Signal-Induced Ubiquitination of IκBα

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DECLARATION

I, Lesley Margaret Thomson, hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for higher degree.

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List of abbreviations

A	Adenine
Akt/PKB	Thymoma viral proto-oncogene/protein kinase B
AMP	Adenosine monophosphate
APC	Anaphase promoting complex
ATP	Adenosine trisphosphate
BAEC	Bovine aortic endothelial cells
bHLH	Basic helix loop helix
bp	Base pairs
BSA	Bovine serum albumin
βTrCP	Beta transducin repeat containing protein
bZIP	Basic leucine zipper
C	Cytosine
cdc53/Cul1	Cell division cycle 53/cullin 1
cDNA	copy DNA
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
DTT	Dithiothreitol
DUB	De-ubiquitinating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase

EBM-2	Endothelial cell basal medium 2
EBV	Eppstein Barr Virus
E.Coli	*Escherichia Coli*
EDTA	Ethylenediaminetetracetic acid
G	Guanine
GST	Glutathione S-transferase
HCl	Hydrochloric acid
HECT	Homologous to E6-AP C-terminus
HIV-1	Human Immunodeficiency virus-1
HMG	High mobility group
HUVECs	Human endothelial vein cells
FBS	Fetal bovine serum
FCS	Fetal calf serum
IκBα	I kappa B alpha
^{32}P-IκBα	IκBα phosphorylated on serines 32 and 36
IKK	I kappa B kinase
IKK1(α)	I kappa B kinase 1 (alpha)
IKK2 (β)	I kappa B kinase 2 (beta)
IKKγ	I kappa B kinase gamma
IKAP	IKK-complex associated protein
IKKAP1	IKK associated protein 1
IL-1β	Interleukin 1 β
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranoside
KCl	Potassium chloride
kDa	Kilodalton
KLH	Keyhole Limpet heamocyanin
LB	Luria broth
LMP-1	Latent membrane protein-1
LPS	Lipopolysaccharide

LTβR	Lyhmphotoxin beta receptor
MAP3K	Mitogen activated protein kinase kinase kinase
MEK	Mitogen extracellular regulated kinase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NEDD-8	Neuronal precursor cell-expressed developmentally downregulated
NEMO	Nuclear factor kappa B essential modulator
NF-κB	Nuclear factor kappa B
NIK	Nuclear factor kappa B inducing kinase
NLS	Nuclear localisation signal
NP-40	Nonidet P 40
PAGE	Polyacrylamide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p.f.u.	Plaque forming units
PI(3)K	Phosphatidyl inositol-3-kinase
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PML	Promyelocytic leukaemia protein
PVDF	Polyvinyldifluoride
PVT	Polyvinyl toluene
Rbx1	RING-box protein 1
RHD	Rel homology domain
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
$SCF^{\beta TrCP}$	Skp1-Cul1/cdc53-F-box (βTrCP) E3 ligase complex
SDS	Sodium dodecyl sulphate
Sf	*Spodoptera frugiperda*
Skp1	S-phase kinase associated protein 1
SPA	Scintillation proximity assay
SPDP	N-succinimidyl 3-(2-pyridyldithio)propionate

SUMO	Small ubiquitin modifier
T	Thymidine
TAFs	TATA associated factors
TANK	TRAF family member associated NFκB
TBP	TATA binding protein
TCR	T cell receptor
TFII	Transcription factor II
TNFα	Tumour necrosis factor alpha
TNFR	TNF receptor
TNT	Transcription/Translated
TRAF-2	Tumour necrosis factor receptor associated factor-2
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
tRNA	Transfer ribonucleic acid
U	Uracil
UCII	Ubiquitin C terminal hydrolase
UCRP	Ubiquitin cross reactive protein
WCE	Whole cell extract
WT	Wild type

AMINO ACIDS and their SYMBOLS

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine

I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

GENETIC CODE

TTT	Phe	TCT	Ser	TAT	Tyr	TGT	Cys
TTC	Phe	TCC	Ser	TAC	Tyr	TGC	Cys
TTA	Leu	TCA	Ser	TAA	STOP	TGA	STOP
TTG	Leu	TCG	Ser	TAG	STOP	TGG	Trp
CTT	Leu	CCT	Pro	CAT	His	CGT	Arg
CTC	Leu	CCC	Pro	CAC	His	CGC	Arg
CTA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
CTG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
ATT	Ile	ACT	Thr	AAT	Asn	AGT	Ser
ATC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
ATA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
ATG	Met	ACG	Thr	AAG	Lys	AGG	Arg
GTT	Val	GCT	Ala	GAT	Asp	GGT	Gly
GTC	Val	GCC	Ala	GAC	Asp	GGC	Gly
GTA	Val	GCA	Ala	GAA	Glu	GGA	Gly
GTG	Val	GCG	Ala	GAG	Glu	GGG	Gly

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"From quiet homes and first beginnings,
Out to the undiscovered ends,
There is nothing worth the wear of winning,
But laughter and the love of friends".

Hilaire Belloc

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For my Dad,

Abstract

The control of gene expression is essential in all living organisms. Many mechanisms exist within the cell in order to carry out this function efficiently and precisely. Transcription factors play an essential role in the activation of transcription and serve as the rate-limiting factors in this process. NF-κB is a transcription factor involved in the expression of a number of genes within the cell. The mechanisms which exist in order to control the activity of this protein exemplifies the importance of co-ordinated and regulated control of gene expression.

NF-κB is held in an inactive form in the cytoplasm of unstimulated cells by association with its inhibitor protein IκBα. Stimulation of cells by various sources, results in the rapid phosphorylation of the inhibitor protein on N-terminal serine residues 32 and 36 by a specific kinase complex called the IKK signalsome. Phosphorylation results in IκBα ubiquitination and subsequent degradation by the 26S proteasome.

The role of the ubiquitin-proteasome pathway in the ubiquitination and degradation of IκBα is well-established. However, the actual events prior to ubiquitination of this protein have not been clearly defined. Investigation into the mechanism of IκBα ubiquitination and recognition of this protein by the $SCF^{\beta TrCP}$ complex was explored. It was demonstrated that IκBα must be phosphorylated on serine residues 32 and 36 before ubiquitination can occur. It was also revealed that each of the four components of the SCF complex must be present in order for efficient ubiquitination of phosphorylated IκBα. Furthermore, βTrCP, the F-box protein responsible for recognition of substrate, can only interact with IκBα when it is phosphorylated on serine residues 32 and 36. The requirement for the presence of other residues within IκBα responsible for the interaction of IκBα with βTrCP and its subsequent ubiquitination was also investigated. Interestingly, both ubiquitination and interaction of phosphorylated IκBα with βTrCP absolutely requires the presence of an acidic residue at position 31, aspartic acid.

Therefore it was revealed that signal induced ubiquitination of IκBα is dependent on the specific phosphorylation of IκBα by a kinase complex containing IKKα (IKK1) and IKKβ (IKK2). Phosphorylation allows recognition of IκBα by the E3 ligase, $SCF^{\beta TrCP}$. Recognition by the E3 ligase results in the subsequent ubiquitination of the phosphorylated substrate. Ubiquitinated substrate can be targeted for degradation via the 26S proteasome. Targeting of IκBα for degradation, releases active NF-κB into the nucleus. Hence, this process of IκBα ubiquitination serves as a critical step in the control of the activation of NF-κB. Investigation of this process will provide further insight and understanding into the complex and highly regulated control of the transcription factor, NF-κB and possibly other transcription factors which are regulated in this manner.

1. INTRODUCTION

1.1 Eukaryotic gene expression.

Multi-cellular organisms function by the regulated and co-ordinated interplay of many cellular processes. These cellular processes could not exist without the functioning of a multitude of proteins. The information contained in the genes of an organism provides the cell with instructions on how to process and express these proteins. Gene expression, itself, is also controlled by the presence and activity of a number of proteins, many of which, are also regulated.

Transcription in eukaryotes is catalysed by three different enzymes, RNA polymerase I, RNA polymerase II and RNA polymerase III (Sentenac, 1985; Young, 1991). Each RNA polymerase is responsible for the transcription of different types of genes. RNA pol I transcribes rRNA genes, RNA pol II transcribes mRNA precursors and RNA pol III is responsible for the transcription of small RNAs such as tRNA and 5sRNA. All three enzymes are very large complexes of approximately 500kDa, containing up to 15 subunits.

Each of the enzymes catalyse the synthesis of RNA from a DNA template. DNA is packaged within the cell in a particular structure to form chromosomes. Packaging of DNA into chromosomes enables the efficient storage of the genetic material of the organism. In order for a gene to be transcribed, however, the DNA of interest must be found within this storage system and be recognised by the transcriptional machinery.

