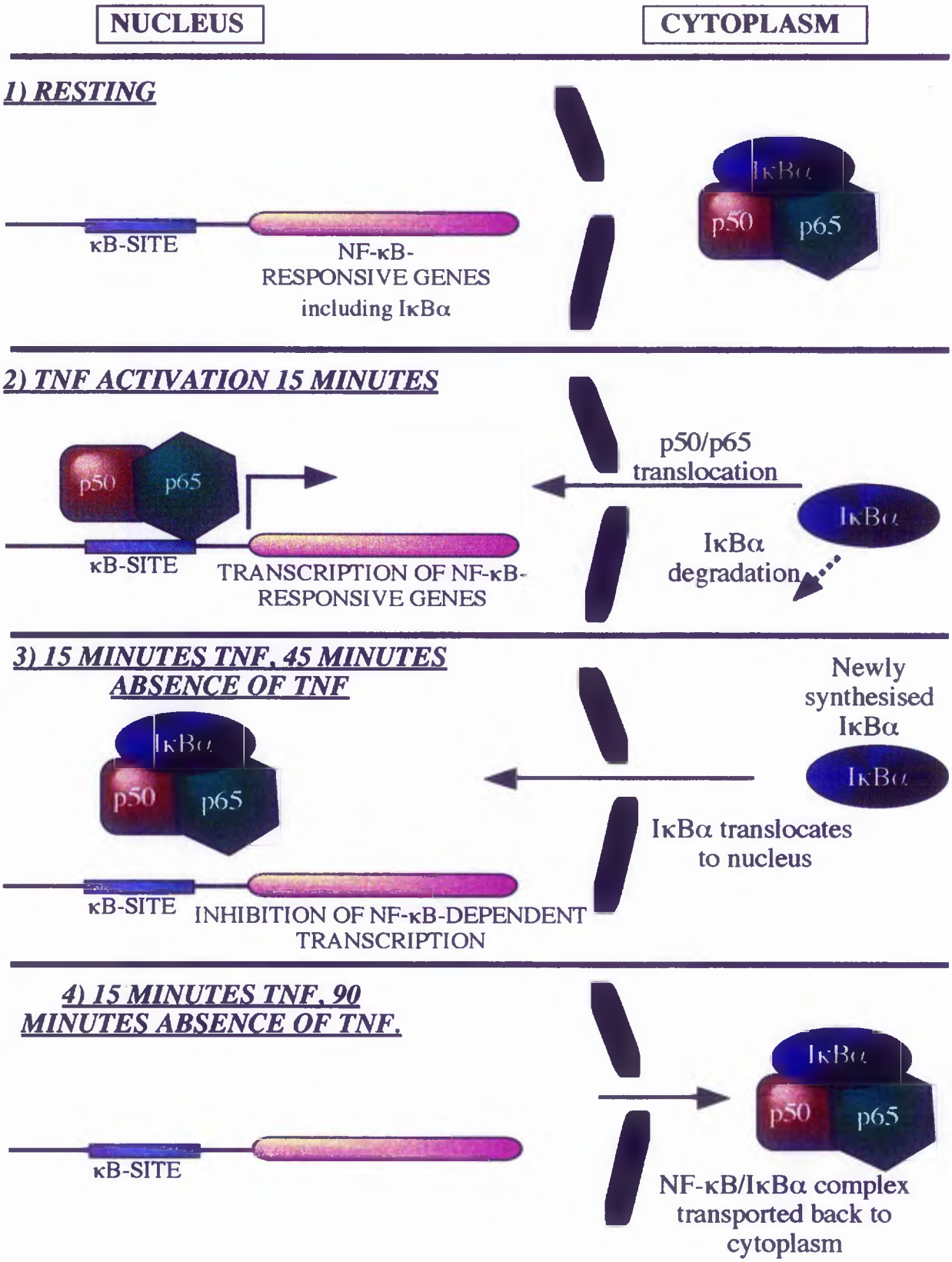


Figure 7: Schematic of the proposed mechanism for re-export of NF-κB- IκBα complexes from the nucleus to cytoplasm to ensure transient activation of NF-κB-dependent gene transcription (adapted from Matthews *et al.*, 1995).

region consists of highly negatively charged amino acids, a feature shared with several other transcription activation domains. The transcription activation domain of pp40 was located between residues 49-229. Surprisingly, a Gal4 fusion protein containing the negatively charged C-terminus, low homology sixth ankyrin repeat and part of the fifth ankyrin repeat (residues 229-318) failed to activate transcription (Morin and Gilmore, 1992).

Since the pp40 ankyrin repeats are required for interaction with c-Rel or p65 containing dimeric complexes, it was suggested that pp40 may activate transcription by associating with endogenous c-Rel or p65 containing complexes. Several findings appeared to indicate that this proposal was correct. For example, a pp40 mutant fused to a Gal4 DNA binding domain which failed to interact with c-Rel was not able to activate transcription in chicken embryonic fibroblasts. However, a pp40 mutant capable of binding to c-Rel, strongly activated transcription. In addition, coexpression of the wild-type pp40 Gal4 fusion protein in chicken embryonic fibroblasts with a c-Rel protein devoid of its transcriptional activation region failed to activate transcription. It was possible that the pp40 mutant which was unable to interact with c-Rel was in a conformation which rendered it inactive in the assays. Similarly, the association of the c-Rel deletion mutant with the wild-type Gal4 fusion protein may have altered the conformation of the pp40 fusion protein to interfere with the pp40 transcriptional activation domain. Alternatively, binding of the mutant c-Rel protein to Gal4-pp40 may have prevented interaction of Gal4-pp40 with some other non-Rel protein (Morin *et al.*, 1993). Significantly, coexpression of p65 or p50 mutant proteins lacking their transcriptional activation domains with the previously described minimal transcriptional activation domain of p105 (residues 727-806) did not inhibit transcriptional activation by the p105 protein. However, coexpression of the wild-type p65 protein with Gal4 p105 (727-806) reduced activation ten fold. This indicated that p105 was a genuine transcriptional activator, probably interacting with a coactivator identical to p65.

Bcl-3 is believed to act as a transcriptional coactivator since it appears to be capable of superactivating p50B (also known as p49 or p52) (Bours *et al.*, 1993) and p50 (Fujita *et al.*, 1993) homodimers during *in vitro* transcription from interferon-β, immunoglobulin and $H2K^b$ κB sites. More recently a report describes that nuclear extracts prepared from the thymocytes

of transgenic mice constitutively expressing Bcl-3 exhibit a ten fold increase in p50 DNA binding activity but no change in p52 binding compared to cells lacking constitutively expressed Bcl-3. In addition, it appeared that the observed increase in p50 DNA binding was dependent on the phosphorylation of Bcl-3. Furthermore, both p52 and p50 were found to be complexed to Bcl-3 in thymocyte nuclear extracts (Caamano *et al.*, 1996). Bcl-3 is thought to form a ternary complex with either p50B and p50 homodimers and their κB motifs to regulate the transcription of target genes through the promoter region. Ternary complexes have also been shown to exist for avian pp40, where the protein was detected in nuclear extracts prepared from WEHI-231 cells as part of a complex comprised of c-Rel and other unidentified κB binding proteins (Kerr *et al.*, 1991). Many *in vitro* studies and transient transfection experiments have described an alternative mechanism of Bcl-3 mediated transcriptional activation where Bcl-3 has been shown to dissociate p50 homodimers from DNA, thus allowing transcriptional activation to occur through NF-κB (Franzoso *et al.*, 1992, 1993; Naumann *et al.*, 1993).

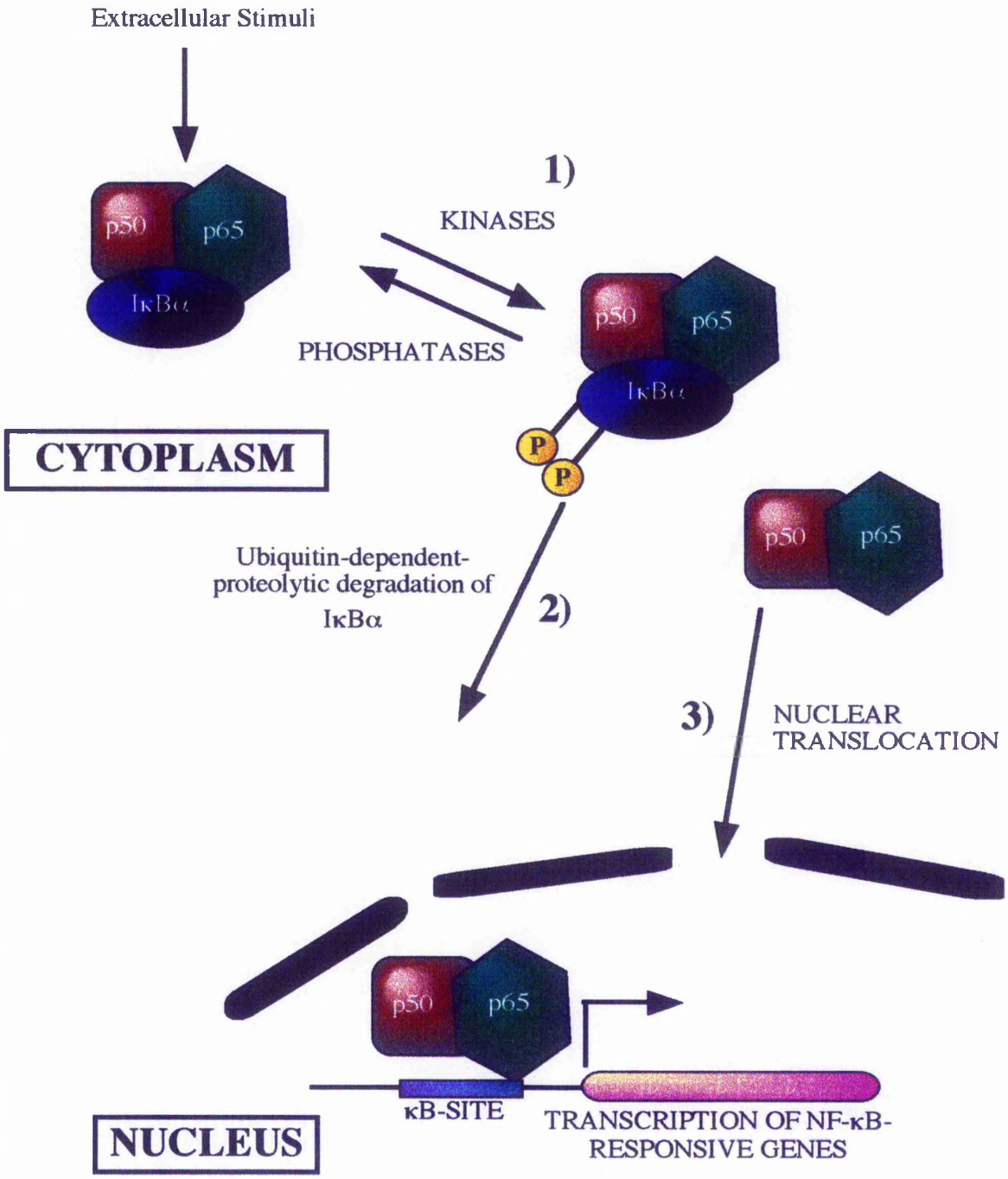
2.234. Inducer-mediated phosphorylation, ubiquitination and degradation of IκBα: a mechanism for inducible NF-κB activation.

Early *in vivo* studies revealed that inducer-mediated stimulation of NF-κB DNA binding activity was accompanied by the rapid, transient hyperphosphorylation and degradation of IκBα (Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Henkel *et al.*, 1993; Mellits *et al.*, 1993; Rice and Ernst, 1993; Sun *et al.*, 1993). Indeed, it was also reported that the activation of NF-κB DNA binding activity was correlated with a slow, transient phosphorylation of p105 and a slow, sustained phosphorylation of both p50 and p65 (Mellits *et al.*, 1993; Mercurio *et al.*, 1993). In addition, the use of certain stimuli such as TNF and double-stranded RNA caused an increase in the processing of the precursor protein, p105 to p50 (Mellits *et al.*, 1993).

The observation that inducibly phosphorylated IκBα did not accumulate but appeared only as a transient species immediately after the stimulation of cells led to the proposal that the inducible phosphorylation of IκBα served as a marker for IκBα degradation. An

alternative proposal was made following the observation that NF-κB bound IκBα was highly stable compared to free, unbound IκBα. In this model it was proposed that inducer-mediated phosphorylation of IκBα caused the dissociation of IκBα from NF-κB leading to the rapid proteolysis of IκBα via constitutively active proteases and the translocation of NF-κB to the nucleus (Beg *et al.*, 1993; reviewed in Siebenlist *et al.*, 1994). However, subsequent reports have revealed that inducibly phosphorylated IκBα is still able to interact with NF-κB demonstrating that signal-induced phosphorylation does not dissociate IκBα from NF-κB. Furthermore, a critical role for the proteases involved in IκBα degradation emerged after the discovery that the protease inhibitors referred to as the peptide aldehydes were able to block the signal induced degradation of IκBα and nuclear translocation of NF-κB but failed to prevent inducible phosphorylation of IκBα (Finco *et al.*, 1994; Miyamota *et al.*, 1994; Palombella *et al.*, 1994; Traenckner *et al.*, 1994; Alkalay *et al.*, 1995a; Didonato *et al.*, 1995; Lin *et al.*, 1995). Therefore, it would appear that the inducible phosphorylation of IκBα tags the protein for proteolytic degradation which then leads to its dissociation from NF-κB (see figure 8 for overview).

Two residues within the N-terminus of IκBα, specifically serine 32 and serine 36 have been shown to be targeted for inducible phosphorylation (Brown *et al.*, 1995; Traenckner *et al.*, 1995). In addition, it appears that serine 19 and serine 23 of IκBβ are required for inducible phosphorylation of the protein (Didonato *et al.*, 1996). For the studies on IκBα, a vector expressing an IκBα mutant containing serine to alanine substitutions at positions 32 and 36 was transfected into either mouse EL-4 lymphocytes or HeLa cells and failed to be inducibly phosphorylated or degraded. Furthermore, cotransfection of the mutant with an NF-κB dependent reporter plasmid into cells, followed by induction of cells with either TNF or PMA resulted in the inhibition of NF-κB mediated transcription. An investigation involving human T-cell leukaemia virus type 1 Tax transactivator protein induction of IκBα, has yielded similar results (Brockman *et al.*, 1995). Phosphopeptide mapping has also been used to show that the two serine residues at positions 32 and 36 are phosphorylated in response to various activators of NF-κB (DiDonato *et al.*, 1996). In addition, two homologous sites in IκBβ at positions 19 and 23 have also been shown to be important for the inducible degradation of the



ure 8. Diagrammatic representation of signal-induced ubiquitin-mediated degradation κBα.

Following signal-induction, IκBα becomes phosphorylated on serine 32 and 36 (1) which marks α for covalent modification through lysine 21 and 22 by a specific ubiquitin ligase (2).After uitination, IκBα dissociates from NF-κB, allowing the translocation of NF-κB to the nucleus where n activate transcription of target genes (3). Ubiquitinated IκBα is rapidly degraded by the easome. Notably phosphorylation and ubiquitination of IκBα are reversible, presumably following ation the forward reactions are favoured.

protein: substitution of both for alanine blocks stimulation dependent degradation of IκBβ. These findings indicate that the N-terminal phosphorylation of both IκBα and IκBβ is essential for their inducible degradation and hence subsequent activation of NF-κB.

However, sequences in and around the C-terminus of IκBα has also been shown to be important for the signal induced degradation of IκBα (Brown *et al.*, 1995; Rodriguez *et al.*, 1995; Beauparlant *et al.*, 1996; Sun *et al.*, 1996). The highly negatively charged C-terminus of IκBα is comprised of a proline, glutamic acid/aspartic acid, serine and threonine rich PEST sequence. Such sequences have been found in other proteins and are associated with rapid protein turnover. Brown *et al* (1995) reported that a deletion mutant lacking 41 C-terminal residues (1-276) was resistant to inducible degradation when a vector expressing the mutant was transfected into mouse EL4 T-lymphocytes. However, the mutant was phosphorylated indicating that the C-terminus is not a target for inducible phosphorylation.

Similarly, deletion mutants of IκBα consisting of either residues 1-256 (Rodriguez *et al.*, 1995), 1-268 or 1-260 (Beauparlant *et al.*, 1996) have been shown to be resistant to signal induced degradation. In addition, mutants 1-268 and 1-260 inhibited NF-κB-dependent transcription in cells cotransfected with vectors expressing either of the mutants and a κB dependent reporter plasmid. However, a deletion mutant corresponding to residues 1-277 was reported by another group to be degraded in response to TNFα (Sun *et al.*, 1996). Furthermore, a mutant comprised of residues 1-287 has also been shown to undergo proteolysis in response to TNFα induction. This group has suggested that the PEST sequences located in the final 40 amino acids of the C-terminal region of IκBα are not involved in the signal induced degradation of IκBα. Instead a glutamine and leucine region in the sixth ankyrin repeat from residues 264-276 is proposed to participate in signal induced degradation (Sun *et al.*, 1996). Results corresponding to this report have recently been published by another group in which the PEST sequences were found to be dispensable for inducible degradation (Aoki *et al.*, 1996).

Several studies have indicated that the 26S multicatalytic proteinase or proteasome is involved in the signal induced degradation of IκBα. Experimental evidence for this suggestion includes the use of a peptide aldehyde inhibitor specific for the chymotrypsin like

activity of the 26S proteasome which prevents the signal induced degradation of IκBα and activation of NF-κB and also leads to the accumulation of hyperphosphorylated IκBα (Traenckner *et al.*, 1994; Palombella *et al.*, 1994).

The 26S proteasome is a multicatalytic, ubiquitous proteinase complex mediating non-lysosomal degradation of ubiquitin-conjugated polypeptides in cells (reviewed in Hochstrasser, 1995; Ciechanover, 1994; Rubin and Finley, 1995). The ability of the proteasome to degrade target proteins generally depends on the substrate having multiple copies of covalently bound ubiquitin (a small, highly stable protein) and requires ATP. However, proteins which are not conjugated to ubiquitin have also been shown to undergo proteasomal dependent degradation including the enzyme, ornithine decarboxylase (Rubin and Finley, 1995).

Ubiquitin dependent proteolysis involves two main steps: The protein is first "marked" by the covalent attachment of multiple molecules of ubiquitin and the secondly the protein is degraded. Degradation is accompanied by the release of free ubiquitin to be reused in subsequent reactions. The addition of ubiquitin to proteins consists of three main stages. Ubiquitin must first be activated by the ubiquitin-activating enzyme, E1 which catalyses the ATP dependent activation of the C-terminal glycine of ubiquitin to a thiol ester intermediate. The second step makes use of the ubiquitin-conjugating enzyme, E2. E2 transfers activated ubiquitin molecules to the substrate which is anchored to a ubiquitin protein-ligase, E3. Finally, an isopeptide bond is formed between an ε-amino group of a lysine residue in the target protein and the C-terminal glycine of ubiquitin. In subsequent reactions the activated ubiquitin molecule is attached to lysine 48 of the previously protein-conjugated ubiquitin molecule, eventually leading to the production of a polyubiquitin chain. The 26S proteasome then degrades the substrate protein and this releases free ubiquitin which can be reused in subsequent reactions. It should be noted that E2 can transfer ubiquitin to the protein substrate itself. However, this is only thought to occur for the addition of single molecules of ubiquitin to the target protein and does not signal the protein for degradation (reviewed in Ciechanover, 1994; Hochstrasser, 1995).

The 26S proteasome comprises a 20S catalytic core referred to as the proteasome. The proteasome appears to require the presence of a 19S particle which is thought to mediate the ATP-dependent recognition of ubiquitinated substrates through its 5S subunit (reviewed in Rubin and Finley, 1995). ATP may also be required for peptide bond cleavage of the protein substrate and for translocation of the substrate along the enzyme from the 19S particle to the 20S catalytic core. In addition, the release of cleavage products during the degradation of the protein in the catalytic core probably utilises ATP. It is thought that the proteasome must unfold the protein substrate in order for it to be efficiently passed through narrow channels into the central cavity of the enzyme which houses the active sites. It has been suggested that the protein substrate is unfolded by the 19S particle since it directly contacts the proteasome channels which open into the central cavity. This view is supported by the observation that the 19S particle contains multiple ATPases. These ATPases could directly contact the protein and destabilise the folded conformation by exhibiting a greater affinity for the unfolded protein.

Degradation of the protein allows free ubiquitin to be regenerated by special de-ubiquitinating enzymes (or ubiquitin carboxy C-terminal hydrolases) (Hochstrasser, 1995). This group of enzymes fall into two distinct families: a set of small proteins which generally remove ubiquitin from small substrates including peptides and lysine, and a set of larger proteins which cleave ubiquitin from a wide variety of protein substrates *in vitro*. It appears that ubiquitin chains are continuously being attached and cleaved from protein substrates so that the regulation of both the enzymes involved in the addition and removal of ubiquitin is vitally important for the process of protein degradation.

Several recent *in vivo* and *in vitro* studies have detected the presence of ubiquitinated forms of IκBα that can be degraded by purified preparations of proteasomes (Chen *et al.*, 1995; Li *et al.*, 1995; Roff *et al.*, 1996; Alkalay *et al.*, 1995b). In addition, it has recently been demonstrated that IκBβ can undergo inducible ubiquitination (DiDonato *et al.*, 1996). In one study, ubiquitinated forms of IκBα were shown to accumulate following TNFα induction of HeLa S3 cells transfected with a vector expressing IκBα and pre-treated with a proteasome inhibitor (Roff *et al.*, 1996). Under the same conditions, an IκBα mutant containing serine to alanine substitutions at positions 32 and 36 failed to be ubiquitinated. An *in vitro* translated C-

terminal deletion mutant of IκBα lacking the final 75 amino acids when incubated with HeLa cell extracts was shown to be successfully ubiquitinated (Chen *et al.*, 1995). Since ubiquitinated forms of IκBα were only detected when cells were pre-treated with the proteasome inhibitor it is likely that such species are relatively transient, reminiscent of inducibly phosphorylated IκBα. It can be envisaged that, following induction of cells, IκBα complexed to NF-κB is phosphorylated on residues 32 and 36 which then targets the protein for ubiquitination and then degradation.

Indeed, only a small proportion of IκBα accumulates when proteasomal degradation is blocked. It has been suggested that this is a direct result of the action of multiple-C-terminal hydrolases which remove ubiquitin bound to target proteins and are responsible for processing the primary products of polyubiquitin gene mRNA translation (Roff *et al.*, 1996). Alternatively, it is possible that both a ubiquitin-independent and dependent mechanism of degradation exists.

Immunoprecipitation studies to determine whether or not ubiquitinated IκBα can associate with NF-κB includes one report where attempts to immunoprecipitate ubiquitinated forms of transfected IκBα from HeLa S3 cytoplasmic cell extracts with an antibody specific for p50 failed (Roff *et al.*, 1996). However, another investigation showed that ubiquitinated forms of *in vitro* translated IκBα incubated in HeLa cell cytoplasmic extracts could be immunoprecipitated with an antibody specific for p65 (RelA) (Chen *et al.*, 1995). Clearly a direct comparison of both studies is difficult since the origin of the expressed IκBα proteins was different and this may explain the apparent contradictory results. Furthermore, the antibodies used to immunoprecipitate IκBα were each for different subunits of NF-κB. It is possible that ubiquitinated forms of IκBα do not associate with p50 but are capable of binding to p65.

Another report isolated a ternary complex of p50, p65 and newly phosphorylated IκBα from stimulated T-lymphocytes pre-treated with a peptide aldehyde proteasome inhibitor. Both inducibly (hyper-) and basal phosphorylated IκBα were detected but only the hyperphosphorylated form could be degraded *in vitro*. In addition, two fractions obtained from reticulocyte extracts, one of which was known to contain the 26S proteasome and

several of the ubiquitin-conjugating enzymes (including the ubiquitin- activating enzyme (E1), many of the ubiquitin-conjugating enzymes and three known E3 ubiquitin ligases) and one of which was comprised of ubiquitin and a few components of the ubiquitin system were only capable of degrading hyperphosphorylated IκBα when they were combined (Alkalay *et al.*, 1995b). Therefore, it appeared that the 26S proteasome required the presence of ubiquitin and other components of the ubiquitin system to achieve degradation of hyperphosphorylated IκBα . *In vivo* analysis of the ubiquitin dependence of hyperphosphorylated IκBα degradation revealed that a cell line containing a temperature sensitive mutation in the E1 ubiquitin-activating enzyme could not support efficient stimulation induced degradation of IκBα at temperatures known to inactivate E1 in the cell line. The report indicated that inducible degradation of hyperphosphorylated IκBα involved the ubiquitin-proteasome pathway (Alkalay *et al.*, 1995b).

Having established that ubiquitinated forms of IκBα exist many groups have investigated the possible target residues for ubiquitination. Protein ubiquitination is known to occur on lysine residues and therefore studies have concentrated on identifying candidate lysine residues around the inducibly mediated phosphoacceptor sites at positions 32 and 36 in the N-terminus of IκBα . It has been demonstrated that the substitution of lysine 21 and 22 for arginine in the N-terminus of IκBα can almost completely block signal induced ubiquitination and degradation of the protein whereas stimulation dependent phosphorylation remains unaffected (Rodriguez *et al.*, 1996; Baldi *et al.*, 1996; Didonato *et al.*, 1996). In addition, NF-κB dependent transcription was drastically reduced following induction of cells cotransfected with a vector expressing the lysine to arginine IκBα mutant and an NF-κB dependent reporter plasmid.

Although it would appear that lysine 21 and 22 are the primary targets for ubiquitination, a more thorough mutational analysis of the N-terminal lysine residues of IκBα revealed that the mutation of lysine residues 38 and 47 together with lysine 21 and 22 completely blocked ubiquitination and NF-κB-dependent transcription whilst still retaining the ability to be inducibly phosphorylated (Rodriguez *et al.*, 1996). However, a mutant containing only substituted lysine 38 and 47 was ubiquitinated but not as extensively as the

wild-type protein. Therefore, it has been proposed that lysines 38 and 47 represent the secondary targets for ubiquitination. These findings taken together suggest that after the signal-induced phosphorylation of IκBα on serine 32 and 36, lysine 21 and 22 are ubiquitinated (and to a lesser extent lysine 38 and 47) which targets the protein for proteolytic degradation.

2.235. Basal phosphorylation and degradation of IκBα

Although the activation of NF-κB is dependent on the inducible phosphorylation and degradation of IκBα, IκBα is also constitutively phosphorylated and turned over in cells. Recent evidence strongly indicates that casein kinase II may be responsible for the constitutive phosphorylation of IκBα in its C-terminal region (Barroga *et al.*, 1995; McElhinny *et al.*, 1996; Lin *et al.*, 1996; Schwarz *et al.*, 1996). Studies have demonstrated that mutating casein kinase II phosphorylation sites in the C-terminus of IκBα block not only constitutive phosphorylation of IκBα but also the basal degradation of the protein. This aspect of IκBα regulation will be detailed in the discussion.

2.236. Inducible phosphorylation, ubiquitination and degradation of the NF-κB subunits: an additional level of control for NF-κB.

Although IκBα appears to be the primary target for modification by various activating signals it has been demonstrated that the subunits of NF-κB and the p105 and p100 precursors can also be modified. Early studies showed that protein kinase A (PKA) was responsible for the rapid inducible phosphorylation of the *Drosophila* dorsal protein (reviewed in Siebenlist *et al.*, 1994) and the p105, p50 and p65 proteins were shown to become phosphorylated in response to activation (Mellits *et al.*, 1993). It appeared that the phosphorylation of p105, p50 and p65 was slower than that observed for IκBα. Furthermore, the phosphorylated p50 and p65 proteins were not short lived like phosphorylated p105 and IκBα, but were detectable 45 minutes after cell stimulation. In addition, activation of cells also appeared to enhance the processing of the p105 to p50, although this conversion exhibited slower kinetics than the loss (degradation) of IκBα (Mellits *et al.*, 1993; Mercurio *et al.*, 1993). The cytokine induced

transient phosphorylation of p65 homodimers has been described more recently (Diehl *et al.*, 1995). As suggested in one report a role for this inducible phosphorylation may be to enhance the DNA binding of p65 containing dimers (Naumann *et al.*, 1994). However, it could equally be required for the release of IκBα, nuclear translocation or for transcriptional activation.

Later investigations demonstrated that the *in vitro* processing of p105 to p50 was dependent on ATP and was accompanied by the degradation of the C-terminus of p105 (Pallombella *et al.*, 1994; Donald *et al.*, 1995). Moreover, ubiquitinated forms of p105 were detectable *in vitro* in the presence of a non-hydrolyzable analogue of ATP known as ATPγS which stabilises ubiquitinated proteins but blocks degradation via the proteasome pathway. The proteasome was also shown to be required for p105 processing *in vitro* and inhibitors of the proteasome also blocked p105 processing both *in vivo* and *in vitro* and prevented the activation of NF-κB. Therefore, it appeared that p105 processing and/or degradation was mediated by a ubiquitin-dependent proteasome pathway. Indeed, the ubiquitin-carrier protein, E2-F1, which participates in the conjugation and degradation of p53, and a novel species of ubiquitin-protein ligase have both been shown to be required for limited processing of the p105 precursor protein to p50 (Orian *et al.*, 1995).

The processing signal of p105 has recently been identified and consists of a 23 amino acid glycine rich region (GRR) (residues 372-398) (Lin and Ghosh, 1996). It appears that the endoproteolytic cleavage of p105 to p50 occurs downstream of this glycine rich sequence. When the C-terminus of p105 was removed (residues 404-917) and replaced with the C-terminal region of IκBα, the processing of the fusion protein *in vivo* (in Cos1 cells) yielded p50 and an intact C-terminal IκBα region. It has been proposed that p105 processing involves an initial GRR-dependent endoproteolytic cleavage event followed by a rapid degradation step.

The PEST sequences (specifically the serine residues) of p105, located in the final 150 C-terminal residues appear to be phosphorylated in response to activation of Jurkat T cells with either PMA (phorbol 12-myristate 13-acetate)/ionomycin or okadaic acid (a serine/threonine phosphatase inhibitor and activator of NF-κB). This has been shown to be accompanied by an increase in the proteolytic processing of p105 to p50. Deletion of the C-

terminal 68 residues of p105 (i.e. part of the PEST-like region) results in a failure of p105 to be phosphorylated or for its processing to p50 to be increased in response to stimulation of cells with PMA/ionomycin (MacKichan *et al.*, 1996). Moreover, the signalling pathway which leads to the phosphorylation and processing of p105 after PMA/ionomycin treatment appears to be have elements distinct from that which controls IκBα phosphorylation and degradation. For example, pre-treatment of Jurkat cells with antioxidants followed by activation with PMA/ionomycin blocks phosphorylation and degradation of IκBα but does not affect either p105 phosphorylation or processing.

Notably, Epstein-Barr immortalised cells contain nuclear p105 complexed to p65. Mitomycin C, which activates NF-κB has been shown to induce processing of p105 to p50 in the nucleus whereas the steady-state levels of both p105 and IκBα in the cytoplasm remain constant. In contrast, phorbol ester induction of cells stimulated the degradation of cytoplasmic p105 and IκBα but did not affect p105 protein levels in the nucleus (Baldassarre *et al.*, 1995). Therefore, it appears, at least in the cell type examined that nuclear processing of p105 to p50 can contribute to the activation of NF-κB.

2.237. Mutual Control of NF-κB and IκBα

As detailed in the previous sections IκBα activity can be mediated through phosphorylation and ubiquitin dependent proteolysis and this in turn controls the activity of NF-κB. However, both IκBα and NF-κB are also regulated at the level of transcription. Early studies demonstrated that the induced loss of IκBα and subsequent translocation of NF-κB to the nucleus was accompanied by an increase in the level of IκBα mRNA (Sun *et al.*, 1993; reviewed in Siebenlist *et al.*, 1994). Later investigations demonstrated that the transfection of a vector expressing p65 into cells resulted in increased levels of IκBα mRNA. Following the identification of several NF-κB binding sites in the promoter of the gene encoding IκBα it was proposed that NF-κB must stimulate the transcription of the gene encoding IκBα (Chiao *et al.*, 1994; LeBail *et al.*, 1993). Therefore, a feedback mechanism exists in the NF-κB-IκBα system whereby newly synthesised IκBα generated through NF-κB mediated transcriptional activation of the IκBα gene, acts to restore NF-κB in the inhibited state in the cytoplasm of

cells. However, more recent experimental findings have indicated that IκBα mRNA levels can be increased via a transcription-independent event during adherence (Lofquist *et al.*, 1995).

Indeed, it appears that the gene promoters of p105 and p100 are also similarly regulated by NF-κB since they too contain κB binding sites. Cotransfection experiments demonstrated that p50 homodimers, p65 homodimers and p50/p65 heterodimers (i.e. NF-κB) could activate the promoter of p105 (and hence p50) (Ten *et al.*, 1992). In addition, the levels of p100 mRNA and protein were increased by nuclear p65 (Sun *et al.*, 1994). The structural and functional characterisation of the NF-κB2 (p100) gene promoter regions has indicated that p65 can enhance the activity of these regions, whereas NF-κB2 p52 can negatively regulate promoter activity (Lombardi *et al.*, 1995). Prior to processing both p100 and p105 can act as inhibitors of NF-κB/Rel proteins in the cytoplasm of cells ensuring, like IκBα a rapid but transient activation of NF-κB. Interestingly, the gene promoter of p65 does not possess any κB elements and has only been shown to be stimulated slightly following induction of cells (reviewed in Siebenlist *et al.*, 1994). It is thought that p65 expression is essentially constitutive.

2.3. Signal transduction pathways involved in the activation of NF-κB.

2.31. Second messengers, kinases and phosphatases implicated in the activation of NF-κB.

NF-κB DNA binding activity can be stimulated by a multitude of agents including cytokines such as tumour necrosis factor α, interleukin-1 (IL-1) and interleukin-2, bacterial lipolysaccharide (LPS), virus infection (HIV-1, HTLV-1 (human T-cell leukaemia virus) and hepatitis B virus)), viral proteins (tax, X and E1A), phytohaemagglutinin (a mitogenic lectin), ultraviolet light, calcium ionophores, phorbol esters, hydrogen peroxide and nitric oxide (Matthews *et al.*, 1996), to name but a few (reviewed in Siebenlist *et al.*, 1994).

A common secondary messenger has not been identified for the range of signals which activate NF-κB, suggesting that several different signalling pathways may regulate NF-κB. However, pretreating cells with antioxidants such as *N*-acetyl-L-cysteine appears to block the majority of these activating signals as demonstrated through the inhibition of NF-κB DNA

binding activity (Schreck *et al.*, 1991) and the suppression of transcriptional activation from the HIV-1 LTR (Staal *et al.*, 1990). Many of the agents which activate NF-κB are thought to do so by generating reactive oxygen intermediates such as the superoxide anion O_2^- and the hydroxyl radical $OH^{\cdot}$. For example, the protein phosphatase inhibitor okadaic acid is thought to inhibit a phosphatase of IκBα indirectly through the production of reactive oxygen intermediates (Schmidt *et al.*, 1995). These reactive oxygen intermediates could result in the activation of various proteases and the degradation of the IκB proteins (Baldwin *et al.*, 1996). Indeed, a recent report has suggested that intracellular reactive oxygen species can mediate the phosphorylation of IκBα in response to TNFα induction of cells (Kretzremy *et al.*, 1996). This study showed that the overexpression of glutathione-peroxidase in cells followed by stimulation with TNFα resulted in the accumulation of the inducibly phosphorylated form of IκBα.

Thioredoxin, a protein involved in redox regulation within cells, can also activate NF-κB and its expression can be increased by the NF-κB-activators, hydrogen peroxide and ultraviolet light. Therefore, it is possible that these stimuli may lead to the activation of NF-κB via thioredoxin (Baldwin *et al.*, 1996; Matthews and Hay, 1995).

Ceramide has also been implicated as second messenger in the pathway to NF-κB activation following stimulation of cells with either TNF and IL-1. It has been proposed that the association of TNF with its receptor on the surface of cells leads to the production of ceramide through the activation of sphingomyelinase by diacylglycerol (generated by a phosphatidylcholine-specific phospholipase C). Sphingomyelinase is capable of inducing the degradation of IκBα *in vitro* and may be involved in the activation of a specific kinase capable of phosphorylating IκBα (reviewed in Baldwin, 1996). However, evidence against ceramide involvement in NF-κB activation does exist. This includes a report on cells chronically activated with PMA. Such activation inhibited the ceramide pathway, however TNF induction of cells still resulted in NF-κB activation (Westwick *et al.*, 1994).

Several kinases have been implicated in the signalling pathways which lead to the activation of NF-κB. For example, inactivation of the interferon-inducible or double-stranded RNA-dependent protein kinase, PKR in cells results in the failure of double-stranded RNA to

induce the DNA binding activity of NF-κB and this is believed to be mediated through the phosphorylation of IκBα (Maran *et al.*, 1994). Protein kinase C ζ, when overexpressed in NIH 3T3 cells, causes the persistent translocation of NF-κB to the nucleus. Furthermore, a dominant negative mutant (devoid of kinase activity) of protein kinase C ζ when cotransfected into cells with an NF-κB-dependent reporter plasmid results in a reduction in transcription from the reporter (Diaz-Meco, 1993). A more recent study has shown that protein kinase C ζ is involved in the activation of NF-κB in human immunodeficiency virus-infected monocytes (Folgueira *et al.*, 1996). The *v-abl* tyrosine kinase (a viral transforming protein) was shown to negatively regulate NF-κB activity in pre-B cells through a post-translational mechanism which increased the stability of IκBα (Klug *et al.*, 1994). The Src family of tyrosine kinases have also been implicated in the activation of NF-κB in response to ultraviolet light (Devary *et al.*, 1993).

Transfection of vectors constitutively expressing the Raf-1 serine/threonine kinase into cells was shown to stimulate transcriptional activation from a κB-dependent reporter plasmid. In addition, when the endogenous activity of Raf-1 was blocked in cells treated with a range of known NF-κB activators by the overexpression of a dominant negative mutant of Raf-1, a reduction in κB-dependent transcription was observed (Finco and Baldwin, 1993). Indeed, the upstream effector, Ras of the so-called Ras-Raf pathway, has also been shown to activate κB-dependent transcription and a recent report has demonstrated that both Ras and Raf-1 are activated in human immunodeficiency virus infected monocytes and stimulate DNA binding of NF-κB (Folgueira *et al.*, 1996). The mitogen-activated protein kinase kinase kinase, MEKK, which stimulates the stress-activated protein kinases/jun kinase-1 (SAPKs/JNK1) was recently shown to participate in the activation of NF-κB through the degradation of IκBα following stimulation of cells with TNFα (Hirano *et al.*, 1996). An additional report has demonstrated that the downstream kinase, JNK-1 and c-Rel can interact *in vivo* but that JNK-1 does not phosphorylate either c-Rel, NF-κB or IκBα *in vitro* . It has been proposed that c-Rel may act as a "docking site" for JNK-1 thereby permitting the kinase to phosphorylate Rel-associated proteins (Meyer *et al.*, 1996).

A novel ubiquitin-dependent protein kinase activity has been discovered in HeLa cell cytoplasmic extracts which specifically phosphorylates IκBα on the N-terminal serine residues at positions 32 and 36 (the targets for inducible phosphorylation). Interestingly the kinase activity appears to require the ubiquitin activating enzyme (E1), a specific ubiquitin carrier protein (E2) and ubiquitin. A ubiquitination step for kinase stimulation is essential and it has been proposed that ubiquitination occurs on a factor associated with the kinase complex. Moreover, this step takes place before the site specific phosphorylation of IκBα on residues 32 and 36 (Chen *et al.*, 1996). Another IκBα associated kinase has been found in the human monocytic cell line, THP-1, which can bind to and phosphorylate IκBα on C-terminal serine and threonine residues (Kuno *et al.*, 1995). A report has recently claimed that neither Raf-1 nor the isozymes of protein kinase C (including ζ) are capable of directly phosphorylating IκBα . Instead Raf-1 associates with casein kinase II and casein kinase II together with other unidentified kinases phosphorylate the C-terminal region of IκBα (Janosch *et al.*, 1996).

Phosphatases are also thought to participate in the pathway leading to NF-κB activation by either dephosphorylating upstream effectors of the signalling cascade or by acting directly on IκBα. For example, an inhibitor of the Ca^{2+}-dependent phosphatase calcineurin referred to as FK506 prevented the activation of c-Rel in both B and T cells (Venkataraman *et al.*, 1995). In addition, the overexpression of calcineurin in T cells from a transfected vector resulted in the activation of NF-κB in T-cells (Franz *et al.*, 1994). Experimental evidence has suggested that the Ca^{2+} -dependent pathway involving calcineurin can synergise with the protein kinase C-dependent and -independent pathways to regulate the phosphorylation and degradation of IκBα (Steffan *et al.*, 1995). The possibility of direct dephosphorylation of IκBα by the serine/threonine protein phosphatase 2A (PP2A) has been suggested through the treatment of human T cells with an inhibitor of PP2A known as calyculin A. Such treatment resulted in the phosphorylation of IκBα at protein phosphatase 2A sensitive sites and activation of NF-κB (Sun *et al.*, 1995). Finally, another study has indicated that a protein-tyrosine phosphatase(s) may participate in the upstream regulation of the TNF signalling pathway which leads to the degradation and subsequent activation of NF-κB (Menon *et al.*, 1995).

2.32. Viral proteins and the activation of NF-κB.

Several studies have revealed the importance of NF-κB in the activation of viral gene expression particularly those genes whose products are required for viral replication. Consequently many viruses regulate the activation of NF-κB through certain viral proteins, a few of which are discussed below.

Recently many studies have focused on the role of the human T-cell leukaemia virus type 1 (HTLV-1) tax protein in NF-κB activation. Tax potently activates transcription from the HTLV-1 long terminal repeat and is also involved in increasing the expression of several viral and cellular genes including the genes encoding c-Fos and interleukin-2. Tax is believed to be essential for the transforming properties of HTLV-1. Numerous routes have been proposed for the activation or suppression of NF-κB by tax. One of these involves p100 (NF-κB2) which has been reported to interact with Tax thereby disrupting Tax mediated activation of NF-κB(Kanno *et al.*, 1994; Murakami *et al.*, 1995). Another occurs through Tax induction of the pathway leading to the degradation of IκBα and IκBβ and the passage of NF-κB to the nucleus (Brockman *et al.*, 1995; Good *et al.*, 1996; McKinsey *et al.*, 1996).

The viral transactivator protein Tat of the human immunodeficiency virus-1 (HIV-1) is thought to interact with NF-κB at the HIV-1 long terminal repeat in order to maintain the elevated level of viral expression which is observed in U937 monocytes infected with HIV-1 (Jacqué *et al*, 1996). The 13S splice variant of the adenovirus type 5 E1A regulatory protein has been shown to activate NF-κB by two routes. The first occurs through the activation of the p65 subunit of NF-κB by 13S E1A. This involves the stimulation of the p65 transcriptional activation domain by direct association of 13S E1A with this region (as demonstrated *in vitro*). The second route leads to the production of reactive oxygen intermediates, phosphorylation of IκBα via serine residues 32 and 36 and the degradation of IκBα. The DNA binding activity of NF-κB can also be inhibited through the adenovirus E1B 19K (19 kilodalton) protein, presumably to counteract the immune stimulatory and apoptotic effects of persistent NF-κB activation (Schmitz *et al.*, 1996).

2.33. Inactivation of NF-κB via specific compounds: a route towards the elucidation of signal transduction pathways and disease therapies .

Many compounds have been shown to inhibit the activity of NF-κB and these provide a useful tool for gaining knowledge on the activation pathways involved in NF-κB stimulation. Moreover, they may enable the identification of possible therapies for those diseases which involve the persistent activation of NF-κB.

Glucocorticoids are powerful anti-inflammatory and immunosuppressive agents and their involvement in NF-κB inhibition has been known for some time (Baldwin, 1996). However, the mechanism of this inhibition was unclear until fairly recently. It now appears that glucocorticoids can block the activation of NF-κB in two ways. Firstly, the receptor for glucocorticoids (a ligand-activated transcription factor), has been reported to interact directly with NF-κB and interfere with the activation of NF-κB-dependent gene expression. The second mechanism of inhibition was discovered following studies on monocytes and lymphocytes and involves the induction of IκBα protein synthesis by glucocorticoids. Newly synthesised IκBα retains NF-κB in the cytoplasm of cells, even after induction with TNF (since degraded IκBα will be quickly replaced by newly synthesised IκBα) (Auphan *et al.*, 1995; Scheinman *et al.*, 1995). It is believed that the suppression of NF-κB DNA binding activity decreases the level of cytokine secretion thereby preventing stimulation of the immune system. However, it appears that in certain cell types such as endothelial cells glucocorticoid-mediated inhibition of NF-κB does not involve an increase in the rate of IκBα protein synthesis (Brostjan *et al.*, 1996).

Nitric oxide acts as a repressor of the immune and inflammatory responses and appears to inhibit the activation of NF-κB in a similar way to the glucocorticoids i.e. through the induction of IκBα protein synthesis (Peng *et al.*, 1995).*In vitro* studies have demonstrated that nitric oxide can inhibit the DNA binding activity of NF-κB (Matthews *et al.*, 1996). Furthermore, NF-κB can activate nitric oxide synthase II (iNOS) gene expression suggesting the existence of a negative feedback loop, whereby nitric oxide (generated by iNOS) can reduce expression of iNOS by inhibiting the DNA binding of NF-κB (Baldwin, 1996).

Many more inhibitors of NF-κB exist including the immunosuppressive fungal metabolite, gliotoxin (Pahl *et al.*, 1996). Gliotoxin is generated as a toxic metabolite in Aspergillus infections, a serious condition often observed in immunocompromised patients. It appears that gliotoxin cannot inhibit the DNA binding activity of NF-κB at physiological concentrations. Instead, it was discovered that gliotoxin acted to inhibit the activation of NF-κB by preventing the degradation of IκBα in intact T cells. The results suggest that the inhibition of NF-κB activity may be important for the immunosuppressive effect of gliotoxin when it is synthesised in during Aspergillus infections.

2.4. NF-κB mediated genes and the physiological role of NF-κB.

2.41. Genes regulated by NF-κB.

NF-κB is responsible for mediating the activity of a plethora of genes, in particular those which encode proteins that are critical for the immune, inflammatory and acute-phase responses (reviewed in Baldwin , 1996; Siebenlist *et al.*, 1994). For example, transcription of the gene encoding the co-stimulatory molecule B7.1 (involved in T cell activation) (Zhao *et al.*, 1996) is controlled by NF-κB together with the genes encoding the proteasome subunit, LMP2 and the peptide transporter, TAP1 (Wright *et al.*, 1995). NF-κB has also been implicated in the control of many cytokine and growth factor genes, such as G-CSF, IL-2, IL-6 and IL-8 (reviewed in Baldwin, 1996).

Extensive studies have been performed on the regulatory role of NF-κB in the expression of genes encoding cell adhesion molecules. The activity of the gene encoding the vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin superfamily, is controlled in part through NF-κB. Following treatment of endothelial cells with TNF, IL-1 or LPS (lipopolysaccharide) VCAM-1 is seen to accumulate on the cell surface. VCAM-1 has been implicated in the recruitment of monocytes and lymphocytes which express α4β1 or α4β7 integrins to an area of tissue injury as well as playing a role in the development of the central nervous system. The promoter of the VCAM-1 gene contains two binding sites for NF-κB (Ahmad *et al.*, 1995) as well as a binding site for IRF-1 (endothelial interferon regulatory factor 1), a phosphoprotein transcription factor implicated in the activation of the

IFN-β gene and other cytokine inducible genes (Neish *et al.*, 1995). NF-κB is necessary but not sufficient for complete cytokine-mediated transcriptional activation of the VCAM-1 gene. However, IRF-1 is cytokine inducible and recombinant IRF-1 and NF-κB can physically interact. Furthermore, IRF-1 can synergise with NF-κB to activate VCAM-1 *in vivo.* Therefore, IRF-1 is thought to interact with NF-κB in order to promote VCAM-1 expression.

The apg gene encoding the α-1-acid glycoprotein/enhancer binding protein (AGP/EBP or C/EBPβ) is also regulated in part through NF-κB. AGP/EBP is a member of the C/EBP family of transcription factors and plays a key role in the induction of a wide variety of genes including a number of those expressed during the acute-phase response. NF-κB, together with a phosphoprotein transcription factor, termed Nopp140 have been shown to interact with two forms of AGP/EBP (possibly generated through alternative translation start sites) which results in transcriptional activation of the agp gene via an AGP/EBP-binding motif. In addition, apg gene expression can be stimulated following exposure to external stimuli which are known to be inducers of NF-κB (Lee *et al.*, 1996).

NF-κB can also regulate the transcription of genes whose products are essential for processes outwith the immune and inflammatory responses. For example, the genes encoding proteins which are important for cell growth such as c-myc (LaRosa *et al.*, 1994). Many viral genes are also mediated by NF-κB including those of the widely studied human immunodeficiency virus (HIV) (reviewed in Roulston *et al.*, 1995).

2.42. Gene knockout studies.

During 1995 and 1996 gene knockout studies have confirmed the important role that the Rel/NF-κB family members play in immune function. In addition, these experiments have resulted in a number of unpredicted discoveries, some of which are summarised below.

NF-κB1: Knockout of the gene encoding p105/p50 (or NF-κB1) in mice resulted in normal development but an impaired immune response due to the failure of B-cells to both proliferate normally in response to LPS or secrete antibodies (Sha *et al.*, 1995). More recently, defects in the maturation of B-cells to immunoglobulin secretion together with IgE, IgA and IgG3 class

switching have been observed in p50 deficient mice (Snapper *et al.*, 1996). This indicates that p50 may be involved in immunoglobulin class switching. In the earlier study by Sha *et al* (1995) mice were not able to clear Listeria and Streptococcus infections but they were more resistant to infection with murine encephalomyocarditis (EMC). Resistance to EMC was correlated with an increase in IFNβ (known to be an antiviral protein). Therefore, in wild-type mice, p50 may act to inhibit the activity of IFNβ.

RelB: RelB deficient mice developed normally but abnormalities existed in the animals immune and hematopoietic systems (Burkly *et al.*, 1995; Weih *et al.*, 1995). It was found that mice lacking RelB showed an impaired development of dendritic cells and hence a defective antigen-presenting function (since dendritic cells from the bone marrow are the primary antigen-presenting cells in the activation of the T-lymphocyte response). Furthermore, the differentiation of thymic medullary epithelial cells was also defective. These cells are very similar to dendritic cells, participating in antigen presentation to T-cells. They have also been implicated in regulating the negative selection of developing thymocytes (Burkly *et al.*, 1995). In previous studies RelB had been implicated in the constitutive expression of κB regulated genes in lymphoid tissues. Weih *et al* (1995) discovered that mice lacking RelB exhibited a large reduction in the level of constitutive κB-dependent DNA binding activity in the spleen and thymus. These mice were also seen to have phenotypic defects including splenomegaly (due to extramedullary haematopoiesis) and reduced cellular immunity. Hence it appears that RelB performs an essential role in immune function as well as in the development of dendritic and thymic medullary epithelial cells. It is also clear that no other member of the Rel/NF-κB family is able to replace the loss of RelB function indicating that RelB performs functions distinct from other family members.

p65(RelA): Disruption of the *relA* locus in mice resulted in embryonic lethality which was thought to be a result of liver degeneration. In addition, mice deficient in p65 lost TNF-mediated inducibility of NF-κB (p50/p65) as well the inducibility of NF-κB-mediated genes such as those encoding IκBα (Beg *et al.*, 1995). The degeneration of the liver observed in

mice lacking p65 occurred through apoptosis and this is similar to the results obtained from studies on c-Jun deficient mice (see Beg *et al.*, 1995). Therefore, it is possible that both p65 and c-Jun both perform overlapping roles in normal liver development.

c-Rel: c-Rel is known to be expressed in high levels within hemopoietic cells. Interestingly, mice containing an inactivated c-Rel gene developed normally and showed normal hematopoietic cell differentiation. However, the mature B and T cells of c-Rel -/- mice failed to be stimulated (to proliferate) by various mitogenic stimuli (Köntgen *et al.*, 1995). More recently, it has been discovered that Rel is required by T-lymphocytes for the production of IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF). However, in Rel-/- lipopolysaccharide macrophages the levels of GM-CSF were higher than normal indicating that in normal cells c-Rel acts as a negative regulator of GM-CSF production (Gerondakis *et al.*, 1996). Mice deficient in c-Rel also exhibited a reduction in T-cell dependent humoral immunity. However, the impaired proliferative response of T-cells to certain membrane receptor mediated stimuli could be overcome by co-activating phorbol-ester and the calcium ionophore (Köntgen *et al.*, 1995). This indicates that T-cell proliferation can be achieved via both c-Rel-dependent and -independent mechanisms.

IκBα & Bcl-3: IκBα (-/-) mice appeared to be normal at birth but exhibited skin defects and extensive granulopoiesis soon after birth, followed by a cessation of growth by 3 days and death by 8 days. The nuclear levels of NF-κB in hematopoietic tissues (the spleen and thymus) were high in IκBα deficient mice and the expression of some NF-κB regulated genes (VCAM-1) was also increased. This suggests that IκBα is predominant in the spleen and thymus of wild-type mice. These phenotypic abnormalities were due to NF-κB elevation because mice deficient in both p50 and IκBα did not develop these abnormalities as rapidly IκBα (-/-) only mice and consequently lived longer. Interestingly, embryonic fibroblasts failed to show abnormally high levels of nuclear NF-κB, indicating that the cytoplasmic retention of NF-κB is mediated by other members of the IκB family of proteins. Indeed, IκBα deficient fibroblasts were also responsive to TNFα treatment, showing NF-κB

activation through the targeted degradation of IκBβ. However, these cells were not able to reduce post-induction levels of nuclear NF-κB as rapidly as cells with IκBα. This signifies the requirement of IκBα for the post-induction repression of nuclear NF-κB (Beg *et al.*, 1995; Klement *et al.*, 1996).

Initial work on bcl-3 gene knockout mice has indicated that mice develop normally and do not show any immunological defects (Baldwin, 1996).

2.43. The importance of NF- κB in disease.

NF-κB has been implicated in many diseases including arthritis, atherosclerosis, cancer and several neurological disorders. Hence an understanding of the mechanisms of activation and regulation of NF-κB represent critical targets for possible therapeutic intervention in these various diseases (reviewed in Baldwin, 1996). NF-κB also plays an important role in the activation of HIV gene expression (reviewed in Roulston *et al.*, 1995). Several other viruses are also partially dependent upon the activity of NF-κB, these include SV40, CMV and influenza virus

Atherosclerosis is a condition which is related to lipid oxidation in low density lipoproteins (LDLs). NF-κB becomes activated when the oxidised lipids accumulate in the extracellular matrix of the subendothelial space. This results in the stimulation of genes which encode proteins participating in the inflammatory response. Furthermore, atherosclerosis leads to the proliferation of the vascular wall, a process thought to be mediated in part by the serine protease, thrombin through the activation of NF-κB (reviewed in Baldwin, 1996).

Since NF-κB is involved in mediating responses to inflammatory cytokines it is perhaps not surprising that it performs a role in the development of acute inflammatory conditions such as arthritis. Indeed, recent experimental evidence has demonstrated that NF-κB is activated in the arthritic synovium (Baldwin, 1996). In addition, many of the treatments which are used for arthritis have been shown to inhibit the activation of NF-κB including gold compounds (Yang *et al.*, 1995).

Recent experimental evidence has strongly indicated that NF-κB performs a crucial function in neurodegenerative processes such as Alzheimer's disease. In Alzheimer's disease

a toxic derivative of the β-amyloid precursor protein known as amyloid β-peptide accumulates in plaques in the brain. Studies have shown that the neurotoxicity of amyloid β-peptide is linked to a mechanism which involves the stimulation of reactive oxygen species (ROS) and high levels of free intracellular Ca^{2+}. Experimental findings have indicated that TNFα and TNFβ can prevent degeneration of hippocampal neurons by amyloid β-peptide through the suppression of ROS and Ca^{2+} levels and that κB-dependent transcription can mediate this effect (Barger *et al.*, 1995). Furthermore, NF-κB can be activated by the β-amyloid precursor protein (Barger and Mattson, 1996). These findings suggest that NF-κB-dependent gene expression, induced by cytokines may be required to protect neurons against the amyloid β-peptide.

A similar protective function for NF-κB in the process of apoptosis has recently been reported in B-cells. It was discovered that following stimulation of B-cells with transforming growth factor-β1 (a factor known to induce apoptosis in several cell types (including B-cells)) a reduction in both NF-κB DNA binding activity and c-myc gene expression was detected together with an increase in IκBα gene expression and protein levels (Asura *et al.*, 1996).The c-myc gene has been previously implicated in mediating cell death in a range of cell types. In B-cells, suppression of c-myc gene expression is correlated with an induction of apoptosis, whereas in myeloid and fibroblast cells an increase in c-myc gene expression has been associated with apoptosis. NF-κB is known to stimulate c-myc gene expression through two κB binding sites (reviewed in Baldwin, 1996). Therefore, it has been proposed that in response to treatment of B-cells with transforming growth factor-β1, apoptosis is induced by inhibiting NF-κB activity through an increase in IκBα protein levels which prevents the translocation of NF-κB to the nucleus and stimulation of c-myc gene expression. Presumably in the absence of apoptotic stimuli NF-κB maintains c-myc levels to avoid cell death.

MATERIALS AND METHODS

1.11. Materials.

Human recombinant TNFα was provided by the MRC ADP reagent programme. The proteasome inhibitor, Z-LLL-H (carbobenzoxyl-leucin-leucin-leucinal) was synthesised as described by Fehrentz *et al.* in 1985 and isolated by reverse phase HPLC (>95% purity). The structure was confirmed by NMR spectroscopy.

1.12. Recombinant proteins.

Human recombinant p65 (amino acids 12-317), GST-p65, p50 (35-381), GST-p50 and wild-type IκBα c-tag were generously supplied by E.Jaffray and J.Nicholson (St.Andrews University). The proteins were expressed as glutathione-S-transferase (GST) fusions in *E.coli* JM101 and cleaved with thrombin as described in Jaffray *et al.*, 1995 and Matthews *et al.*, 1992.

1.13. Bacterial strains.

E. coli K12 strains; XL-1 blues (in house supply) [genotype->*rec* A1, *end* A1, *gyr* A96, *thi* -1, *hsd* R17, *sup* E44, *rel* A1, *lac* , {F` *pro* AB, *lac* I^{q}ZΔM15, Tn *10* (tet^{r})}] or Top 10s (Invitrogen Corporation) [genotype->*mcr* A, Δ(*mrr-hsd* RMS-*mcr* BC), ø80Δ*lac* ΔM15, Δ*lac* X74,*deo* R,*rec* A1, *end* A1, *ara* D139, Δ(*ara, , leu*) 7679, *gal* U, *gal* K, *rps* L(Str^{r}), *nup* G] were used for routine propagation of plasmids. Bacteria were grown in Luria-Bertani (LB) broth with antibiotics added when required.

1.14. Mammalian tissue culture cells and antibodies.

293 cells (transformed human embryonic kidney cells) at low passage number (no. 28) for efficient transfection were a kind gift from F. Graham (M^{c}Master University). 293s were cultured in GMEM supplemented with 10% FCS (GMEM-10F) and passaged weekly. Cos-7s (transformed African Green-monkey kidney cells) were generously donated by F. Arenzana-

Seisdedos (Institut Pasteur) and passaged twice weekly in GMEM-10F. The monoclonal antibody SV5-P-k (Anti-Pk) recognising an epitope on SV5 phospho (P) and V proteins (Randall *et al.*, 1987 and Southern *et al.*, 1991) was supplied by Dr. R.E. Randall, St.Andrews. The monoclonal antibody 10B MAD3 which recognises an epitope between amino acids 21 and 48 of human IκBα (Jaffrey *et al.*, 1995) was prepared by M. Roff (University of St. Andrews).The polyclonal antibody raised against p65 was purchased from Santa Cruz and recognises an epitope between amino acids 531-550 of human p65. Rabbit antiserum raised against p50 (35-381) was prepared by J.Thompson (St. Andrews University).

1.15. Plasmids.

Plasmid pGEX2T was donated by E. Jaffray (University of St.Andrews) and plasmid pGEXcPk was obtained from R.E. Randall (University of St.Andrews). The transient mammalian expression vector pcDNA3 was purchased from Invitrogen Corporation. Plasmid pCT7ßgal which encodes the ß-galactosidase gene under the hCMV promoter/enhancer was generously donated by M.O'Reilly (University of St.Andrews). The luciferase reporter vectors 3EnhConALuc (NF-κB dependent) and LTRΔkBLuc (NF-κB independent) were a kind gift from F. Arenzana-Seisdedos (Institut Pasteur). The 3EnhConALuc vector is driven by three synthetic copies of the NF-κB consensus sequence from the immunoglobulin kappa chain promoter. The LTRΔκBLuc promoter contains regions U3 and R regions of the HIV-1 LTR deleted of the NF-κB consensus sequences.

1.16. Agarose-gel electrophoresis.

DNA samples were separated via horizontal agarose-gel electrophoresis using a mini-gel (10cm x 10cm) apparatus (Bioscience 101). T.B.E. or T.A.E. buffer was used depending on the particular application. The agarose (molecular biology grade, Sigma) concentration ranged from 0.8% to 2% depending on the size of the DNA fragments to be separated. Gels were ran at varying voltages and for differing times, again depending on the application. The DNA was subsequently analysed by ethidium bromide staining and visualisation under U.V.

1.17. Extraction of DNA fragments from agarose-gels.

DNA fragments were separated on an agarose-gel prepared from TAE buffer. Gel fragments containing the DNA band of interest were excised from the agarose gel and processed using Qiaquick gel-extraction spin-columns (Hybaid Ltd.) according to the manufacturers recommendations. To assess recovery of the DNA fragments, $^{1}/_{10}$th aliquots (5µls) were visualised after electrophoresis through agarose-gels.

1.18. DNA linkers and primers.

Oligonucleotides used for the generation of mutant IκBα genes were synthesised in-house using an Applied Biosystems oligonucleotide synthesiser. Oligonucleotides were purified via ethanol precipitation as described in Molecular Cloning by Sambrook, Fritsch and Maniatis. Oligonucleotides used for the generation of Pk-tag-linkers were synthesised and H.P.L.C. purified by Oswell`s DNA service (Chemistry department, University of Edinburgh).

Oligonucleotides of positive and negative sense for the generation of the C-terminal Pk-tag linker were -> **Pkpos** 5`-AAT TCT AGG AAA GCC GAT CCC AAA CCC TTT GCT GGG ATT GGA CTC CAC CTA AGC - 3` and **Pkneg** 5`-GGC CGC TTA GGT GGA GTC CAA TCC CAG CAA AGG GTT TGG GAT CGG CTT TCC TAG- 3`. The two oligonucleotides were annealed together and 5` phosphorylated using standard protocols (Manniatis *etal*). Underlined sequences correspond to the Pk-oligonucleotide sequence.

Primers annealing to the non-coding and coding strands of the human IκBα gene for the generation of mutant IκBα genes, designated **FOR** (forward) and **BACK** (backwards) are as follows;

Two "Universal oligonucleotides":**FOR** 5' - ATT GTA GGA TCC ATG TTC CAG GCG GCC GAG CGC - 3'. **BACK** 5' - GCC AGG AAT TCC TAA CGT CAG ACG CTG GCC TCC - 3'.

Mutants: **W258y, FOR** 5' - TAC CAG CTC ACC **TAC** GGC CGC CCA - 3'. **E275q, FOR** 5' - CAG CTG ACA CTA **CAG** AAC CTT CAG - 3'. **E275s, FOR** 5' - CAG CTG ACA CTA **TCG** AAC CTT CAG -3'. **EDE284-286qnq, FOR** 5'-CTG CCA GAG AGT **CAG AAT CAG** GAG AGCTAT-3'. **EDE284-286sss, FOR** 5' - CTG CCA GAG AGT **TCG TCG TCG** GAG AGC TAT -3'. **EDE300-302qnq, FOR** 5' - ACG GAG TTC ACA **CAG AAT CAG** CTG CCC TAT -

3'. **EDE300-302sss, FOR** 5' - ACG GAG TTC ACA **TCG TCG TCG** CTG CCC TAT - 3'. **265-317, FOR** 5' - ATT GTA GGA TCC ATA CAG CAG CAG CTG GGC CAG - 3'. **275-317, FOR** 5' - ATT GTA GGA TCC GAA AAC CTT CAG ATG CTG CCA - 3'.

Underlined, bold script refers to the mutant type (e.g. **W258y**, **W**= original amino acid, **258** = amino acid location within IκBα and **y** = amino acid after mutation) and the bold letters within the primers represent the areas of mutated sequence.

1.19. Generation of cDNA encoding mutant IκBα c-tag proteins.

A P.C.R. mutagenesis technique was employed (Landt *et al.*, 1990) to generate the cDNA molecules encoding mutant IκBα c-tag proteins. This method used one internal mutagenising primer and two external "Universal" primers (used for all the mutants) in a two step P.C.R.. The first P.C.R. contained 100ng of the linearised (obtained by digestion of the template with EcoRV and dephosphorylation with calf-intestinal alkaline phosphatase) "template" DNA i.e. pGEXcPk+Wild-type IκBα (amino acids 1-317)-c-tag (14 amino acids), 1μM of the "C-terminal" (3') primer **BACK** 5' - GCC AGG AAT TCC TAA CGT CAG ACG CTG GCC TCC - 3' (1μl of 100μM stock), 1μM of the mutant primer (1μl of 100μM stock), 10x dNTP (10μl of a stock containing 0.5mM of each dNTP), 10x P.C.R. reaction buffer (10μl;x1(final): 10mM Tris-Cl pH8.8, 1.5mM $MgCl_2$, 50mM KCl and 0.1% Triton X-100) and 1μl of Dynazyme DNA polymerase (Finnzymes Ltd.) - this mixture was made to a total of 100μl with sterile water and then overlaid with 50μl of liquid paraffin to minimise evaporation. The P.C.R. conditions were as follows: 30 cycles of; 94°C for 1.5 minutes, 50°C for 1.5 minutes and 72°C for 2 minutes with the last cycle being 72°C for 7 minutes. The P.C.R. product was purified using the Qiaquick gel-extraction spin-columns (Hybaid Ltd.) as previously described. However, if the P.C.R. product was around 100 base pairs (the spin columns' limit for small fragments) it was loaded onto a 5% polyacrylamide gel (29:1, acrylamide:bisacrylamide) in 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in sterile water) and ran at 150V in 1xT.B.E. until the marker dyes had separated (2-3 hours). The gel was stained in ethidium bromide (0.5μg/ml in 1x T.B.E.) and the P.C.R. product was excised and eluted from the gel overnight at room temperature in 1-2 volumes of elution buffer (0.5M ammonium acetate,

10mM magnesium acetate, 1mM EDTA, 0.1% SDS). The following day the DNA was recovered by adding 2 volumes of -20°C ethanol and centrifuging at 14, 000 rpm, 4°C for 30 minutes. The DNA was dissolved in 10µl of sterile water and the concentration was checked on a 1% agarose gel.

The second P.C.R. also contained 100ng of linearised pGEXcPk+Wild-type IκBα , 1µM of the "N-terminal" (5') primer **FOR** 5' - ATT GTA GGA TCC ATG TTC CAG GCG GCC GAG CGC - 3" (1µl of 100µM stock), 40ng of the P.C.R. product from the first cycle, 10x dNTP (10µl of a stock containing 0.5mM of each dNTP), 10x P.C.R. reaction buffer (10µl;x1(final): 10mM Tris-Cl pH8.8, 1.5mM $MgCl_2$, 50mM KCl and 0.1% Triton X-100) and 1µl of Dynazyme DNA polymerase (Finnzymes Ltd.)- this mixture was made to a total of 100µl with sterile water and then overlaid with 50µls of liquid paraffin to minimise evaporation. The P.C.R. conditions were as follows: 32 cycles of; 94°C for 1.5 minutes, 55°C for 1.5 minutes and 72°C for 2 minutes with the last cycle being 72°C for 7 minutes. The P.C.R. product was Qiaquick gel-extracted using spin-columns (Hybaid Ltd.) as previously described. The product (the mutant IκBα DNA) was then digested with the appropriate restriction endonucleases (BamHI and EcoRI) for 10-16 hours according to the vendors instructions. The digested products were purified as before and used for subsequent ligations.

1.20. DNA ligations.

Depending on the size of DNA fragments to be ligated, an approximate 3:1 (large DNA inserts, >500bp) to 15:1 (small DNA inserts, <100bp) molar ratio of insert to vector fragments were combined in a total of 10-20µl of ligation buffer (x1: 50mM Tris-Cl pH7.8, 10mM $MgCl_2$, 10mM DTT, 1mM ATP and 25mgml^{-1} BSA) where upon 200-1000U of T4 DNA ligase (New England Biolabs) were added and the ligation reaction incubated at 16°C for 10-16 hours. Half of the ligation reaction was used for subsequent transformation of competent bacteria.

1.21. Preparation of competent bacteria.

E. coli strains were made chemically-competent using $MgCl_2$ and $CaCl_2$ as follows: A 100ml bacterial culture was grown to an OD_{600} = 0.4-0.6, cooled on ice and centrifuged at 5000rpm in a Beckman JA20 rotor for 10 minutes at 4 °C. The pellet was gently resuspended in 10ml of ice-cold, sterile, 0.1M $MgCl_2$ and left on ice for approximately 3minutes. The suspension was centrifuged as above and the pellet resuspended in 2ml of ice-cold, sterile, 0.1M $CaCl_2$.The competent bacteria were used for transformation after approximately 1hour and were optimally competent 12-24 hours after preparation.

1.22. Transformation of competent bacteria.

200µl of competent cells were incubated with 10µl of ligation reaction, on ice, for 30-60 minutes with periodic mixing end-over-end. 1ng of pUC19 or no DNA were added to competent cells as positive and negative controls respectively (typically 10^6 to 10^7 transformants per µg of DNA was observed). After incubation, the bacteria were heat-shocked for 1.5 minutes at 42°C and returned to ice for 5 minutes. The transformation mix was added to 0.8ml of LB-broth and incubated at 37°C for 1hour, to allow expression of ß-lactamase for ampicillin selection, after which the transformed bacteria were plated on LB-agar containing relevant antibiotics.

1.23. Colony hybridisation.

A 9cm diameter nylon membrane (Hybond-N, Amersham International) was placed on top of the agar plate containing the colonies arising from the transformation of DNA ligation reaction into competent *E. coli* . The membrane was left to moisten for approximately 3 minutes, carefully peeled off the plate and placed, for 2 minutes, colony-side up onto a 3MM Whatman filter pre-soaked in lysis solution (10% SDS). The membrane was then transferred for 2 minutes colony-side up, onto a 3MM Whatman filter pre-soaked in denaturing buffer (0.5M NaCl, 1.5M NaCl). Next, the membrane was again transferred colony-side up onto a 3MM filter pre-soaked in neutralisation buffer (1M Tris/HCl, 1.5MNaCl) for 5 minutes, this step being repeated once more. Once the neutralisation step was completed the nylon membrane was washed in 2xSSC (0.3M

NaOH, 0.03M Na citrate). The denatured DNA was cross-linked onto the membrane by illuminating the lysed colonies with U.V light for approximately 2-3 minutes. The membrane was then incubated in pre-hybridisation buffer (6xSSC, 5x Denhardts, 0.1% SDS and 50μg/ml single stranded DNA) for 4 hours at 55°C. A radiolabelled oligonucleotide was prepared by mixing 100ng (2μl) of oligonucleotide, 2μl 10x kinase buffer, 3.5ml γ^{32}P ATP and 11.5μl sterile H_2O and incubating at 37°C for 30 minutes. The labelled solution of oligonucleotide was diluted by adding it to 10ml of hybridisation buffer (6xSSC, 5x Denhardts, 0.1% SDS and 50μg/ml single stranded DNA) and the incubation was continued overnight at 55°C. The following day the filter was washed as follows: 6x SSC, 0.1% SDS for 30 minutes at 50°C, 2x SSC, 0.1% SDS for 30 mins at 50°C, 0.2x SSC, 0.1% SDS for 30 minutes 50°C, 0.2x SSC, 0.1% SDS for 30 minutes at 50°C and 2x SSC briefly. The filter was exposed to X-ray film overnight at -70°C.

1.24. Preparation of plasmid DNA.

Small-scale preparations of DNA, mini-preps., were prepared using the alkaline-lysis method of Birmboim and Doly (1979). Large-scale preparations, maxi-preps., were prepared by utilising Qiagen DNA maxi-prep. ion- exchange columns (Hybaid Ltd.) as used according to the manufacturer. Quality and quantity of DNA was analysed by spectrophotometric readings at 260nm & 280nm and by visualisation on an agarose-gel (Manniatis *et al* .).

1.25. DNA sequencing.

DNA sequencing was performed on double stranded plasmid DNA templates using the dideoxy chain-termination procedure. Plasmid DNA for sequencing was prepared using Qiagen plasmid preparation columns (Hybaid Ltd.), as above. 5μg of plasmid DNA per primer-reaction were sequenced using the Sequenase® version 2.0 sequencing kit (Amersham International), [α^{35}S]dATP (10^3 Ci.mmol^{-1}; Amersham International) and used exactly according to the vendor's recommendations.

1.26. Glutathione-agarose and FPLC Mono-Q column purification of IκBα c-tag from bacterial culture.

A.Triton X-100 method.

A single bacterial colony from a Luria agar plate containing 100μg/ml ampicillin and 12.25μg/ml tetracycline was used to inoculate 50ml of LB-broth containing 100μg/ml amp and 12.25μg/ml tet overnight at 37°C with shaking. 25 -50ml of the overnight culture was then added to 500ml of LB-broth (tet/amp) and incubated with shaking at 37°C until an OD_{600nm} of 0.4-0.6 was reached. The culture was induced with 1mM IPTG for 3-4 hours at 25°C with shaking. The cells were harvested by centrifugation at 10,000rpm, 4°C on a JA14 Rotor in a Beckman J2-21 Centrifuge for 10 minutes and resuspended in 30ml PBS, 0.5MNaCl, 2mM EDTA, 1mM PMSF (Sigma) and 2mM benzamidine (Sigma). The cells were sonicated 6x30 second pulses (and placed on ice between sonications) until homogenous. 10% Triton X-100 was added to a final concentration of 1% and the mixture was centrifuged for 30 minutes at 17,000 rpm, 4°C on a JA17 or JA20 rotor in a Beckman J2-21 Centrifuge. Both the pellet and supernatant were retained.

The supernatant was loaded in a cold room at 4°C onto a 10ml glutathione agarose column (prewashed with 1 column volume of 0.2% NaOH and 6 column volumes of PBS/0.5M NaCl) using a pump at a flow rate of 1ml/min. The column flow-through was retained and the column washed with 2 column volumes of PBS, 0.5MNaCl, 2mM EDTA, 1mM PMSF and 2mM benzamidine. The GST-protein was eluted with 10mM glutathione, 0.5M NaCl and 50mM Tris/HCl pH8.0 and collected in 20x1ml fractions.The protein peak was found using a Bradford's assay (see next method) and an aliquot of each fraction was ran on a 10% SDS polyacrylamide gel and stained with Coomassie Bluestain (2% PAGE blue 83, 40% methanol, 10% acetic acid) to check for purity.The peak fractions were pooled and treated with thrombin (from Sigma, 0.5 units/0.24mg of GST-IκBα) for 1-2 hours at 25°C. The reaction was stopped with 1mM PMSF.

The thrombin cleaved GST and IκBα were diluted by half into 50mMTris/HCl (filtered) and then separated on an FPLC Mono-Q column across a salt gradient ranging from 250mMNaCl in 50mMTris/HCl pH8.0 (filtered) to 1M NaCl in 50mM Tris/HCl pH8.0 (filtered). 30x1 ml fractions were collected and an aliquot of each of the peak protein samples were checked

on a 10% SDS polyacrylamide gel (as described in the next method). The concentration of each of the peak fractions to be retained was estimated using the A_{280} in a quartz cuvette, whereby 1OD=2mg/ml protein. The protein peaks were then aliquoted and stored at -70°C.

B.Triton X-100 and Sarkosyl method.

Overnight cultures were grown, induced with IPTG and harvested by centrifugation as for the Triton X-100 method. After centrifugation the cells were resuspended in 30 ml PBS, 0.5M NaCl, 2mM EDTA, 1mMPMSF, 2mM benzamidine and 100µg/ml of lysozyme (Sigma) and incubated on ice for 15 minutes. This was followed by the addition of dithiothreitol (DTT) to 5mM, N-laurylsarcosine to 1.5%, $MgSO_4$ to 1M and DNase (NEB) to 2mg/ml. This mixture was incubated at 37°C for 15 minutes. The lysate was then clarified by centrifuging for 25-30 minutes at 14, 000 rpm, 4°C on a JA17 or JA20 rotor in a Beckman J2-21 centrifuge. The supernatant and pellet were both retained. The supernatant was transferred to a clean tube and Triton X-100 was added to a final concentration of 2% and EDTA to a final concentration of 8.2mM. The mixture was then centrifuged at 14, 000 rpm, 4°C for 10 minutes on a JA14 rotor in a Beckman J2-21 centrifuge.

The supernatant was diluted 1 part supernatant to 5 parts PBS, 0.5M NaCl, 5mM DTT and 1% Triton X-100. The diluted sample was recirculated overnight on a 25ml glutathione agarose column (prewashed with 1 column volume of 0.2% NaOH and 6 column volumes of PBS/0.5M NaCl) in a cold room at 4°C using a pump set at a flow rate of approximately 1ml/minute. The following morning the column was washed with 100mls PBS, 0.5M NaCl and then the protein was eluted in 20x5ml fractions with 10mM glutathione, 0.5M NaCl and 50mM Tris/HCl pH8.0. The remainder of the procedure was carried out as for the Triton X-100 method.

1.27. Quantitation of protein.

Protein concentrations were determined using either the method of Bradford (1976) or the BCA protein assay system as purchased from Pierce. The Bradford's assay was performed on protein samples where DTT was present, as this interferes with the BCA assay. For the Bradford's assay protein samples of 10ml were mixed with 990ml of Bradford's reagent (100mg coomassie

blue G250, 100ml orthophosphoric acid and 50ml of 100% ethanol made up to 1 litre with distilled water), incubated at room temperature for 5 minutes and the absorbance at 595nm was measured on an LKB Biochrom Ultrospec II spectrophotometer. Protein absorbances were converted to mg/ml concentrations using a standard curve constructed by measuring the absorbances of a range of bovine serum albumin (BSA) concentrations after incubating with Bradford's reagent. The BCA assay was used according to the microtiter plate method in the manufacturers instructions (the BSA standards used for the Bradford's assay were also used for the BCA assay).

1.28. SDS-PAGE and Western blot analysis.

Protein samples were resuspended in disruption buffer (x1; 2% w/v SDS, 5% v/v ß-mercaptoethanol, 2.5% v/v glycerol and 0.25 % w/v bromophenol blue) boiled for 5 minutes and separated through a 10-12% SDS-polyacrylamide gel (29:1, acrylamide: bisacrylamide) using thin (0.75 mm) mini-slab gels of the Bio-Rad mini-protean II electrophoresis system (gel composition varied slightly for some methods but this has been indicated). Separated polypeptides were transferred (transfer buffer for semi-dry blotting- 48mM Tris, 39mM glycine, 20%v/v methanol and 0.0375% SDS. Transfer buffer for wet blotting- 192mM Tris, 25mM glycine, 20%v/v methanol) onto nitrocellulose or P.V.D.F. filters using a semi-dry gel electroblotter (CBS Scientific Co.) or a Wet-blotter (Biorad Systems). The filters were blocked with PBS containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween 20 then incubated with monoclonal or polyclonal antibodies diluted 1:200 to 1:1000 in blocking buffer. After washing in blocking buffer, bound antibodies were detected using HRP-conjugated anti-mouse (for monoclonal primary antibodies) or anti-rabbit (for polyclonal primary antibodies) antibodies (Amersham International) diluted in blocking buffer, washed as above, then developed by the enhanced chemiluminiscence (ECL; Amersham International) protocol according to the manufacturers recommendations.

1.29. Stripping of nitrocellulose or P.V.D.F. membranes.

The membranes were transferred to strip buffer (0.15ml β-mercaptoethanol, 2.00ml 20% SDS, 1.25ml 1M Tris pH6.7, 16.6ml sterile H_2O) and incubated in a hybridisation oven at 72°C for 30 minutes. The membranes were washed x1 quickly (5 mins) and x3 (15 mins) in 250ml PBS containing 0.1% (v/v) Tween 20. The membranes were blocked for 1 hour in PBS containing 5% skimmed milk powder and 0.1% (v/v) Tween 20 and the membranes were probed as explained in the previous method. N.B. after E.C.L. development the membranes may be soaked in PBS containing 0.1% (v/v) Tween 20 and 0.1% NaN_3 and sealed in a polythene bag at 4°C.

1.30. Preparation of a radioactively labelled double stranded DNA probe.

Oligonucleotides were 5' end labelled using the T4 polynucleotide kinase (NEB). 5pmoles (1 µl) of the bottom strand of an oligonucleotide representing the κB motif present in the HIV-1 enhancer was mixed with 1µl 10x kinase buffer (200mM Tris/HCl pH 7.6, 0.1$MgCl_2$, 10 mM dithiothreitol, 1mM spermidine HCl, 1mM EDTA (pH 8.0)), 20µCi $\gamma(^{32}P)$ATP (2µl from a stock of 3000 Ci/mmol, Amersham International), 10 units of T4 polynucleotide kinase (NEB-2µl from a stock of 10, 000 Units/ml) and 4µl sterile water and heated to 100°C for 2 minutes. Immediately after heating 5 pmoles (1µl) of the top κB oligonucleotide strand was added to the mixture. This was allowed to cool over 2 hours in a water bath. DNA dyes (0.25% bromophenol blue, 30% glycerol in sterile water) were added and the mixture was ran on a 12% polyacrylamide (29:1 acrylamide:bisacrylamide) gel. X-ray film was exposed to the gel for 3 minutes in order to detect the position of the radioactively labelled DNA probe. The appropriate gel band was cut out and eluted overnight in 200µl of TEN_{100} at room temperature. The probe was decanted the following morning and stored at 4°C.

(κB 16 - mer Oligonucleotide = 5'-CTGGGGACTTTAGG-3')

1.31. Gel electrophoresis DNA binding assay.

1µl of diluted sample (p65-amounts given in results and discussion) was added to 19µl of binding buffer (85mM NaCl, 8.5% v/v glycerol, 22mM Hepes pH 7.5, 1.3 mg/ml BSA, 0.17%

NP-40, 3.6mM spermidine, 0.85mM DTT, 0.85mM EDTA, 6.1mM $MgCl_2$) which contained a ^{32}P radiolabelled double-stranded 16-mer κB oligonucleotide 5'-CTGGGGACTTTCCAGG-3' (top strand) derived from the human immunodeficiency virus type 1 enhancer. This mixture was left at room temperature for 15 minutes after which the IκBα inhibitor protein was added. This was left for a further 15 minutes at room temperature. 4μl of dye mix (50% glycerol, 10mM Hepes pH 7.5, 0.1% bromophenol blue) was added and each sample was applied to a 6% non-denaturing polyacrylamide (44:0.8, acrylamide bisacrylamide) before electrophoresis at 200Vfor approximately 1 hour. The gel was dried onto DEAE paper and exposed to X-ray film overnight at -70°C to determine the positions of the radioactive species.

1.32. In Vitro phosphorylation assay using recombinant Casein kinase I and II.

Casein kinase assays were performed on mutant and wild-type IκBα c-tag purified proteins, BSA (used as a negative control) and purified casein kinase (Sigma-used as a positive control). The casein kinase I reaction mix was prepared as follows for 10 reactions; 10μl 10x casein kinase I reaction buffer (10x-50mMTris/HCl pH7.5, 10mMMgCl_2, 5mMDTT), 1.18mMATP (0.2μl of a 100mM stock), 4μCi of γ(^{32}P)ATP (0.4μl from a stock of 3000 Ci/mmol, Amersham International) and 6,300 units of casein kinase I (NEB-6.3μl from a stock of $1x10^6$ Units/ml). 1.69μl of this mix was added to each of 10 tubes containing 0.22 pmoles of the appropriate protein (protein dilutions were made in 20mM Tris/HCl pH8.0, 0.5MNaCl) in a volume of no greater than 8.31μl. The final mixture of protein and casein kinase I reaction mix was made up to a total of 10μl with 20mM Tris/HCl pH8.0, 0.5MNaCl and incubated at 30°C for 1 hour. The reaction was quenched in each tube by the addition of 5μl of disruption buffer and the samples were boiled for 2-3 minutes. The contents of each tube were loaded onto a 10% polyacrylamide gel (29:1, acrylamide: bisacrylamide) and ran at 150V for 1 hour. The gel was dried onto DEAE paper and exposed to X-ray film overnight at -70°C to determine the positions of the radioactive species.

The casein kinase II reaction mix was prepared for 10 reactions as follows; 10μl 10x casein kinase II reaction buffer (10x-20mMTris/HCl pH7.5, 50mMKCl, 10mMMgCl_2), 1.18mMATP (0.2μl of a 100mM stock), 4μCi of γ(^{32}P)ATP (0.4μl from a stock of 3000

Ci/mmol, Amersham International) and 210 units of casein kinase II (NEB-0.42µl from a stock of $0.5x10^6$Units/ml). 1.1µl of the casein kinase II reaction mix was added to each of 10 tubes containing 0.22 pmoles of the appropriate protein (protein dilutions were made in 20mM Tris/HCl pH8.0, 0.5MNaCl) in a volume of no greater than 8.9µl. The final mixture of protein and casein kinase I reaction mix was made up to a total of 10µl with 20mM Tris/HCl pH8.0, 0.5MNaCl and incubated at 30°C for 1 hour. The reaction was stopped with 5µl of disruption buffer and the samples were processed as for the casein kinase I samples.

1.33. Protein association experiment.

1mg of GST fusion protein (GST-p65 or GST-p50) was dialysed overnight against PBS, 0.5M NaCl in a cold room at 4°C. 0.4mls of Glutathione-agarose (Sigma) was centrifuged in a 1.5 ml eppendorf tube at 14, 000 rpm for 30 seconds on a microfuge. The supernatant was removed and the beads were washed once with PBS, 0.5M NaCl. The supernatant was removed and the overnight dialysed protein sample or 0.5mg of GST were added to the beads and rotated in a cold room at 4°C for 2-3 hours. The mixture was then centrifuged for 1 minute in a microfuge at 14, 000 rpm and the supernatant was removed followed by x5 washes with PBS, 0.5M NaCl. The beads with bound GST protein were then stored at 4°C in 0.2ml of PBS, 0.5M NaCl, 0.1% sodium azide.

40µl of the agarose beads with bound GST protein in PBS, 0.5M NaCl, 0.1% sodium azide (i.e. 20µl of beads) were pipetted into an eppendorf tube and centrifuged for 1 minute at 14, 000 rpm in a microfuge. The supernatant was discarded and the beads were washed with 1ml of Solution A (for the assay with an [NaCl] variant :- 12mMTris/HCl pH8.0, 1mg/ml protease-free bovine serum albumin (PF- BSA), 0.1% NP-40 and either 60mM, 120mM, 240mM, 480mM or 960mM NaCl. For the assay with pH as the variant :- 12mMMES (free acid) pH5.5, 6.0 or 6.5 or 12mMBis/Tris/propane pH6.5, 7.0, 7.5, 8.0, 8.5 or 9.0, 1mg/ml protease-free bovine serum albumin (PF- BSA), 0.1% NP-40 and 60mM NaCl). The supernatant was discarded and the following components were added to the remaining 20µl of beads: 20µl of 10mg/ml BSA, 160µl of Solution B (12mM Tris/HCl, 0.1%NP-40 and either 60mM, 120mM, 240mM, 480mM or 960mM NaCl) and 2µg of protein sample. This was rotated for 1-1.5 hours at room temperature

and then 1ml of solution A was added. The mixture was centrifuged for 1 minute at 14, 000 rpm, the supernatnt was removed and the beads were washed x4 with 1ml of solution A. This was followed by x2 washes with 1ml of solution B. The supernatant was removed and the beads were resuspended in 20µl of sterile water and 20µl of disruption buffer. The sample was then boiled for 2-3 minutes, centrifuged at 14, 000 rpm for 1 minute and 5µl of the supernatant was ran on a 10-12% polyacrylamide gel.

1.34. Preparation of cationic liposomes for DNA transfection.

Lipofectamine for transfection of Cos 7 cells was purchased from Gibco-BRL. However, lipids for transfection of 293's were prepared using the following procedure , kindly given by R. Elliott (MRC Virology, Glasgow) and yields a lipid mixture equivalent to Gibco-BRL's TransfectACE. Briefly, 1ml DOPE (dioleoyl L-a-phosphatidyl ethanolamine, Sigma) was pipetted into a glass universal where-upon 4mg of DDAB (dimethyldioctadecyl ammonium bromide, Sigma), w/w ratio of 1:2.5 DDAB:DOPE, were added and briefly vortexed till dissolved. The chloroform was evaporated using a stream of nitrogen whilst rotating the universal to ensure an even film of lipid dispersed around the walls. The dried lipids were resuspended in 10ml of sterile distilled water by a 10-15 minute sonication in a sonicating waterbath. Once resuspended, the turbid mixture was sonicated with a soniprobe (15 microns amplitude) using 30 second pulses and incubating on ice in between, until clear (approximately 20-40 minutes). Lipids were tested against previous batches for transfection efficiency using a ß-galactosidase expressing plasmid, pCT7ßgal , then stored in 500µl aliquots at 4°C.

1.35. Staining protocol for detection of ß-galactosidase expressing cells.

Transfected cells were washed once with PBS then fixed for 5 minutes at room temperature by the addition of 1ml of fixative (2% formaldehyde, 2% glutaraldehyde in PBS) for 3.5cm diameter dishes. The fixative was removed and the cells washed with PBS. 1ml of freshly prepared staining solution (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$ and 0.5mgml^{-1} X-gal, in PBS) was added to the washed cells which were then incubated at 37°C for approximately 4 hours or overnight.

1.36.Transient DNA transfections.

Expression from the human CMV I.E. promoter/enhancer.

Mammalian cells were passed into 25cm^2 flats or individual wells of a 6-well plate to obtain 60-70% confluency the next day. Plasmid DNA was introduced into cells using a liposome-mediated procedure. Briefly, for one 3.5 cm diameter well, 1-3μg of DNA was added to 0.5mls of optimem 1 (Gibco-BRL) and gently mixed. 10μl of the liposome mixture was added to the DNA/optimem 1, gently mixed, briefly vortexed and left to stand for 10-15 minutes at room temperature. The volume of the liposome/DNA mixture was adjusted to 1 ml by the addition of 0.5ml of optimem 1 (All steps were carried out in polystyrene tubes due to the affinity of the liposomes for polypropylene). The optimem 1/liposome/ DNA mixture was left on the cells for 12-16 hours after which the cells were washed once with GMEM-10F and incubated for a further 24-72 hours in GMEM-10F.

1.37. Coupling of SV5-P-k (Anti-Pk) antibody to Protein A Sepharose.

250mg of Protein A sepharose beads were swollen in 2ml PBS, 0.1% sodium azide (this yielded a bed volume of 1ml of beads). One hour later 500μl of Protein A sepharose beads were mixed with 1ml of Anti-Pk for 2 hours at room temperature (using a rotator). The mixture of beads and antiserum were centrifuged at 2500rpm on a benchtop centrifuge for 5 minutes, then the supernatant was removed and the beads+bound Anti-Pk were washed x2 in 200mM borate buffer, 3M NaCl pH9.0. After the second wash the beads+bound Anti-Pk were resuspended in 1ml of 20mM dimethylpimelimidate (in 200mM borate buffer, 3M NaCl pH9.0-prepared fresh) and rotated for 30 minutes at room temperature. The beads were then centrifuged for 5 minutes at 2500rpm on a benchtop centrifuge and the supernatant was removed. This was followed by x2 washes in 200mM borate buffer, 3M NaCl pH9.0 and x1 wash in 200mM ethanolamine. The beads were resuspended in 1ml of 200mM ethanolamine and rotated for 2 hours at room temperature followed by centrifugation at 2500rpm for 5 minutes in a benchtop centrifuge. The beads were then washed x2 in PBS, x2 in 200mM glycine (pH2.5), x2 in PBS and the beads were stored in 500μl of PBS+0.1% sodium azide.

1.38. Immunoprecipitation using the Protein A Sepharose beads coupled to antiserum.

200µl of lysis buffer (see preparation of cytoplasmic extracts - no protease inhibitors) were added to 20µl (i.e.10µl of bed volume) of Protein A sepharose beads (Sigma-1.5g of beads/12ml PBS/0.1% sodium azide) coupled to anti-Pk. 50-100µg of cell lysate were added to the beads and buffer and the mixture was rotated for 90 minutes at room temperature. The beads were then centrifuged for 1-2 minutes on a benchtop centrifuge at 14, 000 rpm and the supernatant was removed. The beads were washed x3 with lysis buffer and then resuspended in 10µl of disruption buffer (x1; 2% w/v SDS, 5% v/v ß-mercaptoethanol, 2.5% v/v glycerol and 0.25 % w/v bromophenol blue) and boiled for 10 minutes. The samples were then ready to be loaded on a polyacrylamide gel.

1.39. Luciferase assays.

Cos 7 or 293 cells were grown to 50% confluency in 25cm^2 flats and co-transfected with 1µg (for Cos7s) or 5µg (for 293s) of an NF-κB dependent (3EnhConALuc) or independent (LTRΔκBLuc) luciferase reporter vector and an equal amount of the mutant or wild-type IκBα in pcDNA3cPk plasmid using lipofectamine (for Cos7s) or cationic liposomes (for 293s). Fourty hours later the cells were activated with TNFα (10ng/ml final in medium) for eight hours and then harvested as follows; the cells were washed once with PBS and then trypsinised (for Cos 7s) or treated with versene (for 293s). This was neutralised with GMEM-10 and the cells were pipetted into eppendorfs and centrifuged at 1-2,000 rpm, 4°C for 4 minutes. The supernatant was discarded and the cells were washed once with 1ml of ice cold PBS followed by lysis in 200µl of luciferase lysis buffer (25mMTris phosphate pH7.8, 8mMMgCl$_2$, 1mMDTT, 1% Triton X-100, 15% glycerol) to yield the cytoplasmic extract. Following a thorough vortex 100µl of the cytoplasmic extract was removed and added to 100µl of luciferin buffer (1mM ATP, 0.25mM luciferin, 1% BSA prepared in luciferase lysis buffer) and the luciferase activity was immediately measured using a luminometer (Lumat LB 9501, Berthold). A Bradfords' assay was performed on the remainder of the extract to determine the protein concentration. The results were expressed as relative luciferase units (RLU) per µg of protein. This was determined by subtracting the

background signal from each of the luminometer readings and divising this figure by the amount of cytoplasmic protein contained in the sample.

1.40. Signal-Induced Degradation Assay for IκBα c-tag.

293 cells were grown on 35mm 6 well plates until 50% confluent and then transfected for 12-15 hours with 1-3μg of mutant or wild-type IκBα in pcDNA3cPk as described previously for transient transfections. 36 hours after the transfection had begun the cells were pretreated for 5 minutes with 100μg/ml cycloheximide (Sigma). The cells were then either induced (cycloheximide was maintained in culture until the end of the induction) with 10ng/ml TNFα for 15 or 45 minutes or not induced with TNFα at all. The cells were harvested directly into 125μl lysis buffer (50mM sodium phosphate buffer pH 7.5, 0.5% NP40 and the protease inhibitors (added immediately before use) - 1μM leupeptin, 1μM pepstatin, 1mM pefablock, 20μM TPCK, 40μg/ml bestatin). The cells were centrifuged at 6,000 rpm, 4°C for 10 minutes. The supernatant was removed as the cytoplasmic extract and a BCA assay was used to determine the protein concentration of each sample. 10-20μg of protein was loaded in disruption buffer onto a 10-12% polyacrylamide gel (29:1 acrylamide: biacrylamide) and then a Western blot was carried out as previously described (membranes were probed with either the monoclonal Anti-Pk or 10B antibody).

1.41. Signal-Induced phosphorylation assay of IκBα c-tag.

293 cells were grown as for the degradation assay on 35mm 6 well plates until 50% confluent and then transfected for 12-15 hours with 1-3μg of mutant and wild-type IκBα + pcDNA3cPk as described previously for transient transfections. 36 hours after the transfection had commenced the cells were pretreated with 25μM z-LLL-H for 15 minutes (this was maintained until the end of the culture). The cells were then either induced with 250nM Okadaic acid (OKA from Sigma) alone for 45 minutes or with 250nM Okadaic acid for 45 minutes followed by 10ng/ml TNFα for 15 minutes or not induced with OKA or TNFα. The cells were harvested into 125μl of lysis buffer (as above but with the following phosphatase inhibitors added-50mM sodium fluoride, 5mM tetra sodium pyrophosphate, 1mM sodium orthovanadate, 10 mM β-glycerophosphate and

2 mM EDTA) and centrifuged at 6,000 rpm, 4°C for 10 minutes. The supernatant was removed as the cytoplasmic extract and a BCA assay was used to determine the protein concentration of each sample. 20-30μg of protein was loaded in disruption buffer onto a large 16.5% polyacrylamide/9.4% glycerol gel (48:1.5, acrylamide:bisacrylamide and gel buffer-3MTris/HCl pH8.45, 0.3% SDS) ran in a buffer gradient (Anode buffer-0.2M Tris/HCl, pH9.0. Cathode buffer-0.1M Tris, 0.1M Tricine, 0.1% SDS, adjusted to pH8.25) for 8 hours at 100Vfor the first hour and then 150V thereafter. The gel was then blotted overnight at 50mA on a semi-dry blotter or for 4 hours at 70mA. The gel was probed with Anti-Pk and ECL developed as described previously.

1.42. Analysis of ubiquitination patterns of IκBα c-tag.

293 cells were grown in 35mm 6 well plates until 50% confluent and were transfected for 12-15 hours with 1-3μg of mutant and wild-type IκBα + pcDNA3cPk as described previously for transient transfections. 36 hours after the start of transfection the cells were pretreated with 25μM z-LLL-H for 15 minutes (this was kept in culture until the cells were harvested) and then either induced with 10ng/ml TNFα for 45 minutes or not induced with TNFα. The cells were harvested in 125μl of lysis buffer (with phosphatase inhibitors and fresh 10mM iodoacetamide). The iodoacetamide was quenched 30 minutes later with 10mM DTT and the extracts were centrifuged at 6, 000 rpm, 4°C for 10 minutes. The supernatant was removed as the cytoplasmic extract and a BCA assay was performed to determine the protein concentration. 100-200μg of extract in disruption buffer was loaded onto a 12.5% polyacrylamide gel (12.5% acrylamide: 1.0mg/ml bisacrylamide) and a Western blot was carried out as described previously to detect high molecular weight bands corresponding to covalently bound ubiquitin-IκBα complexes.

1.43. Circular Dichroism Spectroscopy.

CD spectra were recorded using a Jasco J-600 spectropolarimeter for a range of molecules corresponding to the C-terminal region of IκBα by Dr S. Kelly, Stirling University. These were as follows; a chemically synthesised peptide kindly supplied by Francoise Baleux, Institut Pasteur corresponding to amino acids 275-317 of IκBα and containing a chemical

blocking group ($CH_2NHCOCH_3$) at position 308 (a cysteine group which could cross-link molecules of the peptide if it remained unblocked) and three bacterially expressed, glutathione/FPLC purified C-terminal fragments of IκBα - amino acids 265-217 + c-tag (14 amino acids) represented as 265-317c-tag, amino acids 265-317 represented as 265-317 and amino acids 275-317 represented as 275-317.

The dried peptide was resuspended to a concentration of 0.5mg/ml in a solution of 50mM sodium fluoride and 10mM sodium phosphate ranging in pH from 6.0-8.0. The CD spectrum of the peptide at each pH was measured in the far ultraviolet (UV) region (190-260nm). At pH 7.0 the CD spectrum was also measured in the presence of 50% trifluoroethanol (TFE).

The three C-terminal fragments of IκBα, 265-317 c-tag (0.51mg/ml), 265-317 (0.2mg/ml) and 275-317 (0.2mg/ml) were dialysed overnight against 50mM sodium fluoride, 10mMsodium phosphate pH7.0, 0.5mM DTT. Additionally, 1ml aliquots of 265-317 and 275-317 were dialysed overnight against 50mM sodium fluoride and 10mM sodium phosphate pH5.6, pH6.4 (275-317 only), pH7.0 and pH8.0, 0.5mM DTT. Their CD spectra were measured in the far UV region (190-260nm) in the presence and absence of 50% TFE.

RESULTS

CHAPTER 1. CONSTRUCTION, EXPRESSION AND PURIFICATION OF WILD-TYPE AND MUTANT IκBα C-TAG PROTEINS.

Studies performed on deletion mutants of the human IκBα protein (note: there are several homologues of IκBα but in general human IκBα or MAD-3 is referred to as IκBα and it is the human form which is the subject of this study) had revealed that the highly negatively charged C-terminus was required for the association of IκBα with the p65 subunit of NF-κB and for inhibition of p65 DNA binding (Inoue *et al.*, 1992; Hatada *et al.*, 1993). These reports indicated that specific residues in the C-terminal region may be contributing to these functions but that the changes in activity observed in the deletion mutants had perhaps arisen from a conformational change in the mutated proteins. Therefore, it was reasonable to predict that the substitution (rather than deletion) of a smaller number of residues could reduce the probability of inducing a conformational change and may also allow a specific function to be assigned to these residues.

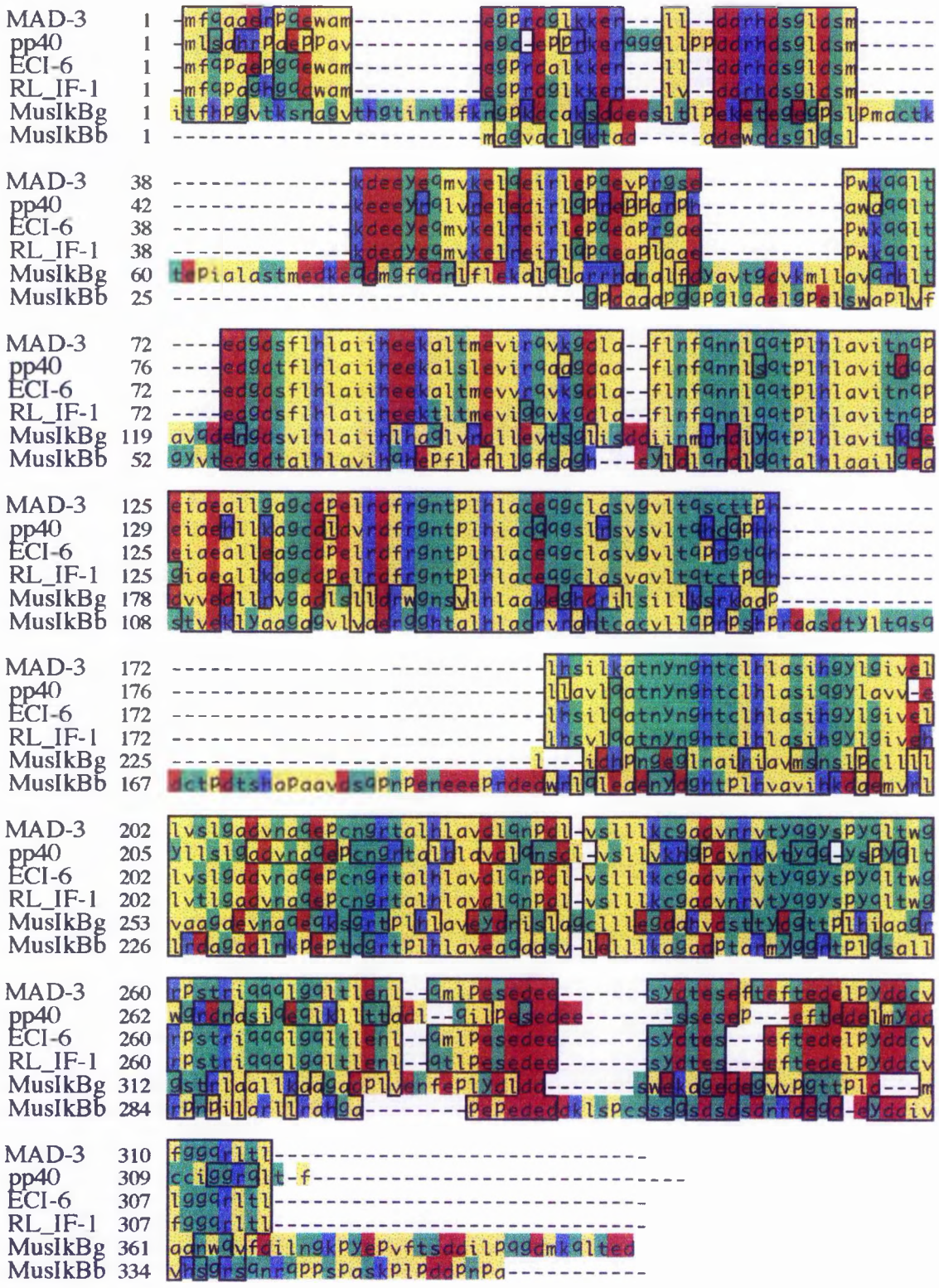
In order to identify suitable target amino acids in the C-terminus of IκBα for mutation, the areas of contact between the p65 subunit of NF-κB and IκBα had to be considered. It had been shown that IκBα could mask the nuclear localisation signal (NLS) of p65 (Ganchi *et al.*, 1992; Zabel *et al.*, 1993). In addition, it had also been suggested that IκBα may bind to this signal sequence since mutation of the 4 basic residues which compose the NLS abolished binding of IκBα (Beg *et al.*, 1992). Available evidence also implied that positive charges residing in the DNA binding region of the related v-Rel protein may contribute to the binding *in vitro* of the chicken homologue of mammalian IκBα, pp40 (Kumar and Gelinas, 1993). It was possible that the binding of IκBα to p65 involved an ionic interaction between the negatively charged C-terminal region of IκBα and the positively charged NLS and DNA binding region of p65. An ionic interaction between IκBα and p65 is supported by the finding that the anionic detergent deoxycholate can dissociate an inactive IκBα-NF-κB complex (Baeuerle and Baltimore, 1988b). Comparison of the C-terminal amino acid sequence of human IκBα (MAD-3) with the equivalent acidic regions in the

avian (pp40), porcine (ECI-6) and rat (RL/IF-1) homologues of IκBα and the related proteins, IκBβ and IκBγ revealed two distinct areas of negatively charged conserved residues, glutamic acid (E) 284, aspartic acid (D) 285 , glutamic acid (E) 286 and glutamic acid (E) 300, aspartic acid (D) 301, glutamic acid (E) 302 (figure 9). The location of E286 was analogous to that of E302 in that it was adjacent to aspartic acid at position 285 and then glutamic acid at position 284. These amino acids were therefore obvious candidates for mutagenesis and it was decided that amino acids 284-286 and 300-302 should be changed to either three serine (S) residues or their uncharged equivalents, glutamine (Q), asparagine (N), glutamine (Q). The nature of the altered amino acids were critical and "safe" amino acid substitutions were selected so that any disturbance of protein folding, both locally and globally, was minimised (Bordo and Argos, 1991). For this reason two different mutants were generated at each site to enable a comparison of the substituted residues effect (if any) on the function of IκBα.

In addition to the construction of C-terminal mutants of IκBα, a second set of mutants within the sixth ankyrin repeat or linker region of the protein were generated. This work followed a report on the protease sensitivity of IκBα and its sites of interaction with p65 (Jaffray *et al.*, 1995). While the central 5 ankyrin repeats and C-terminal domain of IκBα were found to be relatively resistant to proteolysis, the sixth, low-homology ankyrin repeat and the N-terminal region were both susceptible to proteolysis. However, in the presence of bound p65, proteolysis was blocked at tyrosine (Y) 251, tryptophan (W) 258 and glutamic acid (E) 275 in the low homology ankyrin repeat suggesting their involvement in the interaction between IκBα and p65. It was proposed that the sixth ankyrin repeat may exist as a flexible linker region between the highly structured 5 ankyrin repeats and the acidic C-terminal domain of IκBα. W258 and E275 were targeted for mutation and as previously described for the C-terminal mutants, amino acid substitutions were chosen to minimise disruption of IκBα's conformation. W258 was changed to tyrosine and E275 was substituted with either serine or glutamine.

Figure 9. Comparison of the amino acid sequence of human IκBα (MAD-3) with related proteins.

The deduced amino acid sequence of human IκBα (MAD-3) (Haskill *et al.*, 1991) compared with the deduced amino acid sequences of avian IκBα (pp40) (Davis *et al.*, 1991), porcine IκBα (ECI-6) (deMartin *et al.*, 1993), rat IκBα (RL/IF-1, regenerating liver inhibitory factor) (Tewari *et al.*, 1992), mouse IκBγ (MusIkBg) (Inoue *et al.*, 1992) and mouse IκBβ (MusIkBb) (Thompson *et al.*, 1995). Amino acids are numbered to the left of the figure. Dashes indicate spaces introduced to optimise sequence identity during alignment. The amino acids are colour coded according to their charge: Red, acidic (DE); blue, basic (HKR); yellow, non-polar (AILVPFWM) and green, uncharged polar (GSTYCNQ).


MAD-3
pp40
ECI-6
RL_IF-1
MusIkBg
MusIkBb

Both the C-terminal and linker IκBα c-tag (the origin and relevance of the c-tag region will be discussed in section 1.2) mutants are represented diagrammatically in figure 10.