The isolation and recognition of genes to be transcribed can be achieved in two ways. Firstly, DNA binding proteins which recognise specific DNA sequences in the gene help direct RNA polymerases to the transcriptional initiation start site. These proteins, known as transcription factors, bind to DNA sequences in the promoters of genes. Different types of promoters exist for each of the three RNA polymerases. They usually contain short stretches of nucleotides that characterise binding sites which are specific for specific transcription factors. These transcription factors can then associate with proteins

found in the pre-initiation complex and help to place the RNA polymerases over the transcription start site. The method in which DNA is packaged into nucleosomes provides another mechanism of regulating transcription. Specific DNA sequences can be found selectively wrapped around histones, such that the DNA sequences which are recognised by transcription factors are positioned on the outside of the nucleosome accessible to these proteins. Wrapping of DNA around histones can result in DNA sequences, and the proteins that bind these sequences, which would otherwise be far apart into close proximity. Proteins which bind these sequences may also interact with each other, or with other proteins within the transcriptional machinery, thus influencing or activating transcription in some way. The positioning of nucleosomes and post-translational modifications, such as acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination, of some of the histones within the nucleosomes can also effect the accessibility of the DNA to transcription factors. These distinct histone modifications have led to the suggestion of the existence of a "histone code" in which the pattern and appearance of different types of modifications within the histones reveal signals that can be read by other proteins. A single modification or a combination of modifications may allow the specific proteins which recognise these signals to elicit distinct downstream events (Strahl and Allis, 2000). Hence a combination of transcription factor activation and chromatin structure contribute to the overall activation of transcription.

1.1.2 Formation of a transcriptional pre-initiation complex

In the case of protein-coding genes, there are several promoter elements that are necessary for regulated transcription initiation. These can be enhancer elements and/or upstream elements which are located variable distances upstream of the initiation start site. Some genes are also found to contain repressor elements, which are essentially the same as enhancer elements except that they repress rather than activate transcription. Formation of the basal transcriptional machinery is established through a universal set of proteins; RNA

pol II and the general transcription factors, TFIIA, B, D, E, F and H (Pugh, 1996; Roeder, 1996). Common to most eukaryotic RNA pol II genes, is the presence of a TATA box approximately 30 base pairs upstream of the initiation start site. This is the site at which the pre-initiation complex is assembled. The TATA box consists of a consensus motif, 5' TATAT/AAT/A 3, which is the binding site for the TATA binding protein (TBP). TBP is a component of the TFIID multiprotein complex which also contains TATA associated factors (TAFs) as well as TBP. Binding of TFIID to the TATA element is the first step in the formation of a pre-initiation complex. TFIIA then binds and the formation of a minimal initiation complex proceeds by the recruitment of TFIIB via protein-protein interactions with TBP. TFIIB has been shown to be essential for transcription and acts as a bridging molecule between TBP and RNA pol II. RNA pol II in association with TFIIF are then recruited to the complex by binding to the N-terminal putative zinc finger domain of TFIIB. The underphosphorylated form of RNA pol II can now be placed directly over the initiation start site and the recruitment of TFIIE, by RNA pol II, and TFIIH by TFIIE, completes the assembly of the pre-initiation complex. Variations on the above theme have been noted and there is evidence that pre-assembled RNA pol II-general transcription factor-coactivator complexes, or holoenzymes, exist which are capable of promoter recognition and transcription without the necessity of a step by step assembly process. (Koleske and Young, 1994). There are also genes which lack TATA elements, but contain functional initiator elements (Inr). These initiator elements are common elements that are thought to be able to influence the direction of transcription as well as the location of the transcription start site. Basal transcription still requires all of the general transcription factors but also a number of specific initiator element binding proteins. Initiator elements have been found in a number of genes with and without TATA boxes which are able to bind an initiator-binding factor TFII-I and facilitate transcription on TATA-less promoters and TATA- and Inr-containing promoters (Morikawa *et al.*, 2000; Manzano-Winkler *et al.*, 1996; Mobley and Sealy, 2000; Roy *et al.*, 1993). Another initiator transcription factor Ying-Yang-1(YY-

1), has also been identified as participating in initiation of transcription from such promoters (Clark *et al.*, 1998; Mobley and Sealy, 2000).

Basal transcription therefore occurs through the formation of a pre-initiation complex on the DNA. However increased rate of transcription can only occur through the co-ordinated and regulated action of activating proteins. These proteins act by recognising enhancer or upstream elements in the promoters, binding sequence specific regions of the DNA. They are then able to increase the rate of transcription by increasing the rate of stably assembled TFIIB and also by interacting with TAFs within the pre-initiation complex, thereby increasing the rate at which TFIID is recruited to the core promoter and in turn increasing the rate of formation of the pre-initiation complex (Choy and Green, 1993). Specific transcription factors are the key to controlling transcriptional activation. Basal transcription factors are present constitutively, but require the appearance of these specific upstream binding factors in order to selectively and purposefully increase the rate of transcription. These specific transcription factors are the rate-limiting step in the process of transcriptional activation. Due to the importance of these proteins in the overall functioning of the cell, it is essential that these proteins are themselves controlled in order to control the rate at which transcription of genes and hence production of protein is maintained.

1.1.3 Control of transcription

Control of eukaryotic gene expression is achieved in a number of ways. However, transcriptional regulatory mechanisms can be broadly classified into two main groups; control of the proteins involved in the transcriptional apparatus and control at the level of DNA packaging within the cell. Through the integration of both of these events combinatorial control of transcription can be achieved. This provides the cell with a higher-order of transcriptional control due to the fact that a combination of mechanisms may exist in order to control the rate at which a specific gene is transcribed. Consequently

transcription within eukaryotes is a highly complex and regulated mechanism of events which must exist in order for the cell to function properly.

1.1.3.1 Transcription factor control

Sequence specific DNA binding proteins allow the controlled and regulated expression of proteins within the cell. They are the most direct regulators of gene transcription. Therefore regulation of transcription factors is fundamental in controlling cell function. This regulation can be achieved by either controlling the level of concentration of the transcription factor within the cell or by regulating its activity.

Concentration of transcription factors can be regulated at the level of transcription, RNA processing, mRNA degradation, translation or even protein stability. See Figure 1. One of the most obvious ways in controlling transcription factor levels is whether the protein is made. The genes for many transcription factors are expressed in a tissue specific fashion. Hepatic nuclear factor 1 (HNF-1), along with a number of other liver-enriched regulatory proteins, while present in other tissues, are only expressed to significant levels in liver cells where they regulate the expression of hepatic genes (Xanthopoulos *et al.*, 1991).

Transcription factors themselves are also regulated by the combinatorial effect of transcription factors binding to promoter and enhancer sequences within their own gene. Many transcription factors act as auto-regulatory factors being involved in the transcription of their own genes. Pit-1, a transcription factor involved in the expression of growth hormone and prolactin genes, has been shown to positively autoregulate Pit-1 expression by binding to two Pit-1 binding sites in the promoter of its own gene (Chen *et al.*, 1990). MyoD1, a transcription factor involved in muscle differentiation can also stimulate MyoD1 expression in mouse fibroblasts from the MyoD1 promoter, suggesting expression is controlled by a positive feedback loop (Zingg *et al.*, 1994).