1.1. Construction of IκBα c-tag mutant proteins.

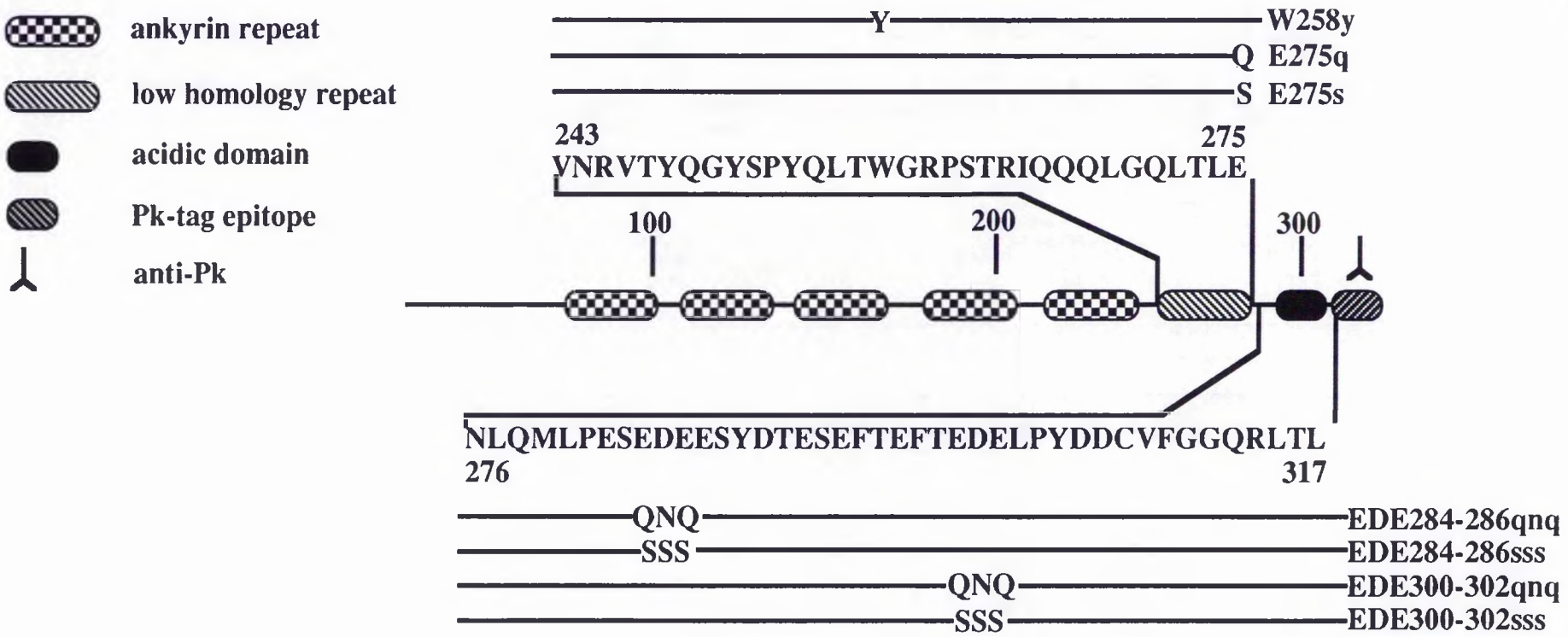
The C-terminal (EDE284-286qnq, EDE284-286sss, EDE300-302qnq, EDE300-302sss) and linker (W258y, E275q, E275s) IκBα c-tag mutant proteins were generated by the PCR mutagenesis technique (Landt *et al.*, 1990) represented schematically in Figure 11. This method utilised one internal mutagenic primer and two external "universal" primers (used for all mutants) in a two-step PCR (described in detail in the Materials and Methods section) as follows:

The first PCR contained the mutagenic primer and the 3' universal primer (this possessed an EcoRI restriction site) which were used to generate a partial length product from the linearised template DNA, pGEXcPk + wild-type IκBα (pGEXcPk will be described in section 1.2). The first PCR product was purified from contaminating primers, nucleotides and PCR by-products and then used as a primer for the second PCR in addition to the 5' universal primer (containing a BamHI restriction site) and the linearised template DNA, pGEXcPk + wild-type IκBα. The final C-terminal and linker mutant PCR products were analysed by separation through a 0.8% agarose gel and revealed that all had the expected size of approximately 951 base pairs (data not shown).

Finally, the mutant DNA was digested with the restriction endonucleases, BamHI and EcoRI, purified and ligated into the pGEXcPk vector (also digested with BamHI and EcoRI) and transformed into competent *E.coli* (XL-blue). Tetracycline and ampicillin resistant *E.coli* transformants were isolated and small scale preparations of their DNA (mini-preps.) were prepared and used as double stranded DNA templates to sequence the cDNA surrounding the sites of mutation (i.e. around the C-terminal region), the N-terminal region and some of the surrounding ankyrin repeats using the dideoxy-chain termination procedure. Figures 12 and 13 show the sequencing data obtained for the cDNA surrounding the areas of mutagenesis only (including the Pk tag region) for the C-terminal and linker mutants respectively and

Figure 10. Schematic representation of IκBα C-terminally linked to the epitope Pk-tag and derivative mutant proteins with amino acid changes in the linker and C-terminal regions.

Wild-type IκBα c-tag is shown with the amino acid sequences, 276 to 317 and 243 to 275 corresponding to the C-terminal and low homology ankyrin repeat or linker regions respectively. The amino acid changes performed in each of the regions to produce the mutant IκBα c-tag proteins are also indicated in addition to the ankyrin repeats (), low homology repeat (), acidic domain () (-this forms most of the C-terminal domain), the Pk-tag epitope () and the anti-Pk antibody ().


ankyrin repeat
low homology repeat
acidic domain
Pk-tag epitope
anti-Pk
Y
W258y
Q E275q
S E275s
243
275
VNRVTYQGYSPYQLTWGRPSTRIQQQLGQLTLE
100
200
300
NLQMLPESEDEESYDTESEFTEFTEDELPYDDCVFGGQRLTL
276
317
QNQ
EDE284-286qnq
SSS
EDE284-286sss
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SSS
EDE300-302sss

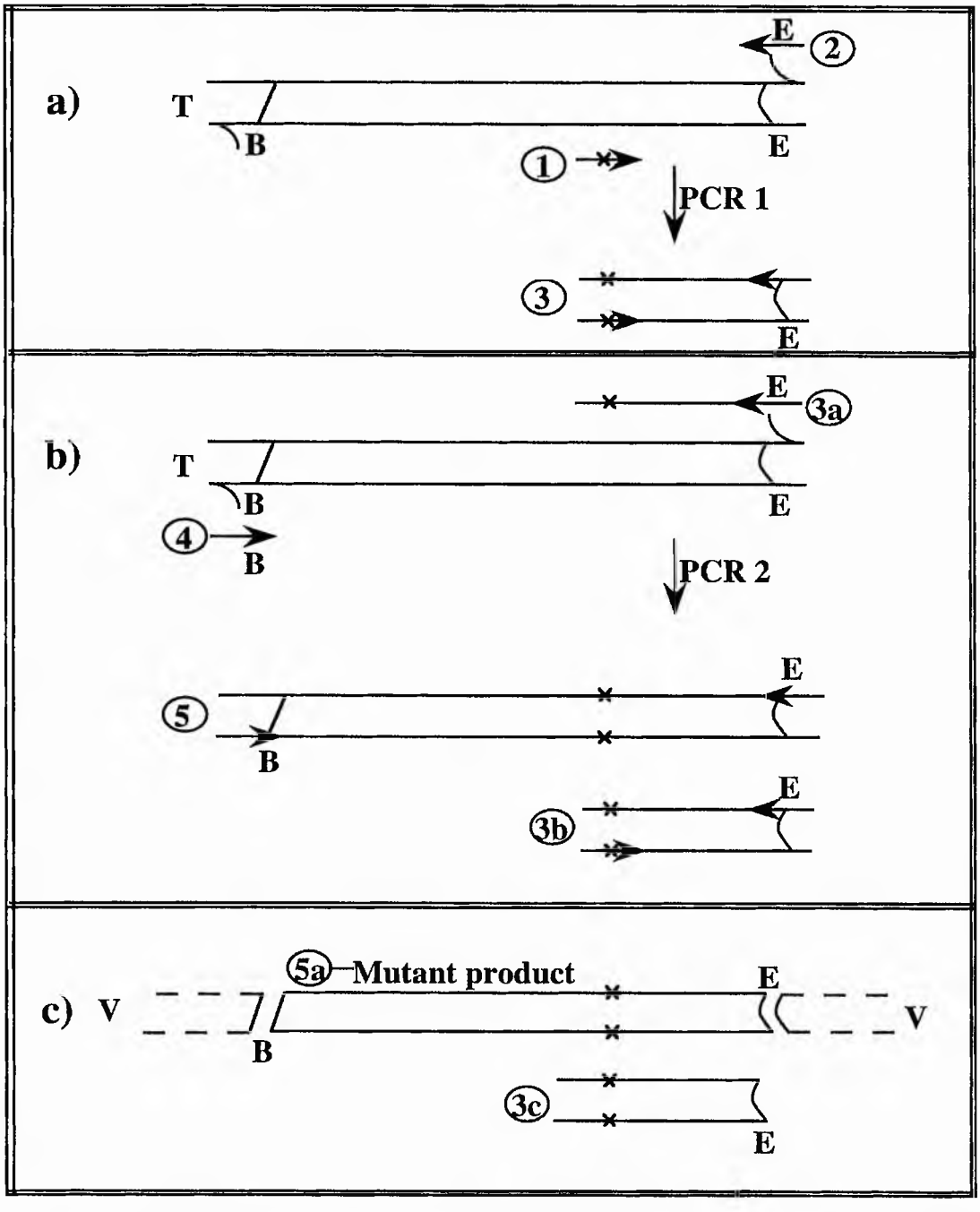
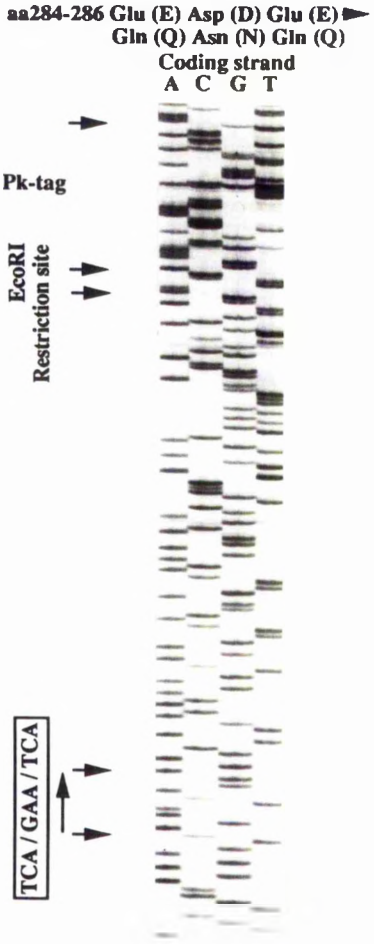


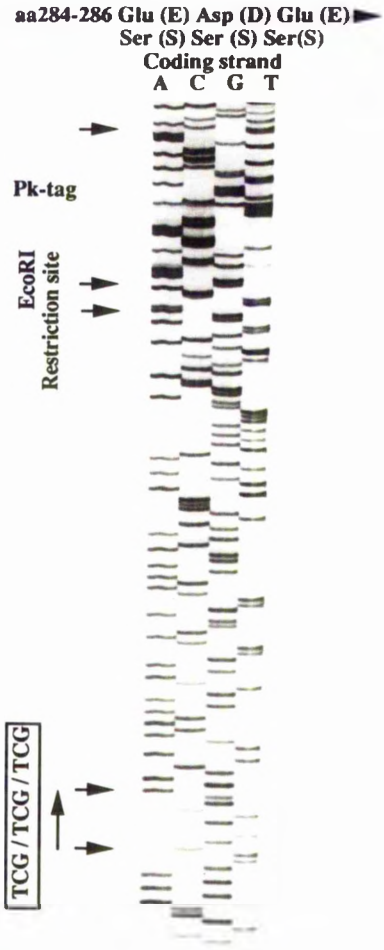
Figure 11. Schematic presentation of the PCR mutagenesis method.

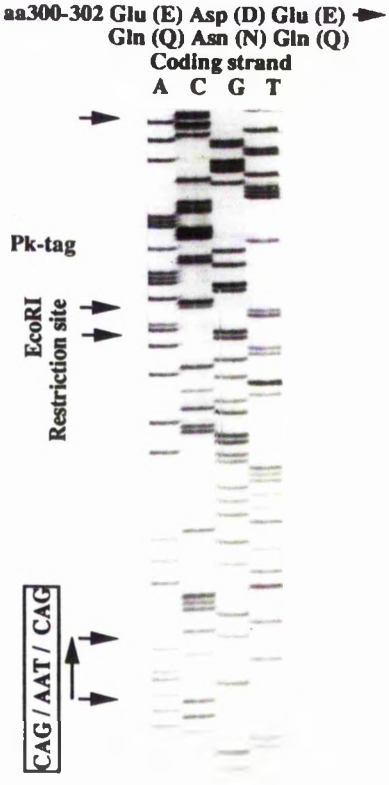
In the first PCR (**a**) the mutagenesis primer (**1**) and the 3' primer (**2**) (containing an EcoRI site **E**) were used to generate the product (**3**) from the template DNA (**T**). Following a purification step (**3**) was taken as the primer (**3a**) together with the 5' primer (**4**) (containing a BamHI site **B**) and the template DNA (**T**) in the second PCR **b**) to yield the products (**5**) and (**3b**). Product (**5**) was purified and in a final step **c**) the mutant gene product (**5a**) was obtained by cleavage of (**5**) with the restriction enzymes BamHI and EcoRI and ligated into the appropriate vector (**V**). Any possible contamination from product (**3b**) during subcloning in step **c**) was reduced because of the size dependent purification of product (**5a**) from an agarose gel before step **c**) and the absence of a BamHI restriction site in **3b**) which would yield a digested product (**3c**) non-viable for the subcloning step in step **c**).

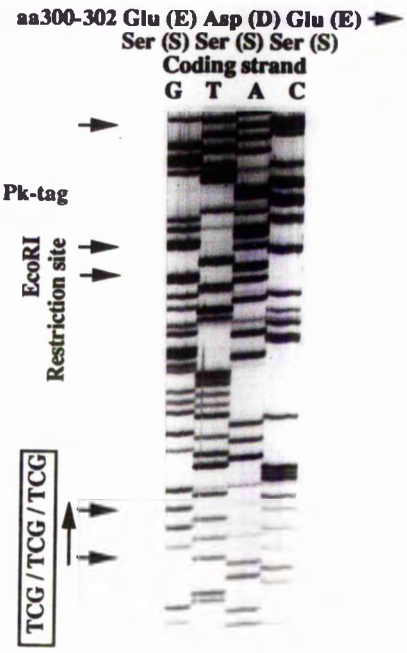
Figure 12. Sequence confirmation of the C-terminal IκB α c-tag mutants.

The dideoxy chain-termination procedure was used to yield the coding DNA strands shown opposite for each of the following C-terminal IκBα c-tag mutants (named at the top of each seguence): EDE284-286qnq i.e. the mutation of amino acids 284-286 where glutamic acid, aspartic acid and glutamic acid were changed to glutamine, asparagine and glutamine (translated from TCA/GAA/TCA); EDE284-286sss i.e. the mutation of amino acids 284-286 where glutamic acid, aspartic acid and glutamic acid were changed to three serine residues (translated from TCG/TCG/TCG); EDE300-302qnq i.e. the mutation of amino acids 300-302 where glutamic acid, aspartic acid and glutamic acid were changed to glutamine, asparagine and glutamine (translated from CAG/AAT/CAG) and EDE300-302sss i.e. the mutation of amino acids 300-302 where glutamic acid, aspartic acid and glutamic acid were changed to three serine residues (translated from TCG/TCG/TCG). The coding DNA sequences are read from bottom to top (5' to 3' on the coding DNA) and the lane location of each DNA base is shown at the top of the gel (A= adenine, C= cytosine, G= guanine and T= thymine). The position and sequence of each mutation is boxed. The DNA sequence for Pk-tag and the restriction site for EcoRI are also indicated.


aa284-286 Glu (E) Asp (D) Glu (E)
Gln (Q) Asn (N) Gln (Q)
Coding strand
A C G T
Pk-tag
EcoRI
Restriction site
TCA / GAA / TCA


aa284-286 Glu (E) Asp (D) Glu (E)
Ser (S) Ser (S) Ser(S)
Coding strand
A C G T
Pk-tag
EcoRI
Restriction site
TCG / TCG / TCG


aa300-302 Glu (E) Asp (D) Glu (E)
Gln (Q) Asn (N) Gln (Q)
Coding strand
A C G T
Pk-tag
EcoRI
Restriction site
CAG / AAT / CAG


aa300-302 Glu (E) Asp (D) Glu (E)
Ser (S) Ser (S) Ser (S)
Coding strand
G T A C
Pk-tag
EcoRI
Restriction site
TCG / TCG / TCG

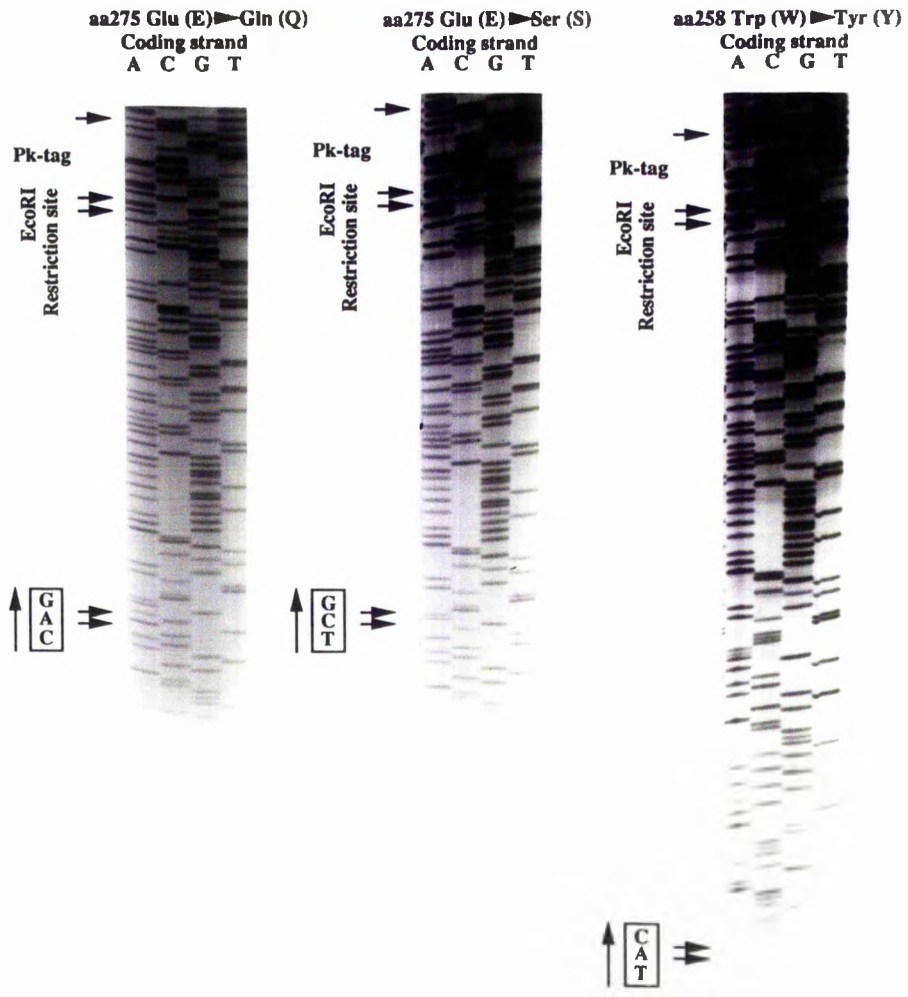


Figure 13. Sequence confirmation of the linker IκBα c-tag mutants.

The dideoxy chain-termination procedure was used to yield the coding DNA strands above for each of the following IκBα c-tag linker mutants (named at the top of each sequence): E275q i.e. the mutation of amino acid 275 where glutamic acid was changed to glutamine (translated from CAG); E275s i.e. the mutation of amino acid 275 where glutamic acid was changed to serine (translated from TCG) and W258y i.e. the mutation of amino acid 258 where tryptophan was changed to tyrosine (translated from TAC). The coding DNA sequences are read from bottom to top (5' to 3' on the coding DNA) and the lane location of each DNA base is shown at the top of the gel (A= adenine, C= cytosine, G= guanine and T= thymine). The position and sequence of each mutation is boxed. The DNA sequence for Pk-tag and the restriction site for EcoRI are also indicated.

indicates that the mutagenesis procedure had generated the expected products with no inadvertent mutations being produced over the region of readable sequence (approximately 150-200 base pairs). The N-terminal region of sequence and ankyrin repeats also showed no inadvertent mutations. It should be noted that the use of Pfu DNA polymerase (purchased from Stratagene) in the polymerase chain reactions minimised the possibility of DNA mismatches because of its 3' to 5' proofreading activity.

1.2 Expression and purification of wild-type and mutant C-terminal and linker IκBα c-tag (aa1-317) proteins in *E.coli.*

For the expression and purification of wild-type and mutant C-terminal and linker IκBα c-tag proteins in *E.coli* (XL-blues) the glutathione-S-transferase (GST)-fusion expression system was used (Smith and Johnson., 1988). The vector employed in this study was pGEXcPk (Hanke *et al.*, 1992) which was generated from the pGEX-2T expression vector (Promega) and allowed the production of high levels of protein with a cleavable N-terminally fused 26 kD enzyme, glutathione-S-transferase (GST) and a 14 amino acid C-terminally fused Pk-tag linker. Generally proteins fused to GST are more soluble in *E.coli* and can be easily recovered from bacterial lysates by affinity chromatography using glutathione bound to agarose. The C-terminally fused Pk-tag linker is recognised by a monoclonal antibody termed SV5-Pk (anti-Pk) and therefore allowed IκBα c-tag to be easily detected by Western blotting. Pk-tag was originally derived from amino acids 95-108 of the phospho (P) and V proteins of simian virus 5 (SV5) . The Pk-tag linker DNA and amino acid sequences are shown below in figure 14.

gly lys pro ile pro asn pro leu leu gly leu asp ser thr
GGA AAG CCG ATC CCA AAC CCT CTG CTG GGA TTG GAC TCC ACC
CCT TTC GGC TAG GGT TTG GGA GAC GAC CCT AAC CTG AGG TGG

Figure 14 The oligonucleotide linker coding for Pk-tag. Coding and non-coding DNA strands are shown and the amino acids in bold represent those derived from SV5 P and V proteins.

Screening for the over-expression of the 63 kD glutathione-S-transferase-wild-type and mutant IκBα c-tag fusion proteins from the pGEXcPk vector was carried out as follows: 1ml overnight cultures of the positively identified wild-type and mutant IκBα c-tag/pGEXcPk *E.coli* (XL-1 blue) clones (i.e. the clones which when sequenced exhibited the expected changes) were diluted the following day, 1:100, into 1ml of LB-broth and grown until an OD_{600nm} was reached. The cultures were then induced with IPTG for 3-4 hours and the cells were harvested by centrifugation, resuspended in PBS/0.5M NaCl and disruption buffer, boiled and then separated through a 10% polyacrylamide gel. An uninduced culture was included as a control. The coomassie blue stained gels were used to identify clones possessing a protein band of around 63kD (corresponding to the GST-IκBα c-tag fusion protein). 1ml overnight cultures of clones positive for the fusion protein were diluted 1:100 into 1litre of LB-broth and grown at 37°C to the appropriate density before induction with IPTG to yield the fusion protein.

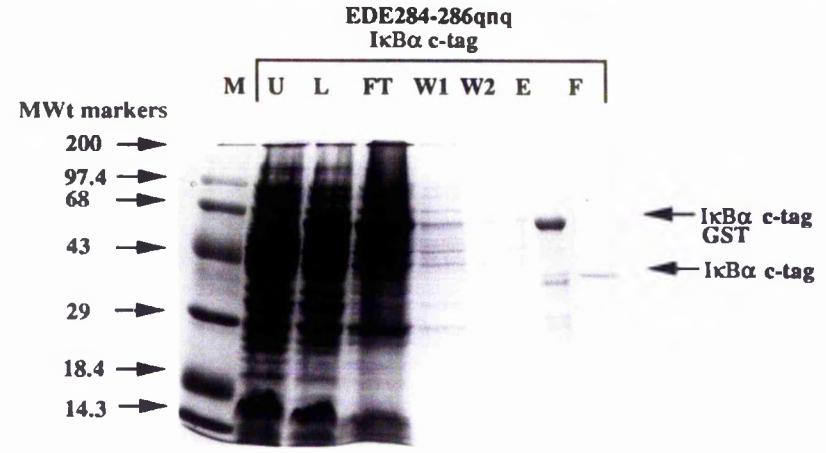
The individual fusion proteins were then purified by glutathione-agarose column chromatography using either the tritonX-100 method or the tritonX-100 and sarkosyl method (see materials and methods). The tritonX-100 method was used for the purification of the C-terminal mutants, EDE300-302qnq and EDE300-302sss only and a supply of tritonX-100 purified wild-type IκBα c-tag was kindly provided by Ellis Jaffray (University of St.Andrews). This method used the mild non-ionic detergent tritonX-100 to achieve protein solubilisation before the protein sample was applied to a glutathione-agarose column. However, the remainder of the GST-fusion mutants (EDE284-286qnq, EDE284-286sss, W258y, E275q and E275s) failed to be solubilised by tritonX-100 alone and a method which employed both tritonX-100 and the stronger ionic detergent, sarkosyl was required (Frangioni *et al.*, 1993). This procedure achieved protein solubilisation through the denaturing action of sarkosyl. The addition of tritonX100 was then thought to orient sarkosyl into micelles thereby allowing the GST-fusion protein to refold (and remain solubilised) and bind to glutathione-agarose. Wild-type IκBα c-tag was also purified using the tritonX-100 and sarkosyl method to act as a direct comparison between wild-type and mutant proteins purified under the same conditions in the experiments which follow in chapter 2.

Finally, the purified GST-fusion proteins were cleaved with thrombin and the wild-type and mutant IκBα c-tag proteins were recovered using ion exchange FPLC on a mono Q column. This purification strategy separated proteins on the basis of charge-the more negatively charged the protein the greater its affinity for the column. The wild-type and mutant IκBα c-tag proteins possessed a highly negatively charged C-terminus and could therefore bind to the column. GST was uncharged and would not bind to the column, it was present in the initial flow-through. Proteins were eluted from the column using a concentration gradient of sodium chloride (250mM to 1M). An additional glutathione-agarose purification step was included if the FPLC purified protein sample contained any undigested fusion protein (which can bind to the FPLC mono-Q column). The fusion protein was absorbed onto the column whereas the thrombin cleaved protein was passed through the column to yield the final wild-type and mutant IκBα c-tag proteins.

Figures 15 and 16 show the results of the purification steps. The thrombin cleaved fusion protein (i.e. the FPLC column load), the FPLC column flow-through (i.e. GST), FPLC column washes and the optional glutathione-agarose purification step are not shown. The polyacrylamide gels illustrate that the glutathione-agarose affinity chromatography yielded virtually pure GST-IκBα c-tag wild-type and mutant (aa1-317) proteins in the various eluates (**E**). The mutant and wild-type IκBα c-tag proteins eluted from the FPLC Mono-Q column (**F**) were shown to be free from most of the contaminating proteins-a small amount of undigested fusion protein was visible in some of the mutants but this was removed by including an additional glutathione-agarose purification step. It should be noted that IκBα c-tag does not stain well with coomassie blue compared to GST, therefore the GST-IκBα c-tag fusion protein appears as a much darker band compared to IκBα c-tag alone. Also evident was a protein band around 30kD which had resulted from the thrombin cleavage step. IκBα c-tag contains a weakly recognised thrombin cleavage site at its N-terminus from amino acids 58-63 (EVPRGS) which, although not identical to the true thrombin site, LVPAGS, it can be cleaved at a slower rate to give the lower molecular weight product of 30kD. The occurence of this product was reduced by performing a "test" thrombin cleavage on a small

Figure 15. Glutathione-agarose and FPLC column purifications of wild-type and C-terminal mutant IκBα c-tag proteins.

Coomassie blue stained 10% SDS polyacrylamide gels of glutathione-agarose and FPLC column purifications are shown above and overleaf for the wild-type IκBα c-tag protein (using the Triton-X100 and sarkosyl purification method-TX+S) and the C-terminal IκBα c-tag mutant proteins - EDE284-286qnq (using method TX+S), EDE284-286sss (using method TX+S), EDE300-302qnq (using the TritonX-100 purification method-T) and EDE300-302sss (using method T) either with GST (GST-IκBα c-tag) or without GST (IκBα c-tag). All sample tracks contained a mix of 15µl of protein solution and 7.5µl of 3x disruption buffer which had been boiled for 2-3 minutes before loading. The following abbreviations were used for the sample tracks shown :- **M** = protein molecular weight standards (units=kDa), **U** = uninduced crude bacterial extract, **L** = IPTG induced crude bacterial load to the glutathione-agarose column, **FT** = flow-through from the glutathione-agarose column, **W1**, **W2** = first (**W1**) and second (**W2**) glutathione-agarose column washes with PBS, 0.5M NaCl, 2mM EDTA, 1mM PMSF, 2mMbenzamidine, **E** = 10mM glutathione, 0.5M NaCl, 50mM Tris/HCl pH8.0 eluate from the glutathione-agarose column, **F** = FPLC Mono-Q column pooled peak fractions in PBS/0.5M NaCl. For lanes **L**, **FT**, **W1**, **W2** and **E** the IκBα c-tag proteins were fused to glutathione-S-transferase (GST) to give a combined molecular weight of approximately 63 kDa. For lane **F** the IκBα c-tag proteins had been cleaved by thrombin to yield the final protein at a molecular weight of 37 kDa.


EDE284-286qnq
IκBα c-tag
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MWt markers
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97.4
68
43
29
18.4
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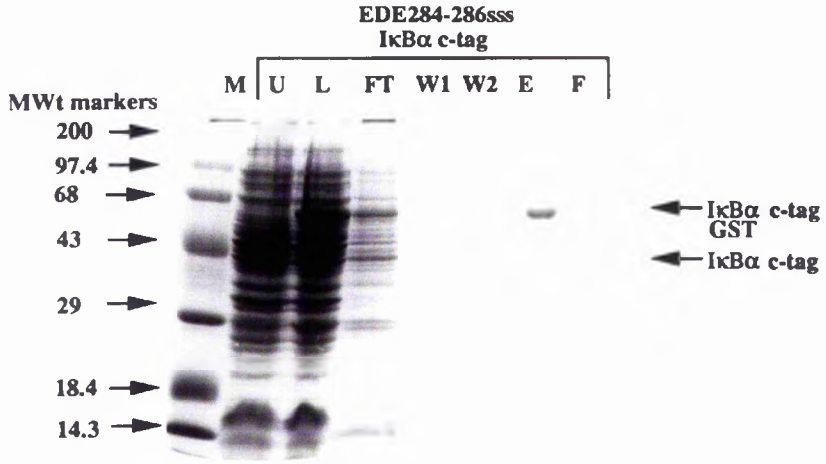
IκBα c-tag GST
IκBα c-tag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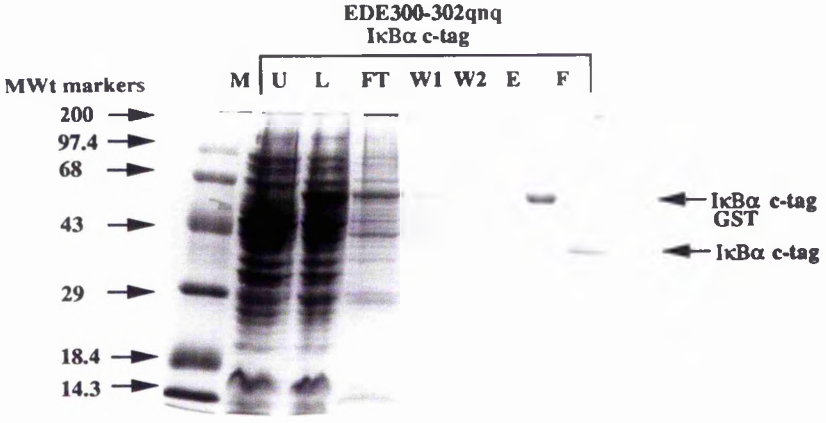
IκBα c-tag GST
IκBα c-tag


EDE300-302qnq
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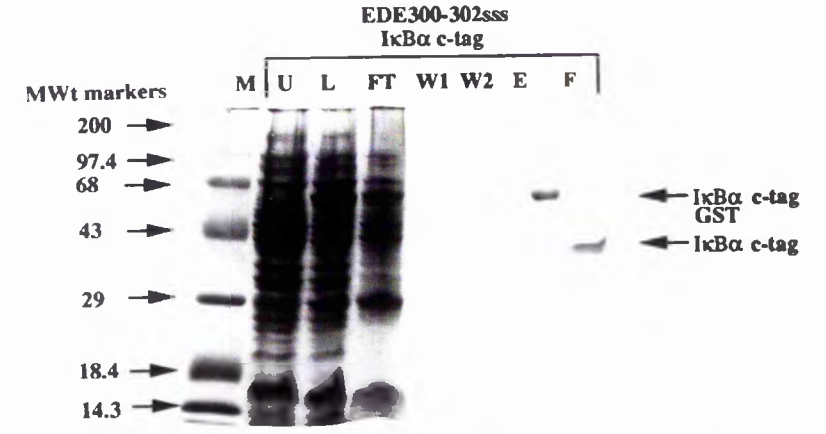
IκBα c-tag GST
IκBα c-tag


EDE300-302sss
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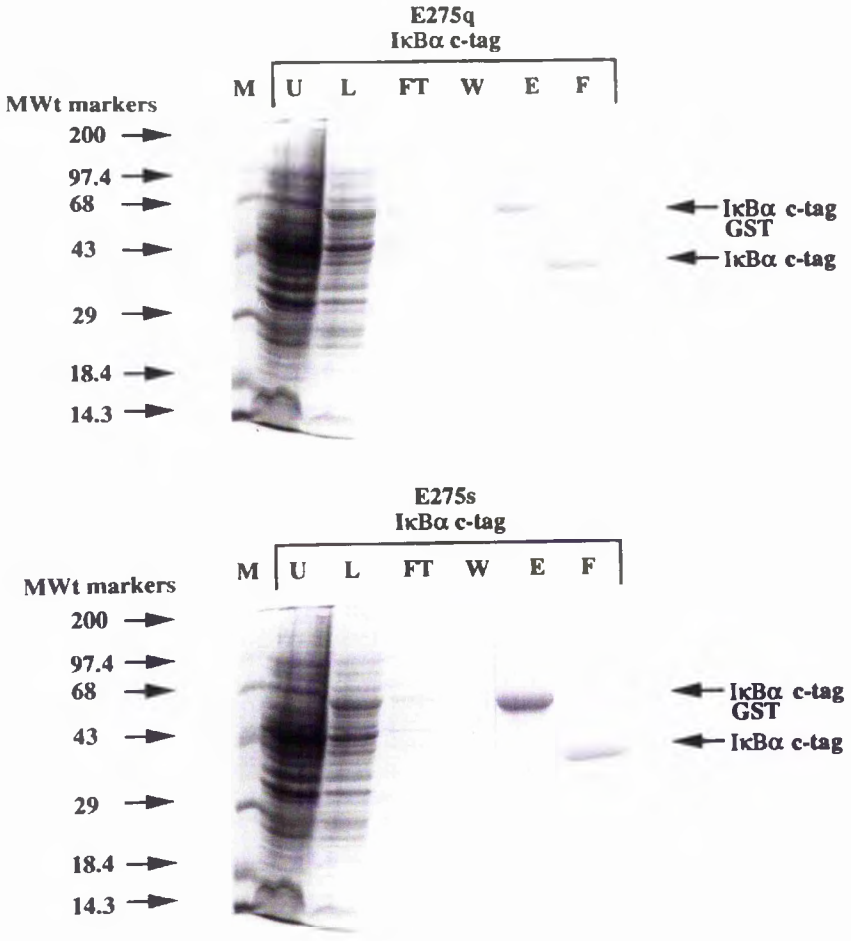
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα c-tag GST
IκBα c-tag

Figure 16. Glutathione-agarose and FPLC column purifications of the linker mutant IκBα c-tag proteins.

Coomassie blue stained 10% SDS polyacrylamide gels of glutathione-agarose and FPLC column purifications are shown above and overleaf for the linker mutant IκBα c-tag proteins-W258y (using the Triton-X100 and sarkosyl method-TX+S), E275q (using method TX+S) and E275s (using method TX+S) either as GST fusions (GST-IκBα c-tag) or without GST (IκBα c-tag).All sample tracks contained a mix of 15µl of protein solution and 7.5µl of 3x disruption buffer which had been boiled for 2-3 minutes before loading. The following abbreviations were used for the sample tracks shown :- **M** = protein molecular weight standards (units=kDa), **U** = uninduced crude bacterial extract, **L** = IPTG induced crude bacterial load to the glutathione-agarose column, **FT** = flow-through from the glutathione-agarose column, **W1**, **W2** or **W** = first (**W1**) and second (**W2**) glutathione-agarose column washes with PBS, 0.5M NaCl, 2mM EDTA, 1mM PMSF, 2mM benzamidine, **E** = 10mM glutathione, 0.5M NaCl, 50mM Tris/HCl pH8.0 eluate from the glutathione-agarose column, **F** = FPLC Mono-Q column pooled peak fractions in PBS/0.5M NaCl. For lanes **L**, **FT**, **W1**, **W2**,**W** and **E** the IκBα c-tag proteins were fused to glutathione-S-transferase (GST) to give a combined molecular weight of approximately 63 kDa. For lane **F** the IκBα c-tag proteins had been cleaved by thrombin to yield the final protein at a molecular weight of 37 kDa.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IκBα c-tag
M
U
L
FT
W
E
F
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα c-tag
GST
IκBα c-tag
E275s
IκBα c-tag
M
U
L
FT
W
E
F
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα c-tag
GST
IκBα c-tag

sample of the fusion protein using a range of time intervals. This determined the minimum incubation time before cleavage at the internal thrombin site became too advanced.

Figures 17 and 18 show the UV absorbance output trace obtained for the FPLC column purification of each of the IκBα c-tag proteins. The initial GST flow-through was observed to the far left of each trace. In general, the IκBα c-tag proteins eluted between 0.40M and 0.55M sodium chloride. The peak fractions were analysed using SDS-PAGE. The most concentrated fractions were combined and stored at -70^{o}C.

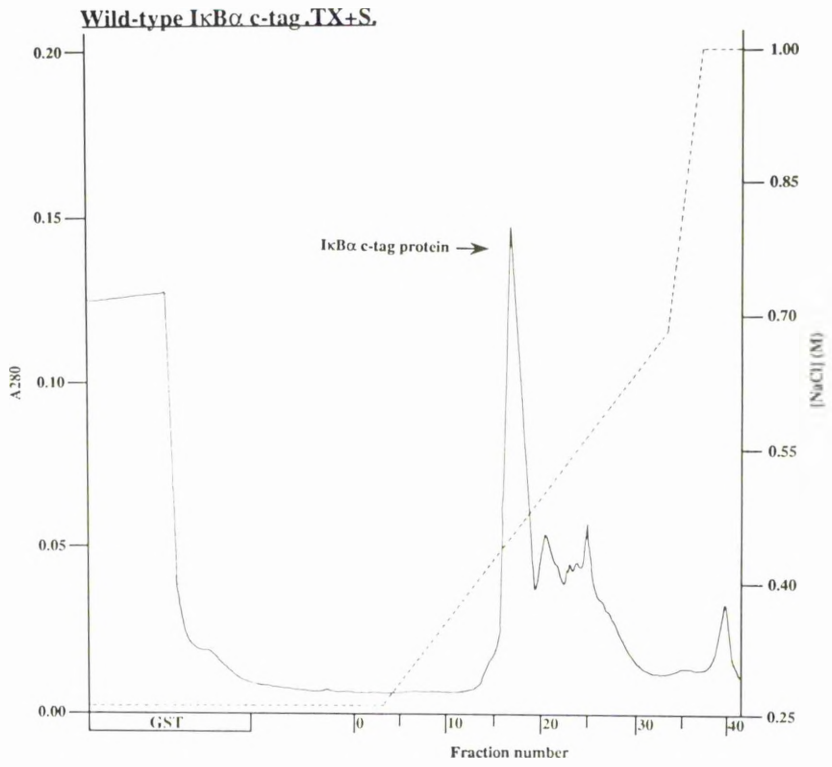
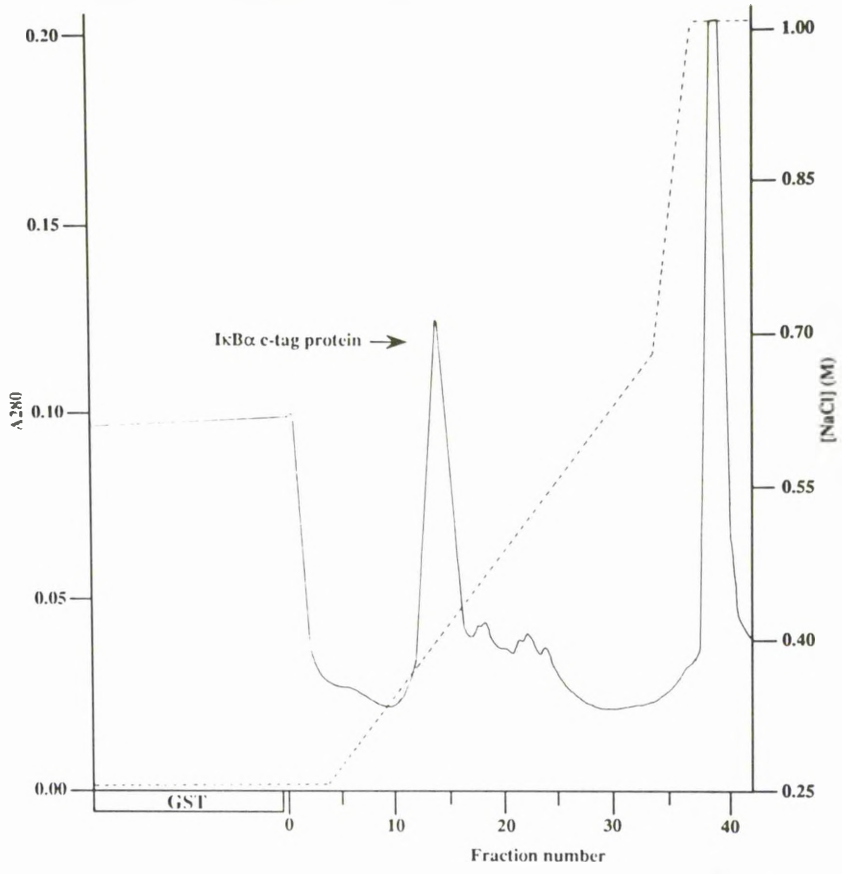


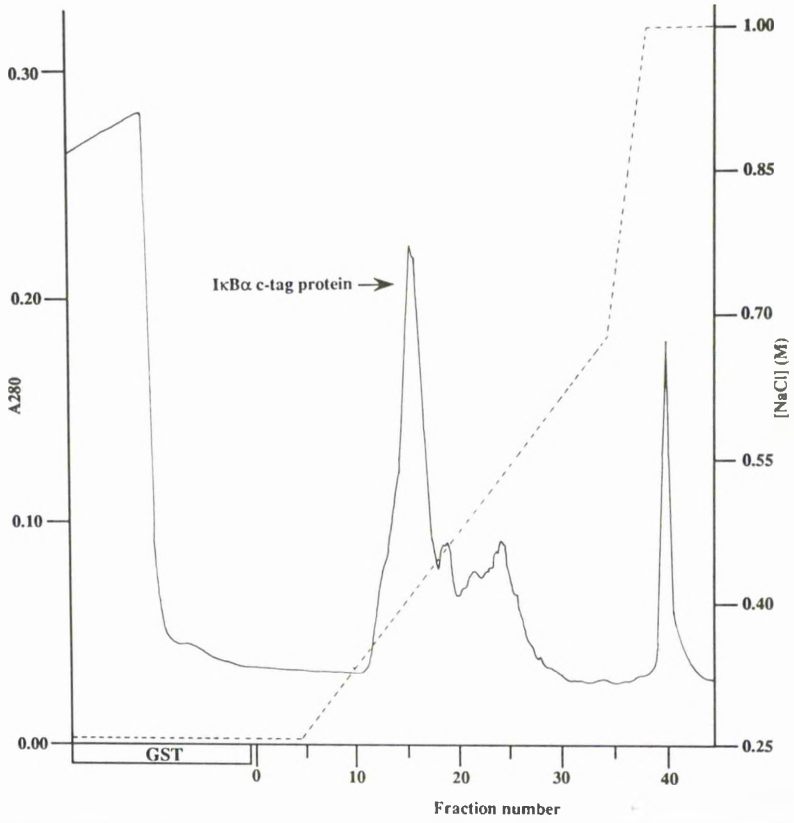
Figure 17. FPLC Mono-Q column purification of wild-type and C-terminal IκBα c-tag proteins.

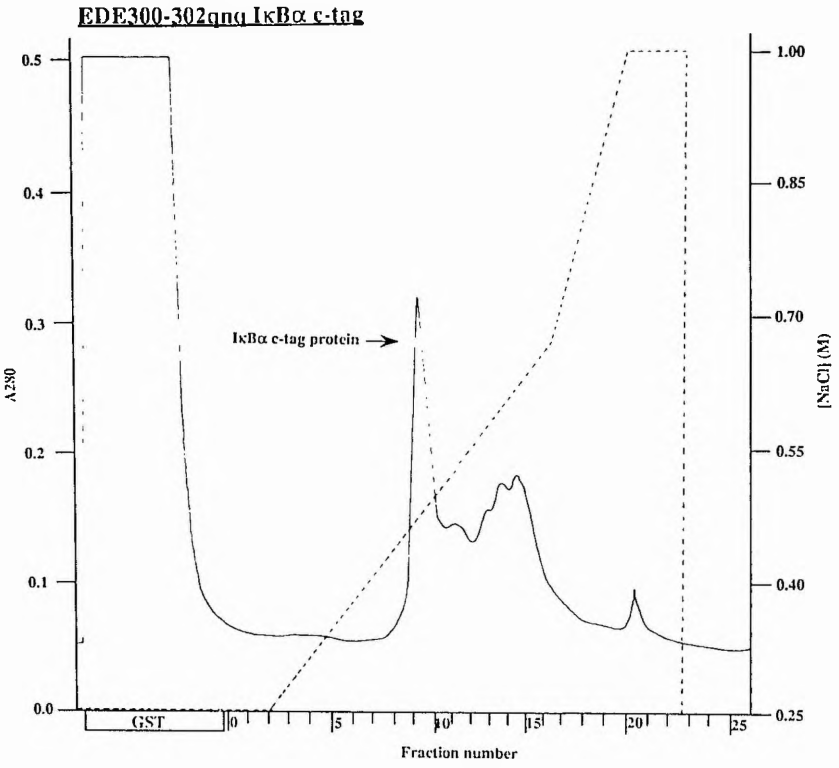
The UV absorbance output traces at 280nm are shown (solid lines) above and on the following two pages for the elution of the wild-type and C-terminal mutant IκBα c-tag proteins (EDE284-286qnq, EDE284-286sss, EDE300-302qnq and EDE300-302sss) from the FPLC Mono-Q column using a salt gradient ranging from 250mM NaCl to 1mM NaCl (dashed lines). The position of the fractions collected from the salt gradient are indicated along the base of the trace. Glutathione-S-Transferase appeared in the initial column flow-through (to left of IκBαc-tag peak) before the salt gradient was applied to the FPLC Mono-Q column.

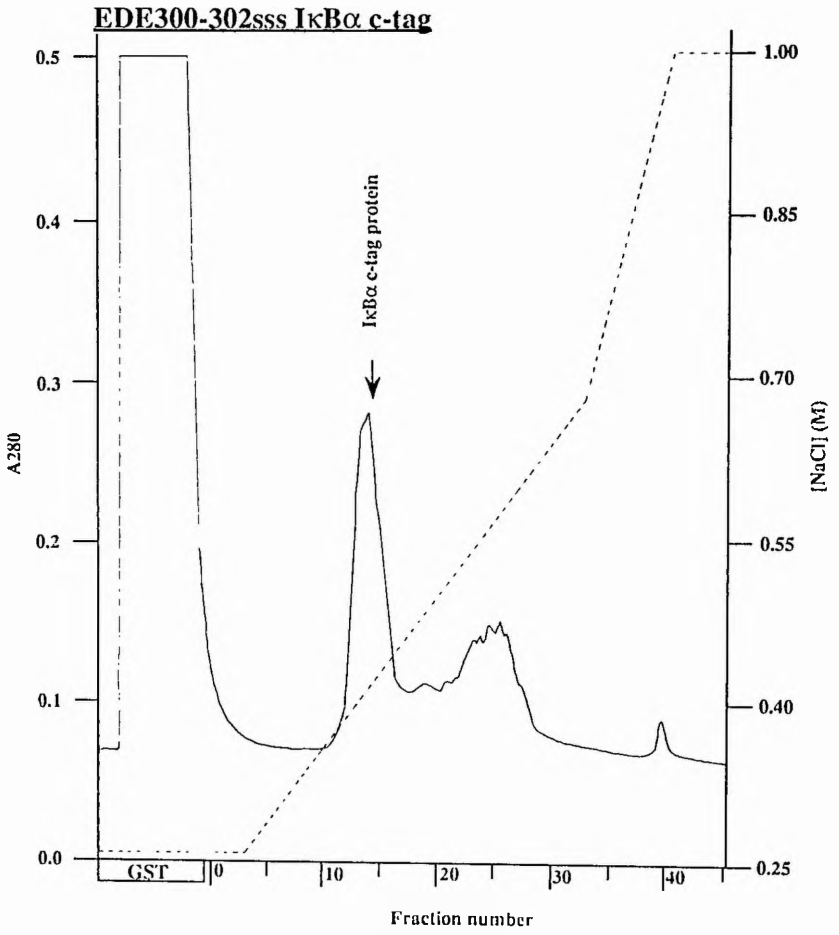
EDE284-286qnq IκBα c-tag



EDE284-286sss IκBα c-tag




EDE300-302qnq IκBα c-tag
IκBα c-tag protein
A280
[NaCl] (M)
GST
Fraction number
0.0
0.1
0.2
0.3
0.4
0.5
0.25
0.40
0.55
0.70
0.85
1.00
0
5
10
15
20
25


EDE300-302sss IκBα c-tag
IκBα c-tag protein
A280
[NaCl] (M)
GST
Fraction number
0.0
0.1
0.2
0.3
0.4
0.5
0.25
0.40
0.55
0.70
0.85
1.00
0
10
20
30
40

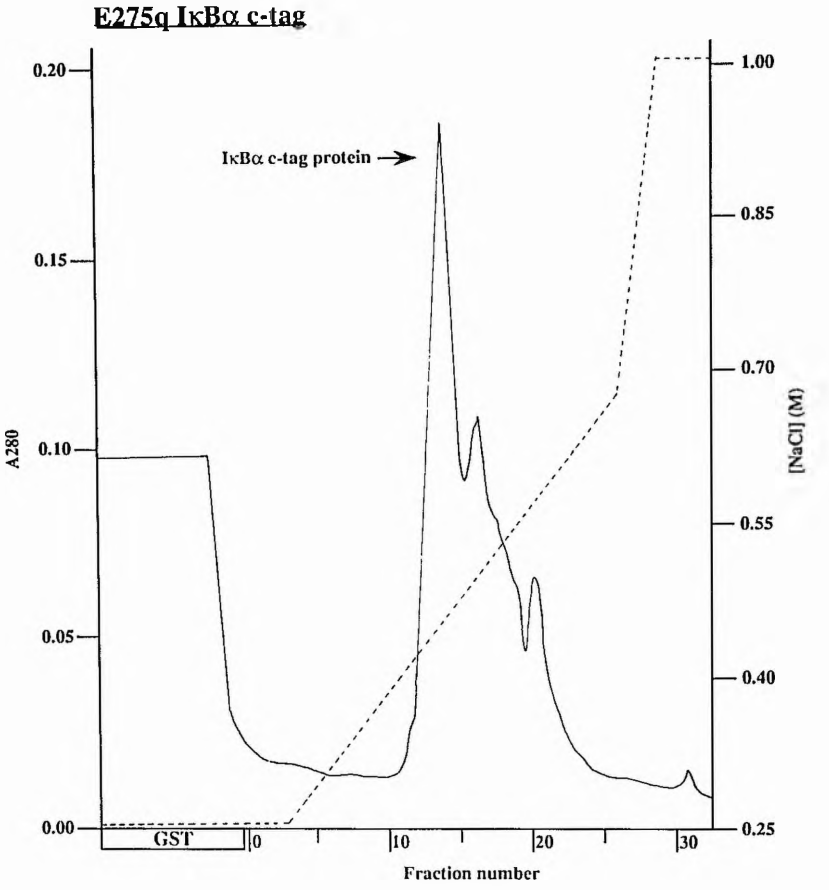
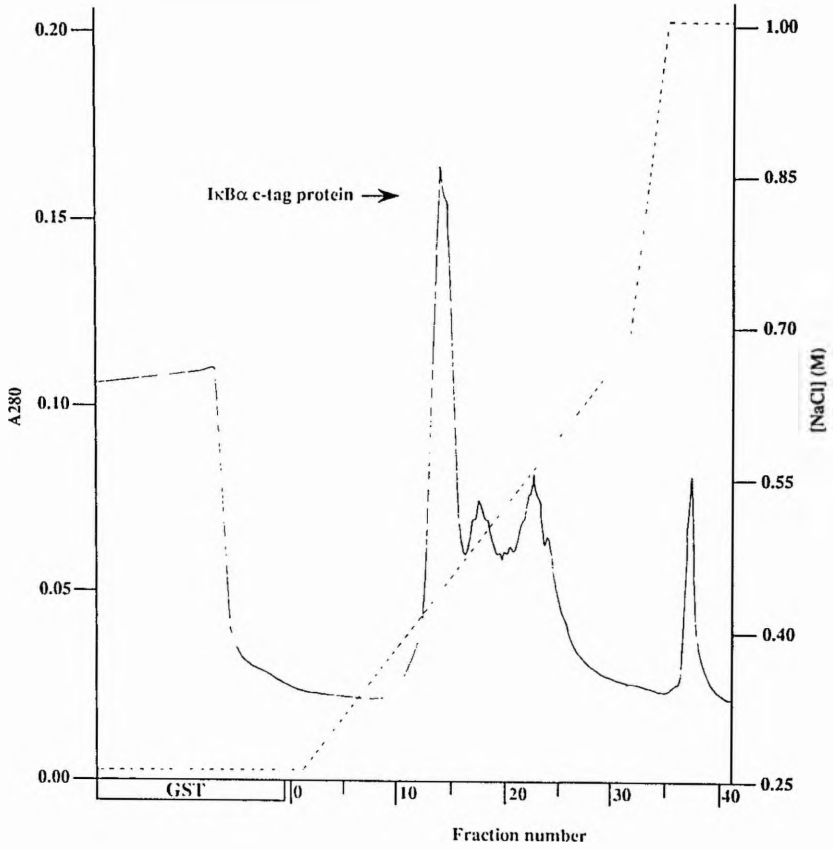


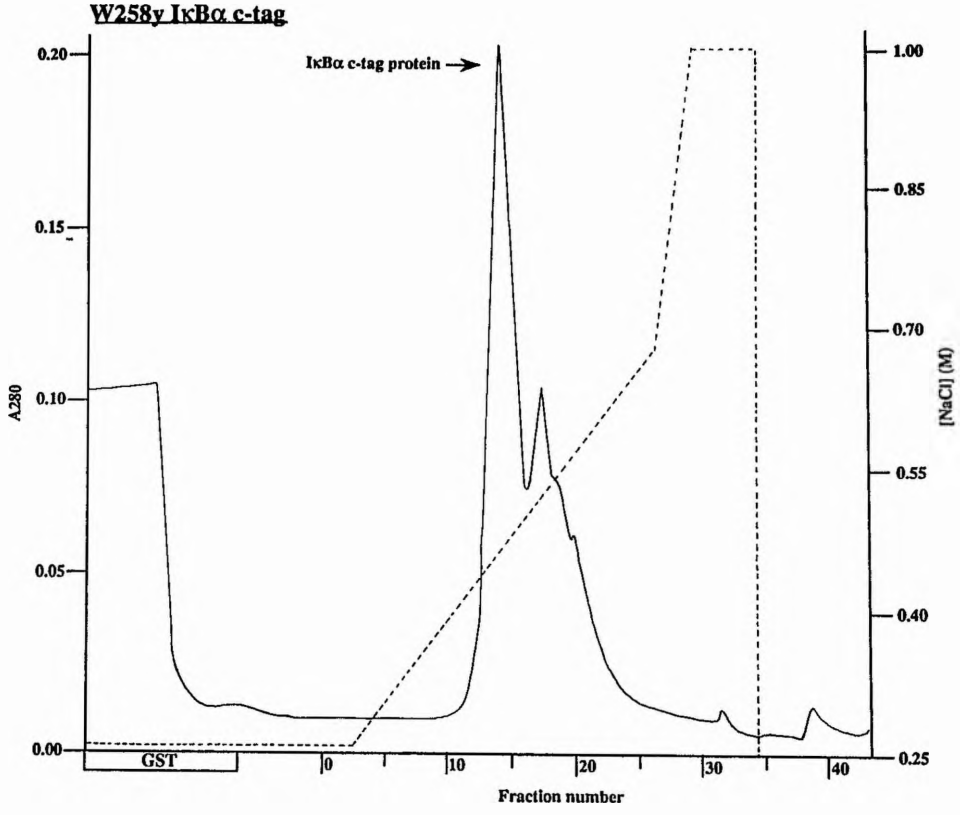
Figure 18. FPLC Mono-Q column purification of the linker mutant IκBα c-tag proteins.

The UV absorbance output traces at 280nm are shown above and on the following page (solid lines) for the elution of linker mutant IκBαc-tag proteins (W258y, E275q and E275s) from the FPLC Mono-Q column using a salt gradient ranging from 250mM NaCl to 1mM NaCl (dashed lines). The position of the fractions collected from the salt gradient are indicated along the base of the trace. Glutathione-S-Transferase appeared in the initial column flow-through (to left of IκBαc-tag peak) before the salt gradient was applied to the FPLC Mono-Q column.

E275s IκBα c-tag



W258y IκBα c-tag



CHAPTER 2. *IN VITRO* CHARACTERISATION OF IκBα C-TAG C-TERMINAL AND LINKER MUTANTS.

As outlined in chapter 1, the IκBα c-tag mutants were constructed to primarily investigate the interaction between specific residues in the linker and C-terminal regions of IκBα and the p65 subunit of NF-κB and to also study their effect (if any) on p65 DNA binding activity. To examine these functions *in vitro*, the purified mutant and wild-type proteins were analysed by a combination of protein association experiments and DNA binding assays (these results are given in sections 2.1 and 2.2). It should be noted at this point that a comparison was made between the activities of wild-type IκBα and IκBα c-tag in both the protein association experiments and the DNA binding assays by M. Rodriguez, Institut Pasteur (unpublished data). The activities of both proteins were found to be the same indicating that the C-terminally fused tag epitope did not affect the function of IκBα.

The residues mutated in the C-terminal region (residues 284-286 and 300-302) of IκBα were also of interest because they were located within two separate consensus phosphorylation sites for the serine/threonine protein kinase, casein kinase II (casein kinase II (CKII) consensus sequence; S/T-X-X-E/D, where S or T is the phosphoacceptor). Comparison of the C-terminal amino acid sequence of human IκBα (MAD-3) with the equivalent acidic regions in the avian (pp40), porcine (ECI-6) and rat (RL/IF-1) homologues of IκBα (figure 9) revealed that the two consensus CKII phosphorylation sites were completely conserved (corresponding to MAD-3 residues 283-286; SEDE and residues 299-302; TEDE) among human, porcine and rat IκBα homologues (MAD-3, ECI-6, RL/IF-1). Further examination of the C-terminus of human IκBα revealed an additional CKII site from residues 291-294 (TESE), the first three residues of which were conserved between the human, porcine and rat IκBα homologues. Moreover, a phosphorylation site for the functionally related protein kinase, casein kinase I (CKI) (S(P)-X-X-S/T, where S or T is the phosphoacceptor) was identified at position 288-291 (SYDT) in human IκBα and was also found to be conserved between the rat and porcine IκBα homologues. Figures 9 and 19 shows another CKI site from residues 293-295 (SEFT) in human IκBα although this does not appear to be conserved between any of the other IκB family members. IκBα also contains an

N-terminal consensus site for tyrosine phosphorylation and a phosphotidylinositol-3-kinase binding site (residues 39-47: DEEYEQMVK). A protein kinase C phosphorylation site also exists in the sixth ankyrin repeat region (residues 260-264: RPSTR).

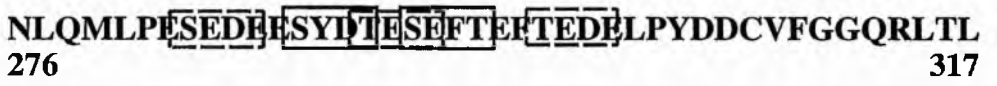


Figure 19. Amino acid sequence of the IκBα C-terminus (amino acids 276-317) and its consensus casein kinase I and casein kinase II phosphorylation sites.

The amino acid sequence for the C-terminus of wild-type IκBα is shown and the consensus phosphorylation sites for casein kinase I (S(P)-X-X-S/T, where S or T is the phosphoacceptor) and casein kinase II (S/T-X-X-E/D, where S or T is the phosphoacceptor). Casein kinase I sites are boxed with a solid line and casein kinase II sites are boxed with a dashed line.

Since phosphorylation is important for the *in vivo* function of IκBα (see section 2.3 for more details) the consensus phosphorylation sites for the various kinases discussed above may play a central role in IκBα activity *in vivo*. Section 2.3 examines the affect of the C-terminal mutations (which alter the CKII consensus sequences), on the ability of IκBα to be phosphorylated *in vitro* by CKII. The relevance of the findings in terms of the *in vivo* function of IκBα is discussed.

2.1. Ability of wild-type and mutant IκBα c-tag purified proteins to associate with NF-κB p65 homodimers at A) a range of NaCl concentrations and B) a range of pH values.

The type of interaction expected between the negatively charged C-terminal residues (including glutamic acid 275 in the linker region) of IκBα and the nuclear localisation signal sequence of p65 was primarily ionic. Therefore, any differences in the affinity of mutant and wild-type IκBα c-tag for p65 may be detected by altering the ionic stength or pH of the solution containing these proteins.

Initial protein association studies were carried out in the presence of a range of sodium chloride concentrations by incubating mutant or wild-type IκBα c-tag proteins with either GST or GST-p65 proteins bound to glutathione-agarose. Following extensive washing

to remove any unbound proteins, the remaining associated proteins were separated by SDS-PAGE and visualised by Western blotting using either anti-Pk (to detect IκBα c-tag) or polyclonal anti-p65 (to detect p65) (Figures 20 and 21). Figure 20 shows that all of the wild-type and mutant IκBα c-tag proteins were able to bind to GST-p65 with similar affinities even at different salt concentrations. However, it appeared that the salt concentration affected the ability of the wild-type and mutant IκBα c-tag proteins to bind to p65: Maximum binding was observed at 60-120mM NaCl; at 480mM NaCl binding was reduced and at 960mM NaCl binding was almost undetectable. The proteins did not bind GST alone (data not shown) which indicated that the GST-p65/IκBα c-tag interaction was achieved through p65 rather than GST. Figure 21 illustrates that p65 remained present throughout the range of salt concentrations used and therefore the loss of IκBα c-tag binding after 120mM NaCl was not due to the dissociation of GST-p65 from the glutathione-agarose beads.

Protein association assays were also performed on purified wild-type or mutant IκBα c-tag proteins incubated with either GST or GST-p65 bound to glutathione-agarose using a range of pH's at a constant salt concentration of 60mM. As previously described this was followed by extensive washing to remove any unbound proteins and the remaining associated proteins were separated by SDS-PAGE and visualised by Western blotting using either anti-Pk (to detect IκBα c-tag) or polyclonal anti-p65 (to detect p65) (Figures 22 and 23). It was observed that changing the pH from 5.5-9.0 did not affect the ability of either wild-type or mutant proteins to interact with p65. In addition, there was no obvious difference in the binding affinities for p65 between the mutant and wild-type proteins over the range of pHs used. Furthermore, the GST-p65/IκBα c-tag interaction was attainable only through p65 and not GST because the proteins were not able to bind GST alone (data not shown). Figure 23 shows that GST-p65 remained bound to the glutathione agarose beads over the entire range of pHs used.

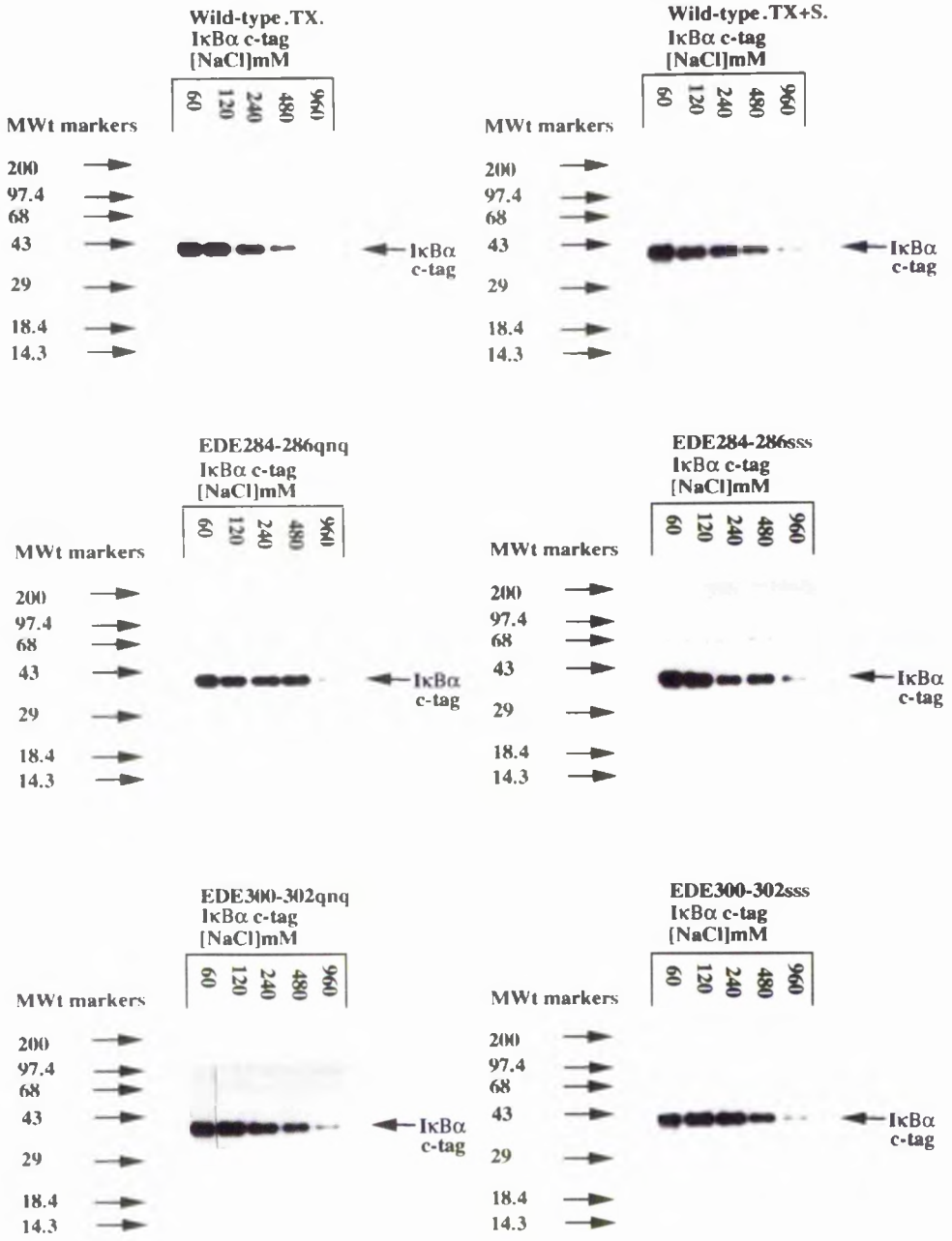
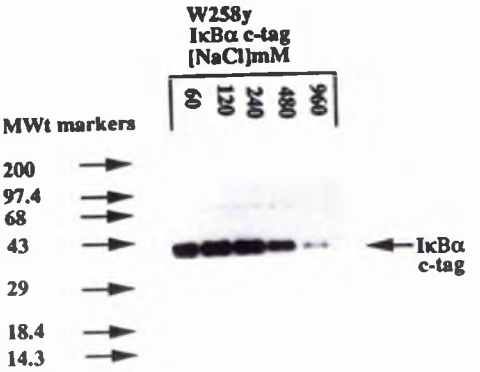
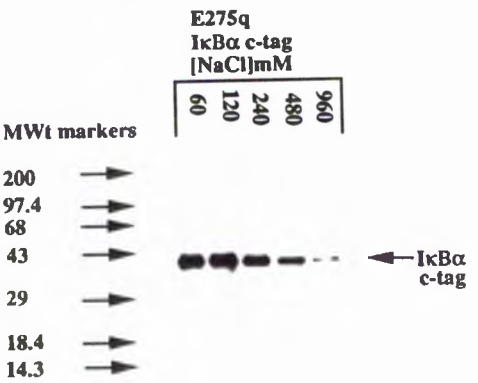
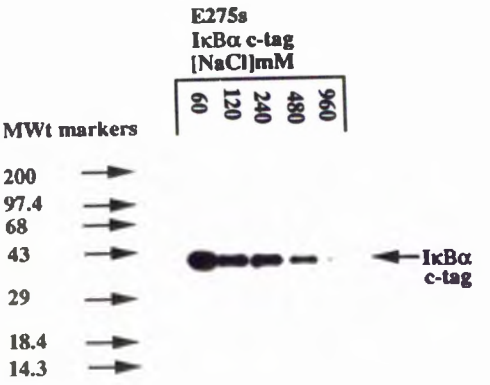


Figure 20. Protein association assays illustrating the ability of wild-type and mutant IκBα c-tag purified proteins to associate with NF-κB p65 homodimers at different NaCl concentrations.

Wild-type IκBα c-tag proteins purified using either the Triton-X100 (TX) or the Triton-X100/Sarkosyl(TX+S) method and the C-terminal and linker mutant IκBα c-tag proteins were incubated with either GST (data not shown) or GST-p65 proteins immobilized on glutathione-agarose in the presence of 60mM, 120mM, 240mM, 480mM or 960mMNaCl. The proteins were separated through a 10% SDS polyacrylamide gel and analysed by Western blotting with anti-Pk (shown above and on the next page). The position of the protein molecular weight standards are indicated (units=kDa). The position of the IκBα c-tag proteins are also given (around 37kDa). The concentration of salt which was used for each assay is shown at the top of each western blot


W258y
IκBα c-tag
[NaCl]mM
60
120
240
480
960
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα
c-tag


E275q
IκBα c-tag
[NaCl]mM
60
120
240
480
960
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα
c-tag


E275s
IκBα c-tag
[NaCl]mM
60
120
240
480
960
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα
c-tag

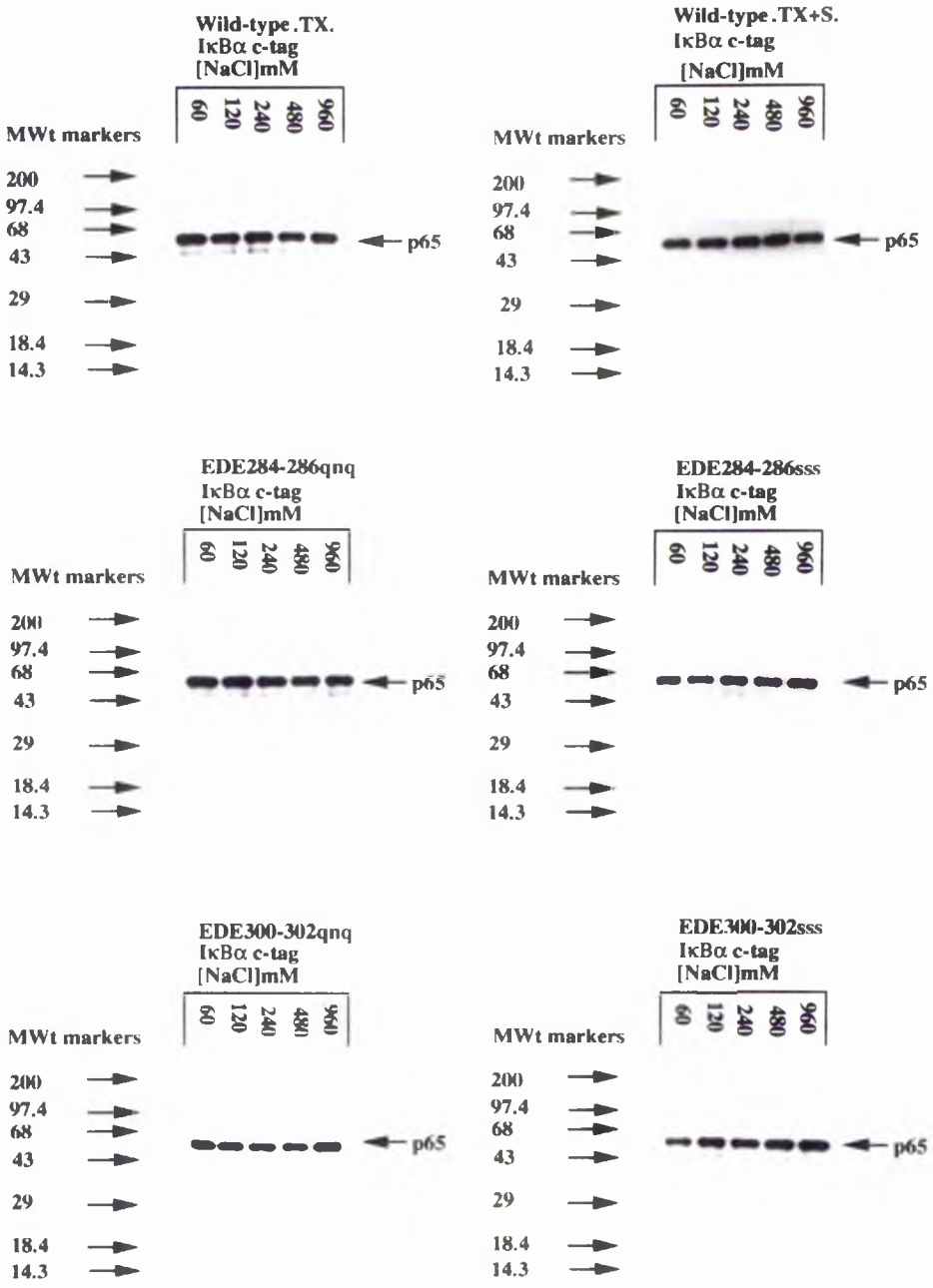
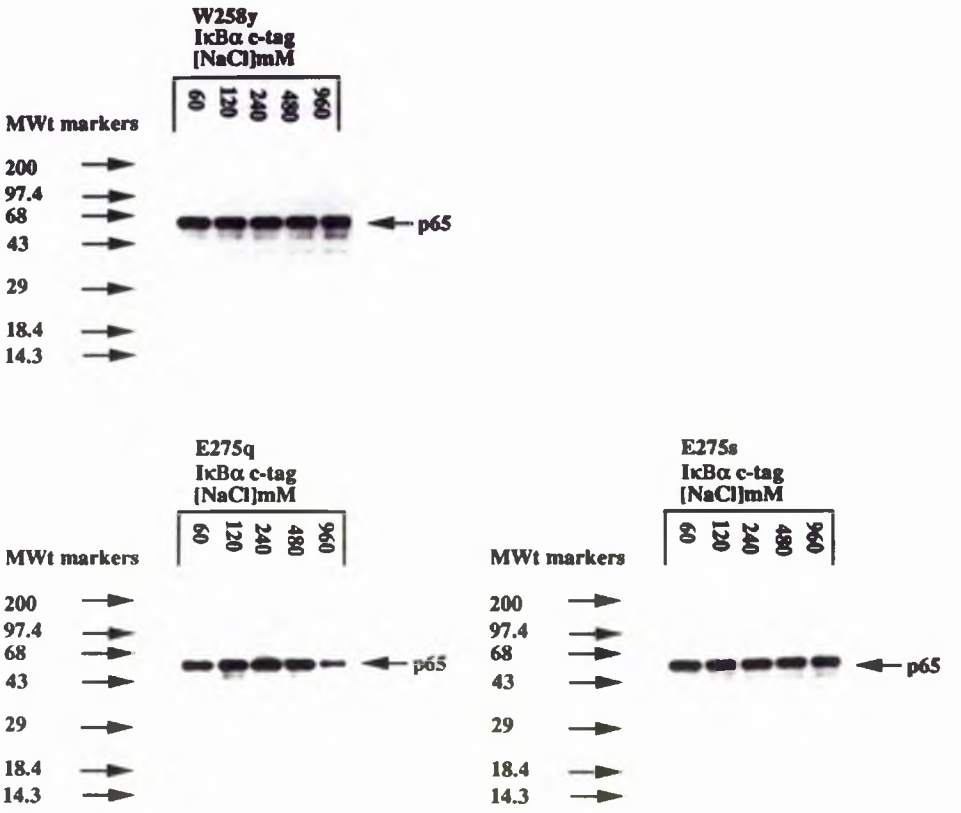


Figure 21. Protein association assays illustrating the presence of NF-κB p65 homodimers at different NaCl concentrations when incubated with wild-type and mutant IκBα c-tag purified proteins.

Wild-type IκBα c-tag proteins purified using either the Triton-X100 (TX) or the Triton-X100/Sarkosyl(TX+S) method and the C-terminal and linker mutant IκBα c-tag proteins were incubated with either GST (data not shown) or GST-p65 proteins immobilized on glutathione-agarose in the presence of 60mM, 120mM, 240mM, 480mM or 960mMNaCl. The proteins were separated through a 10% SDS polyacrylamide gel and analysed by Western blotting with polyclonal anti-p65 (shown above and on the next page). The position of the protein molecular weight standards are indicated (units=kDa). The position of the p65 protein is indicated (around 65kDa). The concentration of salt which was used for each assay is shown at the top of each Western blot


W258y
IκBα c-tag
[NaCl]mM
60
120
240
480
960
MWt markers
200
97.4
68
43
29
18.4
14.3
p65
E275q
IκBα c-tag
[NaCl]mM
60
120
240
480
960
MWt markers
200
97.4
68
43
29
18.4
14.3
p65
E275s
IκBα c-tag
[NaCl]mM
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120
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480
960
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68
43
29
18.4
14.3
p65

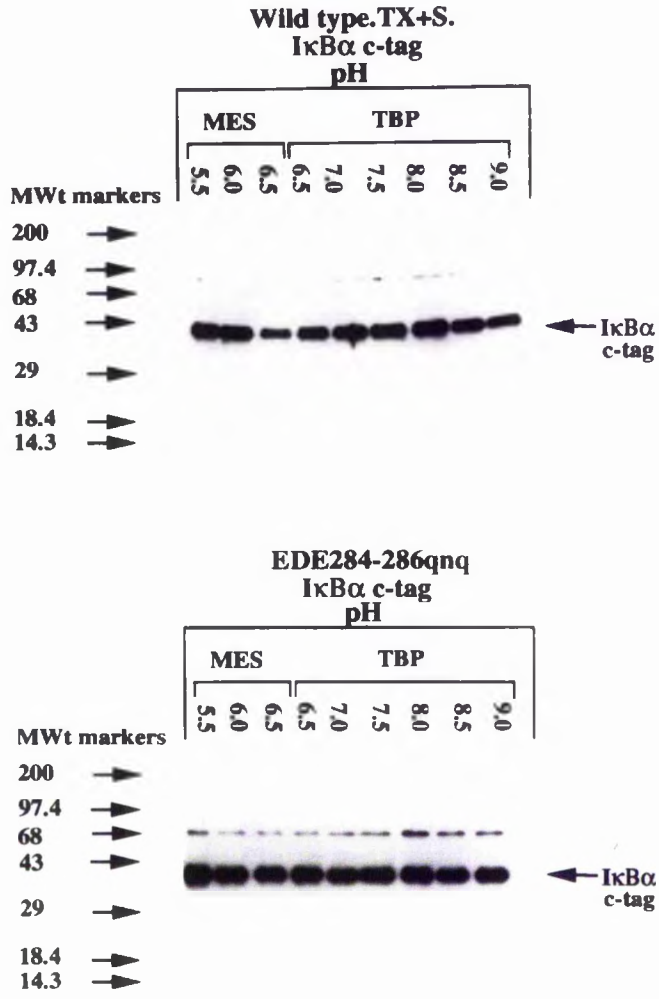
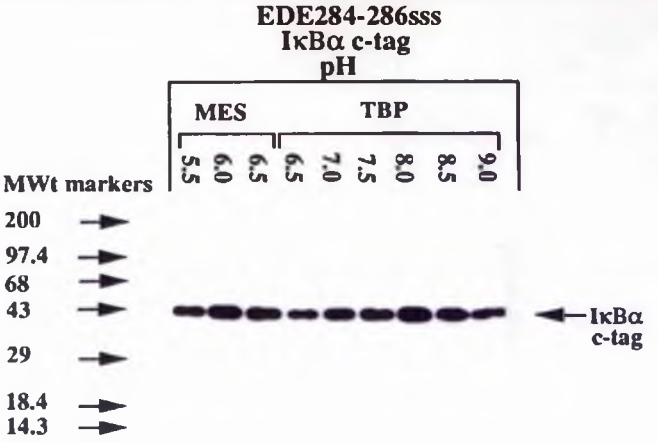
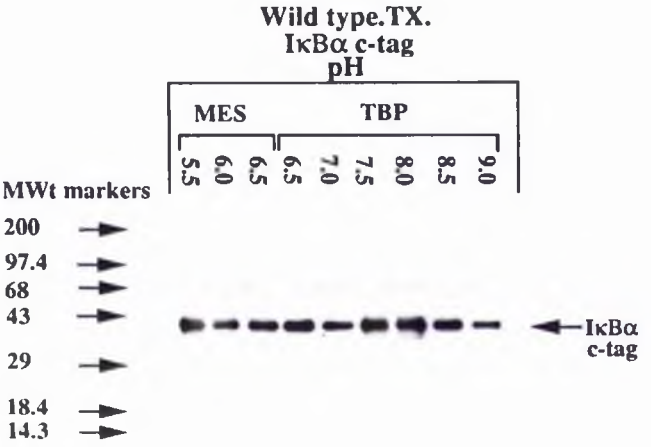


Figure 22. Protein association assays illustrating the ability of wild-type and mutant IκBα c-tag purified proteins to associate with NF-κB p65 homodimers at different pH values.

Wild-type IκBα c-tag protein purified using either the Triton-X100 (TX) or the Triton-X100/Sarkosyl(TX+S) method and the C-terminal and linker mutant IκBα c-tag proteins were bound to either GST (data not shown) or GST-p65 proteins immobilized on glutathione-agarose in the presence of 60mM NaCl and pH5.5, pH6.0, pH6.5 (using the buffer, MES (free acid)), pH6.5, pH7.0, pH7.5, pH8.0, pH8.5 or pH9.0 (using the buffer, Bis/Tris/propane (TBP)). The proteins were separated through a 10% SDS polyacrylamide gel and analysed by Western blotting with anti-Pk and are shown on above and on the following 4 pages. The position of the protein molecular weight standards are indicated (units=kDa). The position of the IκBα c-tag proteins (around 37kDa) are also given. The pH used for each assay is shown at the top of each Western blot.


EDE284-286sss
IκBα c-tag
pH
MES
TBP
5.5
6.0
6.5
6.5
7.0
7.5
8.0
8.5
9.0
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα
c-tag


Wild type.TX.
IκBα c-tag
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TBP
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6.0
6.5
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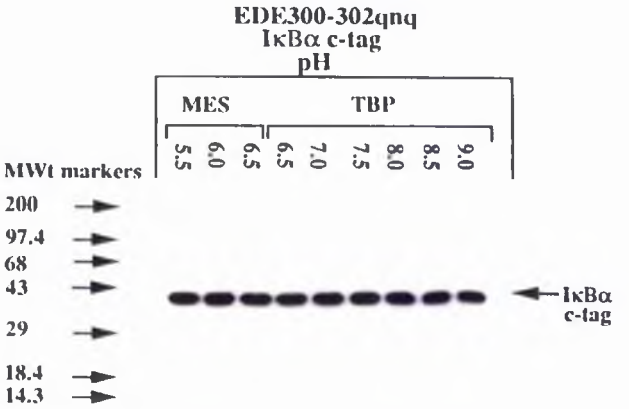
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα
c-tag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IκBα c-tag
pH
MES
TBP
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6.0
6.5
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7.0
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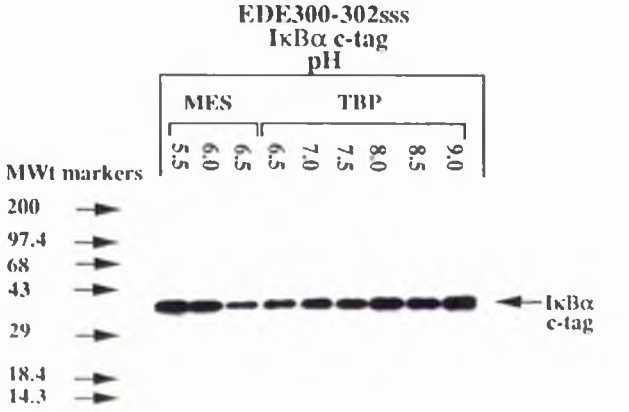
200
97.4
68
43
29
18.4
14.3
IκBα
c-tag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TBP
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6.0
6.5
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7.0
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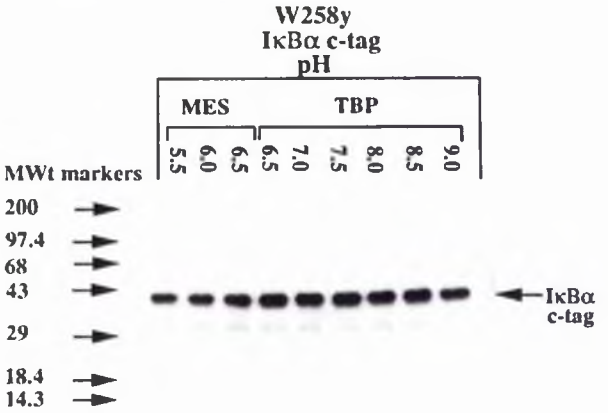
MWt markers
200
97.4
68
43
29
18.4
14.3
IκBα
c-tag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TBP
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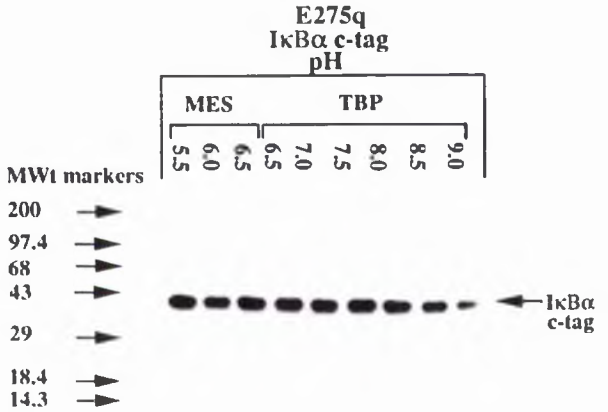
43
29
18.4
14.3
IκBα
c-tag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TBP
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200
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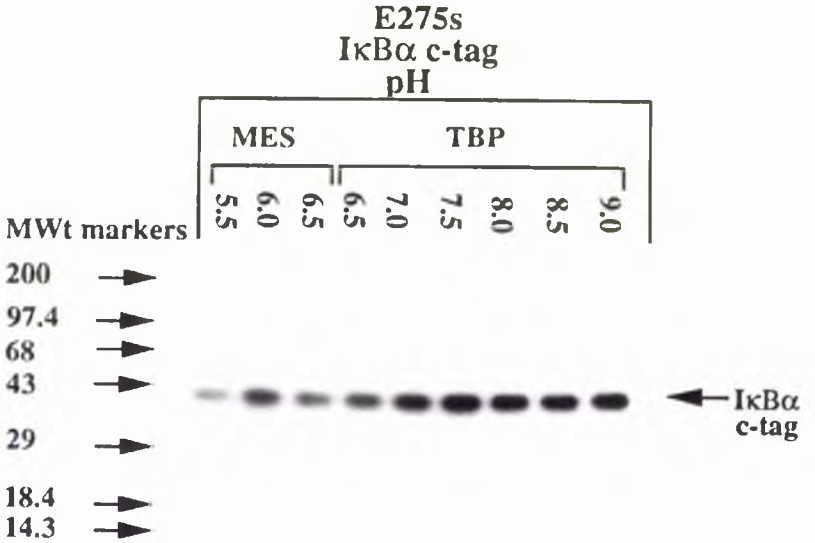
43
29
18.4
14.3
IκBα
c-tag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IκBα
c-tag

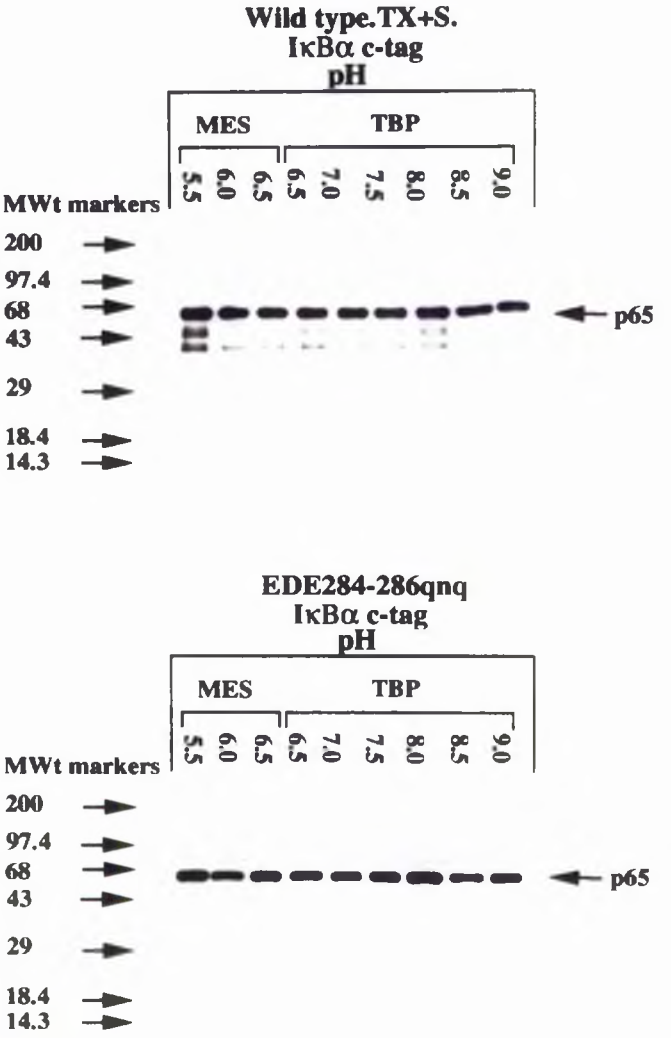
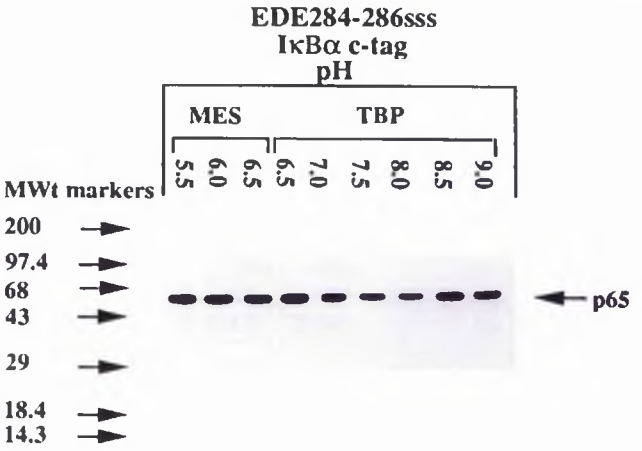


Figure 23. Protein association assays illustrating the presence of NF-κB p65 homodimers at different pH values when incubated with wild-type and mutant IκBα c-tag purified proteins .

Wild-type IκBα c-tag protein purified using either the Triton-X100 (TX) or the Triton-X100/Sarkosyl(TX+S) method and the C-terminal and linker mutant IκBα c-tag proteins were bound to either GST (data not shown) or GST-p65 proteins immobilized on glutathione-agarose in the presence of 60mM NaCl and pH5.5, pH6.0, pH6.5 (using the buffer, MES (free acid)), pH6.5, pH7.0, pH7.5, pH8.0, pH8.5 or pH9.0 (using the buffer, Bis/Tris/propane (BTP)). The proteins were separated through a 10% SDS polyacrylamide gel and analysed by Western blotting with polyclonal anti-p65 and are shown on the following 4 pages. The position of the protein molecular weight standards are indicated (units=kDa). The position of the p65 protein is also given (around 65kDa). The pH used for each assay is shown at the top of each Western blot.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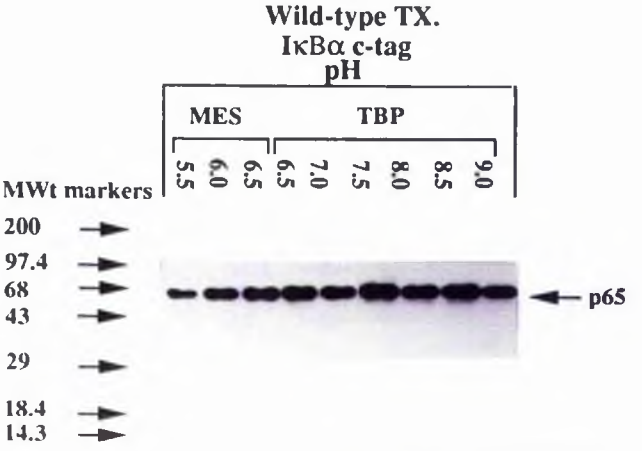
IκBα c-tag
pH
MES
TBP
5.5
6.0
6.5
6.5
7.0
7.5
8.0
8.5
9.0
MWt markers
200
97.4
68
43
29
18.4
14.3
p65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IκBα c-tag
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TBP
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6.5
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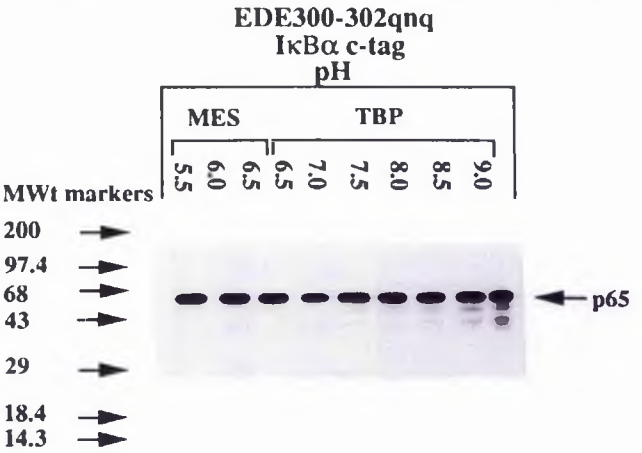
MWt markers
200
97.4
68
43
29
18.4
14.3
p65


EDE300-302qnq
IκBα c-tag
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TBP
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6.5
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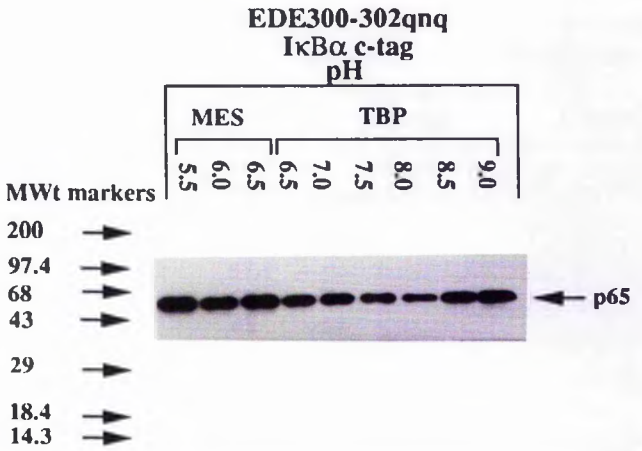
97.4
68
43
29
18.4
14.3
p65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68
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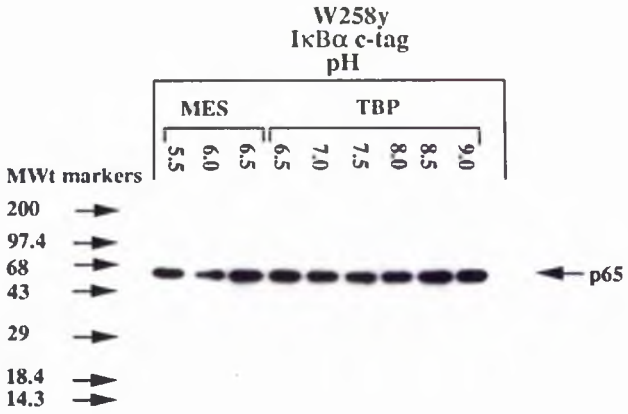
14.3
p65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97.4
68
43
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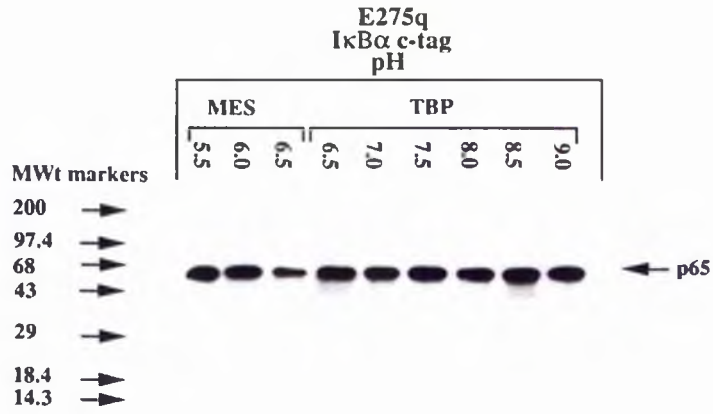
18.4
14.3
p65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TBP
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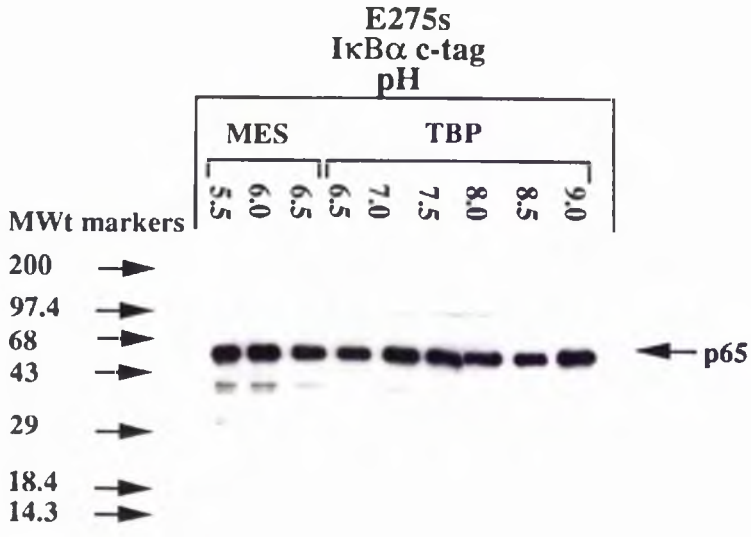
200
97.4
68
43
29
18.4
14.3
p65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7.0
7.5
8.0
8.5
9.0
MWt markers
200
97.4
68
43
29
18.4
14.3
p65

2.2. Ability of wild-type and mutant IκBα c-tag purified proteins to inhibit the DNA binding activity of NF-κB p65 homodimers.

In order for IκBα to inhibit the DNA binding activity of p65 homodimers, IκBα must make physical contact with p65. It is unclear whether the ability of IκBα to inhibit p65 DNA binding activity and bind p65 are separable activities. It is possible that different regions of IκBα are responsible for each function. For example, IκBα can associate with p50, retaining it in the cytoplasm but fails to inhibit p50 DNA binding (Beg *et al.*,1992; Ganchi *et al.*, 1992).

The purified wild-type and mutant IκBα c-tag proteins were analysed for their ability to inhibit the DNA binding activity of the p65 subunit of NF-κB by performing a gel electrophoresis DNA binding assay on equal amounts of recombinant p65 (aa12-317) incubated with a range of IκBα c-tag protein concentrations in the presence of a ^{32}P labelled double-stranded oligonucleotide which contained a recognition site for NF-κB (Figure 24 A)). In the absence of wild-type or mutant IκBα c-tag, p65 formed a stable complex with its DNA binding site (p65 only lane in figure 24 A)).Wild-type IκBα c-tag proteins purified by either the tritonX-100 method (TX) or the tritonX-100 and sarkosyl method (TX+S) were shown to vary in their ability to inhibit the DNA binding activity of p65 homodimers: Wild-type IκBα c-tag purified by the tritonX-100 and sarkosyl method was a more efficient inhibitor of p65 DNA binding than the tritonX-100 purified protein. The C-terminal mutants, EDE284-286qnq and EDE284-286sss, exhibited a reduction in their ability to inhibit the DNA binding of p65 homodimers compared to the wild-type protein whereas the remainder of the C-terminal and linker mutants exhibited wild-type inhibitory characteristics.

The information from the DNA binding assays was represented graphically by quantitating the amounts of ^{32}P radioactivity in each of the DNA-protein (oligonucleotide+p65) complexes using liquid scintillation counting of dried gel slices and expressing the values as a fraction of the total radioactivity within the p65 only control (given an arbitrary value of 1.00) for each set of gel shifts. This data was compared to the amount (fmoles) of wild-type or mutant IκBα c-tag protein used in each binding reaction and the information was plotted on a graph (figure 24 B)). Wild-type IκBα c-tag protein purified

Figure 24.

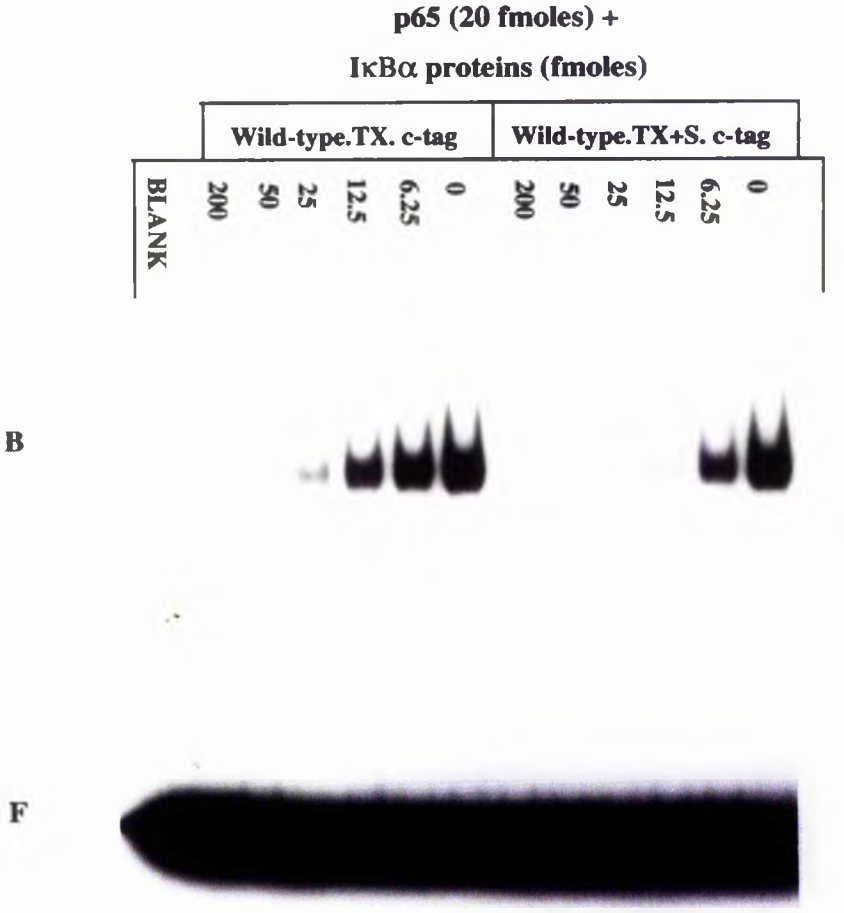
A) Gel electrophoresis DNA binding assays illustrating the ability of wild-type and mutant IκBα c-tag purified proteins to inhibit the DNA binding activity of NF-κB p65 homodimers.

For each binding reaction 20 fmoles of recombinant p65 (amino acids 12-317) were incubated for 15 minutes (at RT) with 0-200 fmoles of the following IκBα c-tag proteins in the presence of binding buffer which contained ^{32}5nM P labelled double-stranded oligonucleotide (5'-CTGGGGACTTTCCAGG-3'):- wild-type IκBα c-tag proteins purified using either the Triton-X100 (TX) or the Triton-X100/Sarkosyl(TX+S) method (shown opposite) and the C-terminal and linker mutant IκBα c-tag proteins (shown on the following two pages). The DNA-protein complexes were resolved on native 6% polyacrylamide gels and visualised by autoradiography of the dried gels. The protein-bound and free radiolabelled oligonucleotide bands are indicated by B and F respectively.

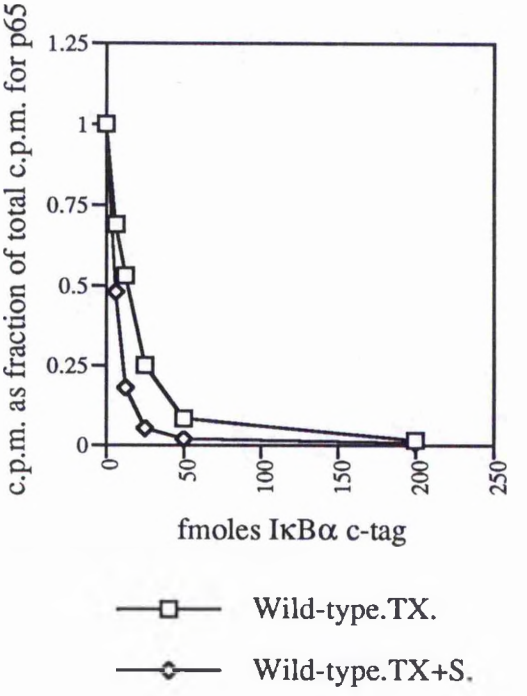
B) Graphical representation of the ability of wild-type and mutant IκBα c-tag purified proteins to inhibit the DNA binding activity of NF-κB p65 homodimers.

Liquid scintillation counting of dried gel slices was used to determine the amount of ^{32}P radioactivity in each of the DNA-protein complexes (oligonucleotide+p65) shown in figure 24 A). The values were expressed as a fraction of the total radioactivity present in the p65 only control (which was given an arbitrary value of 1.00) and plotted on a graph against the number of fmoles of wild-type or mutant IκBα c-tag protein used in each binding reaction (opposite and on the next two pages).

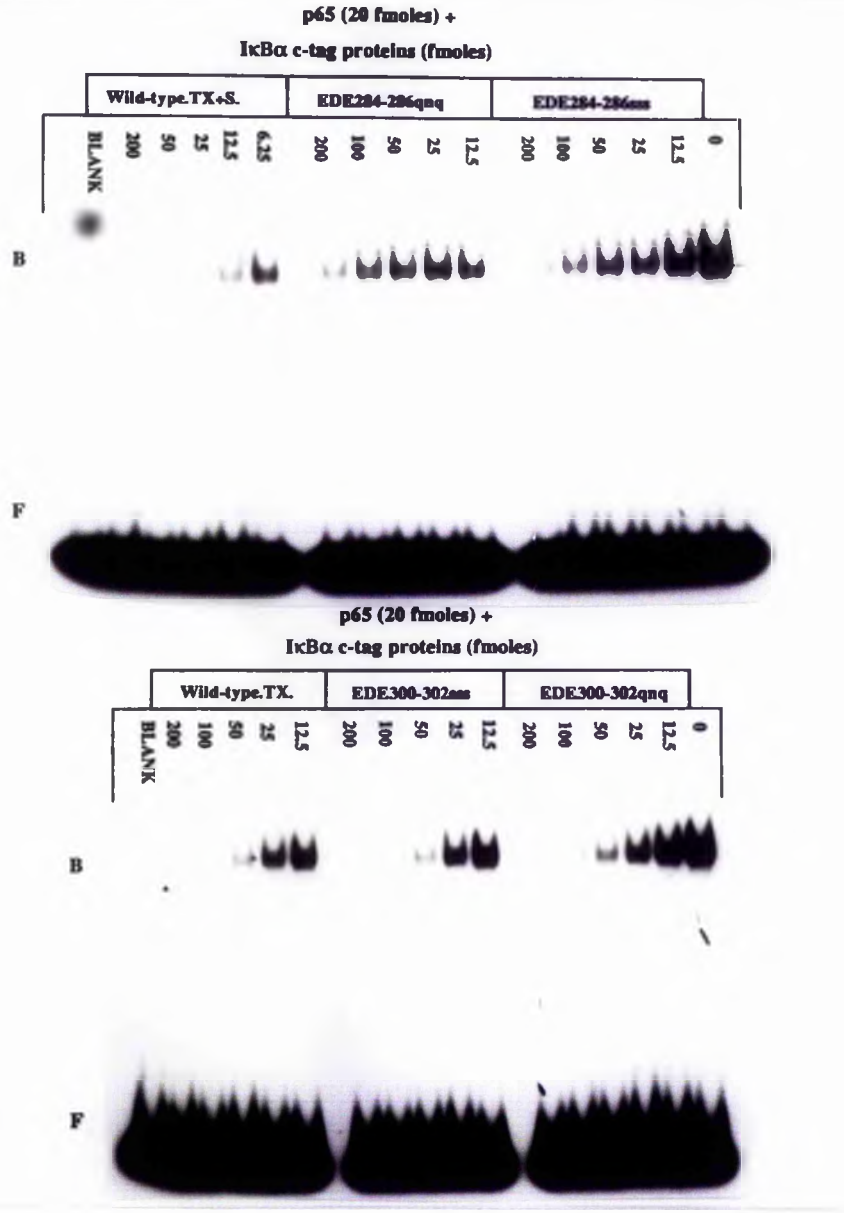
A)


p65 (20 fmoles) +
IκBα proteins (fmoles)
Wild-type.TX. c-tag
Wild-type.TX+S. c-tag
BLANK
200
50
25
12.5
6.25
0
200
50
25
12.5
6.25
0
B
F

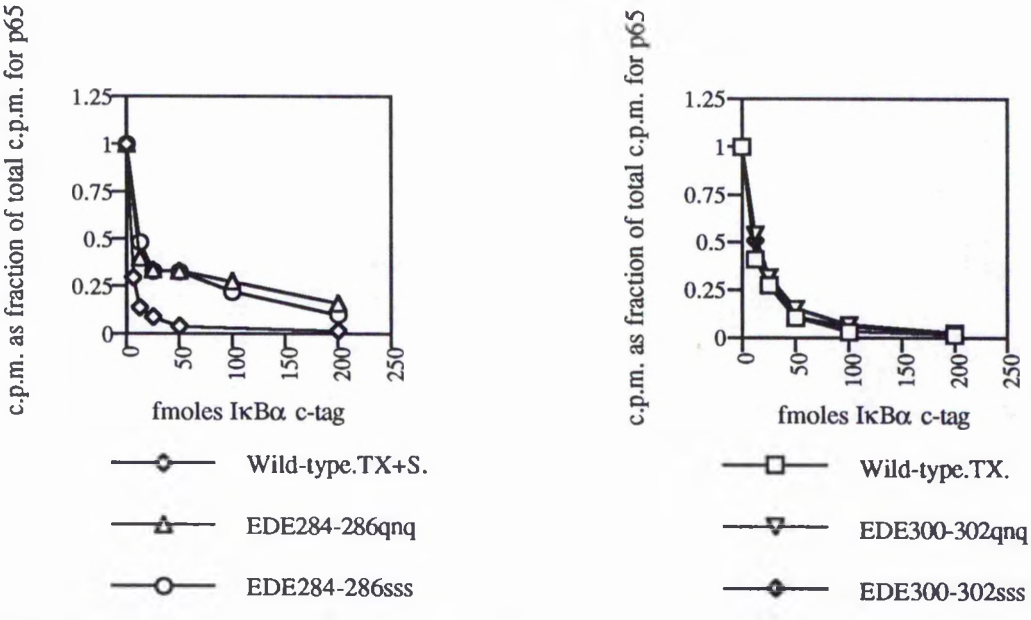
B)


c.p.m. as fraction of total c.p.m. for p65
1.25
1
0.75
0.5
0.25
0
0
50
100
150
200
250
fmoles IκBα c-tag
Wild-type.TX.
Wild-type.TX+S.