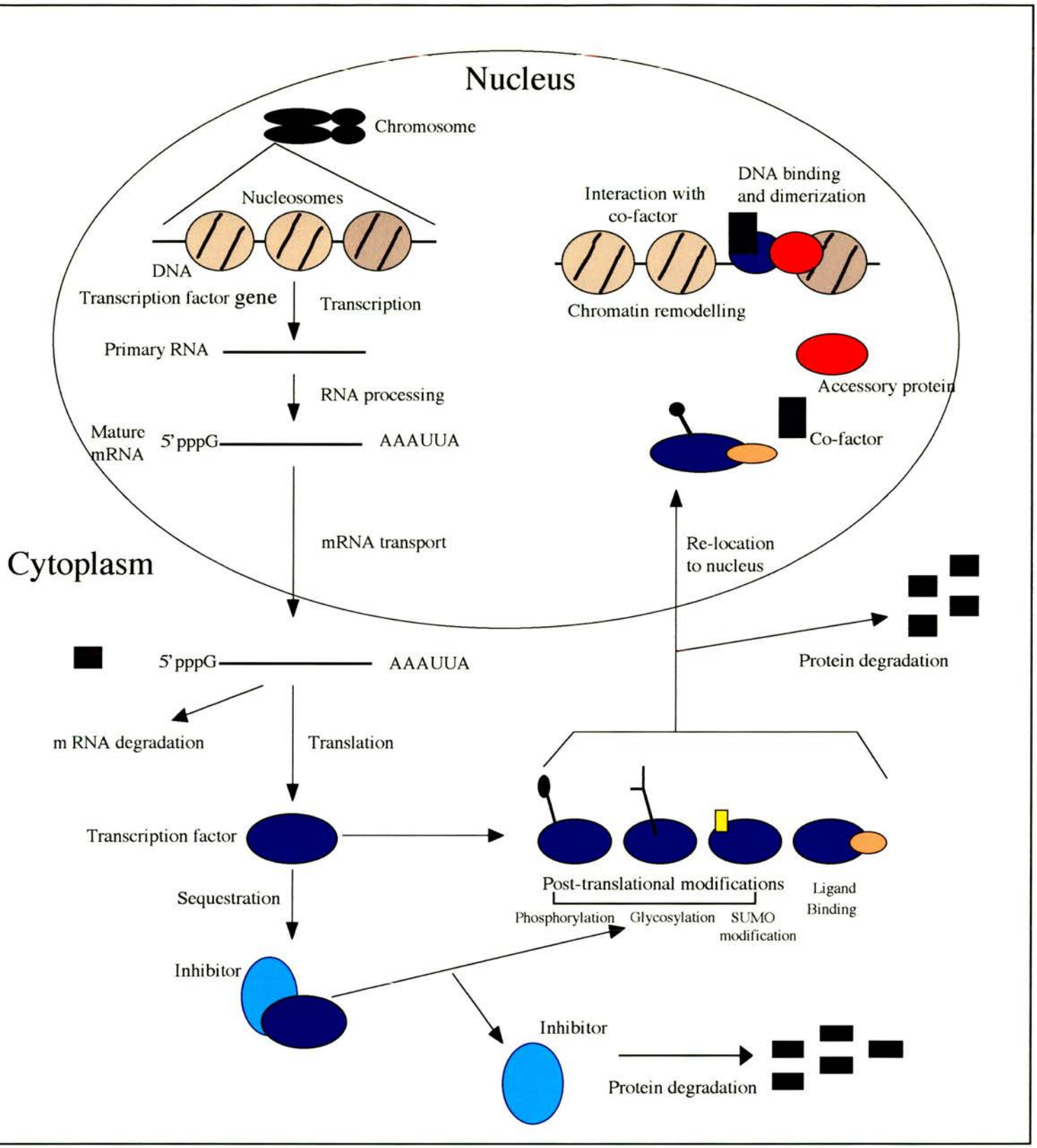


Figure 1 Schematic overview of the mechanisms and controls of eukaryotic Transcription factors

Control of eukaryotic gene expression can be exemplified by the control of transcription factors which regulate gene expression or through the control of the chromatin structure. Control of transcription factors can be regulated in two ways; firstly by regulation of the concentration of protein within the cell through controlling such mechanisms as RNA processing, mRNA degradation, translation or protein degradation, and secondly at the level of transcriptional activtiy. This can be achieved by sequestering the protein in an inactive complex in the cytoplasm; post-translational modifications such as phosphorylation, glycosylation, sumolation; association with other proteins; import into the nucleus and association with co-activators and repressors. These controls along with chromatin packing and regulation contribute to the overall control of eukaryotic gene expression.

Pre-mRNA is processed by the addition of a 5'cap and a 3' poly A tail together with splicing of introns to produce mature mRNA. Alternative splicing of RNA can result in the same RNA molecule being used to create a number of different isoforms of the same protein. This can be constitutive where alternative splicing of the same gene always results in the expression of the same isoforms. Or it can be regulated and different isoforms produced depending on the cell type and circumstances. Alternative splicing of transcription factor pre mRNAs can generate multiple mRNAs that can differ in their untranslated or coding regions. This in turn affects mRNA stability, translation efficiency or intracellular location or yields proteins with different, often opposing activities (Smith *et al.*, 1989).

Eukaryotic mRNAs exist in the cell for a limited period of time once they have been synthesised. Some mRNAs are more stable than others, and the turnover rate can range from days to hours to minutes. Transcription factor mRNA is one of the mRNAs which is found to have a high turnover rate along with other transiently expressed proteins such as cytokines and growth factors. This is illustrated by the transcription factor c-Fos, in which specific sequences are found in the 3'untranslated region (UTR) and in the coding region which facilitate mRNA degradation (Shyu *et al.*, 1989; Bonnieu *et al.*, 1989). These AU-rich instability sequences have also been recognised in the 3'UTR of other mRNAs suggesting that these sequences may serve as a general mechanism of controlling mRNA stability within the cell (Shaw and Kamen, 1986;Caput *et al.*, 1986).

Transcription factor levels, along with other proteins, can also be regulated at the stage of protein translation. Eukaryotic initiation factors are required for the selection of the mRNA to be translated and also for the selection of the inititator codon by the scanning ribosome complex. The *Saccharomyces cerevisiae* gene, GCN 4, has been shown to be controlled at the level of transcriptional initiation through sequences in upstream open reading frames (ORFs) and the actions of initiator factors and protein kinases (Hinnebusch, 1990).

One of the most effective ways in controlling the function of transcription factors within the cell is by regulating transcription factor activity. Many processes have evolved which efficiently carry out this procedure These include regulation by post-translational modifications such as phosphorylation, glycosylation, ubiquitination and sumoylation. Hetero- or homodimerisation of transcription factors or association with other proteins or co-factors, as well as cellular localisation also plays a part in this regulation process.

Phosphorylation, or dephosphorylation, can serve to activate transcription factors which are otherwise inactive. Many extracellular signals are transmitted to the nucleus via a cascade of phosphorylation reactions that ultimately activate their target protein. Phosphorylation or dephosphorylation of transcription factors can result in activation of the proteins in a number of ways. The STAT (signal transducers and activators of transcription) family, which are involved in the immune system and signalling via cytokine receptors, are present in the cytoplasm of unstimulated cells as monomers. Upon ligand binding to receptor, a cascade of events occurs involving recruitment and activation of members of the Janus kinase (JAK) family of tyrosine kinases. Phosphorylation of these transcription factors leads to their homo- or heterodimerisation resulting in their subsequent translocation to the nucleus. Only the dimeric form of the transcription factor is capable of binding to specific DNA sequences and activating transcription of responsive genes (Liu *et al.*, 1998). Not only does phosphorylation affect cellular localisation of transcription factors, it can also modulate DNA binding activity and trans-activation potential. It has been shown that the transcription factor c-Jun can be phosphorylated on three sites located in its N-terminal domain next to the DNA-binding domain, by casein kinase II (CKII) (Lin *et al.*, 1992). Dephosphorylation at these three sites increases its DNA-binding activity. However, two phosphorylation sites in its N-terminal transactivation domain, when phosphorylated in response to mitogenic signals by distinct mitogen activated protein (MAP) kinases and also in response to stress signals by stress-activated protein kinases,

SAPKs, cause an elevation in trans activation potential (Hibi *et al.*, 1993; Derijard *et al.*, 1994).

Other post-translational modifications of transcription factors such as glycosylation, ubiquitination and SUMO conjugation have also been shown to play a part in controlling transcription factor activity (Hart, 1997; Desterro *et al.*, 2000; Melchior, 2000). It is well documented that p53, a transcription factor involved in the transcription of a number of genes involved in apoptosis and cell cycle arrest, can be ubiquitinated and degraded (Maki *et al.*, 1996; Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). It has also been shown that c-Jun, a component of the AP-1 transcription factor can be degraded in a ubiquitin-dependent manner (Fuchs *et al.*, 1996; Karin *et al.*, 1997). SUMO conjugation of transcription factors has also become evident as a method of controlling transcription factor actvity. p53 and c-Jun are targets of this post-translational, ubiquitin-like modification (Rodriguez *et al.*, 1999; Gostissa *et al.*, 1999; Muller *et al.*, 2000) and it has also been shown to modulate the activity of NF-κB, through sumoylation of its inhibitor protein IκBα (Desterro *et al.*, 1998).

One of the many ways of controlling activity of transcription factors is by its association with other proteins. Transcription factors can form homodimers themselves or heterodimers with other transcription factors, or with non-DNA binding proteins which in turn, can cause activation or inhibition of their DNA-binding and transactivation potential. For example, the AP-1 family of transcription factors is composed of homo- or heterodimers of bZIP containing proteins (Jun, Fos and activating transcription factor-2 (ATF-2) amongst others). Dimerisation is needed to form a fully functional DNA-binding domain (Angel and Karin, 1991). Other protein-protein interactions can cause inhibition of transcription factor functioning. This is illustrated by the fact that MyoD, a basic HLH protein, can be inhibited by the binding of Id. MyoD binds to DNA through interactions between its basic domain and the major groove of the DNA molecule. Id is able to heterodimerise with MyoD due to the presence of a HLH motif. However because Id lacks

a basic domain , it cannot bind to DNA, and therefore is able to sequester MyoD in non-functional complexes (Benezra *et al.*, 1990).

Ultimately, transcription factors must be in the nucleus in order to exert their effect on transcriptional activation. Therefore, localisation of the proteins within the cell also serves as a mechanism in controlling transcription factor functioning. As well as employing some of the mechanisms described above, such as phosphorylation and sequestration of the transcription factor by inhibitor proteins, to control localisation, other techniques are also employed. These include masking of nuclear localisation signal/s and modulation of the nuclear import machinery itself (Schmitz M et al 1991; Jans, 1995; Jans and Hubner, 1996).

Transcription factors must be present in the nucleus in order to activate transcription. However, these specific DNA binding proteins must only be present as and when required. Ultimately, transcription factor activity is controlled through the intracellular signalling pathways that are activated in response to external stimuli. Numerous pathways and signalling mechanisms exist, which all in some way or another converge at the activation of their respective transcription factor and transcription of their respective genes. Some of these pathways are known to be interlinked, whereas others are unique to a particular cell type.

Overall, by controlling transcription factor levels and activity within the cell, through a number of unique and universal mechanisms, the process of gene expression can be tightly regulated.

1.1.3.2 Role of chromatin

Not only are transcription factors used to regulate the transcriptional machinery but the actual way DNA is packaged within the cell contributes to the regulation of gene expression.

DNA is found within the nuclei of eukaryotic cells in structures called chromosomes. DNA is first wrapped around a nucleosomal core, which is composed of a tetramer of histones, H3 and H4 with an H2A/H2B dimer added to each face of the tetramer, forming an octameric complex. Core nucleosomes are linked to each other through the linker histone, H1, which makes contacts with both linker DNA and the DNA in the core nucleosome. Electron micrographs of DNA have shown characteristic 10nm 'beads on a string' structure. The nucleosomes package into 30nm solenoid fibres by forming 6 nucleosomes/turn. Solenoid fibres can then package into loops that are attached to an acidic protein scaffold to form chromosomes (Kornberg and Lorch, 1999). The positioning of nucleosomes and post-translational modifications within the histones of the nucleosomes, have been shown to serve as factors in controlling the transcriptional machinery.

By specifically placing nucleosomes at defined sites on DNA, the transcriptional apparatus can be inhibited or activated as and when required. The yeast acid phosphatase gene, PHO5, has in its promoter region two upstream activating sequence (UAS) elements, which are binding sites for the transcription factor Pho4. One of the UAS, UASp1 is situated in a nucleosome free region of DNA, whereas UASp2 is found within an adjacent nucleosome. Under repressing conditions neither site is occupied and the TATA box is buried within nucleosomes. However, when cells are starved of phosphate, pho4 is activated and binds both UASp1 and UASp2, displacing nucleosomes within the promoter region of the PHO5 gene. Disruption of these nucleosomes facilitates the binding of transcription factors and upregulates transcription of the PHO5 gene (Svaren *et al.*, 1994). Similarly, in yeast, the galactose (GAL) gene is inactive in the absence of galactose. The TATA box and transcription start site are buried within nucleosomes whereas the major upstream activation sequence elements are found within open hypersensitive sites. Induction of GAL expression correlates with the disruption of nucleosomes along the promoter region (Bash and Lohr, 2000).

Each of the core histones within the nucleosomes have been shown to be targets for a variety of post-translational modifications. These include acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination. The effect of these modifications is not completely understood, but some have been shown to have an effect on transcriptional activation and repression.

All four histones within the core nucleosome, H2A, H2B, H3 and H4 have been shown to be modified by acetylation of specific lysine residues located within their N-terminal tails. Acetylation of histones has been linked to events which cause the attenuation of DNA-histone contacts within the chromatin as well as affecting the interactions between the histones and non-histone chromatin proteins which in turn modulate chromatin structure (Hansen *et al.*, 1998; Tse *et al.*, 1998; Nightingale *et al.*, 1998).

It has been suggested that acetylation of the N-terminal basic histone tails weakens the interaction of histones with DNA, thus allowing binding of transcription factors to their cognate recognition sites (Puig *et al.*, 1998; Mutskov *et al.*, 1998). Vitolo and colleagues have shown that acetylation of H3 and H4 tails within nucleosomes bound to the promoter region of the 5S RNA gene function to allow binding of TFIIIA (Vitolo *et al.*, 2000). Not only does acetylation allow the binding of transcription factors to their specific DNA sequences, it can also disrupt the interactions between DNA and repressor proteins, alleviating repression by these proteins and facilitating transcriptional activation (Edmondson *et al.*, 1996; Bone and Roth, 2001).

Acetylation of histones is carried out by histone acetyl transferases (HATs) and conversely deacetylation is carried out by histone deacetylases (HDACs). Many HATs can be found to specifically associate with transcription factors. When these proteins are recruited to the transcriptional start site acetylation of the histone tails occur. This results in chromatin remodelling and access of the transcriptional machinery (Kuo *et al.*, 2000; Davie and Chadee, 1998; Martinez-Balbas *et al.*, 1998). Conversely, proteins containing HDAC activity are known to be recruited to genomic sites by repressors, removing acetyl groups

from histones and favouring chromatin packing and inaccessibility by transcriptional activators (Davie and Chadee, 1998; Kouzarides, 1999).

Amongst acetylation of histones, methylation, ubiquitination and phosphorylation also occur (Rea *et al.*, 2000; Chen *et al.*, 1999; Robzyk *et al.*, 2000; Pham and Sauer, 2000; Sassone-Corsi *et al.*, 1999). These modifications have been shown to be involved in many processes of gene expression including mitosis, signalling pathways, chromosomal segregation and transcription.

Non-histone components of chromatin such as HMG domain containing proteins (Thomas and Travers, 2001) and also ATP-dependent chromatin remodelling complexes such as the yeast SWI/SNF complex also contribute to transcriptional control through the ordering of the chromatin structure (Vignali *et al.*, 2000).

Therefore, the activation of transcription is controlled by a number of sequential and/or combinatorial events. This is illustrated by the emergence of a histone code and the ability of transcription factors to rescruit HATs, HDACs and chromatin remodelling complexes. These events allow for the controlled and coordinated regulation of gene expression in eukaryotes.

1.2 NF-κB

The transcription factor NF-κB was first identified as a nuclear factor controlling the transcription of the immunoglobulin κ light chain gene in B lymphocytes (Sen and Baltimore, 1986). It is now well recognised as being an inducible and ubiquitous transcription factor which can be activated by a variety of stimuli including tumour necrosis factor (TNF) (Duh *et al.*, 1989), phorbol myrsitate acetate (PMA) (Griffin *et al.*, 1989), double-stranded ribonucleic acid (dsRNA) (Lenardo *et al.*, 1989) and okadaic acid (Thevenin *et al.*, 1990) as well as a variety of others (Lenardo and Baltimore, 1989). Upon stimulation of cells with these certain signals, activation of NF-κB can occur, allowing it to transcribe certain cellular and even viral genes (Lenardo and Baltimore, 1989;

Baeuerle, 1991). Some of the genes that are under the control of NF-κB, include those involved in the immune and inflammatory process such as interluekin 2 (IL-2), the α chain of the IL-2 receptor, TNFα, and TNFβ; acute phase response genes such as serum amyloid A (SAA) and complement C4 protein; cell adhesion molecules including ICAM-1 and viral genes from HIV, cytomegalovirus and adenovirus (Grilli *et al.*, 1993; Pahl, 1999). The diverse array of genes that are targeted by this transcription factor can be illustrated by the fact that it has also been shown to target a gene called *Twist*, which is known to be involved in chick limb development (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998). Therefore, not only is NF-κB involved in the regulation of the immune system, it can also influence developmental pathways as well.

As for all transcription factors, the activation of NF-κB must be tightly controlled in order to achieve specific regulation of transcription. NF-κB activation and thus activity is regulated by sequestering the protein in the cytoplasm of unstimulated cells. This is acheived by association of NF-κB with inhibitory proteins that contain ankyrin repeats (Haskill *et al.*, 1991). These inhibitory proteins known as inhibitor of NF-κB (IκB), bind to and mask the nuclear localisation signal of NF-κB, preventing it from translocating to the nucleus and binding to its cognate DNA recognition sequences. Upon certain extra-cellular stimulation of cells, IκB is released from NF-κB allowing the transcription factor to enter the nucleus and exert transcription of its responsive genes.