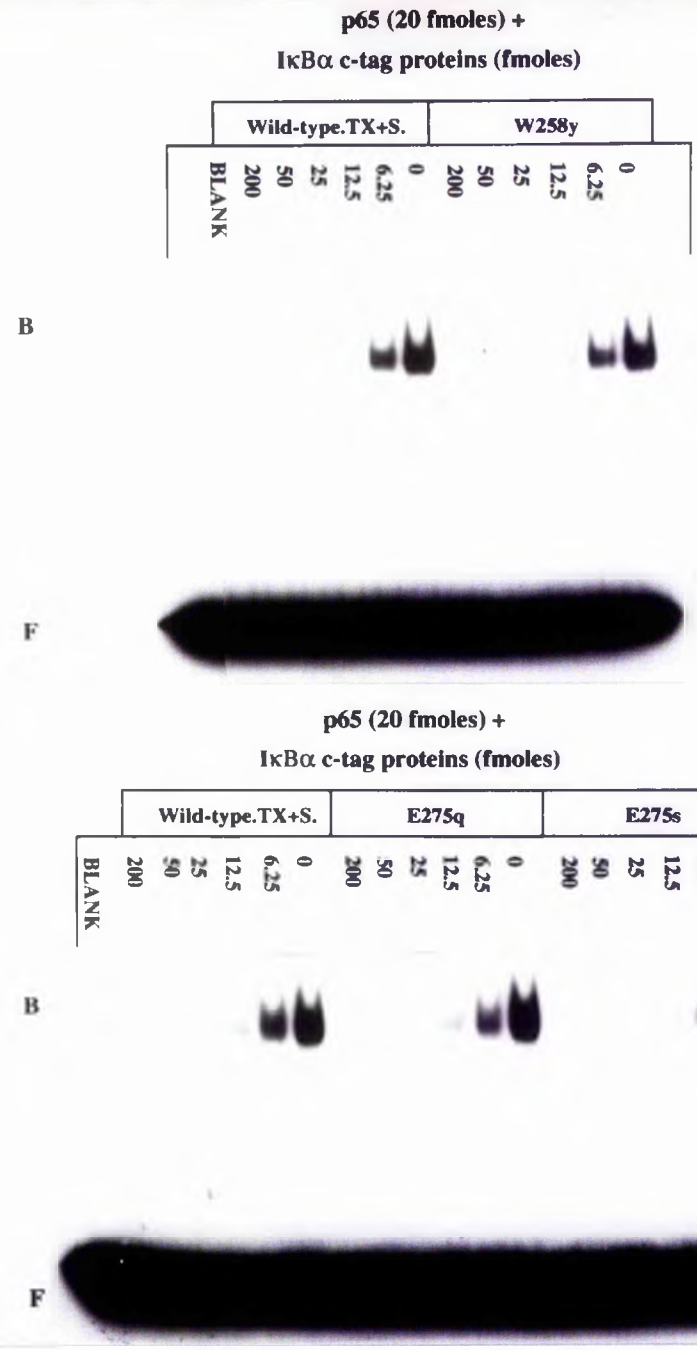
A)


p65 (20 fmoles) +
IκBα c-tag proteins (fmoles)
Wild-type.TX+S.
EDE284-286qnq
EDE284-286sss
BLANK
200
50
25
12.5
6.25
100
0
B
F
p65 (20 fmoles) +
IκBα c-tag proteins (fmoles)
Wild-type.TX.
EDE300-302sss
EDE300-302qnq
BLANK
200
100
50
25
12.5
0
B
F

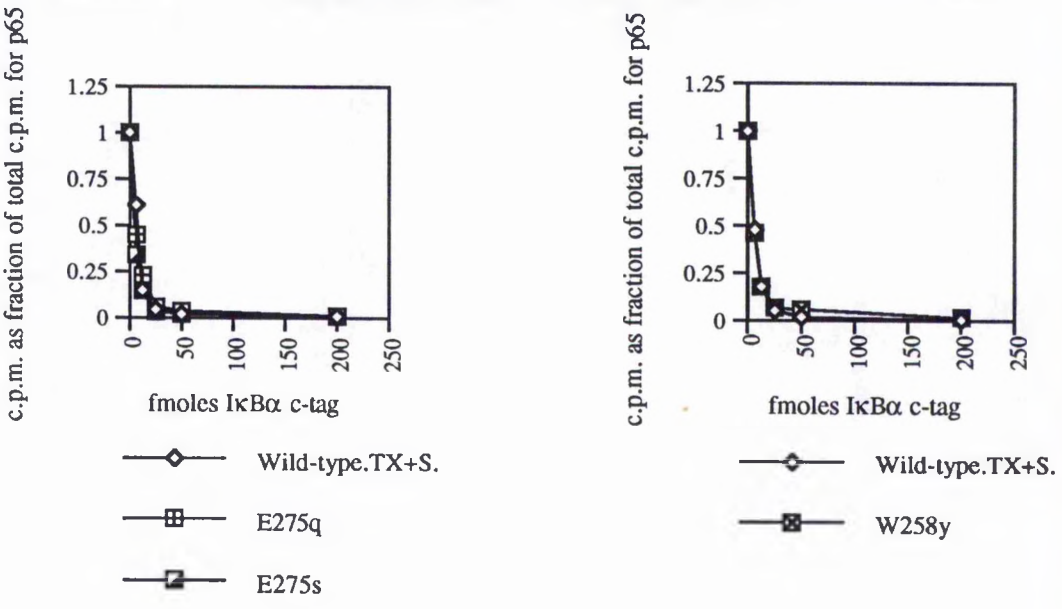
B)


c.p.m. as fraction of total c.p.m. for p65
1.25
1
0.75
0.5
0.25
0
0
50
100
150
200
250
fmoles IκBα c-tag
Wild-type.TX+S.
EDE284-286qnq
EDE284-286sss
c.p.m. as fraction of total c.p.m. for p65
1.25
1
0.75
0.5
0.25
0
0
50
100
150
200
250
fmoles IκBα c-tag
Wild-type.TX.
EDE300-302qnq
EDE300-302sss

A)


p65 (20 fmoles) +
IκBα c-tag proteins (fmoles)
Wild-type.TX+S.
W258y
BLANK
200
50
25
12.5
6.25
0
B
F
p65 (20 fmoles) +
IκBα c-tag proteins (fmoles)
Wild-type.TX+S.
E275q
E275s
BLANK
200
50
25
12.5
6.25
0
B
F

B)


c.p.m. as fraction of total c.p.m. for p65
1.25
1
0.75
0.5
0.25
0
0
50
100
150
200
250
fmoles IκBα c-tag
Wild-type.TX+S.
E275q
E275s
c.p.m. as fraction of total c.p.m. for p65
1.25
1
0.75
0.5
0.25
0
0
50
100
150
200
250
fmoles IκBα c-tag
Wild-type.TX+S.
W258y

using the tritonX-100 and sarkosyl method was able to almost completely inhibit the DNA binding activity of p65 homodimers at 50 fmoles, only 1.5 times the number of moles of p65 (20 fmoles) used for the binding reaction. However, approximately twice the number of moles, 100 fmoles, of the wild-type IκBα c-tag protein purified using the tritonX-100 method were required to achieve similar inhibition of p65 homodimers (figure 24 B)). For the C-terminal mutants, EDE284-286qnq and EDE284-286sss, complete inhibition of p65 DNA binding activity was only being approached at 200 fmoles of protein - the wild-type protein had achieved a similar level of inhibition at only 12.5 fmoles i.e. the mutants exhibited an approximate 16-fold reduction in their ability to inhibit p65 DNA binding activity compared to the wild-type protein. The remainder of the C-terminal and linker mutants were shown to have similar p65 DNA inhibition curves to the wild-type protein.

2.3. The capacity of wild-type and mutant IκBα c-tag purified proteins to be phosphorylated by recombinant casein kinase I and II.

The phosphorylation of IκBα is extremely important for its *in vivo* activity. In resting cells phosphorylated IκBα is associated with NF-κB in the cytoplasm. Upon activation of cells with cytokines or mitogens, IκBα is further phosphorylated prior to proteasome-mediated IκBα degradation and NF-κB translocation (IκBα degradation will be discussed further in chapter 3)(Britta-Mareen Traeckhner *et al.*, 1994; Didinato *et al.*, 1995; Roff *et al.*, 1996) . The *in vitro* phosphorylation of IκBα by kinases including protein kinase C (PKC), protein kinase A (PKA) (Ghosh *et al.*, 1990; Shirakawa *at al.*, 1989), heme regulated eIF2α kinase (Ghosh *et al.*, 1990), p38 (Han *et al.*, 1994), Raf-1 (Li *et al.*, 1993) and a PKC-ζ-associated kinase (Diaz-Meco *et al.*, 1994) has already been shown. As discussed at the beginining of chapter 2, the residues which were mutated in the C-terminal region of IκBα were located within consensus phosphorylation sites for the serine/threonine protein kinase, CKII. Therefore, if it was possible to show a failure or reduction in the ability of the C-terminal IκBα mutants to be phosphorylated *in vitro* by CKII compared to the wild-type protein, then this may indicate the importance of CKII and the mutated residues in the *in vivo*

phosphorylation of IκBα. In addition, assaying for the phosphorylation of the wild-type IκBα by CKI may signify a role for this enzyme in the *in vivo* phosphorylation of IκBα.

In vitro casein kinase assays were performed on mutant and wild-type IκBα c-tag purified proteins and the control proteins, bovine serum albumin (BSA) and purified casein kinase using purified recombinant casein kinase I and II. The extent of phosphorylation was followed by including radioactively labelled ATP ($\gamma(^{32}P)$ATP) in the reaction mixture. The radioactive phosphate group of ATP would be incorporated into the protein if phosphorylation was successful. Full details of the reaction mixtures and reaction conditions are given in the Materials and Methods section.

Figure 25 shows an autoradiograph of the CKI reaction mixtures separated through a 10% polyacrylamide gel. All of the mutant and wild-type IκBα c-tag proteins were phosphorylated by CKI. The extent of phosphorylation observed between the wild-type and mutant proteins appeared to be approximately equal. This was expected since none of the mutant proteins contained amino acid changes within any of the consensus CKI motifs. The activity of CKI was confirmed by the successful phosphorylation of casein kinase, a known substrate for CKI. Casein kinase is composed of four subunits, α1, α2, β and κ, ranging from 19-25kDa. The major subunit, α2 at 25kDa can be clearly seen in figure 25 and the minor subunits can be faintly observed below α2. The specificity of CKI activity and the absence of contaminating kinase activities were verified by the failure of BSA to be phosphorylated, a protein not normally targeted by CKI.

The corresponding autoradiograph obtained from the separation of the CKII reaction mixtures through a 10% polyacrylamide gel is given in Figure 26. The linker mutants appeared to be phosphorylated by CKII to the same extent as the wild-type IκBα c-tag protein. This was predicted since the altered residues in the linker mutants were not located within CKII phosphorylation sites. However, in contrast the phosphorylation of the C-terminal mutants was reduced to approximately 10-15% of the phosphorylation observed for the wild-type IκBα c-tag protein. Interestingly EDE284-286sss was phosphorylated to a greater extent (approximately 50% less phosphorylation than the wild-type IκBα c-tag protein) than the other C-terminal mutants. It is possible that the conformation of the mutant

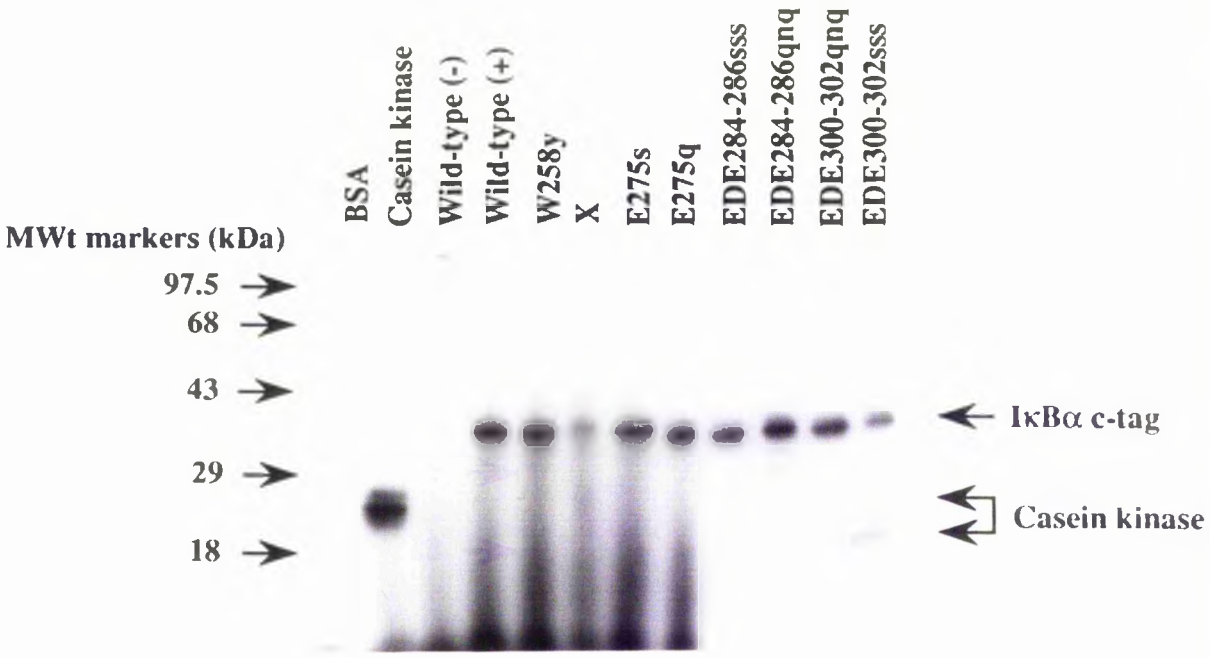


Figure 25. Phosphorylation of wild-type and mutant IκBα c-tag proteins by casein kinase I using γ(^{32}P) ATP.

0.22pmoles of either BSA, casein kinase, wild-type IκBα c-tag or mutant IκBα c-tag were incubated at 30°C for 1 hour in the presence of casein kinase I and γ(32 P) ATP. The reactions were quenched with 5µl of disruption buffer, boiled for 2-3 minutes and then separated through a 10% polyacrylamide gel. The gel was dried and exposed to X-ray film overnight at -70 °C. The position of the molecular weight markers are indicated to the left of the figure. Phosphorylated casein kinase and IκBα c-tag proteins are indicated by arrows. The IκBα c-tag mutant proteins are named at the top of the figure together with BSA, casein kinase and the wild-type IκBα c-tag protein (incubated with casein kinase I (+) and without casein kinase I (-)). The lane marked X should be ignored.

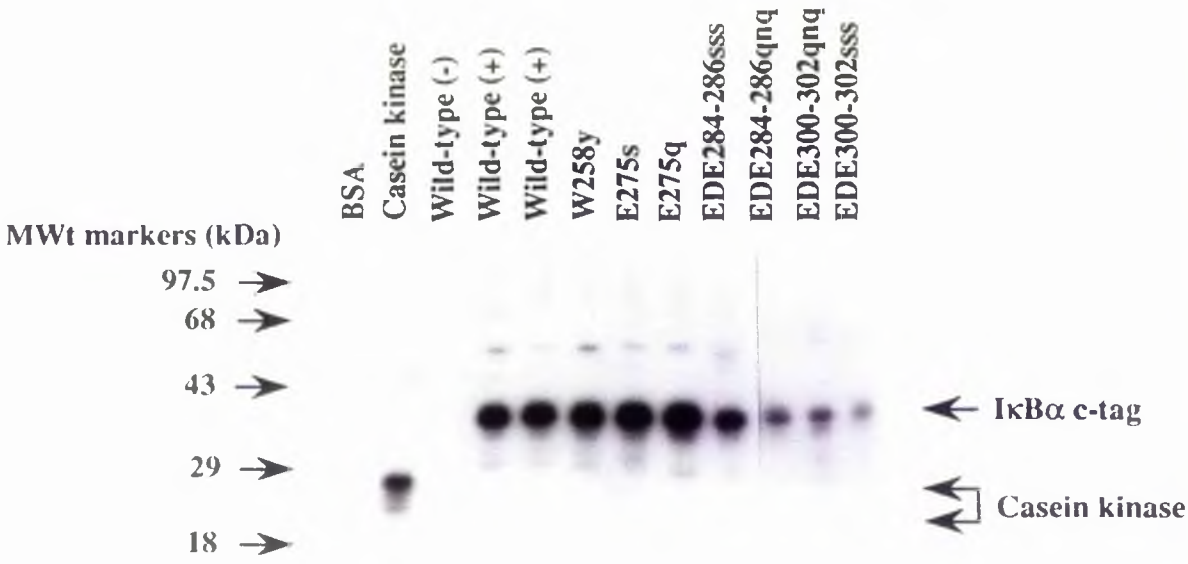


Figure 26. Phosphorylation of wild-type and mutant IκBα c-tag proteins by casein kinase II using γ(^{32}P) ATP.

0.22pmoles of either BSA, casein kinase, wild-type IκBα c-tag or mutant IκBα c-tag were incubated at 30°C for 1 hour in the presence of casein kinase II and γ(32 P) ATP. The reactions were quenched with 5µl of disruption buffer, boiled for 2-3 minutes and then separated through a 10% polyacrylamide gel. The gel was dried and exposed to X-ray film overnight at -70 °C. The position of the molecular weight markers are indicated to the left of the figure. Phosphorylated casein kinase and IκBα c-tag proteins are indicated by arrows. The IκBα c-tag mutant proteins are named at the top of the figure together with BSA, casein kinase and the wild-type IκBα c-tag protein (incubated with casein kinase II (+) and without casein kinase II (-)). The lane marked X should be ignored.

protein exposed other casein kinase II phosphorylation sites in the C-terminal region, allowing more efficient phosphorylation by CKII. Presumably the C-terminal mutants which possessed amino acid changes in CKII consensus sites were now able to prevent the enzyme recognising these sequences as targets for phosphorylation. However, phosphorylation was not completely blocked and this was probably due to phosphorylation at CKII sites other than those changed in the C-terminal mutants. Control proteins identical to those used for CKI were included in the CKII assays. Both the activity and specificity of CKII were confirmed by the phosphorylation of casein kinase and lack of such modification for BSA.

CHAPTER 3. *IN VIVO* CHARACTERISATION OF IκBα C-TAG C-TERMINAL AND LINKER MUTANTS.

Following the *in vitro* characterisation of the mutant IκBα c-tag proteins it was decided to study some of their functional properties *in vivo*. The C-terminal mutants were of particular interest because they possessed amino acid changes within a region rich in proline, glutamic acid, serine and threonine (PEST) residues, which have been associated with rapid protein turnover (Rogers *et al.*, 1986). More detail concerning the C-terminal region and its role in IκBα degradation will be given under the sub-headings which follow. The studies were performed by transient transfection of either 293 or Cos7 cells with the mammalian expression vector pcDNA3Pk (containing the appropriate wild-type or mutant DNA) which was engineered to generate C-terminally tagged proteins. The C-terminal Pk-tag linker (obtained by annealing together two oligonucleotides corresponding to the positive and negative strands of Pk-tag) was cloned into pcDNA3 via EcoRI and NotI restriction sites and sequenced (figure 27).

Also included in this chapter is a section of work which was performed in collaboration with colleagues from the Pasteur Institute, France. These studies involved the identification of lysine residues in the N-terminus of IκBα required for signal-induced ubiquitination and degradation of the protein *in vivo*. More details will be given under the appropriate sub-heading (3.6).

3.1. Assessment of signal-induced degradation of wild-type and mutant IκBα c-tag proteins.

When cells are exposed to tumour necrosis factor (TNF), IκBα is rapidly degraded. However, deletion of the C-terminal region of IκBα has been shown to stabilise the protein (Rodriguez *et al*, 1995) under conditions of activation. Since the C-terminal and linker IκBα c-tag mutants possessed amino acid changes within this region it was decided that these mutants would be tested for their ability to be degraded following activation with TNF α.

Before proceeding with the degradation assays the amounts of expression vectors were standardised such that equivalent quantities of wild-type and mutant IκBα c-tag protein

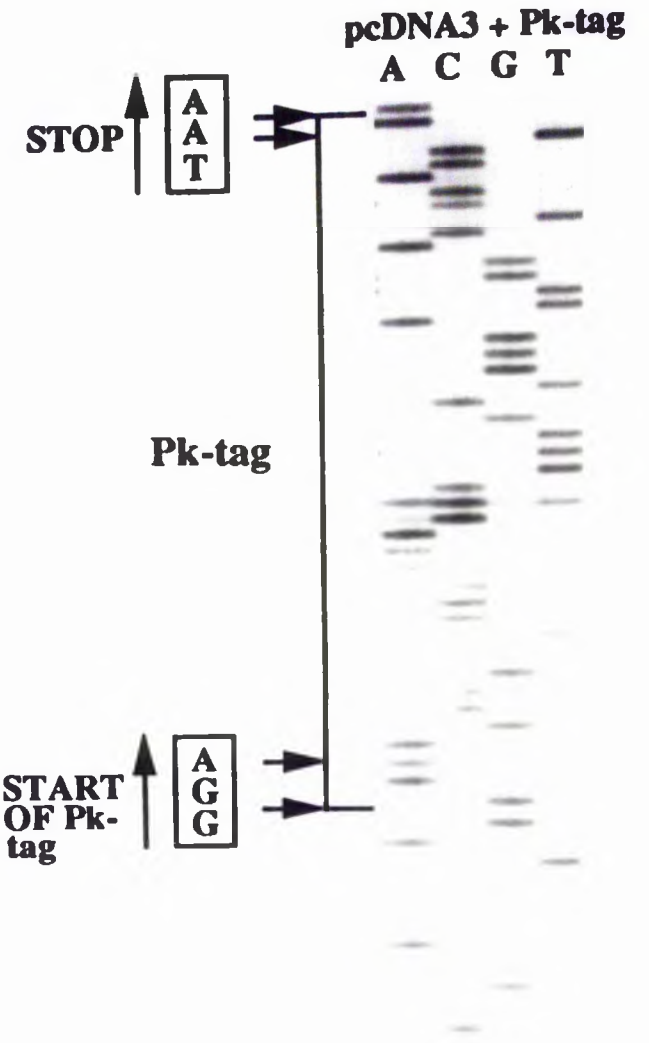


Figure 27. Sequence confirmation of the Pk-tag region cloned into the pcDNA3 plasmid.

The dideoxy chain-termination procedure was used to yield the coding DNA strands shown above for the Pk-tag region and part of the pcDNA3 plasmid (the area of DNA immediately below Pk-tag). The coding DNA sequences are read from bottom to top (5' to 3' on the coding DNA) and the lane location of each DNA base is shown at the top of the gel (A= adenine, C= cytosine, G= guanine and T= thymine). The position and sequence of the first codon of Pk tag is boxed (which is translated into glycine) and the final stop codon.

were generated. Therefore, any differences in the signal induced degradation patterns of the various IκBα c-tag proteins would not be related to variations in the amounts of protein generated from each vector. 293 cells were transfected with wild-type or mutant IκBα pcDNA3cPk and 36 hours later cytoplasmic extracts were prepared from the cells. The extracts were analysed by Western blotting using anti-Pk. The protein bands corresponding to wild-type and mutant IκBα c-tag were compared, and the amount of expression vector which produced comparable protein amounts was chosen (data not shown) and used for the degradation assays.

The degradation assays were performed on 293 cells 36 hours after transfection with wild-type or mutant IκBα pcDNA3cPk. The cells were pretreated for 5 minutes with the protein synthesis inhibitor, cycloheximide (to prevent continued IκBα c-tag protein synthesis from the strong cytomegalovirus promoter which would have prevented visualisation of IκBα c-tag degradation) followed by activation for 15 or 45 minutes with TNFα. The extent of signal-induced degradation was determined by Western blot analysis of cytoplasmic proteins using **A)** anti-Pk or **B)** a monoclonal anti-IκBα antibody, 10B MAD3.

Figure 28 A) shows the proteolytic degradation patterns observed with the anti-Pk antibody which revealed that the linker mutants behaved like the wild-type transfected IκBα c-tag, reaching almost complete degradation after 45 minutes. Interestingly however, the C-terminal mutants appeared to be only partially degraded after 45 minutes treatment with TNFα. However, since the basal turnover of these proteins was not examined (by incubating each set of mutant or wild-type transfected 293 cells for 0-45 minutes with cycloheximide alone) it cannot be concluded that this partial degradation is due solely to a reduction in inducible degradation, it may indicate that the C-terminal mutants have a lower level of basal turnover compared to the wild-type protein (see discussion for further details). The anti-Pk antibody did not show the presence of protein in the untransfected extracts which indicated that the antibody did not cross-react with any of the cellular proteins.

Analysis of the same set of cytoplasmic extracts using the 10B MAD3 monoclonal antibody exposed both the ectopic, tagged and the endogenous IκBα proteins (figure 28 B)) in the transfected extracts and endogenous (cellular) IκBα protein only in the untransfected

A)

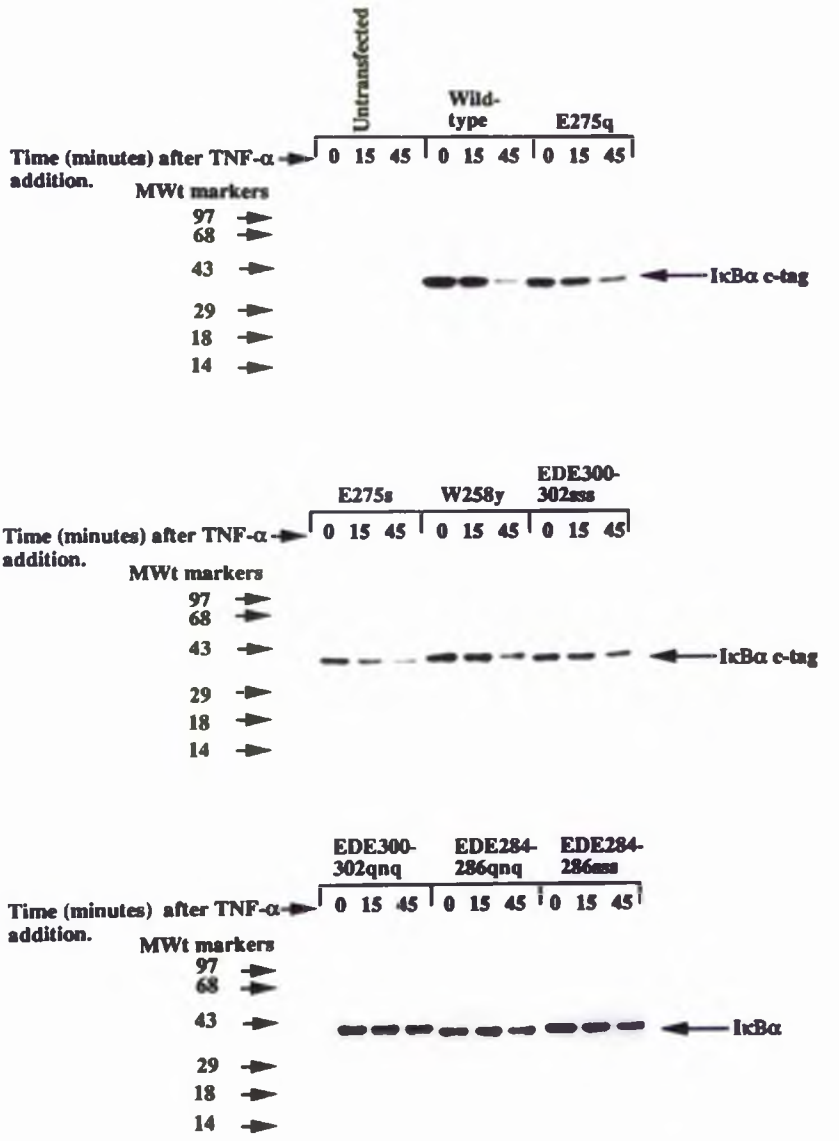
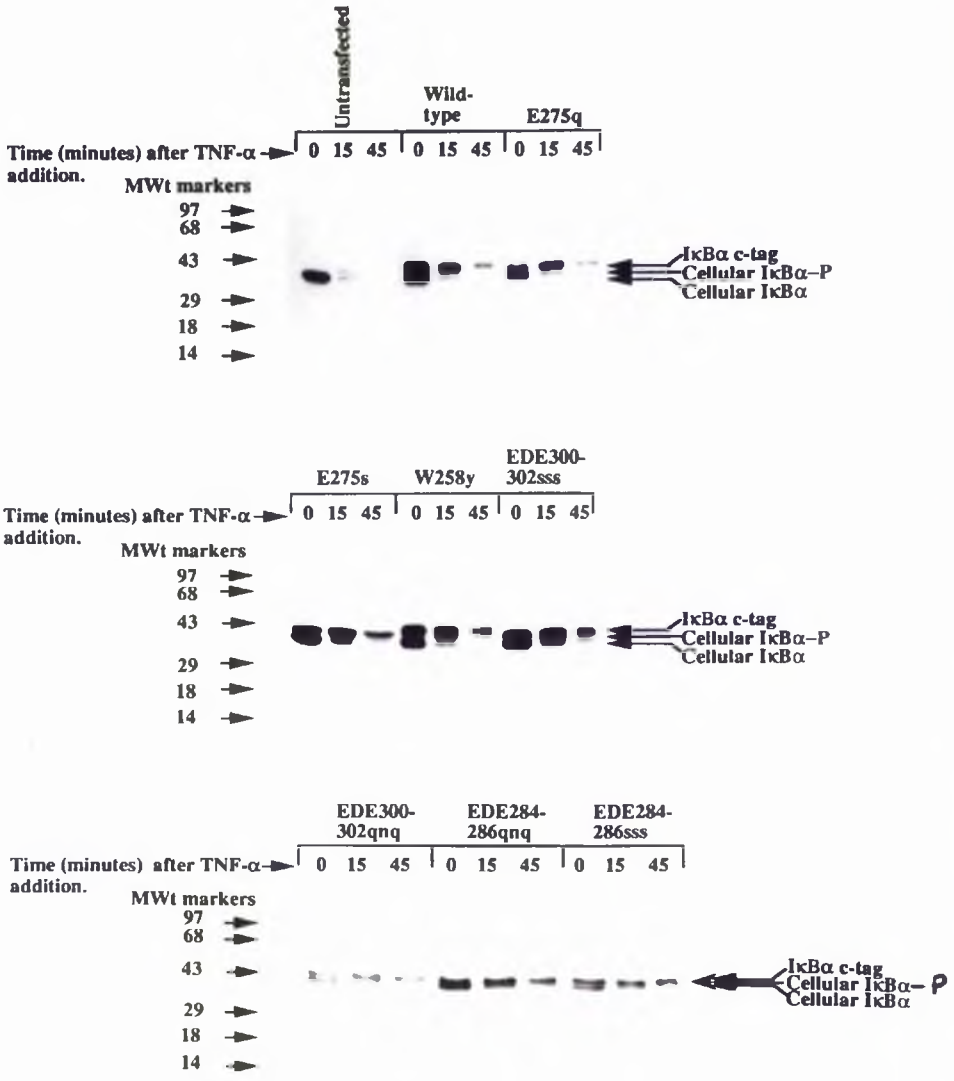


Figure 28. TNFα-induced degradation assays performed on 293 cells transfected with DNA encoding wild-type and mutant IκBα c-tag proteins.

293 cells were transfected with DNA (pcDNA3Pk) encoding either wild-type or C-terminal and linker mutant IκBα c-tag. At 36 hours post-transfection the cells were pretreated for 5 minutes with 100μg/ml cycloheximide and either left unactivated or activated with 50ng/ml TNFα for 15 minutes or 45 minutes. Cytoplasmic extracts were prepared and 20μg of each (in disruption buffer) were separated through a 10% SDS polyacrylamide gel and the proteins were detected by Western blot analysis using either **A)** anti-Pk (shown above) or **B)** a monoclonal anti-IκBα antibody, MAD10B (shown on the next page) followed by E.C.L. The position of the protein molecular weight standards are shown to the left of each figure (units=kDa) and arrows indicate the position of ectopic IκBα c-tag protein and phosphorylated and unphosphorylated endogenous IκBα protein. Untransfected 293 cytoplasmic cell extracts were included as a control. The time in minutes after treatment of cells with TNFα is given at the top of each Western blot in addition to the name of each mutant

B)


Untransfected
Wild-type
E275q
Time (minutes) after TNF-α addition.
0 15 45 0 15 45 0 15 45
MWt markers
97
68
43
29
18
14
IκBα c-tag
Cellular IκBα–P
Cellular IκBα
E275s
W258y
EDE300-302sss
Time (minutes) after TNF-α addition.
0 15 45 0 15 45 0 15 45
MWt markers
97
68
43
29
18
14
IκBα c-tag
Cellular IκBα–P
Cellular IκBα
EDE300-302qnq
EDE284-286qnq
EDE284-286sss
Time (minutes) after TNF-α addition.
0 15 45 0 15 45 0 15 45
MWt markers
97
68
43
29
18
14
IκBα c-tag
Cellular IκBα– P
Cellular IκBα

extracts. The ectopic protein appeared as a slower migrating band on the Western blot as a result of the C-terminally fused epitope, tag of 14 amino acids. As observed with the anti-Pk antibody, 10B MAD3 showed that the degradation of wild-type and linker mutant IκBα c-tag proteins was reached after 45 minutes treatment with TNFα, whereas the C-terminal mutants were only partially degraded after this period of time. It was also evident that the endogenous IκBα protein was degraded more rapidly than the wild-type and linker mutant ectopic, tagged IκBα proteins: After only 15 minutes the endogenous protein was almost completely degraded compared to 45 minutes for the wild-type and linker mutant ectopic, tagged IκBα protein (see discussion for further details).

3.2. Assessment of signal-induced phosphorylation of wild-type and mutant IκBα c-tag proteins.

Since hyperphosphorylation of IκBα is a pre-requisite to its signal-induced degradation (Traenckner *et al.*, 1994; Roff *et al.*, 1995) it was possible that the increased resistance to proteolysis observed for the C-terminal mutant IκBα proteins was a result of incomplete hyperphosphorylation. The ability of the C-terminal mutants along with the linker mutants to be inducibly phosphorylated was therefore examined.

293 cells were transfected with wild-type or mutant IκBα pcDNA3cPk (C-terminal and linker mutants and an IκBα c-tag mutant known to be resistant to proteolysis and possessing serine to alanine changes at positions 32 and 36) and 36 hours later the cells were pretreated for 15 minutes with a proteasome inhibitor, the peptide aldehyde z-LLL-H, to prevent IκBα degradation and stabilise phosphorylated forms of the protein. This was followed by 45 minutes or with OKA for 45 minutes followed by TNFα for 15 minutes or no indu. The proteins were visualised by Western blotting using the anti-Pk antibody (figure 29). Only the results for the co-stimulation by OKA and TNFα of transfected cells are shown. When unstimulated extracts were compared to stimulated extracts it was clear that the stimulated extracts of the wild-type IκBα c-tag protein exhibited an additional slower migrating band which corresponded to the hyperphosphorylated form of the protein. This slower migrating hyperphosphorylated IκBα c-tag species was also observed (somewhat less

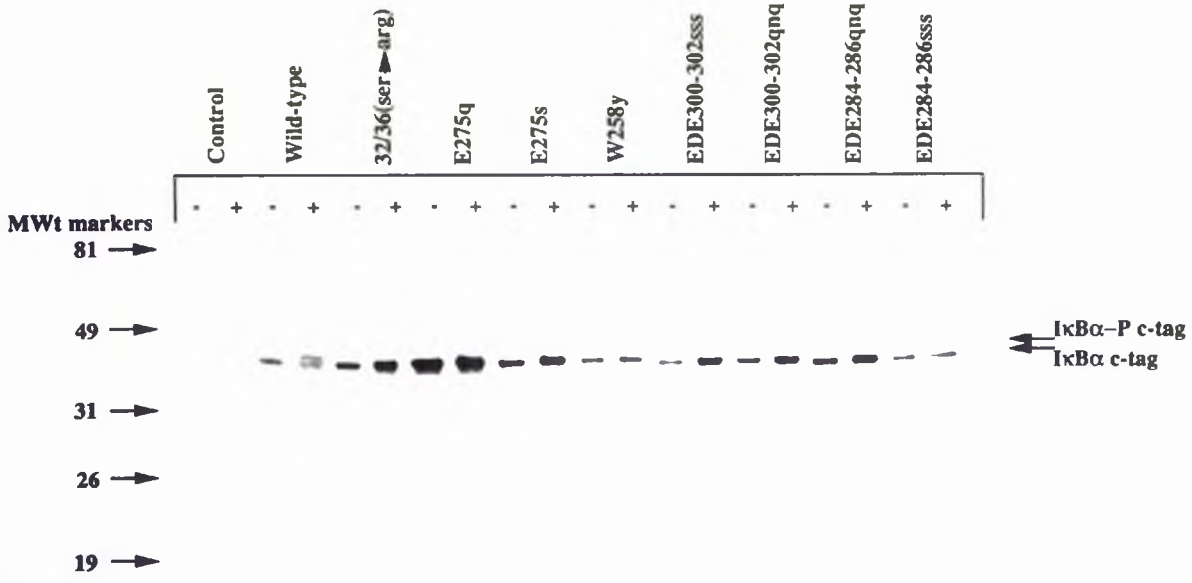


Figure 29. TNFα–induced phosphorylation assays performed on 293 cells transfected with DNA encoding wild-type and mutant IκBα c-tag proteins.

293 cells were transfected with DNA (pcDNA3Pk) encoding wild-type, C-terminal or linker mutant IκBα c-tag. At 36 hours post-transfection the cells were pretreated with 25μM of the proteasome inhibitor z-LLL-H for 15 minutes (this was maintained until the end of the culture). The cells were then either induced with 250nM okadaic acid for 45 minutes followed by TNFα for 15 minutes (indicated by + at the top of the Western blot) or not induced at all (indicated by - at the top of the Western blot). Cytoplasmic cell extracts were prepared in the presence of phosphatase inhibitors. For each sample 30μg of total protein was separated through a 16.5% polyacrylamide/ 9.4% glycerol gel in a buffer gradient and detected by Western blot analysis using anti-Pk followed by E.C.L. (shown above). Arrows indicate the position of the IκBα c-tag protein and the inducibly phosphorylated form of IκBα c-tag (IκBα-P c-tag). The position of the protein molecular weight standards (units=kDa) are given to the left of the figure. Untransfected 293 cytoplasmic extracts are included as a control. Mutants are named at the top of the figure.

clearly) for the stimulated extracts of the linker mutants (E275q, E275s and W258y) and the C-terminal mutants EDE284-286qnq and EDE284-286sss. However, a hyperphosphorylated form of IκBα c-tag was not observed in the stimulated extracts of mutants EDE300-302qnq and EDE300-302sss. These results indicated that the increased resistance to proteolysis exhibited by the C-terminal mutant IκBα c-tag proteins, EDE284-286qnq and EDE284-286sss did not result from a loss of the proteins ability to be hyperphosphorylated in response to cell signalling. However, mutants EDE300-302qnq and EDE300-302sss appeared not to be inducibly phosphorylated and it was therefore possible that the increased resistance to proteolysis exhibited by these mutants was due to an inability to be inducibly phosphorylated. It should be noted that the apparent lack of hyperphosphorylation observed for these C-terminal mutants and the poor shift in mobility exhibited by the other mutants may be due to the nature of the amino acids which were changed in each of the mutants i.e the substitution of charged residues for their uncharged equivalents. In theory, this should not affect the mobility of the mutant proteins because they are analysed by SDS-PAGE and therefore the SDS should render the proteins native charge insignificant. However, it is possible that the substitutions have, in some way, altered the mobility of the mutants so that inducible phosphorylation cannot be detected on the gradient gel.

3.3. Analysis of the ubiquitination patterns of wild-type and mutant IκBα c-tag proteins.

Proteolytic degradation of IκBα in stimulated cells has been shown to involve ubiquitination of two key residues, lysines 21 and 22, located in the N-terminal region of the protein (Rodriguez *et al.*, 1996-see section 3.6). It has been proposed that following signal-induced phosphorylation of IκBα on serine residues 32 and 36 (Brown *et al.*, 1995; Brockman *et al.*, 1995; Traenckner *et al.*, 1995), lysines 21 and 22 are ubiquitinated marking the protein for degradation by the proteasome. Since the C-terminal IκBα c-tag mutants exhibited a partial resistance to signal-induced degradation which was not a result of

incomplete inducible phosphorylation for EDE284-286qnq and EDE284-286sss, it was possible that their ability to be ubiquitinated was compromised.

This was tested by transfecting 293 cells with either wild-type or mutant/pcDNA3Pk and 36 hours later TNF activating the cells in the presence of z-LLL-H. Cytoplasmic extracts from the cells were analysed by Western blotting using the anti-Pk antibody and the proteins were visualised after treatment with E.C.L. reagents using a long exposure time. Figure 30 illustrated the presence of ubiquitin-conjugated forms of all of the mutant IκBα c-tag proteins. The amount of ubiquitination was comparable to that observed for the wild-type protein. Thus, the incomplete signal-induced degradation of the C-terminal IκBα c-tag mutants could not be attributed to any reduction in the ability of these mutants to be ubiquitinated. The C-terminal mutants EDE300-302qnq and EDE300-302sss were also ubiquitinated despite their apparent failure to be inducibly phosphorylated. This appears to agree with the suggestion in section 3.2 that the analysis of the mutant proteins by SDS-PAGE was not sufficient to detect the hyperphosphorylated forms of the proteins.

3.4. The affect of wild-type and mutant IκBα c-tag proteins on NF-κB-dependent transcription in response to TNFα.

It would be predicted from the results of the degradation assays that the C-terminal IκBα mutants (EDE284-286qnq, EDE284-286sss, EDE300-302qnq and EDE300-302sss) which were only partially degraded in response to TNFα should retain NF-κB in the cytoplasm of cells. This would prevent the nuclear translocation of NF-κB and subsequent activation of NF-κB-dependent genes.

To test this prediction a fixed amount of an NF-κB-dependent (3EnhConALuc) luciferase reporter vector was employed to be initially co-transfected with a range of wild-type IκBα/pcDNA3Pk concentrations into 293 or Cos 7 cells. Fourty hours later the cells were activated with TNFα for 8 hours and the relative luciferase units (RLU) per μg of protein was determined.

The results are illustrated in figures 31 and 32 and permit the amount of wild-type IκBα/pcDNA3Pk required to give a reasonable level of NF-κB-dependent transcription to be

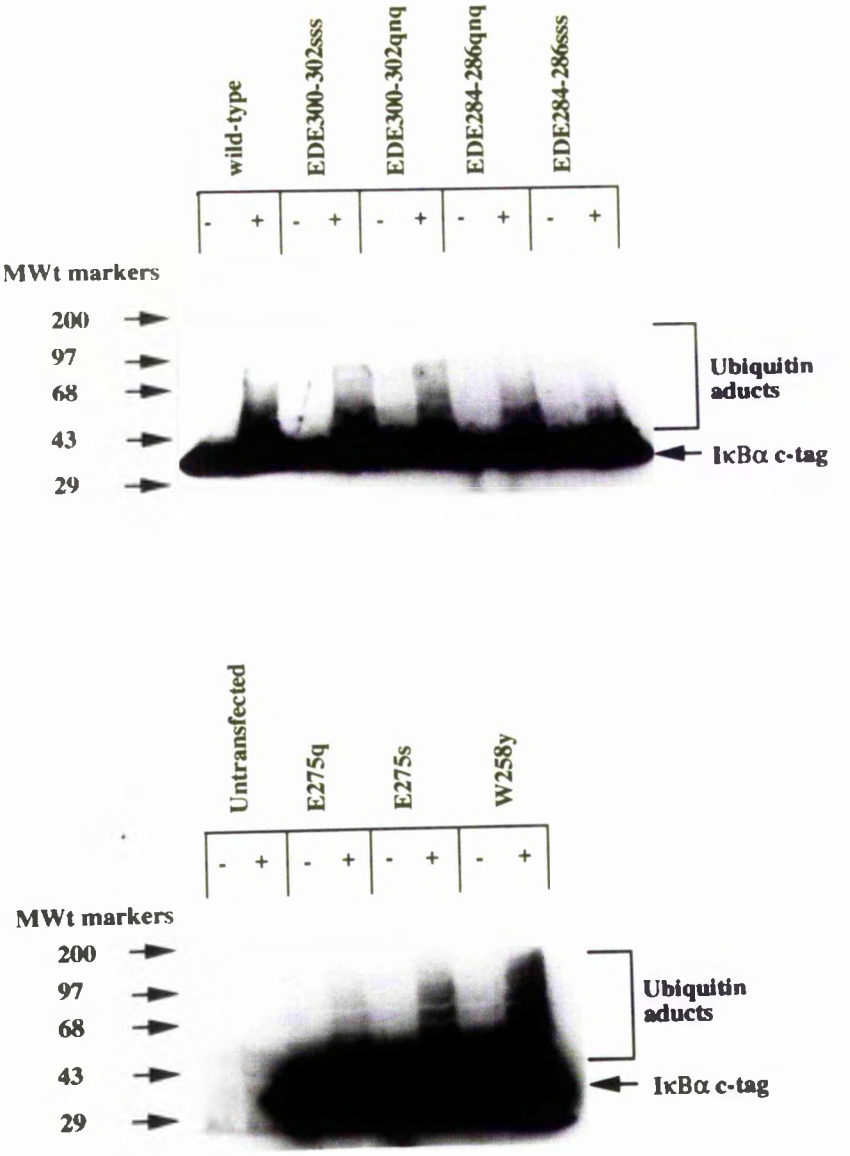


Figure 30. Analysis of the ubiquitination patterns obtained from 293 cells transfected with DNA encoding wild-type and mutant IκBα c-tag proteins.

293 cells were transfected with DNA (pcDNA3Pk) encoding either wild-type or C-terminal and linker mutant IκBα c-tag . At 36 hours post-transfection the cells were pretreated with 25μM of the proteasome inhibitor, zLLLH for 45 minutes and 100μg/ml cycloheximide for 5 minutes, before activation with 50ng/ml TNFα for a further 45 minutes (indicated by + at the top of each Western blot). Unactivated cells (indicated by - at the top of each Western blot) were treated with 100μg/ml cycloheximide only. Cytoplasmic extracts were prepared in the presence of iodoacetamide and 20μg of each (in disruption buffer) were separated through a 10% SDS polyacrylamide gel and the proteins were detected by Western blot analysis with anti-Pk followed by E.C.L. using a long exposure. The position of the protein molecular weight standards are shown to the left of each figure (units=kDa) and arrows indicate the position of ectopic IκBα c-tag protein and its ubiquitin aducts. The mutants are named at the top of each Western blot.

identified. It can be observed that TNFα induced Cos7 cells (figure 31) transfected with only the NF-κB-dependent luciferase reporter showed a 17-fold transactivation of the reporter vector. However, co-transfection with the highest quantity of wild-type IκBα/pcDNA3Pk (1μg) resulted in a complete inhibition of transcription from the luciferase reporter. Presumably transfecting cells with higher amounts of wild-type IκBα/pcDNA3Pk resulted in the expression of more ectopic wild-type IκBα c-tag. Consequently, under conditions of activation the cells degradation machinery would not be able to proteolyse all of the ectopic, tagged wild-type IκBα protein. This would lead to elevated levels of wild-type IκBα c-tag available to bind NF-κB and prevent NF-κB-dependent transcription from the reporter vector.

However, as the amount of wild-type IκBα/pcDNA3Pk co-transfected into cells was reduced to around 0.125μg the inhibitory effect on luciferase expression was diminished to give a 9-fold transactivation of the luciferase reporter. Further reductions in the amount of wild-type IκBα/pcDNA3Pk co-transfected into cells to 0.016μg resulted in a 15-fold transactivation of the reporter, a transactivation similar to that obtained from cells transfected with 3EnhConALuc alone (a positive control included to check for expression from the luciferase reporter) i.e. following activation of cells with TNFα, the lower levels of ectopic, tagged wild-type IκBα protein were not sufficient to replace the degraded IκBα protein which allowed free NF-κB to activate luciferase expression. A luciferase reporter, ConAluc, lacking the critical enhancer region was also transfected into cells to act as a control for enhancer-dependent activation of luciferase expression through NF-κB recognition sites. As expected, expression from the ConAluc plasmid failed indicating the requirement for the enhancer region to achieve luciferase expression.

In contrast to Cos7 cells, TNFα stimulated 293 cells co-transfected with 3EnhConAluc and high quantities of wild-type IκBα/pcDNA3Pk did not exhibit an inhibitory effect on NF-κB-dependent transactivation of the luciferase reporter (figure 32). Instead, a 16-fold transactivation of the reporter was observed (comparable to TNFα activated cells transfected with 3EnhConAluc alone), a level of transactivation which remained unchanged when cells were transfected with smaller quantities of the wild-type

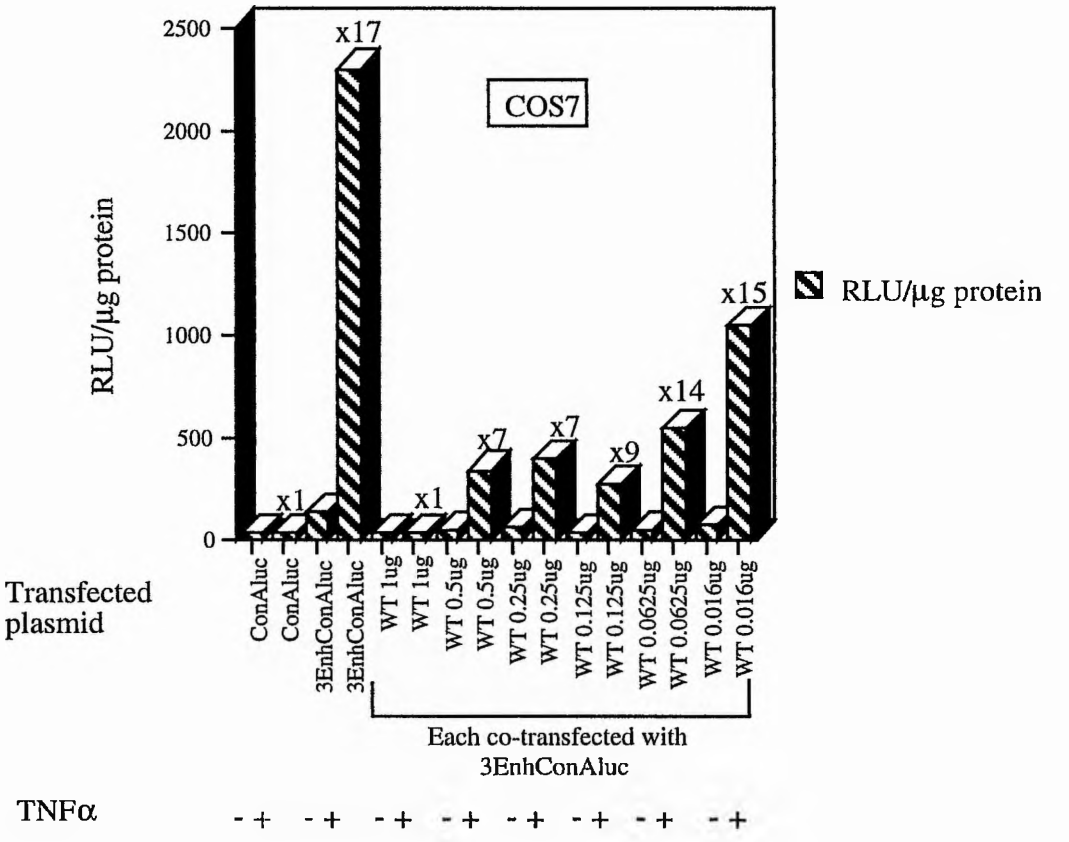


Figure 31. Luciferase assays performed on Cos 7 cells co-transfected with the NF-κB-dependent reporter vector 3EnhConALuc and DNA encoding wild-type IκBα c-tag.

Cos 7 cells were co-transfected with the reporter plasmid 3EnhConALuc and DNA encoding the wild-type pcDNA3Pk expression vector (indicated by WT along the x-axis with the corresponding quantity of DNA used in µg). Two controls were included: the reporter plasmid, 3EnhConALuc transfected alone and the reporter plasmid, ConAluc (which lacked the NF-κB-dependent enhancer) also transfected alone. 40 hours post-transfection the cells were activated with 10ng/ml TNFα for 8 hours (indicated by +) or left unactivated (indicated by -) and the luciferase activity was measured in the cell lysates. Luciferase activity is expressed as RLU/µg of cell lysate protein. The level of luciferase expression in the TNFα activated cell extracts is expressed as a multiple of the corresponding unactivated cell extract (indicated above the + lane for each of the transfected plasmids).