1.2.1 NF-κB and disease

Uncontrolled activation of NF-κB, has been implicated in a variety of disease states such as asthma, rheumatoid arthritis (Barnes and Karin, 1997) and various cancers including Hodgkin's disease and breast cancer (Sovak *et al.*, 1997; Wood *et al.*, 1998). Asthma and rheumatoid arthritis are diseases which are characterised by chronic activation of NF-κB and of the inflammatory process. Chronic stimulation of tissues can ultimately lead to tissue damage that in turn causes severe pain and swelling in the affected tissues of

the individual. The role of NF-κB in some cancers may be explained by the fact that NF-κB has been implicated in the prevention of apoptosis (Beg *et al.*, 1995; Wang *et al.*, 1996; Van Antwerp *et al.*, 1996; Beg and Baltimore, 1996).

Due to its significance in many cellular and viral processes, NF-κB, its activation, structure, function and control has become an area of increasing scientific awareness. The pathways leading to its activation and deactivation and the involvement of many other proteins that govern its control are slowly being revealed. By understanding the processes and proteins by which NF-κB activation is regulated, new insight into the underlying mechanisms of certain life-threatening diseases can be investigated.

1.2.2 NF-κB structure

NF-κB belongs to a group of structurally related proteins which all possess an 300 amino acid conserved domain called the rel homology domain (RHD). See Figure 2. This conserved N-terminal region confers DNA-binding and dimerization and also contains the nuclear localization signal (NLS). To date there are five members including p50, p52, p65 (Rel A), c-Rel and Rel B. Both p50 and p52 are translated as larger non-DNA binding proteins of 105 and 100kDa respectively and are proteolytically processed to the mature transcriptionally active forms after certain stimulation. They also possess C-terminal ankyrin repeat domains. p65, c-Rel and Rel B as well as containing a RHD, also possess a transcriptional activation domain. All members of the family, with the exception of Rel B (Siebenlist *et al.*, 1994) have the ability to form homo- or heterodimers and it is the heterodimer of p50 and p65 which generates the most classical inducible NF-κB form (Baldwin, 1996; Kopp and Ghosh, 1995; Baeuerle and Baltimore, 1989) with the transcriptional activity conferred by a 300 amino acid region found in the C-terminal half of the p65 subunit (Schmitz and Baeuerle, 1991).

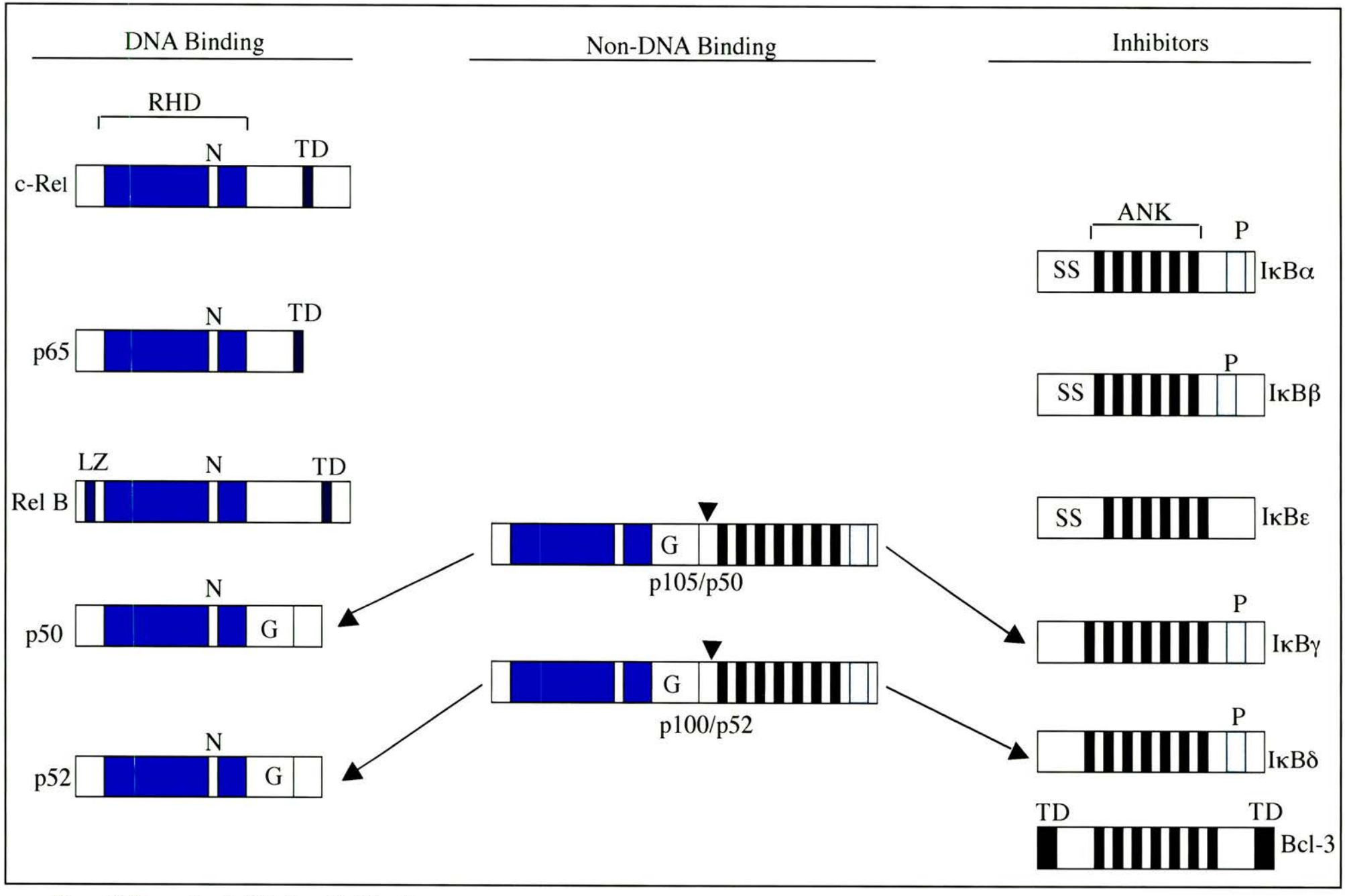


Figure 2 Mammalian Rel/NFκB and IκB families.

NFκB family members are shown on the left, RHD, Rel Homology Domain, TD, Transactivation Domain, LZ, leucine zipper. G, glycine rich regions in p50 and p52. N, nuclear localisation signal. IκB family members are shown on the right. ANK, ankyrin repeat domains., TD, transactivation domains. P, PEST domain. Serine residues required for signal-induced degradtion of IκB are shown, SS. p105/p50 and p100/p52 contain sites of endoproteolytic cleavage as indicated by arrows.

1.2.3 NF-κB function

The transcriptionally active p50-p65 heterodimer binds κB binding motifs in a wide variety of genes. The first κB binding motif was identified as a 10 nucleotide sequence, 5'GGGACTTTCC 3', present in the gene for the immunoglobulin κ light chain. Other κB binding motifs are not identical to this sequence but most can be found to have 3 guanines in the 5' position and 2 cytosines in the 3' position (Grilli *et al.*, 1993).

Both p50 and p65 can bind DNA as homodimers or as a p50-p65 heterodimer. As shown by Urban *et al* (Urban *et al.*, 1991), the p50 subunit prefers to bind the 5' half site with the p65 subunit binding the less conserved 3' half site. Clark and Hay also showed that NF-κB recognises its target sequence by binding to it through base and backbone contacts over one complete turn of the DNA double helix (Clark and Hay, 1989; Clark *et al.*, 1989). Both the structure of the p50 homodimer (Muller *et al.*, 1995; Ghosh *et al.*, 1995) and p65 homodimer (Chen *et al.*, 1998b) bound to DNA has been determined and reveals that the rel homology domain (RHD) is composed of two immunogloblin-like folds with the C-terminal domain being responsible for subunit dimerization and the N-terminal domain being responsible for making sequence specific contacts with the DNA. Huang and colleagues, by solving the crystal structures of the murine p50 and p65 dimerization domains, reveal that these domains are not altered upon binding DNA (Huang *et al.*, 1997). The 2.9 angstrom crystal structure of a p50/p65 heterodimer bound to DNA reveals that both subunits adopt variable conformations on binding DNA and that they also bind to the DNA in a sequence dependent manner. The two domains behave essentially independently of each other and thus the p50/p65 heterodimer when binding DNA can be viewed as the sum of the specificity of the individual monomers. p50 contacts a 5 base-pair 5'subsite whereas p65 recognises a 4 base-pair 3'subsite. Interaction between the monomers occurs via hydrophobic and polar residues within the carboxy-terminus of both subunits. The side chains involved in the heterodimerization of p50 and p65 exhibit a higher degree of chemical complementarity compared with either p50 or p65 homodimers. These

observations suggest why a p50/p65 heterodimer is more thermodynamically stable than the homodimer (Chen *et al.*, 1998a).