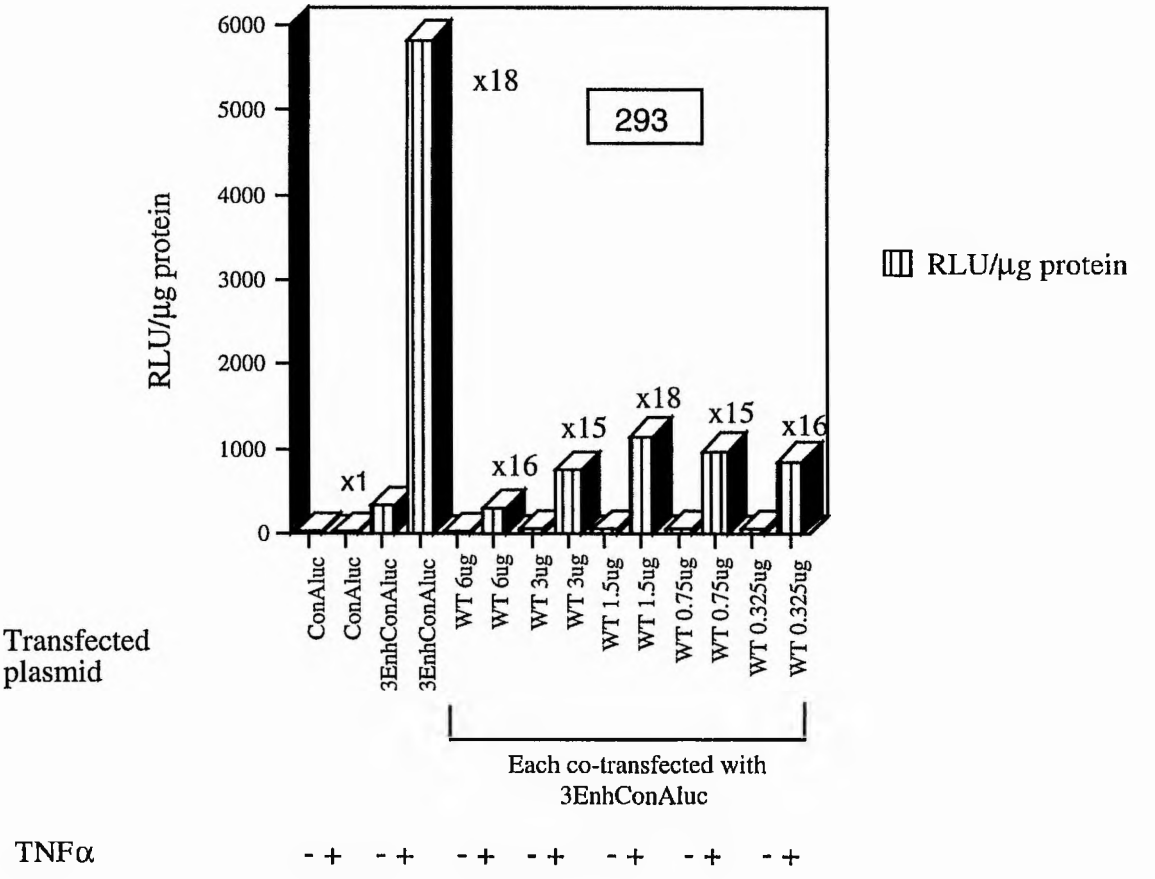


Figure 32. Luciferase assays performed on 293 cells co-transfected with the NF-κB-dependent reporter vector 3EnhConALuc and DNA encoding wild-type IκBα c-tag.

293 cells were co-transfected with the reporter plasmid 3EnhConALuc and DNA encoding the wild-type pcDNA3Pk expression vector (indicated by WT along the x-axis with the corresponding quantity of DNA used in υg). Two controls were included: the reporter plasmid, 3EnhConALuc transfected alone and the reporter plasmid, ConAluc (which lacked the NF-κB-dependent enhancer) also transfected alone. 40 hours post-transfection the cells were activated with 10ng/ml TNFα for 8 hours (indicated by +) or left unactivated (indicated by -) and the luciferase activity was measured in the cell lysates. Luciferase activity is expressed as RLU/µg of cell lysate protein. The level of luciferase expression in the TNFα activated cell extracts is expressed as a multiple of the corresponding unactivated cell extract (indicated above the + lane for each of the transfected plasmids).

IκBα c-tag expression vector. This was a consequence of the lower expression levels of transfected IκBα in 293 cells compared to Cos7 cells. Indeed, 293 cells transfected with wild-type or mutant (C-terminal and linker) pcDNA3Pk were found to express lower levels of transfected IκBα c-tag than endogenous (cellular) IκBα protein (see figure 33).

The level of transactivation from the luciferase reporter in cells expressing elevated levels of transfected IκBα should be lower compared to cells transfected with 3EnhConAluc alone (since there will be more IκBα present to retain NF-κB in the cytoplasm, preventing its passage to the nucleus and activation of luciferase expression). For example, the 9-fold transactivation of the luciferase reporter observed in Cos7 cells transfected with 0.125μg wild-type IκBα/pcDNA3Pk (figure 31) would reflect this situation. Therefore, it was decided that these conditions would be used for the luciferase assays which followed.

Before proceeding to test the mutant proteins in this system, the amount of protein generated from their respective expression vectors was standardised. The levels of mutant IκBα expression were standardised to that obtained for the wild-type protein which had produced a 9-fold transactivation of the luciferase reporter (0.125μg). Thus, any differences in the mutants ability to affect signal-induced NF-κB-dependent transcription would not be related to variations in the amount of protein generated from each expression vector. Western blot analyses from cells transfected with different amounts of the expression vectors encoding the mutant proteins were carried out (data not shown) to determine the amount of construct required to yield comparable protein levels. Expression vectors encoding mutant or wild-type proteins were then co-transfected with the NF-κB-dependent (3EnhConALuc) luciferase reporter vector into Cos 7 cells. Fourty hours later the cells were activated with TNFα for 8 hours and the RLU per μg of protein was determined.

Shown in figure 34 are the results of this experiment. TNFα activated cells transfected with the NF-κB-dependent luciferase reporter alone showed an 45 fold transactivation of the luciferase reporter. Co-transfection of cells with wild-type IκBα/pcDNA3Pk promoted a 24 fold transactivation. An IκBα mutant known to be resistant to proteolysis and possessing serine to alanine changes at positions 32 and 36, was co-transfected with the luciferase reporter. As anticipated, the mutant caused a massive decrease

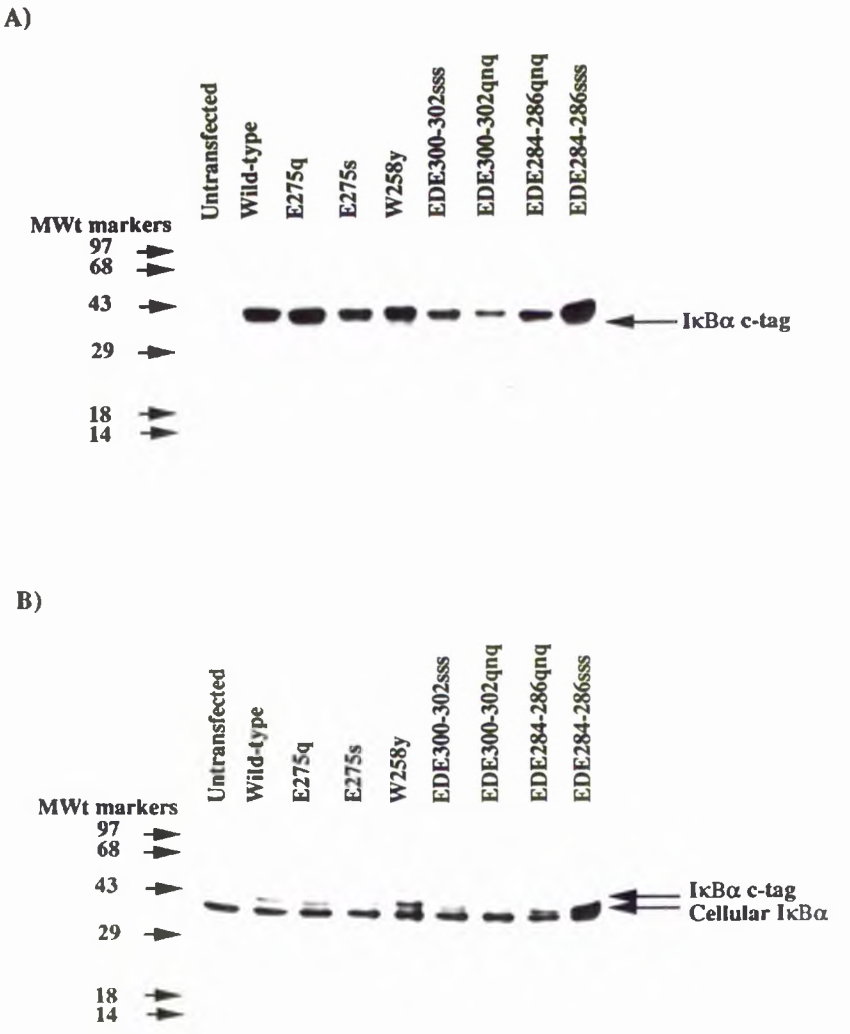


Figure 33. Western blot of 293 cell extracts prepared from cells transfected with DNA encoding either wild-type or mutant IκBα c-tag proteins illustrating the different expression levels of transfected and cellular IκBα .

293 cells were transfected with DNA encoding either wild-type or mutant pcDNA3Pk expression vectors (shown at the top of the figure). Untransfected 293 cells were also included as a control. At 36 hours post-transfection cytoplasmic extracts were prepared and 20µg of each (in disruption buffer) were separated through a 10% SDS polyacrylamide gel. The proteins were detected by Western blot analysis with either **A)** anti-Pk or **B)** a monoclonal anti-IκBα antibody, MAD10B, followed by E.C.L. Arrows indicate the position of transfected IκBα c-tag and cellular IκBα. The position of the protein molecular weight standards are also shown (units=kDa). Mutants are named at the top of the figure.

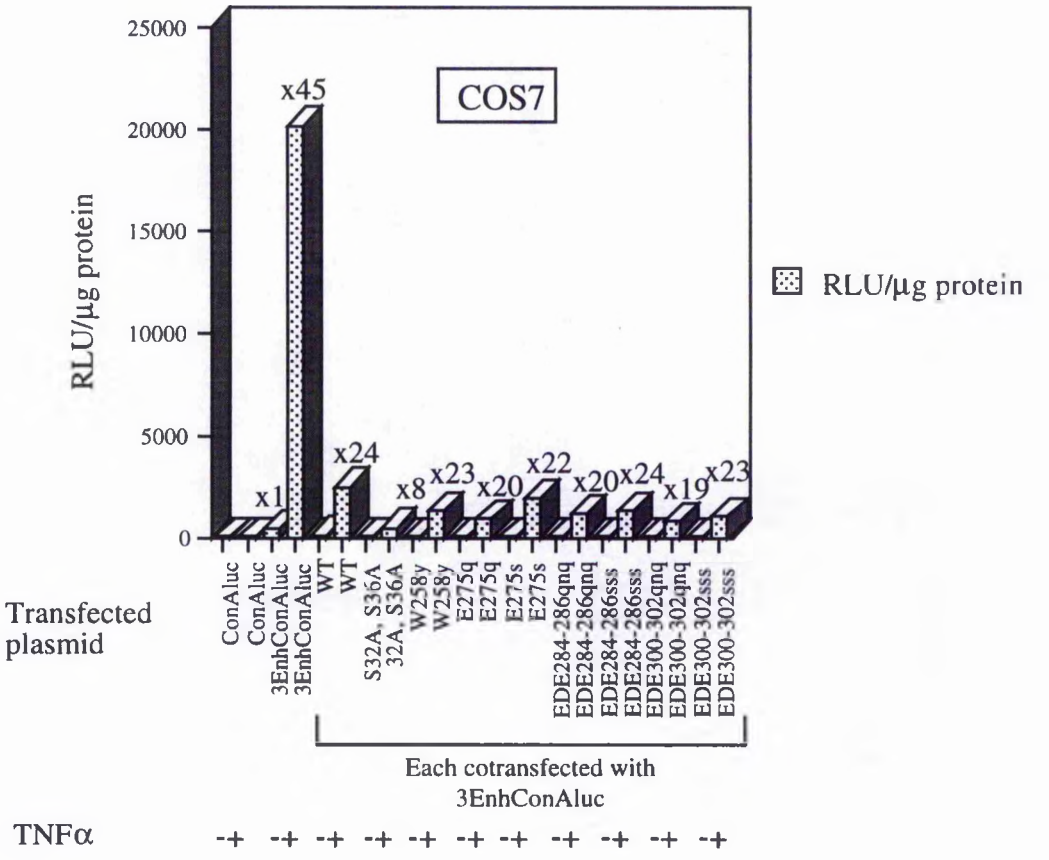


Figure 34. Luciferase assays performed on Cos 7 cells co-transfected with the NF-κB-dependent reporter vector 3EnhConALuc and DNA encoding either wild-type or mutant IκBα c-tag.

Cos 7 cells were co-transfected with the reporter plasmid 3EnhConALuc and DNA encoding either wild-type, C-terminal or linker mutant pcDNA3Pk expression vectors (indicated along the x-axis with the corresponding quantity of DNA used in υg). Two controls were included: the reporter plasmid, 3EnhConALuc transfected alone and the reporter plasmid, ConAluc (which lacked the NF-κB-dependent enhancer) also transfected alone. 40 hours post-transfection the cells were activated with 10ng/ml TNFα for 8 hours (indicated by +) or left unactivated (indicated by -) and the luciferase activity was measured in the cell lysates. Luciferase activity is expressed as RLU/µg of cell lysate protein. The level of luciferase expression in the TNFα activated cell extracts is expressed as a multiple of the corresponding unactivated cell extract (indicated above the + lane for each of the transfected plasmids).

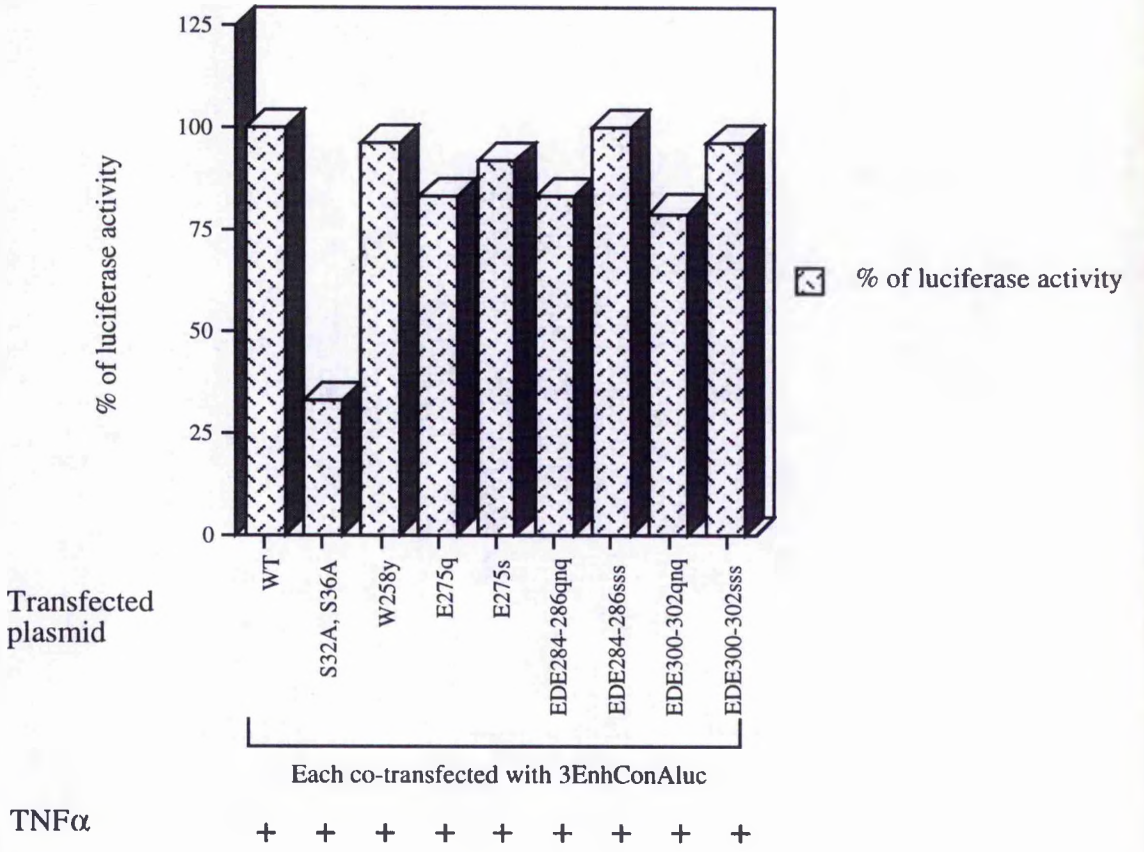


Figure 35. Percentage of luciferase activity obtained from TNF-activated 293 cells co-transfected with the NF-κB-dependent reporter vector 3EnhConALuc and DNA encoding wild-type and mutant IκBα c-tag .

The luciferase activity obtained from 293 cells co-transfected with the reporter plasmid 3EnhConALuc and DNA encoding the mutant pcDNA3Pk expression vector (indicated along the x-axis) was expressed as a percentage of the luciferase activity of cells transfected with the wild-type construct (indicated by WT along the x-axis). The luciferase activity for the wild-type transfected cells was given an arbitrary value of 100%.

in luciferase expression to around 65% of the level observed for the wild-type protein (figure 35). This can be explained by its ability to resist signal-induced degradation thus allowing it to retain NF-κB in the cytoplasm and prevent its nuclear translocation and transactivation. Surprisingly, the C-terminal and linker mutants did not show any inhibitory effect on luciferase expression. Their levels of transactivation were similar to those observed for the wild-type protein. The reason for this is unclear although it is possible that the degradation characteristics of the mutant proteins were different in Cos7 cells compared to 293 cells. The cell types do have different origins (Cos7 cells are transformed African Green-monkey kidney cells whereas 293 cells are transformed human embryonic kidney cells) and it possible that some post-translational modification of the mutant proteins may occur in Cos7 cells but not in 293 cells to alter the degradation of the mutants or perhaps there ability to interact with NF-κB. ConALuc was transfected into cells to act as a control for enhancer-dependent activation of luciferase expression through NF-κB recognition sites. The results clearly showed that ConALuc transfected cells were not able to express luciferase under conditions of activation indicating the requirement for the enhancer region. Therefore, it would appear that ubiquitination of the C-terminal and linker IκBα c-tag proteins releases NF-κB for translocation to the nucleus where it can upregulate gene expression.

3.5 Binding efficiency of wild-type and mutant IκBα c-tag proteins to cellular NF-κB p65 homodimers.

In order to determine whether the wild-type and mutant IκBα c-tag proteins were able to interact *in vivo* with NF-κB cytoplasmic extracts were prepared from 293 cells 36 hours after transfection with pcDNA3Pk encoding wild-type or mutant IκBα c-tag proteins. This was followed by immunoprecipitation of the cell lysates with the anti-Pk antibody and Western blot analysis using **A)** anti-Pk and **B)** polyclonal p65 antibody. (figure 36). Both the wild-type and mutant proteins were seen to bind p65 with equal efficiency indicating that the proteins were indeed capable of binding to NF-κB.

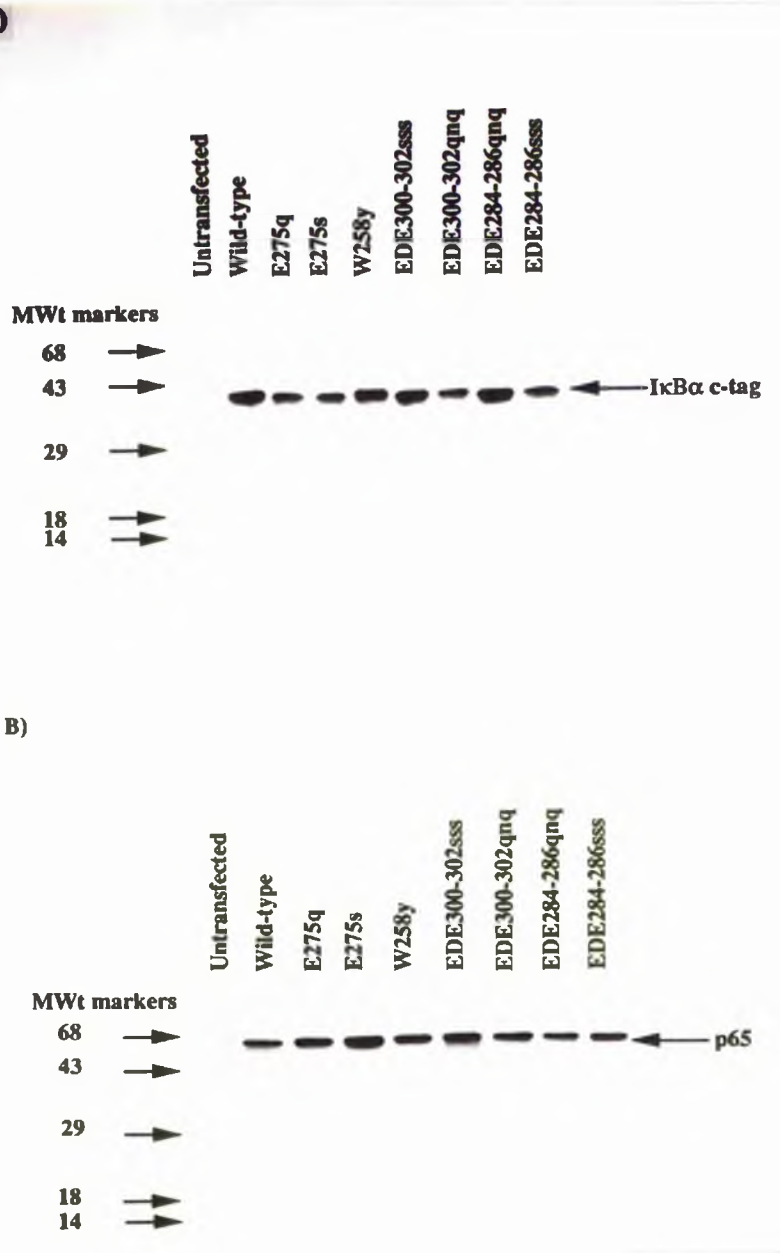


Figure 36. Immunoprecipitations by the SV5-P-k (anti-Pk) antibody of ectopic wild-type and mutant IκBα c-tag proteins and endogenous p65 protein obtained from 293 cells transfected with DNA encoding wild-type and mutant IκBα c-tag proteins .

293 cells were transfected with DNA (pcDNA3Pk) encoding wild-type, C-terminal or linker mutant IκBα c-tag. At 36 hours post-transfection the cells were harvested and an immunoprecipitation using protein A sepharose beads bound to anti-Pk was carried out on 50-100µg of the cytoplasmic cell lysate. The immunoprecipitated proteins in x1 disruption buffer were then separated through a 10% SDS polyacrylamide gel and detected by Western blot analysis using either **A)** anti-Pk (shown at the top of the figure) or **B)** polyclonal p65 antibody (shown at the base of the figure) followed by E.C.L. The position of the protein molecular weight standards are indicated to the left of each figure (units=kDa) and arrows show the ectopic IκBα c-tag and endogenous p65 proteins. Untransfected 293 cytoplasmic cell extracts were immunoprecipitated with anti-Pk and included as a control. Mutants are named at the top of each Western blot.

3.6. The identification of lysine residues required for signal-induced ubiquitination of IκBα.

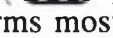
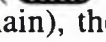
When the activity of the 26S proteasome is inhibited, signal-induced degradation of IκBα is blocked together with NF-κB activation. Furthermore, inhibition of proteosomal activity and subsequent stimulation of cells with TNF results in the detection of a hyper-phosphorylated form of IκBα and ubiquitinated forms of the protein (Pallombella *et al.*, 1994; Traenckner *et al.*, 1995; Roff *et al.*, 1996). The ubiquitinated forms of IκBα are not detected when inducible phosphorylation and degradation of IκBα is blocked by the mutation of serine 32 and serine 36 in the N-terminus of IκBα (Chen *et al.*, 1995; Roff *et al.*, 1996). This indicates that signal-induced phosphorylation is required for both *in vitro* and *in vivo* ubiquitination of IκBα.

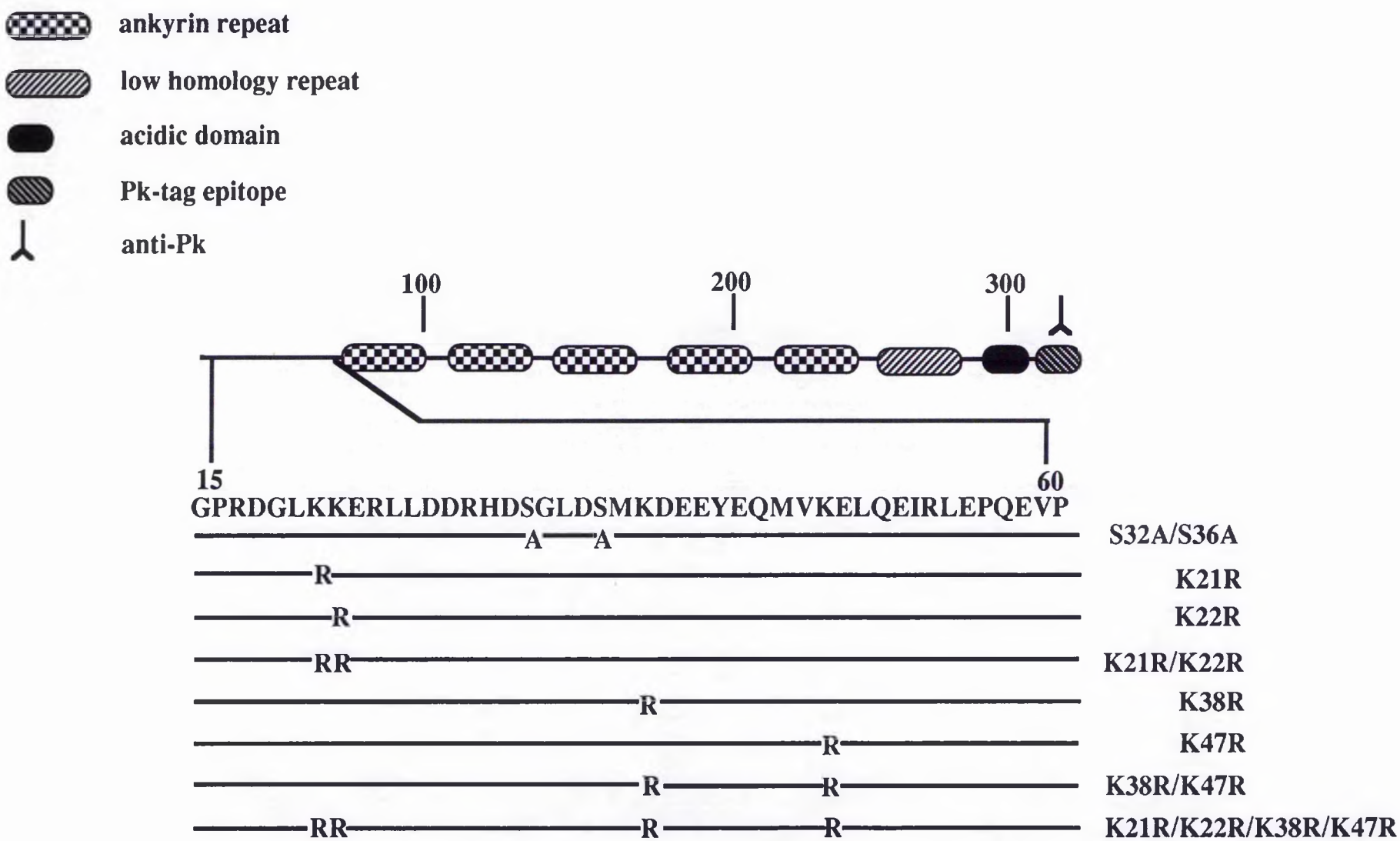
Ubiquitin chains are added to the ε-amino group of lysine residues in the target protein marking the protein for degradation by the proteasome. The following studies set out to identify which residues were responsible for the signal-induced ubiquitination and degradation of IκBα. A panel of seven IκBα mutants containing single and multiple lysine to arginine substitutions and a mutant possessing serine to arginine changes at residues 32 and 36 were generated by M. Rodriguez (Pasteur Institute, France) (figure 37) using a polymerase chain reaction strategy reported in Rodriguez *et al.*, 1995. The mutants were engineered to contain a C-terminal tag and cloned into the mammalian expression vector, pcDNA3 for transient transfection into 293 or Cos7 cells.

The experiments carried out by colleagues at the Pasteur Institute on the lysine IκBα mutants were similar to those performed on the C-terminal and linker IκBα mutants, detailed in sections 3.1, 3.2, 3.4 and 3.5 of this chapter and section 2.2 of chapter 2. A summary of the results obtained from these experiments will be given below together with any experimental conditions not detailed in the previously mentioned sections. In addition , the findings of the ubiquitination experiment (for details see section 3.3) performed in St. Andrews will be given.

An initial investigation of the ability of the lysine to arginine IκBα mutants to interfere with TNF induced transactivation of an NF-κB-dependent reporter gene

Figure 37. Schematic representation of IκBα C-terminally linked to the epitope Pk-tag and derivative mutant proteins with amino acid changes in the N-terminus region.

Wild-type IκBα c-tag is shown with the N-terminal amino acid sequence encompassing residues 15-60. Also illustrated are the amino acid changes introduced into the wild-type sequence to generate the lysine to arginine IκBα mutants and the ankyrin repeats (), low homology repeat () and acidic domain ()(-this forms most of the C-terminal domain), the Pk-tag epitope () and the anti-Pk antibody ( ).


ankyrin repeat
low homology repeat
acidic domain
Pk-tag epitope
anti-Pk
100
200
300
15
60
GPRDGLKKERLLDDRHDSGLDSMKDEEYEQMVKELQEIRLEPQEVP
A A
S32A/S36A
R
K21R
R
K22R
RR
K21R/K22R
R
K38R
R
K47R
R R
K38R/K47R
RR R R
K21R/K22R/K38R/K47R

(3EnhConAluc) was performed in Cos7 cells (see section 3.4). This revealed that co-transfection of the IκBα mutants containing the double substitutions, K21R and K22R with the luciferase reporter (mutant K21R/K22R and mutant K21R/K22R/K38R /K47R) severely impaired NF-κB-dependent transcription. However, co-transfection of either mutant K21, mutant K22, mutant K38R or mutant K47R with the luciferase reporter resulted in a far greater level of luciferase expression (similar to that observed for the wild-type IκBα protein but lower than the level observed in cells transfected with 3EnhConAluc alone). The inhibitory effect shown for mutants K21R/K22R and K21R/K22R/K38R /K47R was also detected using two other NF-κB inducers, IL1 and OKA and in 293 cells. In addition, the specificity of the NF-κB inhibitory effect was proven through the failure of both mutant and wild-type proteins IκBα proteins to prevent transcription from an inducible, but NF-κB-independent promoter, LTRΔκBLuc. It appeared that the level of inhibition of NF-κB activation observed for mutant K21R/K22R was less dramatic than that detected for the proteolysis resistant S32A/S36A mutant. However, the mutant containing two additional changes at K38 and K47 exhibited an inhibitory capacity comparable to the IκBα mutant, S32A/S36A.

In order to check for possible disruptions to the structure of the IκBα lysine mutants as a result of their amino acid substitutions, mutants were examined for their ability to interact with the p65 subunit of NF-κB. This was achieved by analysing the capacity of the mutants *in vitro* to prevent p65-DNA interactions in a gel electrophoresis DNA binding assay and their ability to bind *in vivo* (in Cos7 cells) overexpressed p65 (co-transfected with the pcDNA3 expression vectors containing the various lysine mutants, followed by immunoprecipitation of the IκBα mutants as in section 3.5). For the DNA binding assays the IκBα lysine mutants and p65 protein were generated *in vitro* individually using a coupled transcription/translation system (for more details see Rodriguez *et al.*, 1996). The mutants shown in figure 38 were able to inhibit the DNA binding of p65 to the same extent as the wild-type IκBα protein and to bind p65 with a similar affinity to the wild-type IκBα protein. Therefore, the inhibitory effect of the lysine mutants, K21R/K22R and

K21R/K22R/K38R/K47R on NF-κB-dependent transcription was not a result of any variation in the capacity of the mutants to interact with NF-κB.

Studies to determine whether the transfected lysine IκBα mutants were degraded in response to treatment of Cos7 cells with TNF (see section 3.1), revealed that mutant K21R/K22R was stable to proteolysis, reflecting its ability to inhibit NF-κB-dependent transcription. However, mutant K21R, mutant K22R and mutant K38R/K47R were inducibly degraded to the same extent as the wild-type IκBα protein which appeared to be in agreement with the higher level of NF-κB-dependent transcription observed for these IκBα proteins. Mutants K21R/K22R and K21R/K22R/K38R/K47R were also shown to be inducibly phosphorylated in Cos7 cells (see section 3.2) indicating that the increased resistance of K21R/K22R to proteolysis was not due to an impaired capacity of the mutant to be phosphorylated.

Since mutant K21R/K22R lacked two potential ubiquitin targets (lysine 21 and 22) it was possible that its inability to be inducibly degraded was due to a reduction in the ability of the mutant to be ubiquitinated. Mutant K21R/K22R was therefore transfected into 293 cells and analysed for its ability to be ubiquitinated under conditions of TNF activation and in the presence of the proteasome inhibitor, Z-LLL-H (see section 3.3 for details). As predicted the ubiquitination of mutant K21R/K22R was almost eliminated (figure 38). The proteolysis resistant S32A/S36A IκBα mutant and an IκBα mutant in which all the lysine residues(all K,R) had been substituted with arginine failed to be ubiquitinated. Mutant K21R/K22R/K38R/K47R was also not targeted for ubiquitination whereas mutant K38R/K47R was ubiquitinated but, to a lesser extent than the wild-type IκBα protein. Since the ubiquitination of mutant K21R/K22R was greatly reduced but not completely eliminated until lysines 38 and 47 were substituted for arginine residues in mutant K21R/K22R/K38R/K47R, lysines 21 and 22 must be critical targets for ubiquitination but lysines 38 and 47 must also play an auxillary role. This view is supported by the slightly poorer ubiquitination of mutant K38R/K47R compared to wild-type IκBα.

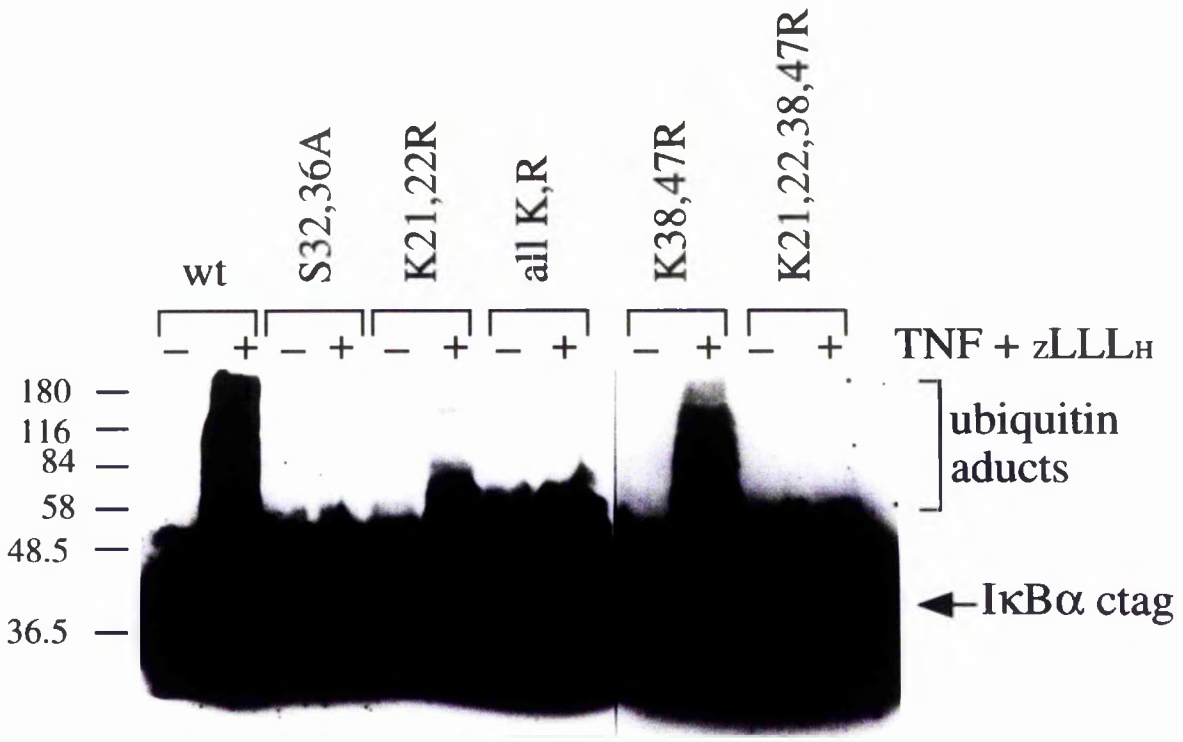


Figure 38. ***In vivo*** **ubiquitination of IκBα and lysine to arginine mutants.**

Human 293 cells were transfected the indicated IκBα constructs and maintained in culture for 24 hours. For the last 60 min in culture, cells were either incubated with control medium (-) or with 10μM z-LLL-H followed by 10ng/ml TNF. Cell extracts were prepared in the presence of iodoacetamide to inhibit the action of ubiquitin C-terminal hydrolases and analysed by Western blot with the SV5 PK tag Mab. The positions of the molecular mass markers, ectopic IκBα and its ubiquitin aducts are indicated (Rodriguez *et al.*, 1996).

CHAPTER 4. STRUCTURAL ANALYSIS OF THE C--TERMINAL REGION OF IκBα USING CIRCULAR DICHROISM (CD) SPECTRO--SCOPY.

Proteolysis studies had allowed IκBα to be arranged into three distinct domains, an unstructured N-terminal region and a highly organised central core consisting of 5 ankyrin repeats connected to the acidic C-terminal region by a protease sensitive linker (Jaffray *et al.*, 1995). It appeared that both the ankyrin repeats and the C-terminal region were generally resistant to proteolysis compared to the N-terminal and linker domains. This suggested that the central core and C-terminus possessed tightly organised structures. Indeed, circular dichroism spectroscopy had previously revealed that the ankyrin repeats of the IκB-like protein, cactus adopted an alpha- helical secondary structure (Gay *et al.*, 1993). It was possible that the ankyrin repeats existed as a single folded unit of alpha helices.

The functional integrity of IκBα was apparently reflected in the domain organisation of the protein, in that loss of either part of the ankyrin repeats or the C-terminus left the protein unable to inhibit p65 DNA binding and/or interact with p65 (Inoue *et al.*, 1992; Hatada *et al.*, 1993; Jaffray *et al.*, 1995; Rodriguez *et al.*, 1995). It was conceivable that the C-terminal region of IκBα, like the ankyrin repeat domain could form an independently folded unit and that any disruption of this structure (such as the deletion of residues within the C-terminus) would be deleterious to the activity of IκBα.

In order to determine whether the C-terminal region of IκBα could form a self-contained structure a series of four molecules corresponding to the C-terminal domain were generated; a chemically synthesised peptide corresponding to residues 275-317 of IκBα (supplied by Francoise Baleux, France) and three bacterially expressed, glutathione/FPLC purified C-terminal proteins-amino acids 265-317 c-tag (provided by E. Jaffray, St. Andrews University), amino acids 265-317 and amino acids 275-317 (construction and purification details are given in the following sections). These were analysed under different pH conditions and in the presence of trifluoroethanol (TFE), an agent known to promote α-helix, using the technique of circular dichroism (CD) spectroscopy. This method examines the interaction between polarised light and optically active molecules. Specifically, the technique

measures the wavelength dependence of the differential absorption of right and left circularly polarised light from an optically active molecule in order to elucidate the conformation of a macromolecule or macromolecular complex in solution. The technique can be used to identify elements of secondary structure in proteins, nucleic acids and nucleoproteins.

4.1. Construction of GST fusion proteins, 265-317 and 275-317 containing the C-terminal region of IκBα.

The coding sequence of the C-terminal region (265-317 and 275-317) of IκBα was generated by the PCR technique (Landt *et al.*, 1990) represented schematically in Figure xx, chapter 1, section 1.1 (for further details on this technique refer to chapter 1, section 1.1). The PCR products were analysed by separation through a 0.8% agarose gel and showed that the 265-317 construct had the expected size of approximately 159 base pairs and the 275-317 construct had the expected size of approximately 129 base pairs (data not shown).

PCR amplified DNA was digested with the restriction endonucleases, BamHI and EcoRI, purified and ligated into the pGEX2T vector (also digested with BamHI and EcoRI) and transformed into competent *E.coli* (XL-blue). Tetracycline and ampicillin resistant *E.coli* transformants were isolated and small scale preparations of their DNA (mini-preps.), were prepared and used as double stranded DNA templates to sequence the cDNA of the 265-317 and 275-317 constructs using the dideoxy-chain termination procedure. Figure 39 shows the sequencing data obtained for the cDNA of constructs 265-317 and 275-317 and indicates that the cloning procedure had generated the expected products with no inadvertent mutations being produced.

4.2 Expression and purification of the proteins, 265-317 and 275-317 containing the C-terminal region of IκBα in *E.coli.*

For the expression and purification of the proteins, 265-317 and 275-317 (containing the C-terminus of IκBα) in *E.coli* (XL-blues) the glutathione-S-transferase (GST)-fusion expression system was used (Smith and Johnson., 1988) (Refer to Chapter 1, section 1.2 for further details). The vector employed in this study was the pGEX-2T expression vector


amino acids 265-317
coding strand
ACGT
STOP
A
G
T
AMINO
ACID 265
A
T
A
amino acids 275-317
coding strand
A C G T
STOP
A
G
T
AMINO
ACID 275
A
A
G

Figure 39. Sequence confirmation of the constructs, 265-317 and 275-317, corresponding to the C-terminal region of IκBα.

The dideoxy chain-termination procedure was used to yield the coding DNA strands shown opposite for each of the two constructs, 265-317 and 275-317. The coding DNA sequences are read from bottom to top (5' to 3' on the coding DNA) and the lane location of each DNA base is shown at the top of the gel (A= adenine, C= cytosine, G= guanine and T= thymine). The position and sequence of the first codon in each construct is boxed (ATA for 265-317 which codes for isoleucine and GAA for 275-317 which codes for glutamic acid). The stop codon, TGA is also indicated.

(Promega) which allows the production of high levels of protein with a cleavable N-terminally fused 26 kD enzyme, glutathione-S-transferase (GST).

Screening for the over-expression of glutathione-S-transferase fusion proteins, 265-317 and 275-317, from the pGEX-2T vector was carried out as follows: 1ml overnight cultures of the positively identified 265-317/pGEX-2T and 275-317/pGEX-2T *E.coli* (XL-1 blue) clones (i.e. the clones which when sequenced exhibited the expected changes) were diluted the following day, 1:100, into 1ml of LB-broth and grown until an OD_{600nm} was reached. The cultures were then induced with IPTG for 3-4 hours and the cells were harvested by centrifugation, resuspended in PBS/0.5M NaCl and disruption buffer, boiled and then separated through a 10% polyacrylamide gel. An uninduced culture was included as a control. The construct, 265-317 was estimated have a molecular weight of 6171Da and the construct, 275-317 was estimated to have a molecular weight of 5049Da (as calculated through a DNA strider computer programme). The combined molecular weight of the GST fusions proteins would therefore be estimated at 32.2kDa for 265-317 and 31kDa for 275-317. The coomassie blue stained gels were used to identify clones possessing appropriately sized protein bands. 1ml overnight cultures of clones positive for the fusion proteins were diluted 1:100 into 1litre of LB-broth and grown at 37°C to the appropriate density before induction with IPTG to yield the fusion protein.

The individual fusion proteins were then purified by glutathione-agarose column chromatography using the tritonX-100 and sarkosyl method (see materials and methods), cleaved with thrombin and recovered using FPLC chromatography. Proteins were eluted from the column using a concentration gradient of sodium chloride (250mM to 1M). An additional glutathione-agarose purification step was included if the FPLC purified protein sample contained any undigested fusion protein (which can bind to the FPLC mono-Q column). The fusion protein was absorbed onto the column whereas the thrombin cleaved protein was passed through the column to yield the final mutant IκBα c-tag proteins.

Figures 40 and 41(A) show the results of the purification steps. The thrombin cleaved fusion protein (i.e. the FPLC column load), the FPLC column flow-through (i.e. GST), FPLC column washes and the optional glutathione-agarose purification step are not shown. The

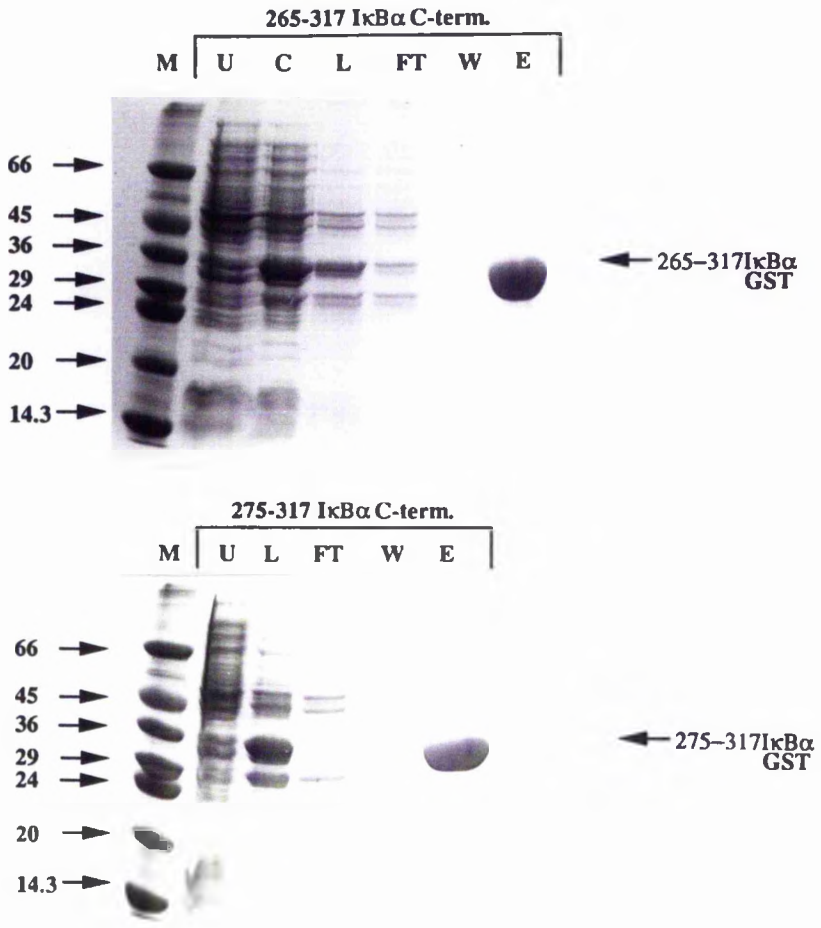


Figure 40. Glutathione-agarose column purifications of the proteins, 265-317 and 275-317 corresponding to the C-terminal region of IκBα.

Coomassie blue stained 10% SDS polyacrylamide gels of glutathione-agarose column purifications (using the tritonX100 and sarkosyl method) are shown for the proteins, 265-317 and 275-317 fused to GST . All sample tracks contained a mix of 15µl of protein solution and 7.5µl of 3x disruption buffer which had been boiled for 2-3 minutes before loading. The following abbreviations were used for the sample tracks shown :- **M** = protein molecular weight standards (units = kDa), **U** = uninduced crude bacterial extract, **C** = induced crude bacterial extract, **L** = IPTG induced crude bacterial load to the glutathione-agarose column, **FT** = flow-through from the glutathione-agarose column, **W** = glutathione-agarose column washes with PBS, 0.5M NaCl, 2mM EDTA, 1mM PMSF, 2mMbenzamidine, **E** = 10mM glutathione, 0.5M NaCl, 50mM Tris/HCl pH8.0 eluate from the glutathione-agarose column. For lanes **L**, **FT**, **W** and **E** the proteins were fused to glutathione-S-transferase (GST) to give a combined molecular weight of approximately 30-32kD.

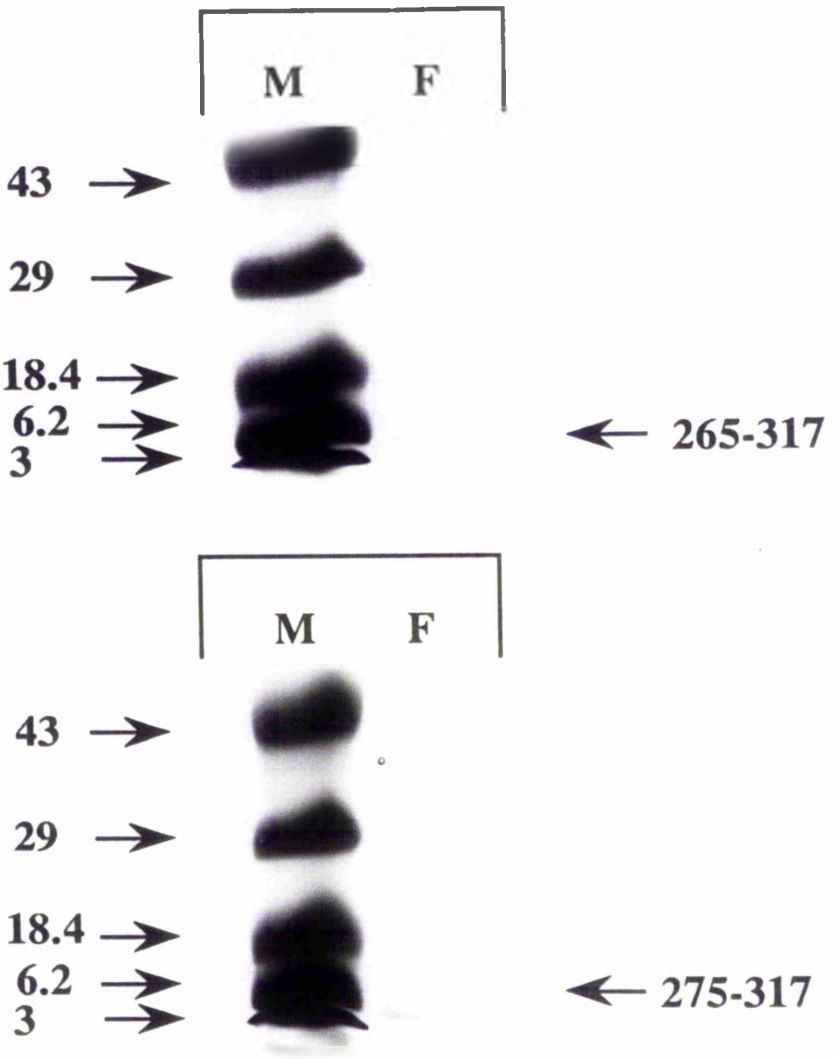


Figure 41A. FPLC purified proteins, 265-317 and 275-317 corresponding to the C-terminal region of IκBα.

Coomassie blue stained 10% SDS polyacrylamide gels of the FPLC purified proteins, 265-317 and 275-317 are shown (4-6kDa). All sample tracks contained a mix of 15µl of protein solution and 7.5µl of 3x disruption buffer which had been boiled for 2-3 minutes before loading. The following abbreviations were used for the sample tracks shown :- **M** = protein molecular weight standards (units=kDa), **F** = FPLC Mono-Q column pooled peak fractions in PBS/0.5M NaCl.

polyacrylamide gels illustrate that the glutathione-agarose affinity chromatography yielded virtually pure GST-fusion proteins in the various eluates (**E**). The proteins, 265-317 and 275-317 containing the C-terminal region of IκBα eluted from the FPLC Mono-Q column (**F**) were shown to be free from contaminating proteins. It should be noted that the IκBα C-terminal proteins do not stain well with coomassie blue compared to GST, therefore the GST-IκBα C-terminal fusion proteins appear as much darker bands compared to the IκBα C-terminal proteins alone.

Figure 42(B) shows the UV absorbance output trace obtained for the FPLC column purification of the IκBα C-terminal proteins (265-317 and 275-317). The initial GST flow-through was observed to the far left of each trace. 265-317 was eluted at the end of the salt gradient (1M) whereas 275-317 was eluted between 0.40M and 0.55M sodium chloride. The peak fractions were analysed using SDS-PAGE. The most concentrated fractions were combined and stored at -70°C.

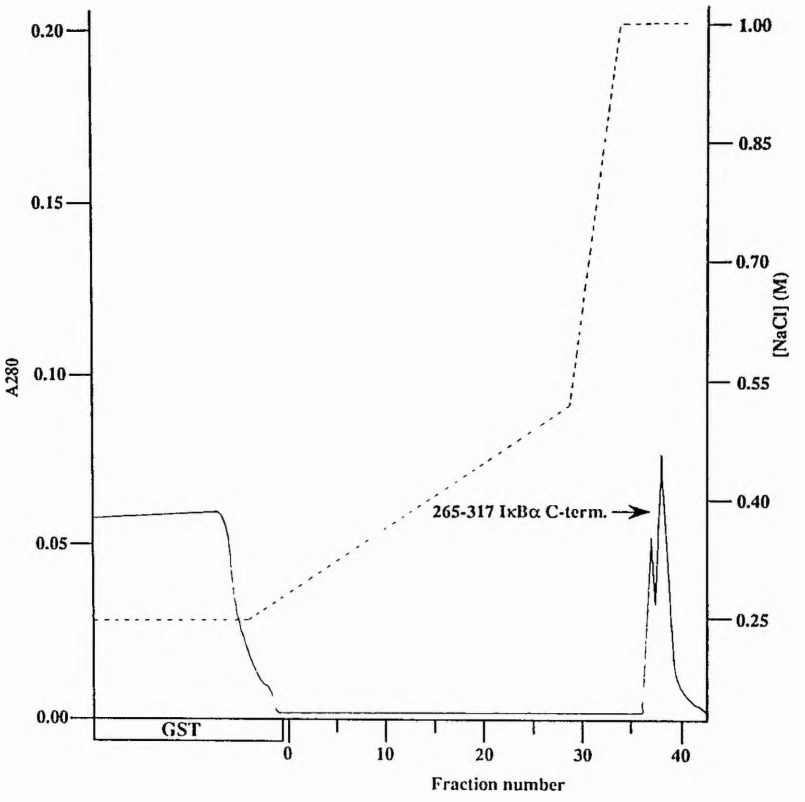
4.3. Circular Dichroism spectroscopy of C-terminal IκBα proteins using different pH conditions and in the presence of trifluoroethanol (TFE).

CD spectroscopy was performed in the far ultraviolet (UV) region (190-260nm) on a chemically synthesised peptide corresponding to residues 275-317 of IκBα (supplied by Francoise Baleux, France) and three bacterially expressed, glutathione/FPLC purified C-terminal proteins-265-317 c-tag (corresponding to the C-terminal region, part of the low homology sixth ankyrin repeat (265-317) and the c-tag region of wild-type IκBα c-tag and provided by E. Jaffray, St. Andrews University), 265-317 (corresponding to the C-terminal region and part of the low homology sixth ankyrin repeat (265-317) of wild-type IκBα) and 275-317 (corresponding to the C-terminal region (275-317) of wild-type IκBα) (sections 4.1 and 4.2) using different pH conditions and in the presence of an α-helix stabilising agent, triflouroethanol (TFE) (figures A-L). All proteins were analysed in a buffer of relatively low UV absorbance: The dried C-terminal peptide was resuspended to a concentration of 0.5mg/ml in a solution of 50mM sodium fluoride and 10mM sodium phosphate ranging in pH from 6.0-8.0. The three C-terminal fragments of IκBα, 265-317 c-tag (0.51mg/ml), 265-

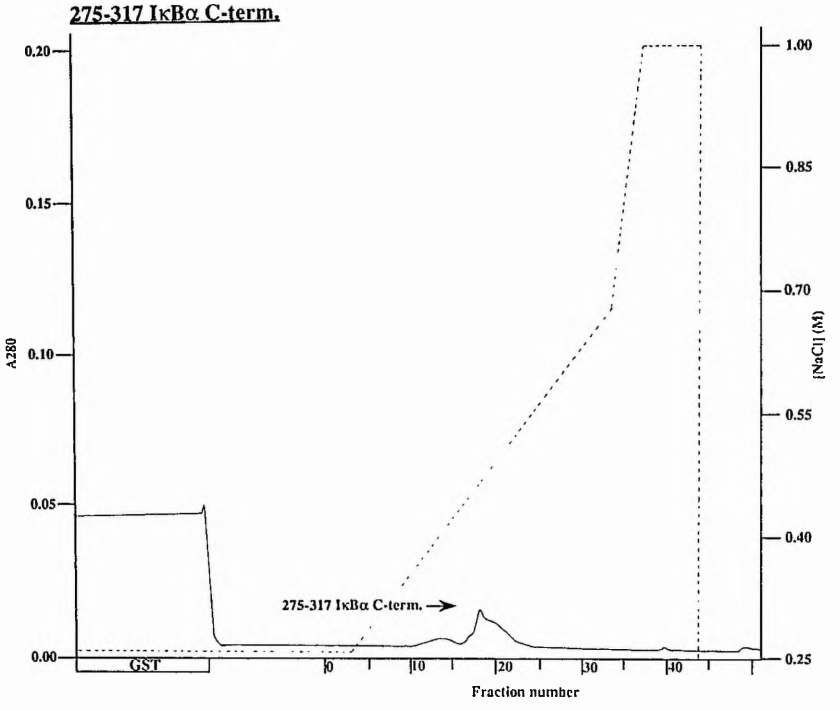
Figure 42A. FPLC Mono-Q column purification of the C-terminal IκBα proteins, 265-317 and 275-317.

The UV absorbance output traces at 280nm are shown (solid lines) opposite for the C-terminal IκBα proteins, 265-317 and 275-317 from the FPLC Mono-Q column using a salt gradient ranging from 250mM NaCl to 1mM NaCl (dashed lines). The position of the fractions collected from the salt gradient are indicated along the base of the trace. Glutathione-S-Transferase appeared in the initial column flow-through (to left of IκBαpeak) before the salt gradient was applied to the FPLC Mono-Q column.

265-317 IκBα C-term.



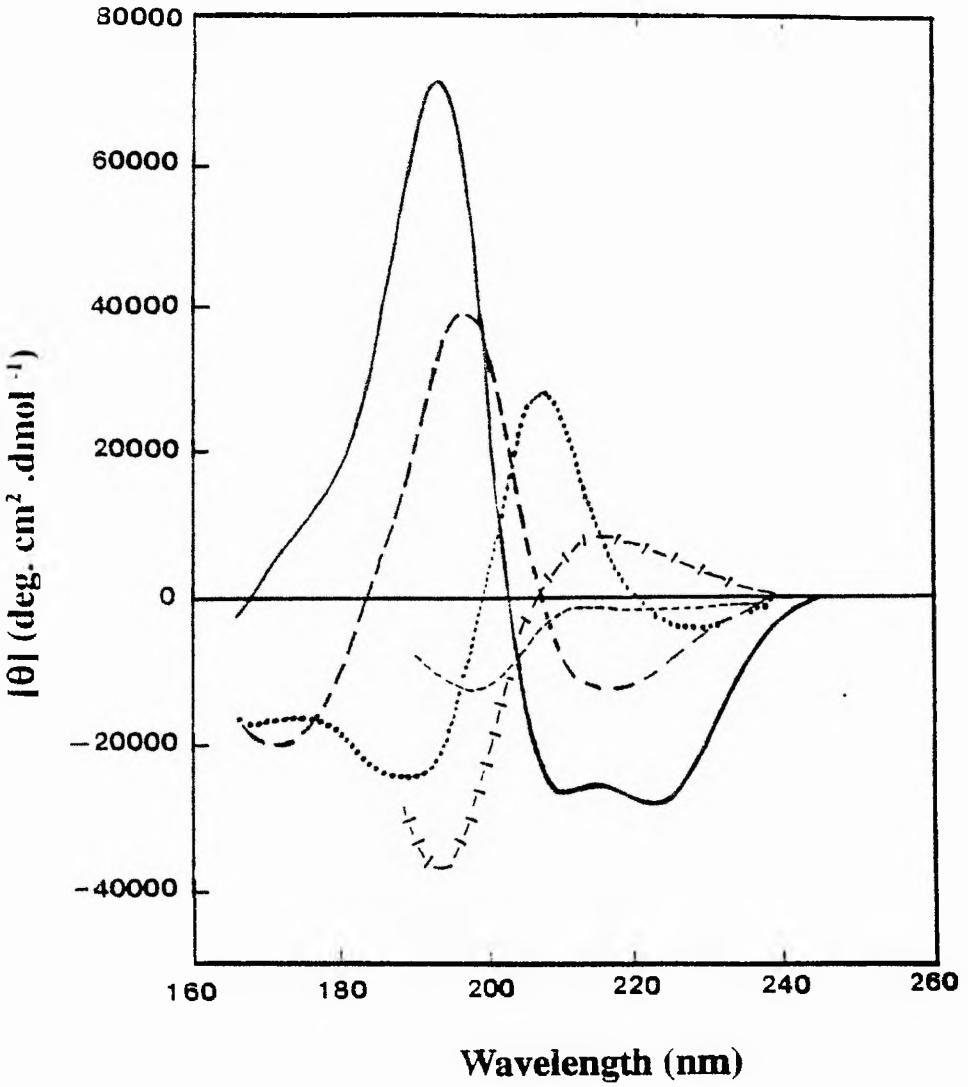
275-317 IκBα C-term.



317 (0.2mg/ml) and 275-317 (0.2mg/ml) were dialysed overnight against 50mM sodium fluoride, 10mMsodium phosphate pH7.0, 0.5mM DTT. Additionally, 1ml aliquots of 265-317 and 275-317 were dialysed overnight against 50mMsodium fluoride, 10mMsodium phosphate pH5.6, pH6.4 (275-317 only), pH7.0 and pH8.0 and 0.5mM DTT.

The various forms of regular secondary structure in proteins and peptides display distinct CD patterns in the far ultraviolet region as shown in figure 41(B). In order to assess the contributions of the various secondary structure constituents from the CD spectrum this study employed the CONTIN procedure of Provencher and Glöckner (1981). This method directly analyses the CD spectrum of a protein or peptide by comparing it to the CD spectra of 16 proteins whose structures have been determined by X-ray crystallography to high resolution (Price, 1995). The use of CD data over the range of 190-240nm (as used in this study) only accurately determines the amount of α-helix and β-sheet in the protein or peptide whereas the contributions of β-turns, 3_{10} helix and random structure are much less well estimated.

The CD spectrum of the C-terminally tagged (this is the 14 amino acid tag region which was also fused to the full-length mutant IκBα proteins-see chapter 1) protein referred to as 265-317 c-tag in either the absence or presence of 50% TFE is shown in figure 42(B). The CD spectrum of 265-317 c-tag in buffered aqueous solution (pH7.0) exhibited a single negative absorption at 197-200nm, indicative of a conformationally mobile species (possibly random coil) (dithiothreitol was included in the solution to prevent a cysteine residue at position 308 in the full length protein from cross-linking molecules of 265-317 c-tag). However, in the presence of 50% TFE the spectrum of 265-317 c-tag altered to display a maximum at 190nm and a minimum at 208nm signifying an increase in α-helical content. α-helical structures usually exhibit a minimum at 222nm. There is a small plateau of absorption around 222nm for 265-317 c-tag but no obvious trough. Application of the CONTIN procedure (Provencher and Glöckner, 1981) to the spectrum recorded in the absence of 50% TFE (table 1-note, remaining secondary structure may include random coil) indicated that the predominant secondary structure was β-sheet (62%). However, after the addition of 50% TFE to 265-317 c-tag the level of α-helix was raised (11%), although the major secondary



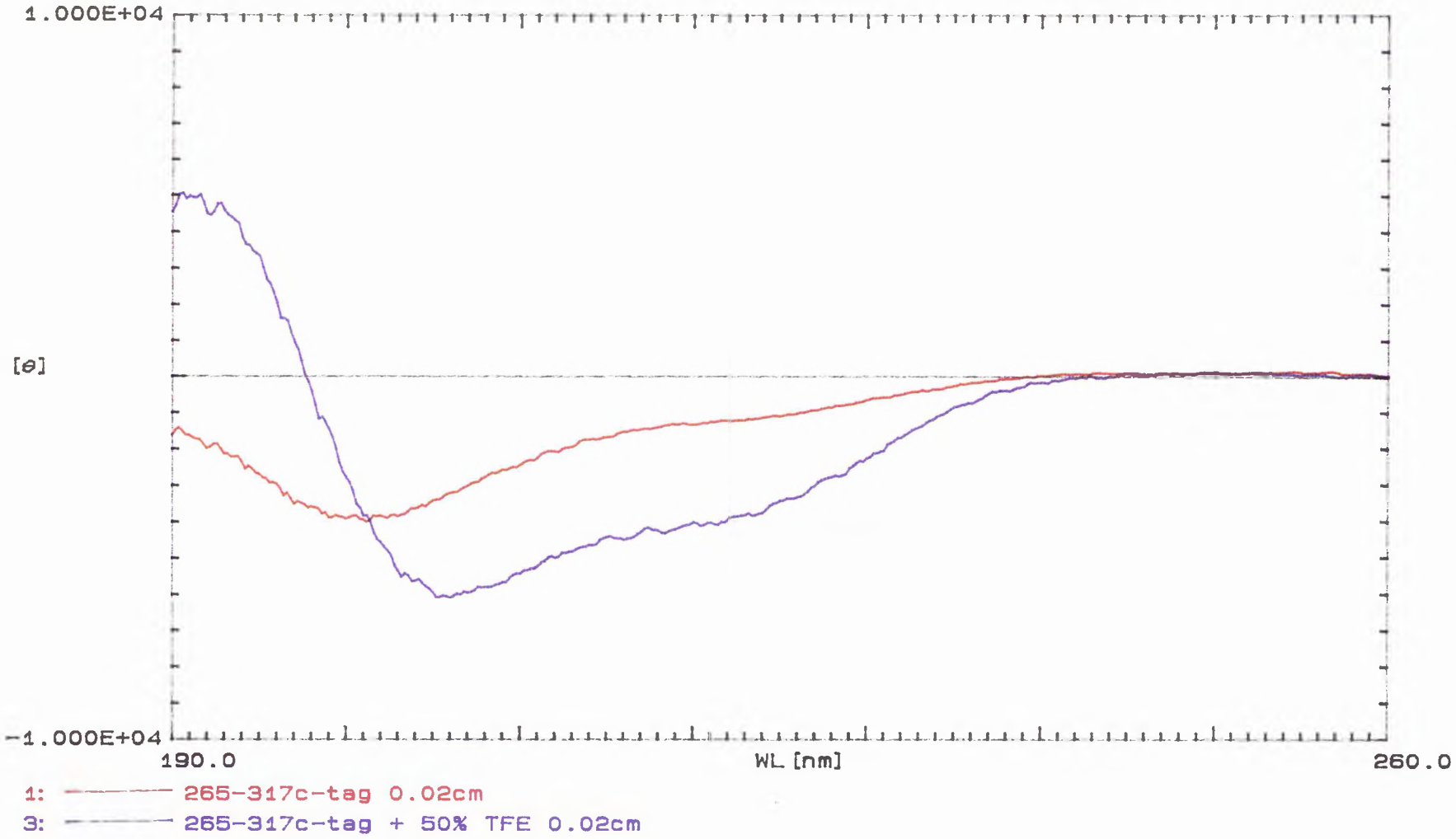
(B)

Figure 41. CD spectra associated with various secondary structures. α-helix (——), anti-parallel β-sheet (– – –), β-turn type I (.....), left-handed extended 3_{10} helix (-/-/-/), irregular structure (——) (from Price, 1995).

(B)

Figure 42: Circular dichroism spectroscopy of 265-317 c-tag in the presence or absence of 50% trifluoroethanol (TFE) at pH7.0.

The CD spectrum of 265-317 c-tag (0.51mg/ml in pH7.0 50mM sodium fluoride/10mM sodium phosphate buffer, 0.5mM dithiothreitol (DTT)) was recorded in the presence (purple line) or absence (red line) of 50% TFE, a helix stabilising agent. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.


1.000E+04
[θ]
-1.000E+04
190.0
WL [nm]
260.0
1: 265-317c-tag 0.02cm
3: 265-317c-tag + 50% TFE 0.02cm

structure component was still β-sheet (60%). Therefore, it appeared that 265-317 c-tag was predominantly β-sheet.

Table 1. Predicted secondary structure content of 265-317 c-tag in the presence and absence of 50% TFE (pH7.0).

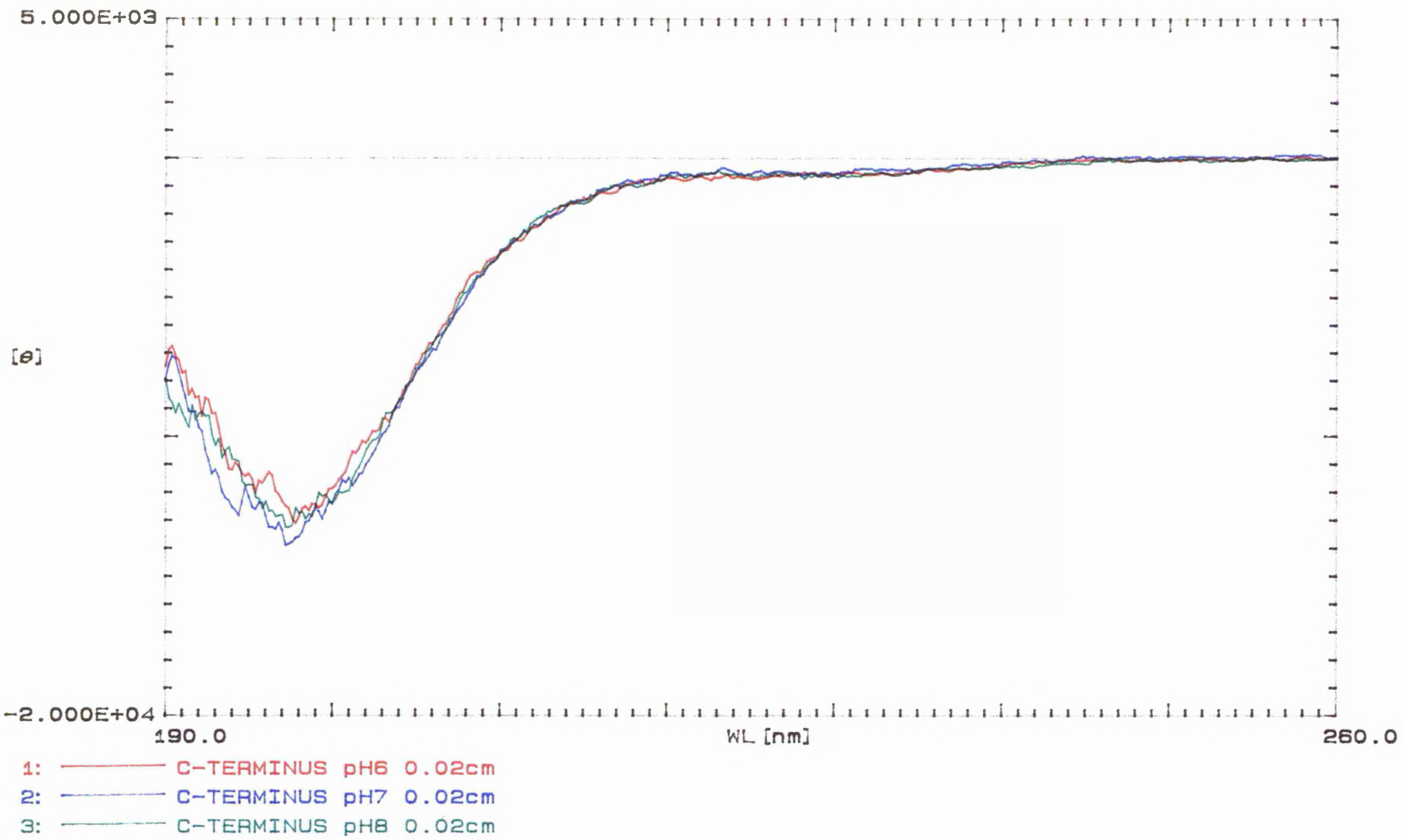
	Secondary Structure Content		
	α-helix	**β-sheet**	**Remaining secondary structure e.g. β-turn, 3_{10} helix**
No TFE	0%	62%	38%
+50% TFE	11%	60%	29%

Since 265-317 c-tag contained a C-terminal tag region, this could dramatically affect the structure of the protein (although the functional characteristics of the full-length IκBα proteins possessing a C-terminal tag were identical to that of non-tagged IκBα). Furthermore, 265-317 c-tag included part of the low homology sixth ankyrin repeat which may also have affected the secondary structure of the protein. Therefore, it was decided that a chemically synthesised peptide corresponding to residues 275-317 of IκBα would be analysed by CD spectroscopy. The peptide, referred to as either C-terminus or C-terminal peptide, contained a chemical blocking group at a cysteine residue (position 308, full length IκBα) to prevent any cross-linking between peptide molecules.

Since the association between IκBα and NF-κB could involve a partial quenching of a selection of negatively charged carboxyl groups in the C-terminal region of IκBα it was decided to examine the conformation of the C-terminal peptide over a range of pH values. The CD spectrum of the C-terminal peptide in buffered solutions of pH6.0, pH7.0 and pH8.0 (in the absence of TFE) is shown in figure 43. No change in the conformation of the C-

Figure 43: Circular dichroism spectroscopy of the C-terminal peptide (amino acids 275-317) at pH6.0, pH7.0 and pH8.0.

The CD spectrum of the C-terminal peptide (amino acids 275-317)(0.5mg/ml) was recorded in either 50mM sodium fluoride/10mM sodium phosphate buffer pH6.0 (red line), pH7.0 (blue line) or pH8.0 (green line). WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.


5.000E+03
[e]
-2.000E+04
190.0
WL [nm]
260.0
1: C-TERMINUS pH6 0.02cm
2: C-TERMINUS pH7 0.02cm
3: C-TERMINUS pH8 0.02cm

terminal peptide was observed over the range of pH's used and the peptide appeared to adopt a random conformation as indicated by the characteristic trough at 197nm. At pH7.0 in the presence of 50% TFE there was a slight shift in the negative absorption to around 204nm but the spectrum indicated that 275-317 was predominantly a random coil (figure 44). Similar results were obtained at pH6.0 and pH8.0 (data not shown). Secondary structure predictions (through the application of the CONTIN procedure to the spectra) suggested that the C-terminal peptide comprised mainly β-sheet or remaining secondary structure constituents such as β-turns, 3_{10} helix or random coil (data not shown).

Evidently, the bacterially expressed protein, 265-317 c-tag was able to assume a more ordered structure than the C-terminal peptide. There were several explanations for the less ordered conformation of the C-terminal peptide: The loss of part of the low homology sixth ankyrin repeat (10 amino acids); the loss of the C-terminal tag region or the presence of the large blocking group ($CH_2NHCOCH_3$) on a cysteine residue to prevent cross-linking. Therefore, it was decided that the bacterially expressed proteins referred to as 265-317 and 275-317 (containing neither a tag region nor a chemical blocking group) would be examined using circular dichroism spectroscopy in an attempt to clarify these points.

Both of the proteins, 265-317 and 275-317 were analysed in the presence and absence of 50% TFE in a buffered solution at pH5.6, pH7.0 and pH8.0 and 275-317 was also examined at pH6.4 using CD spectroscopy. The results are illustrated in figures 45-48. Figures 45-47 show that at pH5.6, pH7.0 and pH8.0 265-317 assumed a random conformation (this may include β-turns and 3_{10} helix) in the absence of TFE as indicated by the absorption minimum at 190nm. However, in the presence of 50% TFE the α-helical content of 265-317 increased. This was also the case when the pH was lowered in the presence of 50% TFE as shown by the appearance of minima at 204-208nm and 222nm and a slight positive absorption at 190nm (Figure 48 shows this more clearly). A comparison of the secondary structure predictions for 265-317 c-tag at pH7.0 in the presence of TFE (table 1 and figure 42B)with the predictions for 265-317 at pH7.0 in the presence of TFE (table 2 and figure 46, note remaining secondary structure may also include random coil) revealed that the C-terminally tagged protein contained more β-sheet and α-helix but less of the remaining

Figure 44: Circular dichroism spectroscopy of the C-terminal peptide (amino acids 275-317) in the presence or absence of 50% triflouroethanol (TFE) at pH7.0.

The CD spectrum of the C-terminal peptide (amino acids 275-317)(0.5mg/ml) was recorded in 50mM sodium fluoride/10mM sodium phosphate buffer, pH7.0 in either the presence (blue line) or absence (red line) of 50% TFE. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.

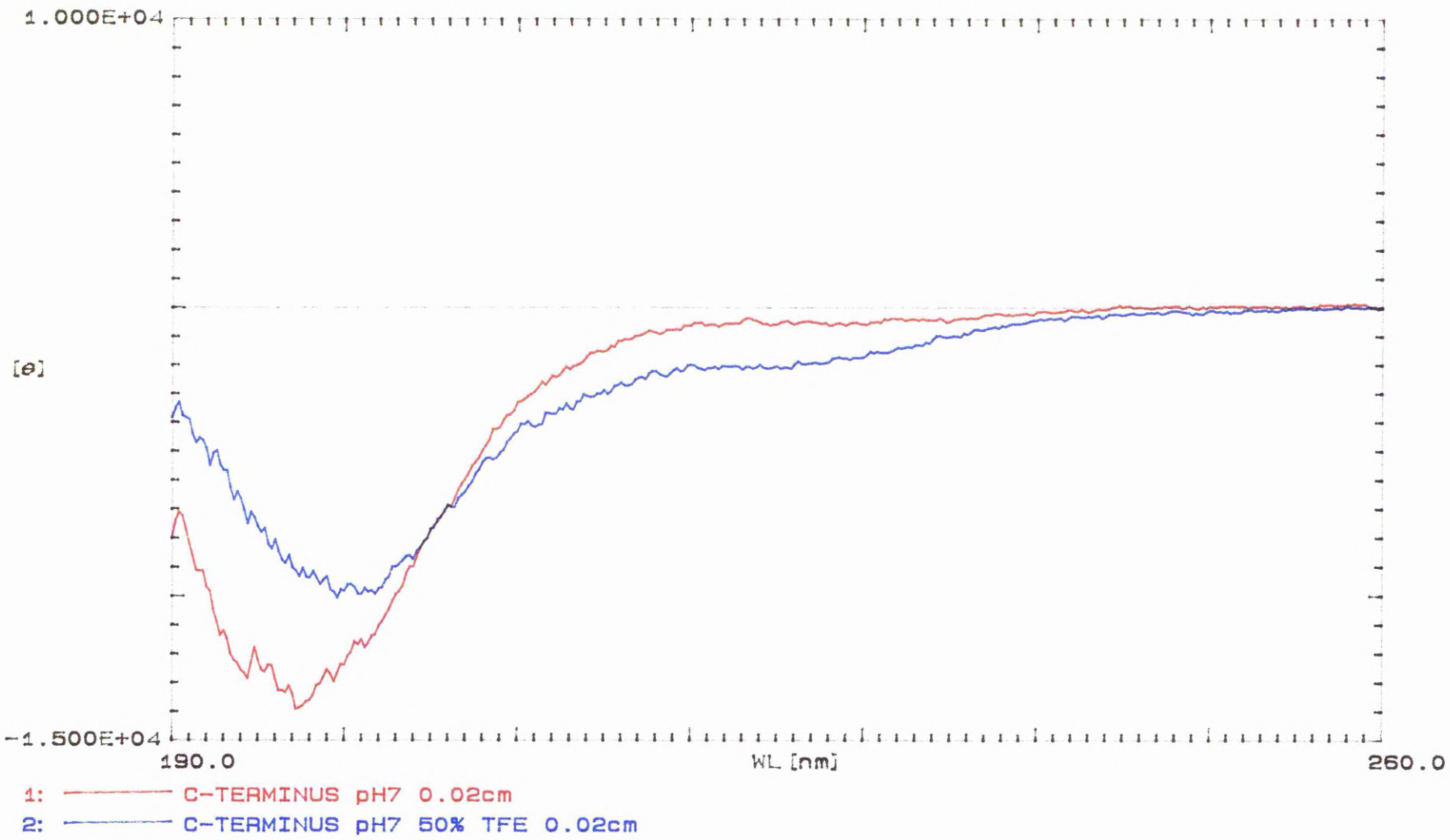

1.000E+04
[θ]
-1.500E+04
190.0
WL [nm]
260.0
1: C-TERMINUS pH7 0.02cm
2: C-TERMINUS pH7 50% TFE 0.02cm

Figure 45: Circular dichroism spectroscopy of 265-317 in the presence or absence of 50% triflouroethanol (TFE) at pH5.6.

The CD spectrum of 265-317 (0.2mg/ml) was recorded in the presence (blue line) or absence (red line) of 50% TFE in 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH5.6. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.

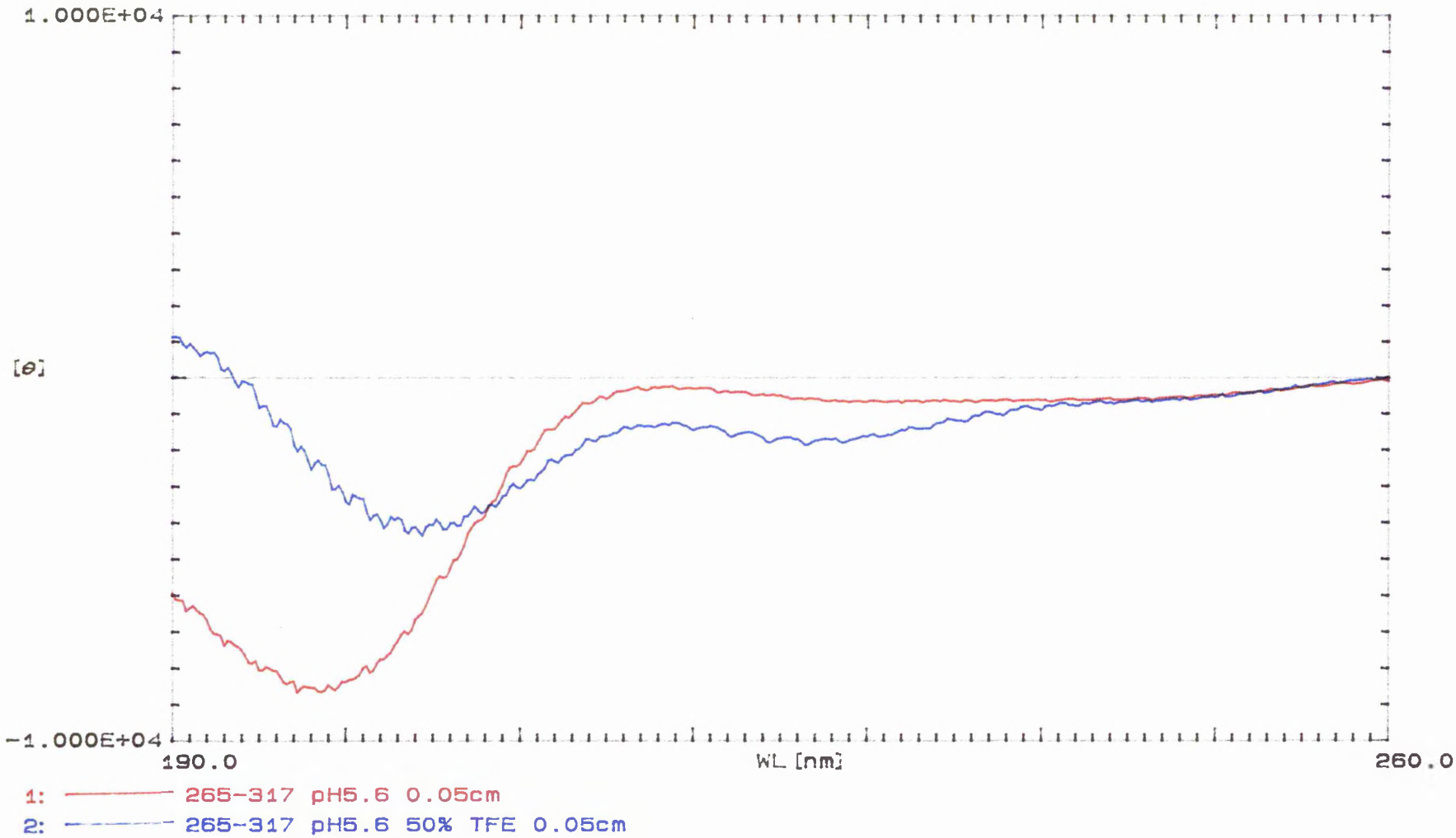

1.000E+04
[θ]
-1.000E+04
190.0
WL [nm]
260.0
1: 265-317 pH5.6 0.05cm
2: 265-317 pH5.6 50% TFE 0.05cm

Figure 46: Circular dichroism spectroscopy of 265-317 in the presence or absence of 50% triflouroethanol (TFE) at pH7.0.

The CD spectrum of 265-317 (0.2mg/ml) was recorded in the presence (blue line) or absence (red line) of 50% TFE in 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH7.0. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.

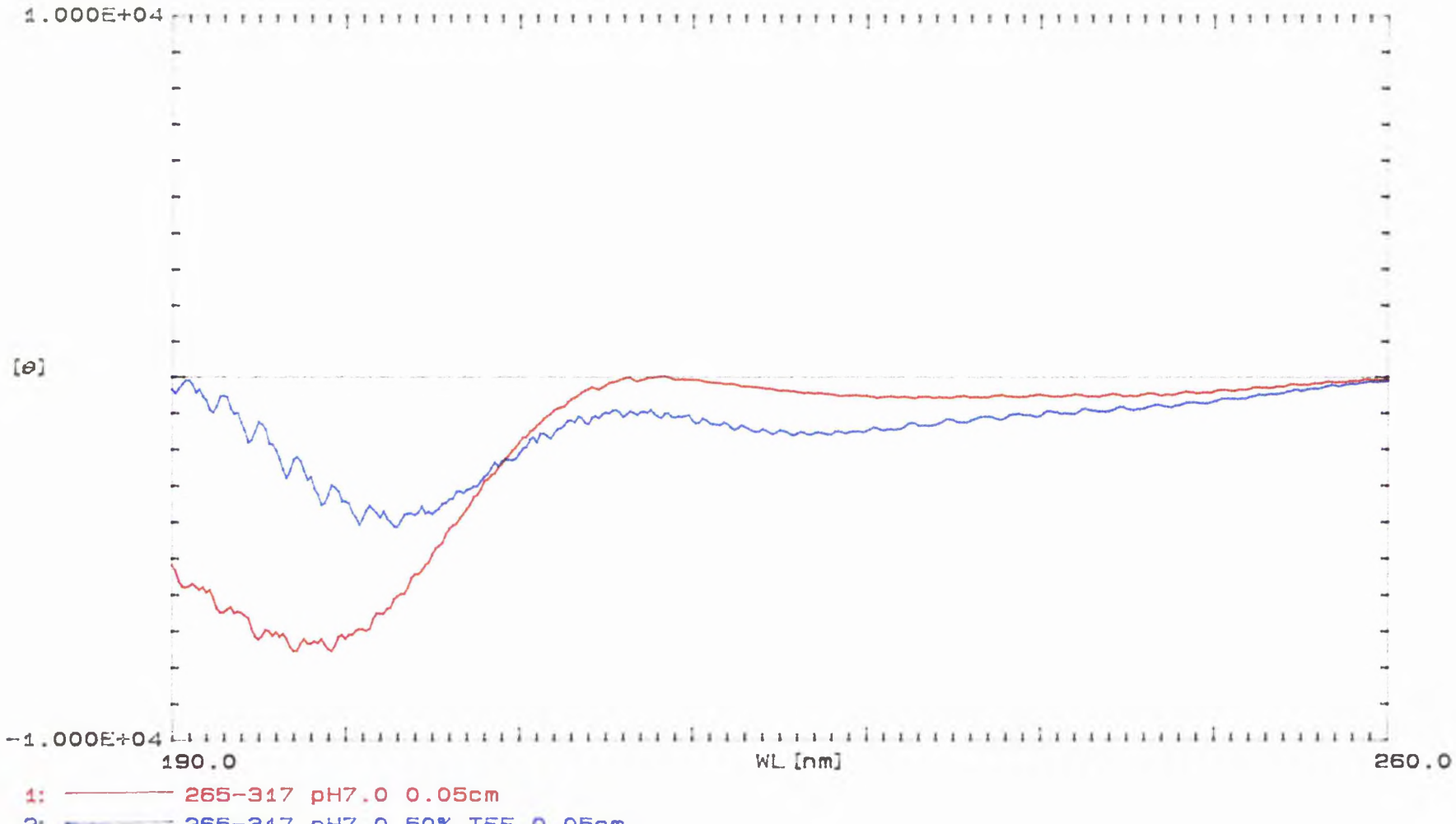

1.000E+04
[θ]
-1.000E+04
190.0
WL [nm]
260.0
1: 265-317 pH7.0 0.05cm
2: 265-317 pH7.0 50% TFE 0.05cm

Figure 47: Circular dichroism spectroscopy of 265-317 in the presence or absence of 50% triflouroethanol (TFE) at pH8.0.

The CD spectrum of 265-317 (0.2mg/ml) was recorded in the presence (blue line) or absence (red line) of 50% TFE in 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH8.0. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.

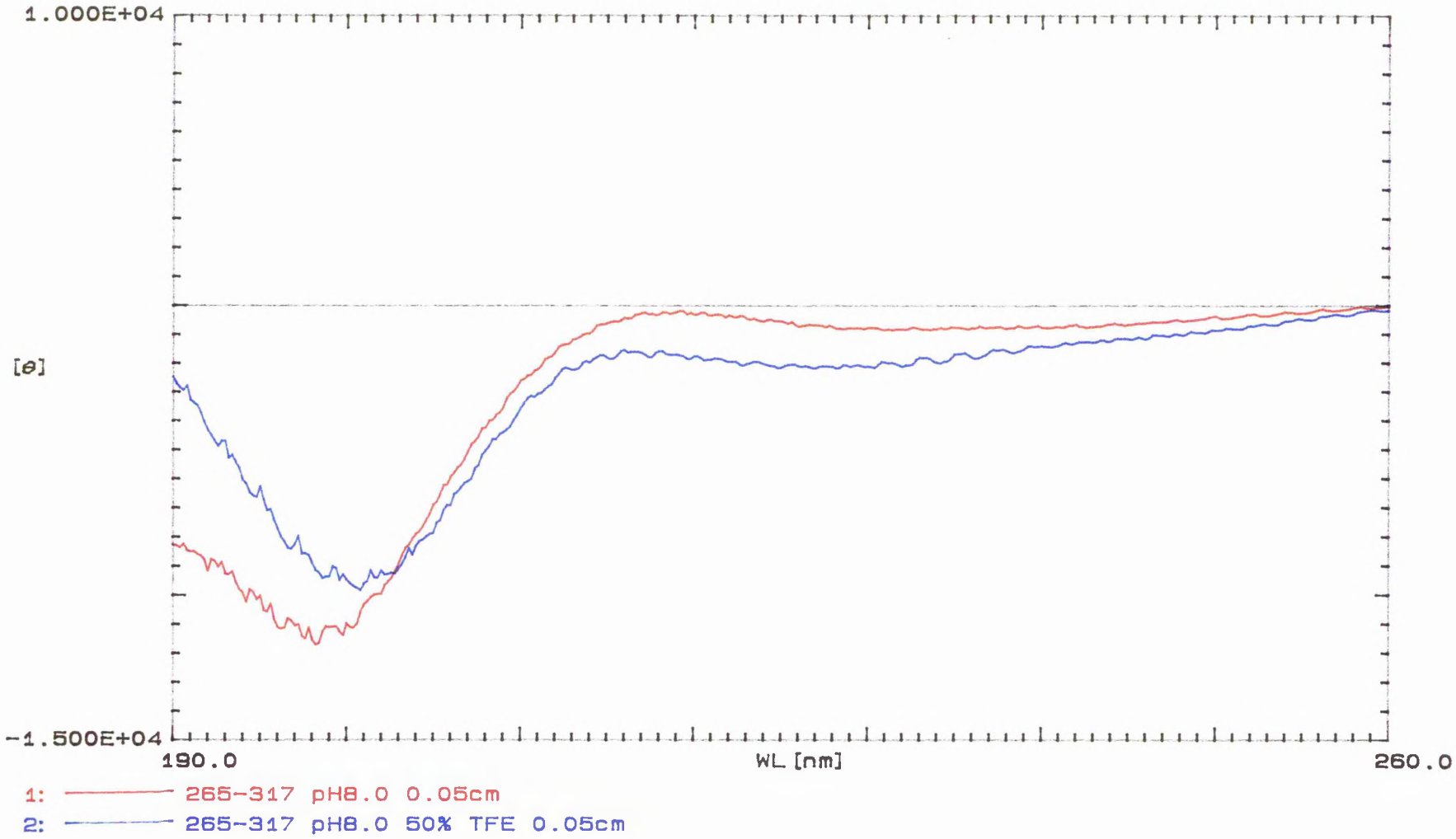
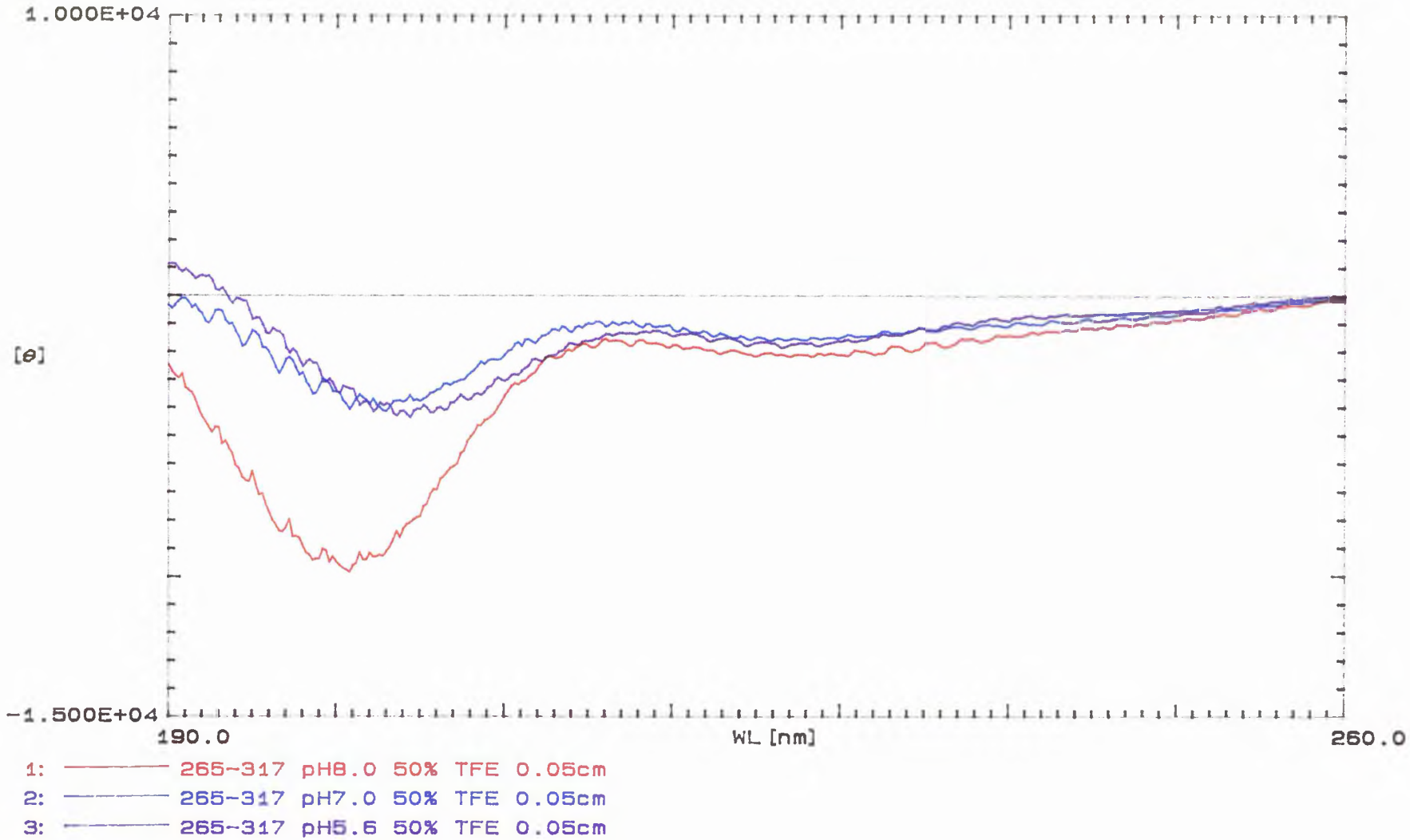

1.000E+04
[θ]
-1.500E+04
190.0
WL [nm]
260.0
1: 265-317 pH8.0 0.05cm
2: 265-317 pH8.0 50% TFE 0.05cm

Figure 48: Circular dichroism spectroscopy of 265-317 in the presence of 50% triflouroethanol (TFE) at pH5.6, pH7.0 and pH8.0.

The CD spectrum of 265-317 (0.2mg/ml) was recorded in the presence of 50% TFE in either 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH5.6 (purple line), pH7.0 (blue line) or pH8.0 (red line). WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.


1.000E+04
[θ]
-1.500E+04
190.0
WL [nm]
260.0
1: 265-317 pH8.0 50% TFE 0.05cm
2: 265-317 pH7.0 50% TFE 0.05cm
3: 265-317 pH5.6 50% TFE 0.05cm

secondary structure constituents than the non-tagged protein. The results suggest that the C-terminal tag serves to stabilise the protein in a more ordered conformation comprising predominantly of β-sheet. Application of the CONTIN procedure to the spectra (table 2) illustrated that the α-helical content of 265-317 at each pH (pH5.6, 7%; pH7.0, 5% and pH8.0, 6%) was raised in the presence of 50% TFE. However, in both the presence and absence of TFE the overall structure was predominantly β-sheet or remaining secondary structure constituents such as β-turns, 3_{10} helix or random coil.

Figures 49-53 show that at pH5.6, pH6.4, pH7.0 and pH8.0 275-317 adopted a random conformation (which may include β-turns and 3_{10} helix) in the absence of TFE as indicated by the absorption minimum at 190nm. However, in the presence of 50% TFE 275-317 shifted its conformation slightly to that of an α-helix. It appeared that as the pH was lowered in the presence of 50% TFE, 275-317 adopted a more α-helical conformation as shown by the appearance of an almost positive absorption at 190nm (Figure 53 shows this more clearly). A comparison of the spectrum of the C-terminal peptide at pH7.0 in the presence and absence of TFE (figure 44) with 275-317 at pH7.0 in the presence and absence of TFE (figure 51) reveals a striking similarity suggesting that the blocking group in the C-terminal peptide did not affect the structure of the protein. Application of the CONTIN procedure to the spectra (table 3) showed that the α-helical content of 275-317 at each pH (pH5.6, 5%; pH6.4, 6%; pH7.0, 2% and pH8.0, 6%) was raised in the presence of 50% TFE. However, in both the presence and absence of TFE the overall structure was predominantly β-sheet or remaining secondary structure constituents such as β-turns, 3_{10} helix or random coil. Surprisingly, a comparison of the secondary structure predictions for 275-317 in the absence of TFE at pH5.6 with those for 265-317 under the same conditions (table 2) revealed that 275-317 contained more β-sheet (63%) and less of the remaining secondary structure constituents (37%) than 265-317 (β-sheet, 49% and random coil, 50%). This suggests that the extra ten amino acids contained in the protein, 265-317 do not stabilise the structure at lower pH's but instead appear to induce a less ordered conformation in the protein.

Figure 49: Circular dichroism spectroscopy of 275-317 in the presence or absence of 50% triflouroethanol (TFE) at pH5.6.

The CD spectrum of 275-317 (0.2mg/ml) was recorded in the presence (blue line) or absence (red line) of 50% TFE in 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH5.6. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.

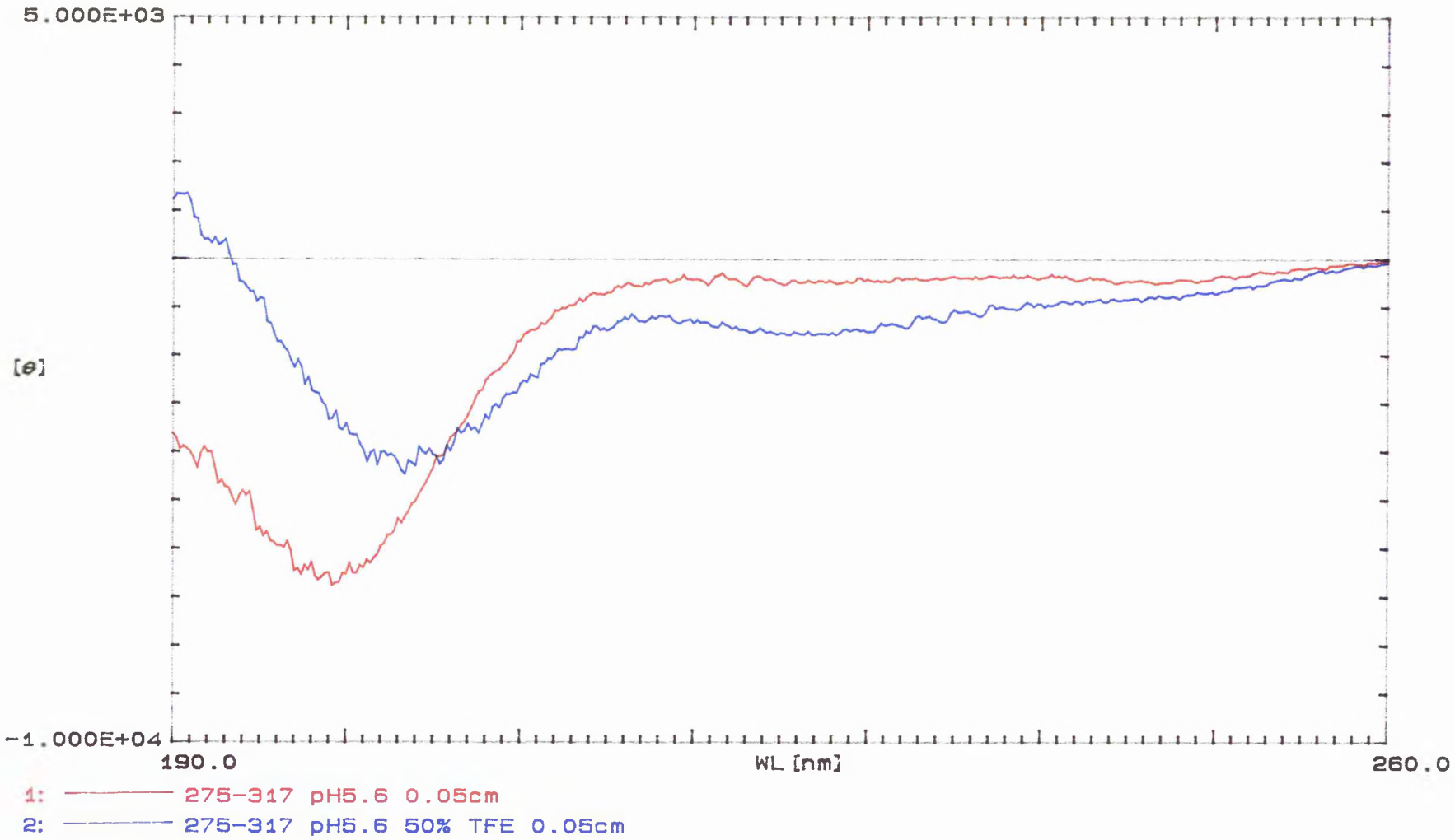

5.000E+03
[θ]
-1.000E+04
190.0
WL [nm]
260.0
1: 275-317 pH5.6 0.05cm
2: 275-317 pH5.6 50% TFE 0.05cm

Figure 50: Circular dichroism spectroscopy of 275-317 in the presence or absence of 50% triflouroethanol (TFE) at pH6.4.

The CD spectrum of 275-317 (0.2mg/ml) was recorded in the presence (blue line) or absence (red line) of 50% TFE in 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH6.4. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.

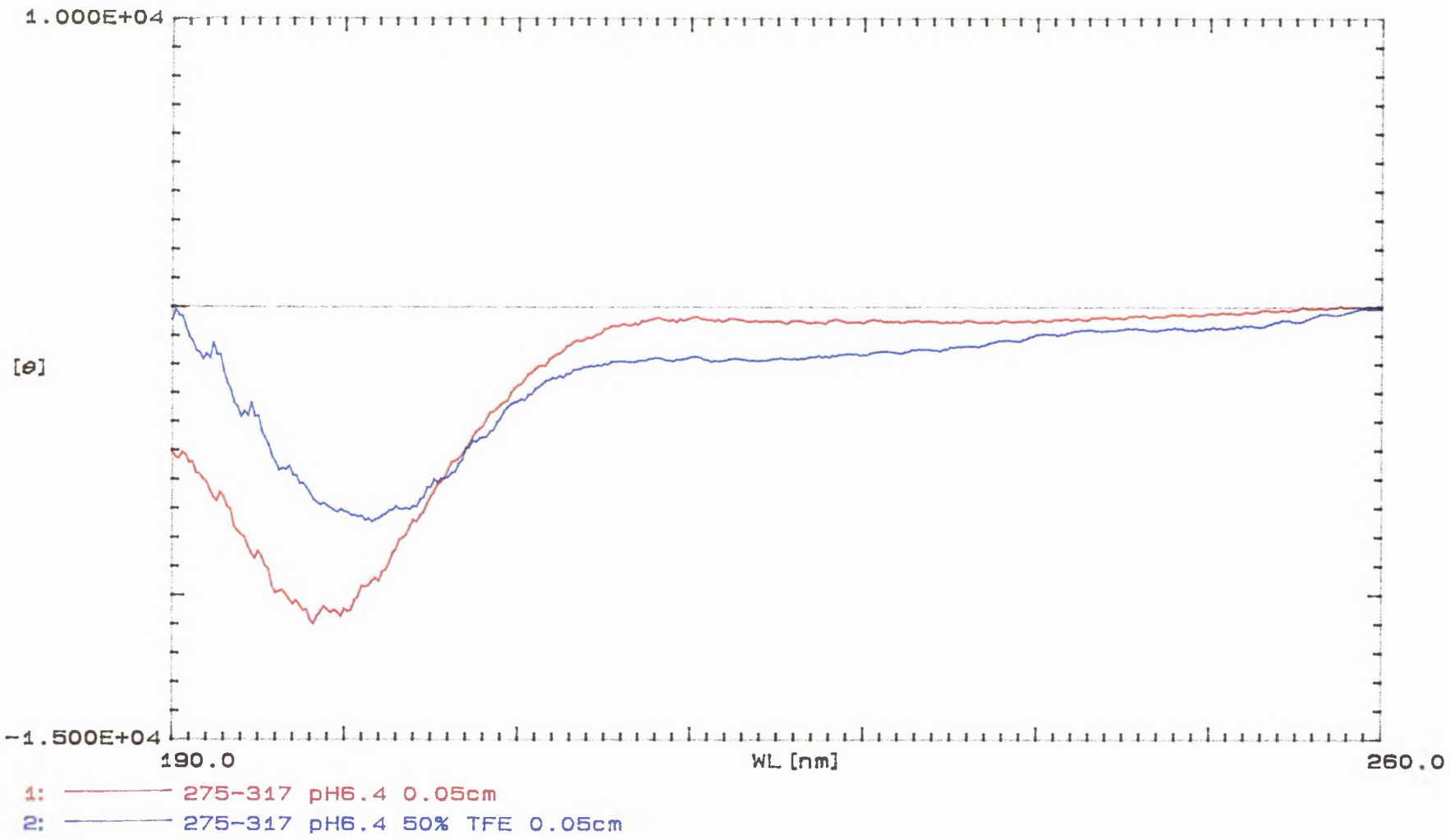
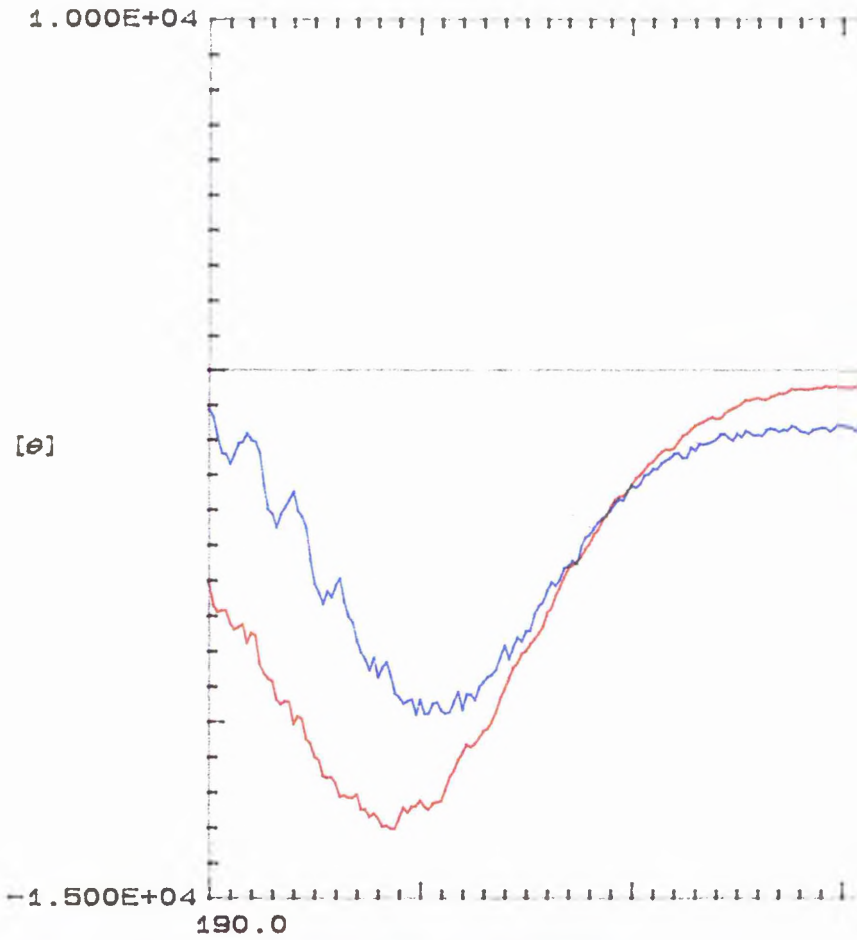

1.000E+04
[θ]
-1.500E+04
190.0
WL [nm]
260.0
1: 275-317 pH6.4 0.05cm
2: 275-317 pH6.4 50% TFE 0.05cm

Figure 51: Circular dichroism spectroscopy of 275-317 in the presence or absence of 50% triflouroethanol (TFE) at pH7.0.