1.2.4 IκB - inhibitor of NF-κB

By treating cells with cycloheximide, a protein synthesis inhibitor, NF-κB DNA binding activity could be observed (Sen and Baltimore, 1986). It was therefore postulated that NF-κB must be present in the cell, but must be inactive until certain stimuli causes its activation. In cytosolic fractions of unstimulated pre-B cells an inhibitor protein termed inhibitor of κB (IκB) could be fractionated from NF-κB by dissociating agents such as sodium deoxycholate. Electrophoretic mobility shift assay (EMSA) demonstrated that fractionated IκB was able to inhibit NF-κB binding to its DNA target sequence (Baeuerle and Baltimore, 1988a; Baeuerle and Baltimore, 1988b). Furthermore, this protein seemed to be specific for NF-κB alone as IκB had no effect on the DNA binding activity of other defined nuclear factors. IκB was identified within these fractions as a 60-70kDa protein that was rendered inactive upon treatment with trypsin. Further analysis of IκB isolated from human placental cytosolic extracts revealed two isoforms of 35-37kDa and 43kDa. These were termed IκBα and IκBβ respectively (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990). The previously identified 60kDa form of IκB most likely represented IκBγ, present specifically in lymphoid tissues (Inoue *et al.*, 1992).

1.2.5 IκB structure

IκBα is a member of a family of proteins, which include IκBβ, IκBε, Bcl-3 and the drosophila protein, cactus. Two other members are IκBγ and IκBδ which are the C-terminal domains of the larger precursor proteins p100 and p105 respectively. Figure 2. Repeats of a 33 amino acid sequence previously found in cell-cycle control proteins and the erythrocyte ankyrin protein (Lux *et al.*, 1990) are found in all of the IκB family members. Specificity of inhibition can be achieved by different family members inhibiting NF-κB/Rel

family members to varying degrees. IκBα and IκBβ predominantly associate with p50-p65 and p50-c-Rel heterodimers (Thompson *et al.*, 1995) whereas IκBε binds p65 and c-Rel homodimers (Whiteside *et al.*, 1997), Bcl-3 interacts with p50 and p52 homodimers (Franzoso *et al.*, 1992; Nolan *et al.*, 1993; Wulczyn *et al.*, 1992) and IκBγ is more effective at inhibiting p50 homodimers than p50-p65 heterodimers or p65 homodimers (Hay, 1993). IκBα, β and ε function in the regulation of stimulus-dependent activation of NF-κB, whereas the other family members have other functions (Ghosh *et al.*, 1998).

The best characterised member of the IκB family is IκBα. The domain organisation and sites of interaction of IκBα with p65 were revealed by Jaffray *et al* using partial proteolysis (Jaffray *et al.*, 1995). They demonstrated that IκBα has a tripartite structure consisting of an N-terminal domain, which confers signal-induced degradation of the molecule, an ankyrin repeat domain and a C-terminal domain containing a PEST (proline, glutamate, serine and threonine rich) region, nuclear export signal (NES) and casein kinase II (CKII) phosphorylation sites. IκBα molecules lacking the unstructured N-terminal region can still interact with and inhibit the DNA binding of the p65 subunit of NF-κB but mutants lacking both the N-terminal and the compact, highly acidic C-terminal domain are unable to do so. The PEST domain has been implicated in basal turnover of the protein (Lin *et al.*, 1996; Schwarz *et al.*, 1996) however both the N-terminal and C-terminal domains are required for signal-induced degradation of the molecule (Brown *et al.*, 1997; Kroll *et al.*, 1997; Sun *et al.*, 1996). It has also been demonstrated by a number of groups that deletion of the acidic C-terminal domain is sufficient to render the mutant IκBα protein resistant to proteolytic degradation (Rodriguez *et al.*, 1995; Beauparlant *et al.*, 1996).

IκB family members act by binding to and inhibiting the nuclear translocation of NF-κB family members. It has been shown that IκBγ, the C-terminal region of p105, which contains the ankyrin repeat motifs, masks the NLS of p50 (Matthews *et al.*, 1993). IκBα has also been shown to mask the NLSs of NF-κB and c-Rel and hence retain them in the cytoplasm (Beg *et al.*, 1992). In fact, the structure of the ankyrin repeats of IκBα

bound to NF-κB (p50/p65) have been solved by x-ray crystallography and reveal the amino acid residues which comprise the NLS of both p50 and p65 are bound to IκB through its N-terminal ankyrin repeats. The structure verifies the mechanism by which IκBα is able to impede the recognition of NF-κB by the nuclear import machinery (Huxford *et al.*, 1998; Jacobs and Harrison, 1998).

Although IκBα and IκBβ share similar overall structural properties they appear to differ somewhat in their inhibitory properties. IκBα is rapidly degraded upon exposure of cells to all known inducers of NF-κB whereas IκBβ is only degraded by a subset of these stimuli (Thompson *et al.*, 1995; McKinsey *et al.*, 1996). The kinetics of degradation of the two proteins also differs, with IκBβ being degraded much slower than IκBα. However, knock-in mice studies in which the IκBβ gene was expressed under the control of the IκBα promoter have demonstrated that, although IκBβ appears to be functionally distinct from IκBα, *in vivo* it is able to functionally replace the IκBα protein (Cheng *et al.*, 1998). Therefore although the two family members have acquired different functions, it may be due to divergence of gene expression rather than to divergence of biochemical properties.

1.2.6 IκBα degradation - mechanism of NF-κB activation

The role of ubiquitination in signal-induced activation of NF-κB was revealed by Roff and colleagues (Roff *et al.*, 1996) who demonstrated that in the presence of a proteasome inhibitor, cells stimulated with TNFα, accumulate IκBα that is multiply conjugated to ubiquitin. The importance of phosphorylation of serine residues 32 and 36 prior to ubiquitination was also illustrated when cell lines expressing an S32A/S36A IκBα protein were used ubiquitination and degradation of this mutant was blocked, whereas wild-type IκBα was efficiently ubiquitinated and degraded. This indicates that phosphorylation of residues S32 and S36 must occur before degradation of IκBα can occur.

It was further demonstrated by using site-directed mutagenesis that lysine residues 21 and 22 were the primary targets of the ubiquitination enzymes (Rodriguez *et al.*, 1996;

Scherer *et al.*, 1995; Baldi *et al.*, 1996). Mutation of these lysine residues to arginine results in reduction of NF-κB activity upon exposure of cells to extracellular stimuli such as TNFα or IL-1. These results indicated the importance of ubiquitination in the activation of NF-κB.

Overall, a general mechanism of NF-κB activation can be described where (a) extracellular stimuli cause phosphorylation of IκBα on serines 32 and 36, (b) ubiquitination then occurs on lysines 21 and 22, followed by (c) degradation of the protein by the 26S proteasome and translocation of active NF-κB into the nucleus allowing transcription of responsive genes.

It should be noted that one of the most significant genes under NF-κB control is IκBα itself. This displays a unique mechanism of deactivation of NF-κB as when IκBα is expressed and translated in the cytoplasm it can re-enter the nuclear compartment and inhibit NF-κB binding to its target sequence. This then abrogates further transcription and activation of NF-κB. IκBα also transports NF-κB back to the cytoplasm where it is sequestered in a p50-p65-IκBα ternary complex until signal-induced degradation of IκBα occurs again (Arenzana-Seisdedos *et al.*, 1995; Arenzana-Seisdedos *et al.*, 1997).

1.2.7 The IκB kinase

Due to the fact that signal-induced degradation of IκBα and subsequent activation of NF-κB occurred following phosphorylation of IκB, the enzyme or enzymes responsible for such phosphorylation were actively sought. Apparent IκB kinase activity was discovered by Chen *et al*, where a large multisubunit kinase of approximately 700kDa was discovered that could specifically phosphorylate IκBα on serines 32 and 36 (Chen *et al.*, 1996). The complex required ubiquitination for activity as when certain ubiquitin-conjugating molecules, for example Ubc4/Ubc5 were separated from the complex during purification, the kinase activity was inhibited. However, no associated kinase subunit has been identified within the multisubunit complex.

The molecular cloning of murine CHUK (conserved helix-loop-helix [HLH] ubiquitous kinase) by Connelly and Marcu revealed that this protein, although belonging to members of the HLH and leucine zipper gene families, also contained an N-Terminal kinase domain with serine/threonine kinase activity (Connelly and Marcu, 1995). Purification of an IκBα kinase complex by Didonato *et al* revealed a protein kinase complex of approximately 900kDa that could specifically phosphorylate IκBα on serines 32 and 36 and IκBβ on serines 19 and 23 and was cytokine inducible (DiDonato *et al.*, 1997). 85 and 87kDa polypeptides within this complex co-eluted with IκB kinase activity and cloning of the 85kDa polypeptide revealed that it contained CHUK sequences. Hence, CHUK, which had previously unknown function was re-named IκB kinase or IKK.

Simultaneously, Regnier *et al*, using a yeast two-hybrid screen, identified CHUK as being a protein that could interact with NIK (NF-κB-inducing kinase) (Regnier *et al.*, 1997). NIK, a member of the MAP kinase kinase kinase (MAP3K) family, has been shown to be a TRAF-2 interacting protein (Malinin *et al.*, 1997). Activation of NF-κB by IL-1 and TNFα have been shown to involve members of the TNF receptor-associated factor (TRAF) family of adaptor proteins which are some of the proteins involved in the upstream signalling pathway leading to NF-κB activation via IL-1 and TNFα (Rothe *et al.*, 1995; Arch *et al.*, 1998)

Within the kinase complex discovered by Didonato and colleagues (DiDonato *et al.*, 1997), a polypeptide of 87kDa also showing kinase activity was later cloned by Zandi *et al* (Zandi *et al.*, 1997). This polypeptide was 50% identical to IKK and hence IKK was re-named IKKα and the 87kDa polypeptide was named IKKβ. IKKα and IKKβ are also known as IKK1 and IKK2 respectively.

1.2.7.1 IKK structure

IKK1 and IKK2 share 52% amino acid identity and both are composed of an N-terminal kinase domain followed by a leucine zipper domain and a C-terminal helix-loop-

helix domain (Karin, 1999; Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997; Regnier *et al.*, 1997; DiDonato *et al.*, 1997). Homo- or heterodimers can exist through interactions between leucine zipper domains and in fact kinase activity is augmented when both IKK1 and IKK2 are co-transfected. Both kinases are phosphorylated on serine residues within an activation loop in kinase subdomains VII and VIII upon stimulation with IL-1 and TNFα (Delhase *et al.*, 1999). This region resembles an activation loop also found in the MAPKK (MEK) family of proteins. IKK1 is phosphorylated on serine residues 176 and 180 whereas IKK2 is phosphorylated on serines 177 and 181 (Mercurio *et al.*, 1997). Homo- and heterodimeric actions are mediated through the leucine zipper motifs (Zandi *et al.*, 1997; Woronicz *et al.*, 1997) and kinase activity is regulated by the HLH motifs (Zandi *et al.*, 1997; Zandi *et al.*, 1998). It has been postulated that upon pro-inflammatory stimuli, the kinase complex is activated through phosphorylation of serine residues in the activation loop of IKK2 that is then able to trans-phosphorylate the other subunit, IKKα. Activated IKK2 undergoes autophosphorylation at the C-terminus resulting in localisation of negative charges at these sites causing electrostatic repulsion and alteration of the interaction between the HLH domain and the kinase domain. A change in conformation of the kinase complex renders it inactive (Karin and Delhase, 2000).

The IKK complex also appears to contain a 48kDa structural protein termed IKKγ, also known as NF-κB Essential Modulator (NEMO) and IKK Associated Protein 1 (IKKAP1) (Rothwarf *et al.*, 1998; Yamaoka *et al.*, 1998; Mercurio *et al.*, 1999). IKKγ/NEMO/IKKAP1 contains a C-terminal leucine zipper domain and two N-terminal coiled-coil motifs. It has been shown to be able to interact with IKK1/IKK2 complexes and the importance of its presence has been demonstrated by the fact that it can rescue NF-κB activation by various stimuli of cells which are devoid of NEMO (Yamaoka *et al.*, 1998). Studies have also suggested that its C-terminal domain acts as a docking site for upstream stimuli such as the Tax protein from human T-cell leukemia virus type I (HTLV-

I) (Harhaj and Sun, 1999) and it has also shown to play a role in assembling the IKK complex (Li *et al.*, 2001).

Another protein found associated with the IKK complex, termed IKK-complex Associated Protein (IKAP), was identified through purification on an IκBα affinity column of IL-1 stimulated cell extracts (Cohen *et al.*, 1998). This 150kDa protein appears to be able to bind NIK and the IKKs and assemble them into active kinase complexes. However, further studies have shown that this protein is not part of the functional IKK complex and does not participate in NF-κB signalling (Krappmann *et al.*, 2000).

1.2.7.2 IKK function

The function of the IKK signalsome is to phosphorylate IκBα on serines 32 and 36, IκBβ on serines 19 and 23 or IκBε on serines 18 and 22. By phosphorylating these residues, the molecules are tagged for ubiquitination and degradation by the 26S proteasome. Phosphorylation by the IKK signalsome is also very specific as when serines 32 and 36 of IκBα are substituted with threonine, phosphorylation is greatly reduced (DiDonato *et al.*, 1997).

Gene knockout studies have revealed unique functions for both IKK1 and IKK2 *in vivo*. IKK1 -/- mice die just after birth and exhibit multiple skin and skeletal abnormalities. IKK1 -/- cells, however, retain NF-κB signalling via IL-1 and TNFα (Li *et al.*, 1999a; Hu *et al.*, 1999; Takeda *et al.*, 1999). IKK2 -/- mice however, die at embryonic stage 12.5-13 due to massive liver degeneration caused by TNF induced apoptosis (Li *et al.*, 1999c; Li *et al.*, 1999b; Tanaka *et al.*, 1999). This implies that IKK2 is the main kinase involved in the pro-inflammatory NF-κB signalling pathway and that IKK1 may play a role in transducing other types of signals, such as those required for limb development. However, these results are complicated by earlier studies demonstrating that kinase inactive forms of IKK1 (K44M), can inhibit NF-κB activation by TNFα, IL-1, Tax, NIK, Cot/Tpl2 and MEKK1 (DiDonato *et al.*, 1997; Fischer *et al.*, 1999; Geleziunas *et al.*, 1998; Lee *et al.*, 1998;

Nakano *et al.*, 1998; Uhlik *et al.*, 1998; Woronicz *et al.*, 1997). This suggests that, although IKK2 is the more active kinase, IKK1 also contributes to NF-κB activation by these stimuli.