The CD spectrum of 275-317 (0.2mg/ml) was recorded in the presence (blue line) or absence (red line) of 50% TFE in 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH7.0. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.


1.000E+04
[θ]
-1.500E+04
190.0
1: 275-317 pH7.0 0.05cm
2: 275-317 pH7.0 50% TFE 0.05cm

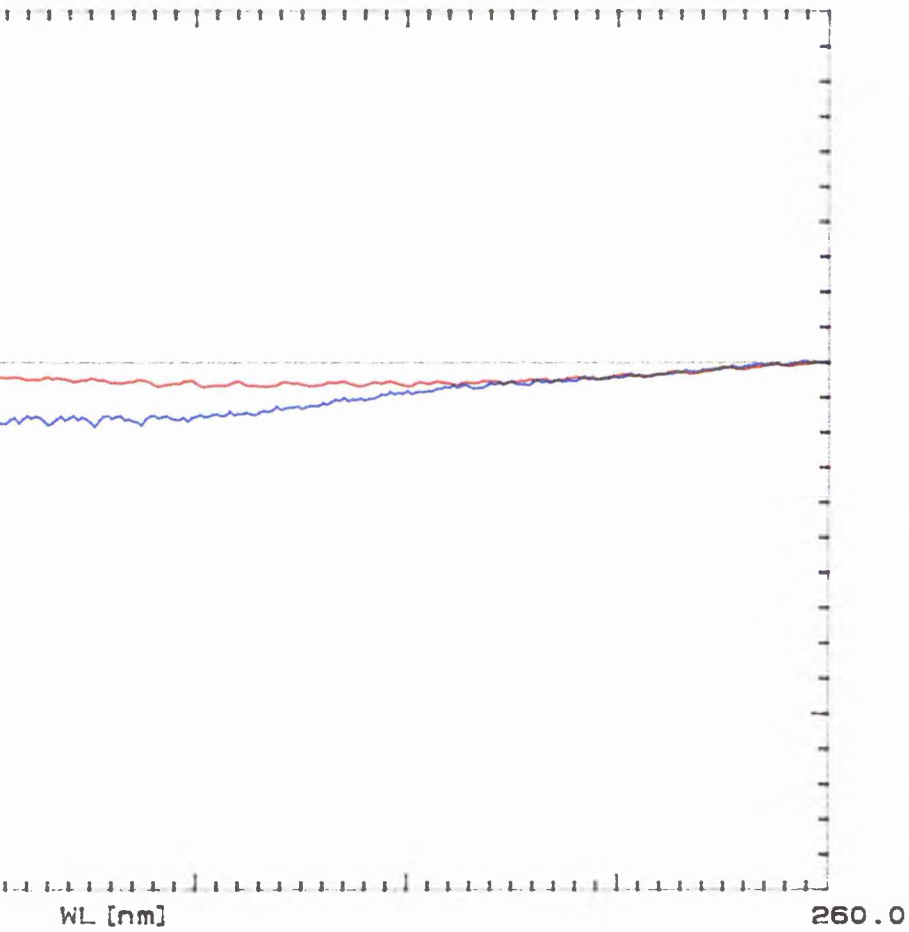

WL [nm]
260.0

Figure 52: Circular dichroism spectroscopy of 275-317 in the presence or absence of 50% triflouroethanol (TFE) at pH8.0.

The CD spectrum of 275-317 (0.2mg/ml) was recorded in the presence (blue line) or absence (red line) of 50% TFE in 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH8.0. WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.

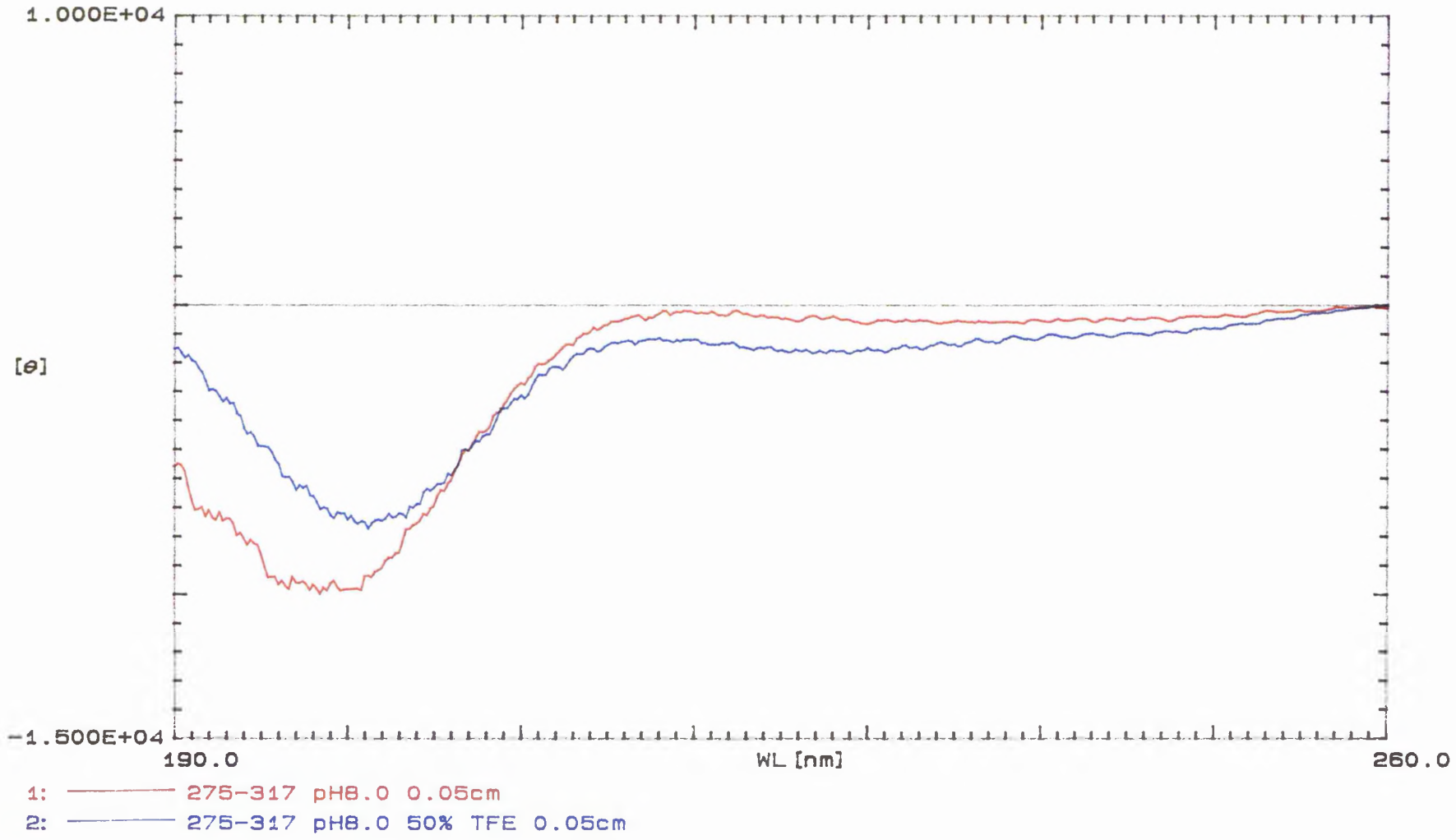

1.000E+04
[θ]
-1.500E+04
190.0
WL [nm]
260.0
1: 275-317 pH8.0 0.05cm
2: 275-317 pH8.0 50% TFE 0.05cm

Figure 53: Circular dichroism spectroscopy of 275-317 in the presence of 50% triflouroethanol (TFE) at pH5.6, pH7.0 and pH8.0.

The CD spectrum of 275-317 (0.2mg/ml) was recorded in the presence of 50% TFE in either 0.5mM dithiothreitol, 50mM sodium fluoride/10mM sodium phosphate buffer, pH5.6 (orange line), pH6.4 (purple line), pH7.0 (blue line) or pH8.0 (red line). WL along the x-axis represents the wavelength (nm) at which the spectra were recorded. The units for the molar ellipticity, theta (θ) along the y-axis are degrees x cm^2 x $dmol^{-1}$ and are given in terms of the mean residue weight of the protein.

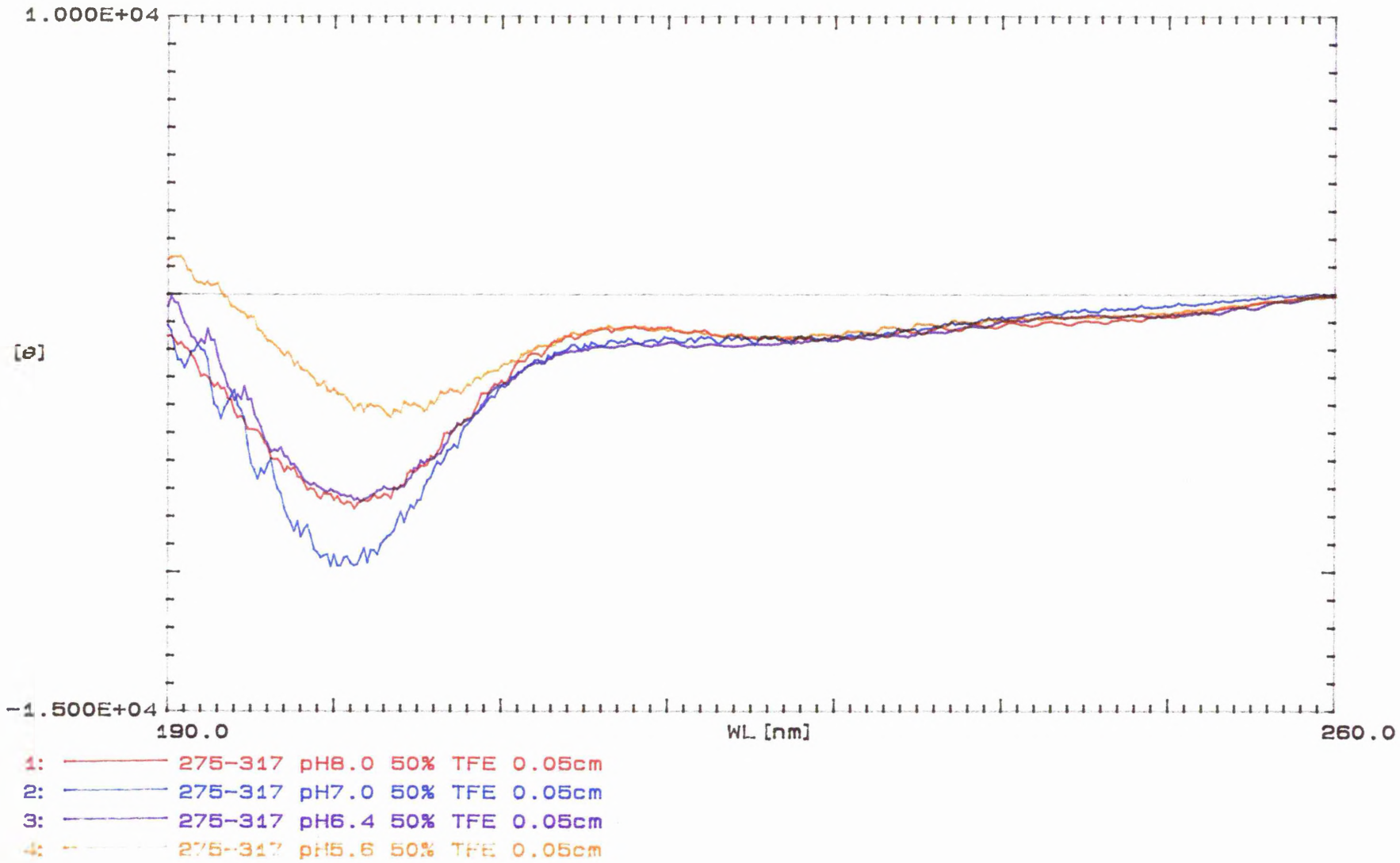

1.000E+04
[θ]
-1.500E+04
190.0
WL [nm]
260.0
1: 275-317 pH8.0 50% TFE 0.05cm
2: 275-317 pH7.0 50% TFE 0.05cm
3: 275-317 pH6.4 50% TFE 0.05cm
4: 275-317 pH5.6 50% TFE 0.05cm

Table 2. Predicted secondary structure content of 265-317 in the presence and absence of 50% TFE (pH5.6, pH7.0 and pH8.0).

	Secondary Structure Content		
pH	**α-helix**	**β-sheet**	**Remaining secondary structure e.g. β-turn, 3_{10} helix**
5.6 (No TFE)	1%	49%	50%
5.6 (+50% TFE)	7%	50%	43%
7.0 (No TFE)	0%	53%	47%
7.0 (+50% TFE)	5%	51%	44%
8.0 (No TFE)	1%	50%	49%
8.0 (+50% TFE)	6%	36%	58%

Table 3. Predicted secondary structure content of 275-317 in the presence and absence of 50% TFE (pH5.6, pH7.0 and pH8.0).

	Secondary Structure Content		
pH	**α-helix**	**β-sheet**	**Remaining secondary structure e.g. β-turn, 3_{10} helix**
5.6 (No TFE)	0%	63%	37%
5.6 (+50% TFE)	5%	50%	45%
6.4 (No TFE)	1%	57%	42%
6.4 (+50% TFE)	6%	45%	49%
7.0 (No TFE)	1%	52%	47%
7.0 (+50% TFE)	2%	51%	47%
8.0 (No TFE)	0%	48%	52%
8.0 (+50% TFE)	6%	44%	50%

DISCUSSION

1. IκBα: Inhibition of p65 homodimer DNA binding and association with p65 *in vitro*.

Experimental findings have indicated that IκBα inhibits the DNA binding activity of the p65 subunit rather than the p50 subunit of NF-κB (reviewed in Grimm and Baeuerle, 1993). Therefore, studies have mainly focused on the interaction between the p65 subunit of NF-κB and IκBα. Deletion analysis of the avian homologue of human IκBα, pp40 had suggested that the C-terminal region and ankyrin repeats were essential for association with c-Rel homodimers and for inhibition of c-Rel and NF-κB DNA binding activity *in vitro* (Inoue *et al.*, 1992). In addition, a later investigation using deletion mutants of human IκBα also demonstrated that the C-terminal domain and the ankyrin repeats were critical for association with p65 and for preventing p65 DNA binding *in vitro* (Hatada *et al.*, 1993). Furthermore, both studies showed that the N-terminal region of IκBα was dispensable for interaction with c-Rel or p65 and for inhibition of c-Rel, p65 or NF-κB DNA binding activity.

It was possible that the apparent functional requirement for the C-terminal region of IκBα was a consequence of conformational changes induced in the protein through the deletion of the C-terminal domain. Therefore, the purpose of this study was to examine the contribution of smaller areas of protein sequence to the function of IκBα (in particular, its relationship with p65) through the substitution, rather than deletion of a lower number of residues. As indicated in the results section the potential areas of contact within the p65 protein for IκBα were considered. Studies had indicated that the nuclear localisation signal of p65 was required for association with IκBα (Beg *et al.*, 1992). In addition, it had been reported that that a C-terminal domain in p65 assisted in the IκBα-mediated inhibition of DNA binding (Ganchi *et al.*, 1992). Since the NLS was positively charged it was reasoned that the interaction between IκBα and p65 may be ionic. Therefore, conserved areas of negatively charged residues were chosen for substitution in the C-terminal region of human IκBα, namely residues 284-286 and residues 300-302. A second set of IκBα mutants were

also generated with amino acid substitutions in the low homology sixth ankyrin repeat as this area had been shown to contact p65 through protease sensitivity studies (Jaffray *et al.*, 1995). Single residue substitutions were produced at positions 258 and 275.

Protein association assays carried out *in vitro* on bacterially expressed and purified C-terminal and linker IκBα c-tag mutant and p65 proteins revealed that the amino acid substitutions at positions 258, 275, 284-286 and 300-302 did not affect the ability of IκBα c-tag to interact with p65. However, it appeared that the substitution at position 284-286 in the C-terminus of IκBα c-tag reduced the inhibitory capacity of the protein *in vitro*: Both EDE284-286qnq and EDE284-286sss displayed a 16-fold decrease in their ability to inhibit the DNA binding of p65 homodimers. The results suggest that the ability of IκBα to inhibit the DNA binding activity of p65 homodimers can be mediated by a region (around residues 284-286) of the protein which is distinct from the area which controls its association with p65.

These results have since been confirmed by other groups. This includes a study on the C-terminal domain of IκBα which identified the region between residues 283 and 286 as being critical for inhibiting the DNA binding of p65 or c-Rel but dispensable for association with either p65 or c-Rel (Ernst *et al.*, 1995). Further analysis of the acidic stretch of amino acids from 284-286 revealed that the residues were located in a consensus casein kinase II phosphorylation site. The mutation of a serine residue at position 283 to an uncharged amino acid (alanine) resulted in a slight loss of inhibitory activity, whereas substituting serine with aspartic acid did not affect the inhibitory capacity of the protein. The study suggested that a negative charge at position 283, supplied by phosphorylation of serine *in vivo*, may contribute to the proteins inhibitory capacity.

Similar results were obtained from another study using avian pp40 where substitution of four C-terminal serine residues to alanine abolished pp40 mediated inhibition of DNA binding by c-Rel but serine to aspartic acid substitutions did not affect the inhibitory function of pp40 (Sachdev *et al.*, 1995).

Therefore, it appears that the inhibitory function of IκBα resides in the C-terminal region, whereas the area responsible for association with p65 is located outwith this domain.

Although the single amino acid substitutions introduced at positions 258 and 275 in the low homology ankyrin repeat did not affect the ability of IκBα c-tag to interact with p65 several recent studies have shown that the deletion of part of the low homology ankyrin repeat with the C-terminal region (residues 261-317, 263-317 or 269-317) prevents p65-IκBα interaction *in vivo* (Sun *et al.*, 1996; Beauparlant *et al.*, 1996). It is possible that these results were due to a conformational change in IκBα, induced following the removal of a large number of residues but proteolysis sensitivity studies have also demonstrated that residues within the low homology sixth ankyrin repeat of IκBα are protected from proteolysis in the presence of p65 *in vitro* (Jaffray *et al.*, 1995).

Although it is clear that the acidic C-terminus of IκBα is critical for inhibiting the DNA binding activity of NF-κB, the actual mechanism of this inhibition has not been elucidated. However, a recent protein footprinting study has demonstrated that IκBγ and DNA appear to protect the same set of lysine residues in p50 homodimers from chemical modification (Bell *et al.*, 1996) but that IκBα failed to protect these residues in p50 homodimers. These findings indicate that IκBγ can inhibit the DNA binding of p50 homodimers by directly interacting with residues critical for p50 DNA binding. In contrast, IκBα does not contact these critical DNA binding residues and therefore cannot inhibit p50 DNA binding, although it can physically interact with p50, presumably at a site distinct from the DNA binding region of p50. It is possible that the mode of IκBγ inhibition on p50 DNA binding is similar to the mechanism of IκBα inhibition on p65. The acidic C-terminus of IκBα may form an intramolecular interaction with regions in or around the DNA binding domain of NF-κB. This could induce a conformational change in the DNA binding region so that NF-κB is no longer able to recognise its specific DNA binding site as suggested by Ernst *et al* (1995). Indeed early studies demonstrated that the N-terminal DNA binding domain of c-Rel was both necessary and sufficient for association with IκBα (Kerr *et al.*, 1991).

A mutational analysis of *Drosophila* Dorsal in yeast has suggested that sites outwith the DNA binding region are required for Cactus-mediated (an IκB protein) inhibition of Dorsal. This study isolated two Dorsal mutants from yeast cells possessing amino acid changes at either residues 233 or 234 corresponding to an area close to the nuclear

localisation signal in p50. Both mutations were found to prevent Cactus-mediated inhibition of Dorsal *in vivo*. However, the nature of the genetic screen used in this study meant that the Dorsal mutants had to translocate to the nucleus, bind DNA and activate transcription before they could be inhibited by Cactus (Lehming *et al.*, 1995). Therefore, Dorsal mutants containing alterations in, for example their nuclear localisation signal were not detected and this conclude that only residues 233 and 234 are required for cactus inhibition. It is likely that other regions of dorsal are also important for Cactus-mediated inhibition.

The effect of sodium chloride and pH on the association between IκBα c-tag and p65 was also examined in this study. It was reasoned that in the event of their being an ionic interaction between p65 and IκBα c-tag, changing the ionic strength or pH of the solution containing these proteins should disrupt this interaction. Furthermore, if the negatively charged residues at positions 275, 284-286 and 300-302 in IκBα were required for binding to p65 then their substitution with uncharged residues may partially remove any dependence on the ionic strength or pH of their environment.

Raising the concentration of sodium chloride (from 60mM to 960mM) dramatically reduced the ability of IκBα c-tag to interact with p65, whereas varying the pH conditions (between pH5.5 and pH9.0) had no effect on IκBα c-tag-p65 association. Both the mutant and wild-type IκBα c-tag proteins behaved similarly over the range of salt concentrations and pH's used. This indicated that the amino acid substitutions at positions 275, 284-286 and 300-302 in IκBα c-tag were not required for specific interactions with p65. In addition, it would appear that other interactions besides those of an ionic nature facilitate IκBα-p65 association since varying the pH alone (from pH5.5 to 9.0) did not affect this interaction. Indeed, the observation that high salt concentrations almost abolished IκBα-p65 binding supports this proposal since ionic strength affects both ionic interactions and hydrogen bonding. This is achieved through the association of sodium or chloride ions with charged groups or with dipoles (peptide bonds) within the proteins which can compete out protein-protein interactions (and intermolecular interactions).

2. Phosphorylation, ubiquitination and degradation of IκBα.

2.1. The role of the C-terminal region of IκBα

Several early studies had shown that the induction of cells with agents such as tumour necrosis factor resulted in the stimulation of NF-κB DNA binding activity. In addition, NF-κB activation was accompanied by the rapid, transient hyperphosphorylation and degradation of IκBα (Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Henkel *et al.*, 1993; Mellits *et al.*, 1993; Rice and Ernst, 1993; Sun *et al.*, 1993). More recently, experimental evidence has indicated that the inducible phosphorylation of IκBα marks the protein for ubiquitin-mediated proteolytic degradation which then leads to its dissociation from NF-κB (Finco *et al.*, 1994; Miyamota *et al.*, 1994; Palombella *et al.*, 1994; Traenckner *et al.*, 1994; Alkalay *et al.*, 1995a; Didonato *et al.*, 1995; Lin *et al.*, 1995; Chen *et al.*, 1995; Lin *et al.*, 1995; Roff *et al.*, 1996; Alkalay *et al.*, 1995b).

Although it was demonstrated that the N-terminal serine residues at positions 32 and 36 in IκBα were targeted for inducible phosphorylation and that this event was essential for inducer-mediated degradation (Brown *et al.*, 1995; Traenckner *et al.*, 1995), other studies had demonstrated that residues in and around the C-terminal region (residues 257-317) were also important for inducible degradation of IκBα (Rodriguez *et al.*, 1995). Indeed, the C-terminal region of IκBα contains a PEST sequence (proline, glutamic acid/aspartic acid, serine and threonine) which is associated with rapid protein turnover. Since the C-terminal and linker IκBα c-tag mutants generated in this study contained amino acid substitutions within this domain it was proposed that they may play a role in the inducer-mediated degradation of IκBα.

Interestingly, the amino acid substitutions at positions 258 or 275 in the linker region (or low homology sixth ankyrin repeat) of IκBα c-tag did not affect the ability of the protein to be degraded in response to tumour necrosis factor α (TNFα) (in 293 cells) but the C-terminal substitutions at positions 284-286 or 300-302 appeared to partially block TNFα-inducible degradation. However, since the basal turnover of these mutants was not investigated their apparent failure to be degraded in response to TNFα may have been a result of a slower rate of basal degradation. Indeed, this was strongly suggested by the

observation that in order to achieve similar levels of protein expression for the various mutant and wild-type IκBα c-tag proteins in 293 cells, far lower quantities of the vectors expressing the C-terminal IκBα c-tag mutants were required.

The degradation assays also revealed that transiently transfected wild-type or linker mutant IκBα c-tag was not degraded in response to TNFα as rapidly as endogenous (cellular) IκBα. However, other groups have observed (M. Rodriguez, Pasteur Institute) that the stable transfection of cells with a vector expressing IκBα c-tag shows a similar rate of inducible degradation to the endogenous protein. Perhaps the stable transfection of cells with IκBα c-tag gives the protein time to establish itself in the cells ubiquitin-dependent degradation pathway (which will be initially turning over mainly endogenous IκBα). The cells degradation machinery will presumably take time to either readjust its rate or increase the level of its component proteins to cope with the influx of exogenous IκBα. Increasing the level of the proteins comprising the degradation pathway may be achieved through the induction of their coding genes.

Studies have shown that the degradation of IκBα is enhanced in mature B-cell lines and that this may account for the presence of constitutively active NF-κB (i.e. nuclear) (Miyamoto *et al.*, 1994). Indeed, it is tempting to speculate that the expression of various components of the constitutive degradation pathway for IκBα are increased by NF-κB in order achieve a more efficient turnover of IκBα..

Inducibly phosphorylated forms of all the mutants except EDE300-302qnq and EDE300-302sss were detected on a gradient gel but all of the mutants appeared to be targeted for inducible ubiquitination. Studies have shown that blocking the inducible phosphorylation of IκBα through the mutation of the N-terminal residues, serine 32 and 36 prevents ubiquitination (Roff *et al.*, 1996; Rodriguez *et al.*, 1996). Therefore, it would be predicted that mutants EDE300-302qnq and EDE300-302sss should not be ubiquitinated. However, it is possible that both of these mutants are being inducibly phosphorylated but that the gradient gel is not detecting the phosphorylated proteins. An explanation for this possibility lies within the nature of the amino acids which were altered in these mutants i.e. charged residues were replaced with uncharged amino acids. This may have affected the mobility of these mutants

on the gradient gel so as to occlude inducibly phosphorylated forms of the proteins (presumably there are instances where the native charge of a protein, if it is high enough, will affect its mobility on a gel even in the presence of SDS). Indeed, it was noted in the results section that the shift in mobility observed for the inducibly phosphorylated forms of the other mutants, which also contained charged to uncharged amino acid substitutions was poor compared to the wild-type protein. Attempts were made to separate the inducibly phosphorylated forms of the various mutants by isoelectric focusing without success. If time had permitted it would have been useful to try and radioactively label the inducibly phosphorylated forms of the wild-type and mutant IκBα c-tag proteins in 293 cells. However, since IκBα is heavily constitutively phosphorylated this may have masked any inducible phosphorylation.

Since the C-terminal mutants appeared to be phosphorylated (observed at least for mutants EDE284-286qnq and EDE284-286sss) and ubiquitinated in response to TNFα (in 293 cells) this indicated that the proteins should undergo inducible degradation. Therefore, as explained earlier this may suggest that the C-terminal mutants are being inducibly degraded to the same extent as the wild-type protein and that it is the basal turnover of the C-terminal mutants which is slower. Alternatively, some other ubiquitin-independent degradation pathway could exist which may or may not require IκBα to be inducibly phosphorylated but that the amino acid changes introduced into the C-terminal mutants are sufficient to block their degradation via this pathway. Perhaps these amino acids are required for a post-translational modification which is recognised by the components of this pathway.

Indeed a very recent study on pervanadate (a protein tyrosine inhibitor and T cell activator) induced Jurkat T cells has shown that NF-κB is activated via tyrosine phosphorylation but not degradation of IκBα. This proteolysis-independent pathway for NF-κB activation appears to require the expression of a tyrosine kinase, $p56^{lck}$. In addition, it was suggested that reactive oxygen intermediates may induce IκBα tyrosine phosphorylation since the reoxygenation of hypoxic cells stimulated tyrosine phosphorylation of IκBα (Imbert *et al.*, 1996). Furthermore, a study on the p50 precursor protein, p105 has revealed that alternative signalling pathways exist for p105 phosphorylation and processing and for IκBα

phosphorylation and degradation following PMA/ionomycin treatment (in Jurkat T cells) (MacKichan *et al.*, 1996). Therefore, it is possible that different signalling pathways exist for TNFα induction of IκBα itself, and that these direct the protein to different degradation pathways.

However, recent data support the idea that the C-terminal region of IκBα is not required for inducible degradation. Instead it appears to be essential for the basal turnover of the protein. This includes a report which generated a C-terminal deletion mutant corresponding to residues 1-277 that was degraded in response to TNFα (Sun *et al.*, 1996). In contrast, the removal of residues 264-317 rendered IκBα resistant to TNFα-induced degradation. The group proposed that a glutamine and leucine rich region in the low homology sixth ankyrin repeat of IκBα participates in signal-induced degradation.

Further studies on the C-terminal region of IκBα have revealed that the substitution of serine to alanine at position 283 and a threonine to alanine substitution at positions 291 and 299 did not affect signal-induced degradation of the protein but increased the intrinsic stability of the protein (Lin *et al.*, 1996). Another investigation utilising a transient transfection system in 293 cells which allowed the stability of free IκBα to be studied (i.e. IκBα not bound to NF-κB) reported that the mutation of serine 283, 288, 293 and threonine 291 to alanine doubled the half-life of free IκBα (Schwarz *et al.*, 1996). Interestingly, Lin *et al* (1996) also reported that following TNFα induction of cells phosphorylation of the IκBα mutant containing the three C-terminal amino acid substitutions (S283A, T291A and T299A) was reduced by about 10-fold. Therefore, it is possible that the C-terminus does contribute to the hyperphosphorylation of IκBα.

The reports on IκBα degradation have also demonstrated that casein kinase II may be responsible for the constitutive phosphorylation of IκBα in its C-terminal region (Barroga *et al.*, 1995; McElhinny *et al.*, 1996; Lin *et al.*, 1996; Schwarz *et al.*, 1996). Casein kinase II is a found in the nucleus and cytoplasm of all eukaryotic cells studied to date. Although the enzyme can phosphorylate several cellular proteins including Fos, Jun, serum response factor, p53 and Myc, its physiological role is unclear. However, many of the proteins targeted for phosphorylation by casein kinase II are involved in DNA replication and transcription.

Furthermore, several of the casein kinase II protein substrates are also substrates for kinases participating in cell cycle regulation. Moreover, the activity of casein kinase II has been shown to be regulated in several cell-types by growth-stimulatory factors suggesting that the enzyme may be important for mitogenesis (reviewed in Allende *et al.*, 1995). The consequences of casein kinase II phosphorylation on the activity of different proteins varies extensively. For example, mutations in the casein kinase II phosphorylation sites or dephosphorylation of the nuclear phosphoprotein, serum response factor reduces the rates of association and dissociation from the serum response element DNA binding sites. Conversely, c-Jun DNA binding activity is reduced through casein kinase II phosphorylation (reviewed in Allende *et al.*, 1995).

The role of casein kinase II in the constitutive phosphorylation of IκBα (in its C-terminal region) (Barroga *et al.*, 1995; McElhinny *et al.*, 1996; Lin *et al.*, 1996; Schwarz *et al.*, 1996) support the observation in this study that the *in vitro* casein kinase II phosphorylation of the C-terminal mutants containing changes in their consensus casein kinase II phosphorylation sites was drastically reduced:

Barroga *et al* (1995) showed that casein kinase II physically associates with and phosphorylates IκBα *in vivo* and *in vitro*. Furthermore, the mutation of potential casein kinase II phosphorylation sites in the C-terminal region of IκBα, namely serine 283, 288 and 293 and threonine 291 and 296 was shown to abolish casein kinase II dependent phosphorylation of IκBα. McElhinney *et al* (1996) performed a sequence analysis of IκBα phosphoisoforms obtained following incubation of purified IκBα with either purified casein kinase II or cellular extracts from U937 cells (known to contain a casein kinase II-like activity). The C-terminus of IκBα was found to be phosphorylated on serine 283, 288 and 293 and threonine 291. Furthermore, the study linked the basal turnover of IκBα to its basal (or constitutive) phosphorylation through casein kinase II in U937 cells.

The investigation performed by Lin *et al* (1996) revealed that in NIH 3T3 cells stably transfected with a vector expressing an IκBα mutant containing a serine to alanine substitution at position 283 and a threonine to alanine substitution at positions 291 and 299, the constitutive phosphorylation of the mutant was blocked. Importantly these residues were

the constitutive phosphorylation of the mutant was blocked. Importantly these residues were all located within consensus casein kinase II phosphorylation sites (S/T-X-X-E/D). Indeed, phosphoamino acid analysis of the purified mutant protein following incubation with either purified casein kinase II or a partially purified kinase activity from Jurkat cells (suspected to be casein kinase II) revealed that its phosphorylation was reduced by 10- to 20-fold. Under the same conditions, a single amino acid substitution at position 291 (threonine to alanine) also reduced phosphorylation by two- to four-fold whereas a substitution at position 283 (serine to alanine) did not affect phosphorylation. It has been proposed that threonine 291 may serve as a "docking site" for casein kinase II where it can sequentially phosphorylate other serine and threonine residues in the C-terminal region of IκBα.

In contrast, Schwartz *et al* (1996) have shown that serine 293 of IκBα is the preferred site for casein kinase II phosphorylation whereas adjacent serine and threonine residues act as secondary phosphorylation targets. However, Lin *et al* (1996) used a different cell type to the one employed in this study and therefore it is possible that distinct patterns of casein kinase II phosphorylation exist between different cell types.

Interestingly, unpublished data in the report by Lin *et al* (1996) and results from our laboratory (Jaffrey, 1995) failed to demonstrate a role for casein kinase II phosphorylation in the N-terminal region of IκBα. This is surprising since the two N-terminal serine residues (S32 and S36) which have been previously shown to be targets for inducible phosphorylation are located within consensus casein kinase II phosphorylation sites. Since Lin *et al* (1996) also showed that the triple mutant (S283A, T291A and T299A) completely blocked constitutive phosphorylation *in vivo* it is likely that the N-terminal serine residues at positions 32 and 36 do not undergo casein kinase II-dependent phosphorylation *in vivo*.

The C-terminal of the *Drosophila* IκB protein, Cactus has also been shown to be the target for basal phosphorylation and degradation in the *Drosophila* embryo (Belvin *et al*., 1995). In parallel to the study carried out by Schwarz *et al* (1996) on IκBα, the degradation characteristics of free Cactus were examined. The removal of the PEST region of Cactus increased the half-life of the free protein. It has been proposed that the C-terminal region of Cactus mediates signal-independent degradation of free Cactus, perhaps via basal

IκBα system. Indeed, it has been demonstrated that free wild-type IκBα is basally phosphorylated and turned over rapidly (but much less rapidly (half-life ~32 minutes) than TNFα induced degradation of NF-κB bound IκBα (half-life ~5 minutes)) (Sun *et al.*, 1993; Schwarz *et al.*, 1996), whereas unpublished data indicates that an IκBα mutant (free) containing mutations in all of the potential C-terminal casein kinase II phosphorylation sites (of which there are five, S283, S288, T291, S293 and T296) is not phosphorylated and has a longer half-life (see Verma *et al.*, 1995). Furthermore, this mutant was not phosphorylated *in vivo* when transiently transfected into 293 cells but was still able to bind p65, indicating that basal phoshorylation is not a requirement for NF-κB-IκBα association. The results also revealed that the mutant was inducibly phosphorylated and degraded in response to TNFα. This suggests that basal phosphorylation is not essential for induced phosphorylation and degradation of IκBα.

The *in vivo* characterisation of the C-terminal and linker mutants generated in this study also demonstrated that all of the IκBα c-tag mutants were able to interact with p65 in 293 cells. In addition, both the linker and C-terminal mutants allowed NF-κB-dependent transcription from a luciferase reporter following the activation of Cos7 cells with TNFα. This result was surprising since the C-terminal mutants were more stable than either the wild-type or linker mutant proteins and consequently they would have been predicted to bind NF-κB, thereby reducing NF-κB-dependent transcription in the nucleus. However, this result may be due to differences in the degradation characteristics of the mutant proteins in Cos7 cells compared to 293 cells. Alternatively the mutants could undergo a post-translational modification in Cos7 cells which prevents them from interacting with NF-κB.

It is worth noting that the level of transactivation from the transfected luciferase reporter in activated Cos7 cells was greater in the absence of both transfected wild-type and mutant IκBα c-tag. Presumably overexpressing wild-type or mutant IκBα c-tag will, under conditions of activation overload the cells degradation machinery leading to a build-up of exogenous IκBα c-tag. This will be available to bind NF-κB and therefore lower the level of NF-κB-dependent transcription.

2.2. The role of the N-terminal region of IκBα.

It had been shown that inhibition of proteasomal activity followed by TNFα induction of cells resulted in the detection of a hyperphosphorylated form and ubiquitinated forms of IκBα (Pallombella *et al.*, 1994; Traenckner *et al.*, 1995; Roff *et al.*, 1996). Furthermore, a mutant containing N-terminal serine to alanine substitutions at positions 32 and 36 was not only resistant to signal induced degradation but was unable to undergo either inducible phosphorylation or ubiquitination (Chen *et al.*, 1995; Roff *et al.*, 1996). This indicates that inducible phosphorylation of residues 32 and 36 is a prerequisite for signal induced ubiquitination and degradation of IκBα. Indeed, the experiments performed with colleagues at the Pasteur Institute have provided additional evidence for the involvement of the N-terminal region in the inducible degradation of IκBα.

The study set out to identify residues within the N-terminal region of IκBα which may represent targets for ubiquitination. Since ubiquitin chains are added to the ε-amino group of lysine residues the study focused on the nine lysine residues within the N-terminal region of IκBα. Although not mentioned in the results section, transiently overexpressed IκBα mutants containing only single lysine to arginine substitutions were ubiquitinated to the same extent as wild-type IκBα in response to TNFα induction of cells. However, the generation of mutants containing multiple lysine to arginine substitutions in the N-terminal region at positions 21, 22, 38 and 47 completely blocked TNFα induced ubiquitination together with NF-κB-dependent transcription but did not affect inducible phosphorylation. The generation of two double mutants containing either lysine to arginine substitutions at positions 21 and 22 or 38 and 47 revealed that lysines 21 and 22 were probably the primary targets for ubiquitination whereas lysines 38 and 47 represented the secondary targets.

The discovery that more than one lysine residue was required for efficient ubiquitination of IκBα is comparable to the situation observed for the c-Jun transcription factor (Treier *et al.*, 1994). In this study ubiquitination of c-Jun but not its viral counterpart, v-Jun was observed *in vitro*. Attempts to identify a single lysine residue responsible for the ubiquitination of c-Jun failed. Indeed double and triple mutations also had no affect on ubiquitination. It was proposed that either ubiquitination was occurring at multiple sites or

that the ubiquitin-attachment machinery was able to move from a lysine residue which had been mutated and begin adding ubiquitin chains to an alternative lysine. However, in the case of IκBα ubiquitination it appears that lysines 21 and 22 are specific targets for ubiquitination and that their removal is only partially compensated for by the secondary ubiquitination sites at positions 38 and 47.

Therefore, it would appear that the N-terminal region of IκBα is essential for both inducible degradation and ubiquitination. It has been proposed that after the signal-induced phosphorylation of IκBα on serine 32 and 36, the protein is recognised by the ubiquitin-attachment machinery. Having covalently attached multiple ubiquitin molecules to lysines 21 and 22 (and possibly to lysines 38 and 47) the protein is then degraded by the 26S proteasome. The importance of lysine 21 and 22 for signal induced ubiquitination and degradation of IκBα has been confirmed by other groups (Baldi *et al.*, 1996; Didonato *et al.*, 1996).

Interestingly, the N-terminal domain of IκBα has been shown to be highly susceptible to proteolytic degradation *in vitro* as determined via protease sensitivity studies (Jaffray *et al.*, 1996). This suggests that the region is probably highly unstructured and therefore ideal for a signal response domain so that it can allow easy access of kinases (and possible phosphatases) and the ubiquitin-proteasome machinery. However, one would think that the apparently unstructured nature of the region would increase the probability of degradation by non-specific proteases *in vivo*. Perhaps the C-terminal region of IκBα can mask this region in unstimulated cells to prevent such degradation. Indeed, the protease sensitivity studies indicated that the low homology sixth ankyrin repeat was also highly susceptible to proteolysis *in vitro* and it was suggested that the region may act as a flexible linker between the fifth ankyrin repeat and the C-terminus (Jaffray *et al.*, 1995). This hinge-like domain would therefore permit the free movement of the C-terminal "arm" allowing it to lie over the N-terminal region.

2.3. Model for basal and signal- induced I κB α degradation.

Represented schematically in figure 54 is one possible model for the basal (i.e. constitutive) and inducible degradation of IκBα. It appears that IκBα protein stability is regulated at two levels. The first involves the signal-dependent turnover of IκBα. Following activation of cells, kinase(s) phosphorylate IκBα on the N-terminal residues, serine 32 and 36 (step 1). It is likely that this event marks IκBα for covalent modification by a specific ubiquitin ligase (step 2). After the ligase catalysed addition of multiple molecules of ubiquitin to lysine 21 and 22 (and possibly the secondary ubiquitination sites at residues 38 and 47) of IκBα, the protein dissociates from NF-κB, allowing the translocation of NF-κB to the nucleus where it can activate transcription of target genes (step 3). Ubiquitinated IκBα is rapidly degraded by the proteasome (step 4). It should be noted that steps 1 and 2 are reversible by phosphatase(s) and C-terminal hydrolase(s) respectively. Presumably following activation the equilibrium of each reaction is pushed forward. As reported by Verma *et al* (1995) recent unpublished work indicates that constitutive phosphorylation of IκBα is not necessarily required for interaction with NF-κB and this is indicated with a question mark.

The second level of regulation exerted on IκBα is signal-independent degradation. This pathway involves constitutive phosphorylation of the C-terminal region of IκBα via casein kinase II. As in the inducible degradation pathway, phosphorylation may target the protein for constitutive degradation via a ubiquitin-dependent or -independent proteolytic pathway. IκBα may or may not be bound to NF-κB prior to the phosphorylation and degradation events. If this pathway represents the degradation of free IκBα it could represent a mechanism for the removal of excess IκBα in cells. This would be particularly important during the activation of NF-κB, where excess IκBα could prevent the passage of NF-κB to the nucleus and the subsequent induction of target genes.

In conclusion, both pathways are probably crucial for regulating the activity of NF-κB. It will be important to elucidate the mechanism of the basal degradation pathway and to identify other pathways involved in IκBα turnover in cells in order to gain a better incite into the global regulation of NF-κB.

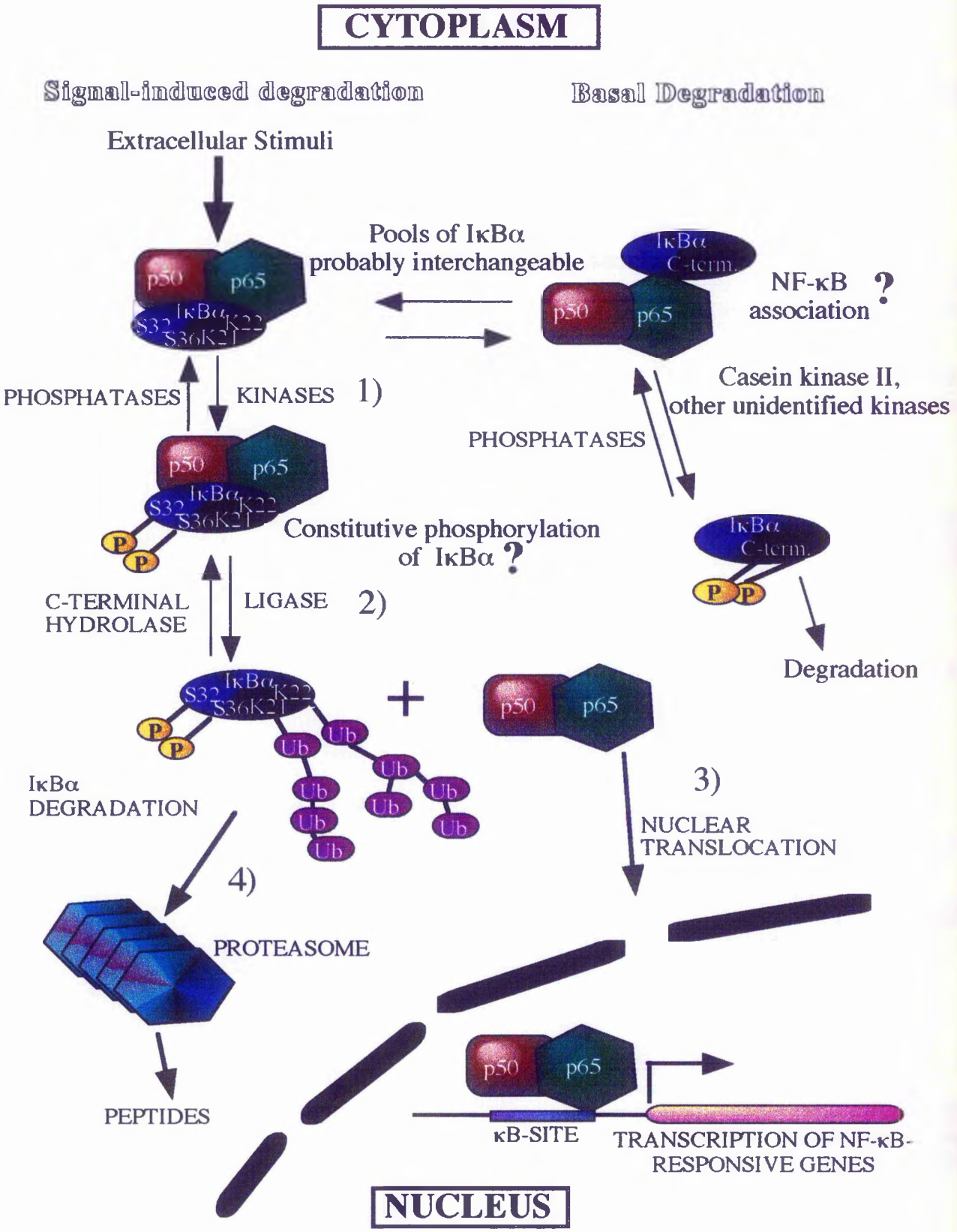


Figure 54. Proposed model for signal-induced and basal degradation of IκBα.
(see discussion, page 135 for details). C-term, refers to C-terminus of IκBα.

3. Structure of the C-terminal region of IκBα.

Proteolysis sensitivity studies on IκBα had revealed that the C-terminal region, in contrast to the N-terminus was highly resistant to proteolysis, indicative of a tightly organised structure (Jaffray *et al.*, 1995). This study set out to determine whether or not the C-terminus of IκBα was able to exist as an independently folded domain. The technique of Circular Dichroism (CD) Spectroscopy was used to predict the secondary structure content of the C-terminal domain. However, as indicated in the results section CD spectroscopy can only accurately determine the amount of β-sheet and α-helix and the remaining secondary structure constituents are averaged (e.g. β-turns and 3_{10} helix).

The secondary structure predictions (calculated by applying the CONTIN procedure to the CD spectra) indicated that the C-terminal region of IκBα (amino acids 275-317) was comprised mainly of β-sheet and remaining secondary structure constituents such as β-turns and 3_{10} helix. The α-helical content of the region was very low in comparison (around 6% compared to 40-50% β-sheet). The presence of a ten amino acid section from the low homology sixth ankyrin repeat of IκBα (residues 265-317) did not appear to alter the level of secondary structure content. However, at lower pH's and in the absence of TFE, the increase in β-sheet content observed for the region corresponding to residues 275-317 of IκBα was not accompanied by a similar increase in the β-sheet content of the region corresponding to residues 265-317.

It is possible that the increase in β-sheet content observed at lower pH's for the C-terminal region (275-317) reflects the conditions produced by the proposed interaction of the C-terminus of IκBα with the DNA binding domain of p65 i.e. at lower pH's carboxyl groups will be protonated and therefore available for hydrogen bonding to p65. The results indicate that the sixth low homology region may slightly destabilise this interaction. A CD study on the acidic activation domains of the yeast GCN4 and GAL4 proteins also revealed that lower pH's increased their β-sheet content (Van Hoy *et al.*, 1993).

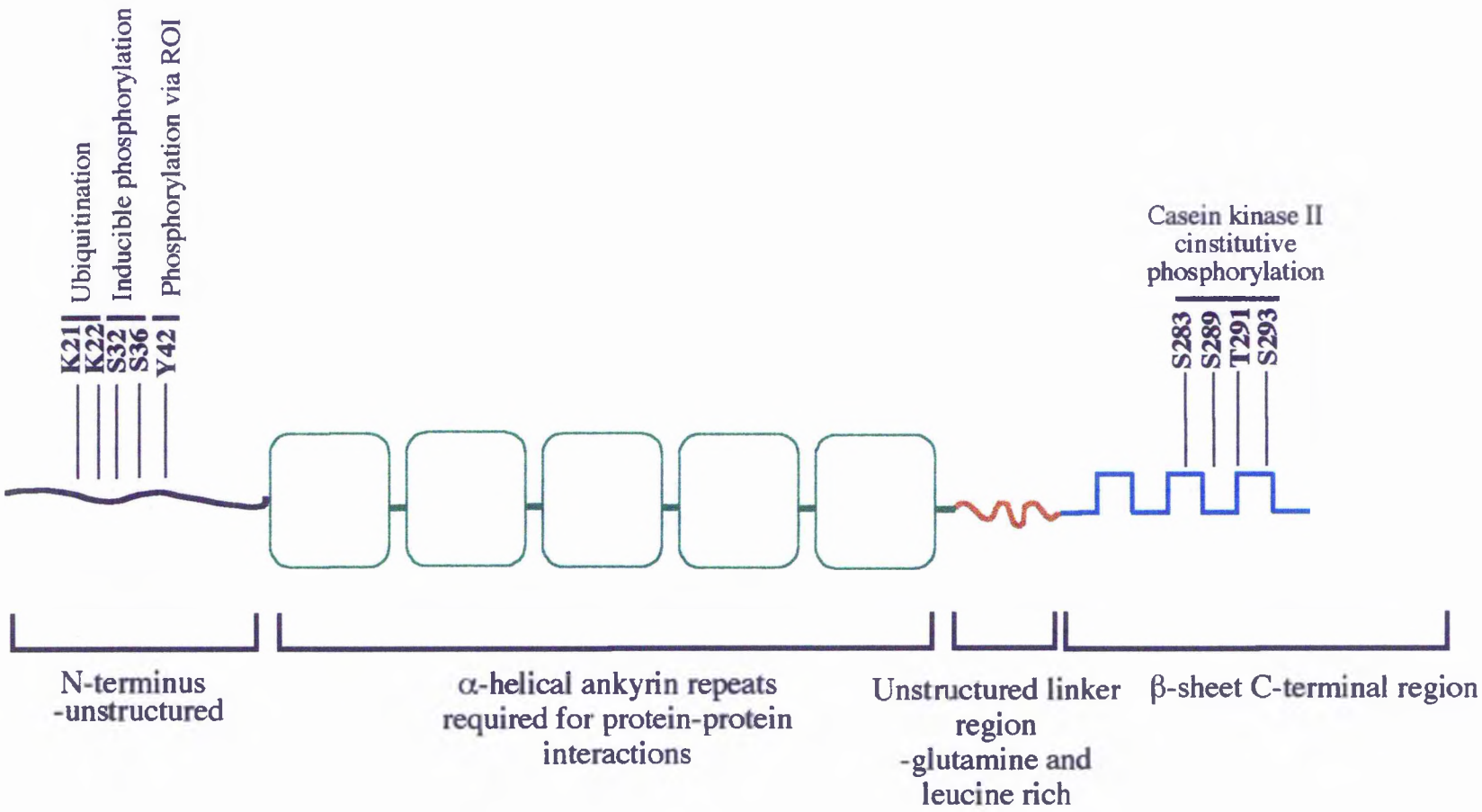
The fusion of a C-terminally fused 14 amino acid tag region to amino acids 265-317 appeared to increase the β-sheet content of the protein. This suggests that the tag region can structurally stabilise the C-terminal region and part of the low homology sixth ankyrin repeat.

It is possible that tag acts as a stabilising influence in the proposed interaction between the C-terminal region of IκBα with the DNA binding domain of p65. However, it is likely that this influence is probably minimal as the tag region when fused to full-length IκBα does not improve IκBα's ability to inhibit the DNA binding of p65. This raises an important point concerning the purification of IκBα c-tag, whereby the inclusion of an additional step in the purification procedure (the triton X100 and sarkosyl procedure) resulted in IκBα c-tag acting as a more efficient inhibitor of p65 DNA binding. This could indicate that the purification technique stabilises the structure of the C-terminal region (possibly by raising the level of β-sheet) so that it can interact more strongly with the DNA binding region of p65. It is also worth noting that the CD studies were performed on independent C-terminal fragments of IκBα and that their conformation in the full-length protein is probably affected (possibly stabilised) by the N-terminal domain and ankyrin repeats.

To summarise, a schematic for the proposed secondary structure of IκBα is given in figure 55. The structure is based on the data obtained from this CD study, proteolysis sensitivity work (Jaffray *et al.*, 1995) and secondary structure prediction programmes (Gay and Ntwasa, 1993). The functional role of the various domains within IκBα are also indicated.

Figure 55: The proposed secondary structure of IκBα.

IκBα can be divided into three main domains: an unstructured N-terminal signal response domain shown to be critical for inducible phosphorylation and degradation of IκBα; a central α-helical region of five ankyrin repeats, responsible for protein-protein interations; a C-terminal region of high β-sheet content required for constitutive phosphorylation and degradation of IκBα as well as for the inhibition of p65 DNA binding activity. In addition, a fourth domain may exist- the so-called flexible linker region proposed to represent the sixth ankyrin repeat and implicated in protein-protein interactions and signal-induced degradation of IκBα.The positions of the amino acids implicated in constitutive and inducer-mediated phosphorylation and degradation are indicated. Key: ROI-reactive oxygen intermediates.


Ubiquitination
Inducible phosphorylation
Phosphorylation via ROI
K21
K22
S32
S36
Y42
Casein kinase II
cinstitutive
phosphorylation
S283
S289
T291
S293
N-terminus
-unstructured
α-helical ankyrin repeats
required for protein-protein
interactions
Unstructured linker
region
-glutamine and
leucine rich
β-sheet C-terminal region

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