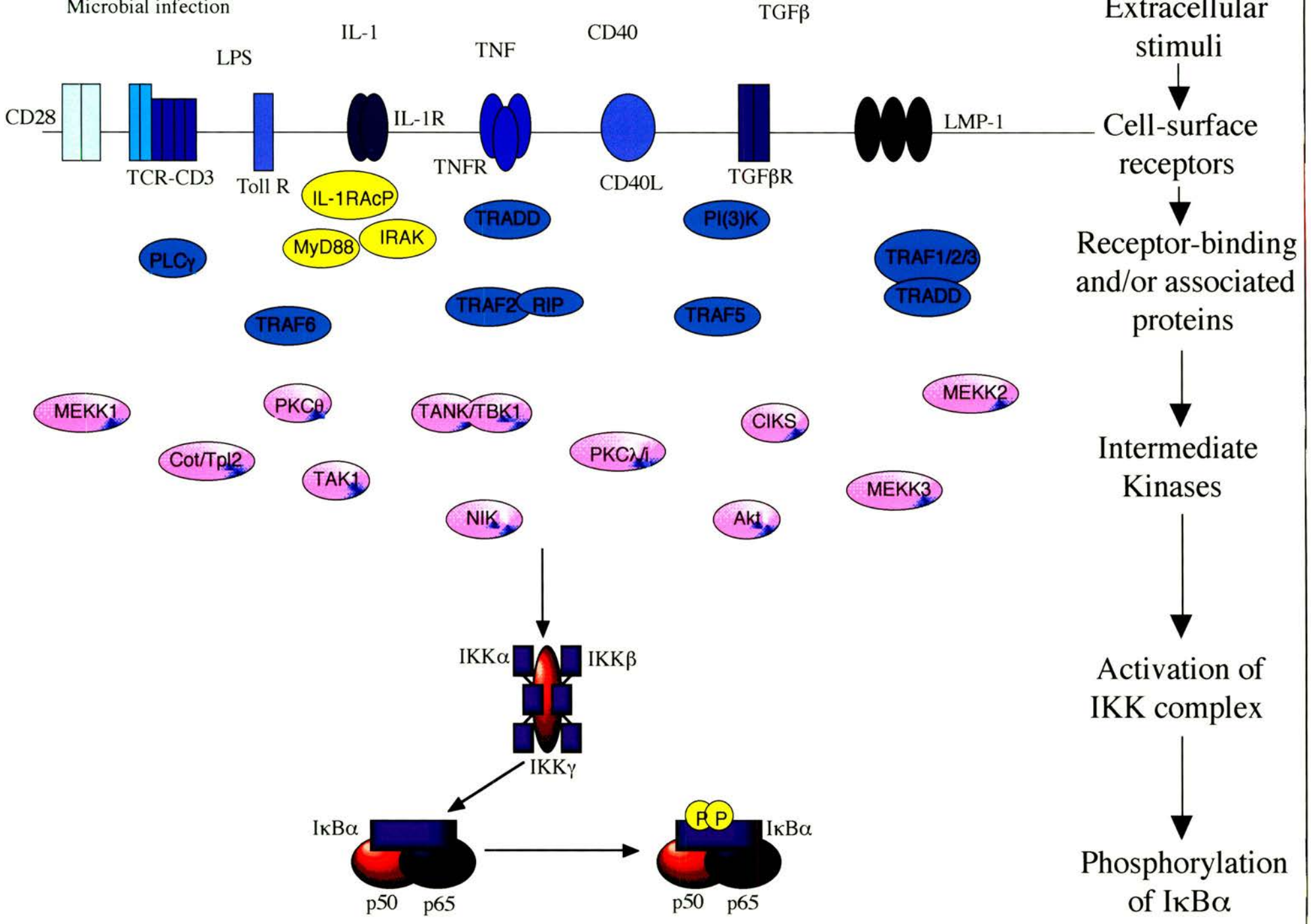
The inactivation of the IKKγ/NEMO/IKKAP1 gene has also demonstrated the role the non-catalytic component of the IKK complex plays. IKKγ -/- mice die at embryonic day 12.5-13 due to severe liver damage caused by apoptosis (Rudolph *et al.*, 2000). IKKγ -/- mouse embryonic fibroblasts (MEFs) lack detectable NF-κB binding activity in response to TNFα and IL-1. Phosphorylation and degradation of IκBα and IKK activity was also absent from these cells, indicating that IKKγ is important in NF-κB signalling through these stimuli. Furthermore IKKγ knockout mice have illustrated the role this protein, and NF-κB activation, plays towards the development of a rare X-linked dominant genetic disorder called incontinentia pigmenti (IP). IP is characterised by multiple congenital defects that affect the hair, teeth, nails eyes and central nervous system. Several mutations and deletions within the human gene for IKKγ have been found in DNA samples from affected patients (Smahi *et al.*, 2000). Clinical features of the human form of the disease have been manifested in the IKKγ -/- knockout mice such that affected male mice die prenatally and females exhibit skin lesions and elevated levels of apoptosis (Makris *et al.*, 2000; Schmidt-Supprian *et al.*, 2000).

1.2.7.3 IKK activation

Many pathways have been implicated in controlling NF-κB activation. The discovery of the IKK signalsome served as a point where these pathways could converge. A summary of the pathways that have been implicated in IKK activation by various stimuli is shown in Figure 3. This is not a defined model with many intermediate signalling molecules as yet to be identified.

Upon extracellular stimuli, cell surface receptors are activated. Receptor ligation results in the transduction of the signal via a cascade of protein-protein interactions and/or

Figure 3 Mechanisims of IKK activation.
Signalling pathways which are postulated to be involved in the activation of the IKK signalsome. Extracellular stimuli activate cell surface receptors that recruit and/or activate receptor-binding proteins. Activation of intermediate kinases follows, resulting in the activation of the IKK signalsome. The activated IKK complex is then able to phosphorylate IκB.


Microbial infection
LPS
IL-1
TNF
CD40
TGFβ
Extracellular stimuli
CD28
TCR-CD3
Toll R
IL-1R
TNFR
CD40L
TGFβR
LMP-1
Cell-surface receptors
IL-1RAcP
MyD88
IRAK
TRADD
PI(3)K
PLCγ
TRAF1/2/3
TRADD
TRAF2
RIP
TRAF5
TRAF6
Receptor-binding and/or associated proteins
MEKK1
PKCθ
TANK/TBK1
CIKS
MEKK2
Cot/Tpl2
PKCλ/ι
TAK1
MEKK3
NIK
Akt
Intermediate Kinases
IKKα
IKKβ
IKKγ
Activation of IKK complex
IκBα
p50
p65
P
P
IκBα
p50
p65
Phosphorylation of IκBα

protein activation. These signals culminate in activation of the IKK complex and activation of NF-κB. Within the plethora of proteins involved in such signal cascades, receptor binding proteins such as TNF receptor associated factors (TRAFs), TNF receptor associated death domains (TRADDs) or IL-1 receptor associated protein (IL-1AcP) associate and/or activate downstream molecules. Some of these downstream molecules have been identified as intermediate kinases. These include the upstream serine/threonine kinase NIK, a MAP3K family member. Evidence for the involvement of NIK in NF-κB signalling has arisen from studies which demonstrated that NIK preferentially phosphorylates IKK1 compared with IKK2, is able to potentiate the activity of the IKK heterodimer and participates in the activation of NF-κB by various stimuli including TNFα and IL-1 (Ling *et al.*, 1998; Rothwarf *et al.*, 1998; Nakano *et al.*, 1998; Nemoto *et al.*, 1998; Malinin *et al.*, 1997). Although NIK does activate the IKK complex, the extracellular stimuli that cause the activation of NIK itself has proved somewhat controversial. Recent studies from NIK -/- deficient mice have suggested that NIK is invovled in the activation of NF-κB by lymphotoxin β (LTβ) and not by TNF or IL-1. No difference in NF-κB DNA binding activity is observed in NIK-/- mouse embryonic fibroblasts (MEFs) treated with IL-1 or TNF, compared to wild-type cells. IKK activity is also normal within these mutant cells along with the transcription of NF-κB responsive genes. Treatment of cells, however with LTβ, demonstrates that, although NF-κB DNA binding activity is normal, transcription of some NF-κB responsive genes is affected (Yin *et al.*, 2001).

Other intermediate kinases which have been demonstrated as participating in the activation of the IKK complex include MAP3K family members, MEKK1 (mitogen activated protein kinase/ERK kinase kinase1), MEKK2, MEKK3, Cot kinase/Tpl2 (tumour progression locus 2) and TAK 1(TGFβ activated kinase 1). MEKK1 has also been demonstrated as a protein which participates in the activation of c-Jun N-terminal kinase (JNK) (Nakano *et al.*, 1998; Lee *et al.*, 1998; Lee *et al.*, 1997; Nemoto *et al.*, 1998). TAK

1 (TGFβ activated kinase 1), has been demonstrated to activate the IKK complex upon stimulation of cells with TGFβ (Sakurai *et al.*, 1999). TAK 1 activation has also been identified in IL-1 signalling pathways (Ninomiya-Tsuji *et al.*, 1999; Takaesu *et al.*, 2001) and upon stimulation of cells with lipopolysaccharide (LPS) (Lee *et al.*, 2000), a component of bacterial cell walls which is capable of binding and activating Toll-like receptors (Kirschning *et al.*, 1998; Muzio *et al.*, 1998). The proto-oncogene Cot kinase/Tpl-2 was originally identified as a rat oncoprotein, 90% identical to the human oncoprotein Cot. Its catalytic domain is homologous to the kinase domain of MAP3K family members and has been demonstrated as participating in NF-κB activation through T-cell signalling (Lin *et al.*, 1999). It has also been demonstrated as being a requirement for the proteolytic processing of p105 into p50 (Belich *et al.*, 1999). Studies from Tpl-2 -/- knockout mice have also suggested that this kinase plays a major role in LPS signalling. However, mutant mice cells, upon stimulation with LPS exhibit no difference in NF-κB DNA binding activity compared to wild type cells. Hence this suggests that NF-κB activation by LPS does not involve Tpl-2 (Dumitru *et al.*, 2000).

Other intermediate kinases identified to date in the activation of the IKK complex include NAK (NF-κB activating kinase), also known as TBK1 (TANK binding kinase-1) and T2K. This kinase was originally identified and demonstrated to phoshorylate the IKK complex via TRAF-2 and TANK (Pomerantz and Baltimore, 1999). The catalytic domain of NAK is 30% identical to IKKα/β and contains leucine zipper and helix-loop-helix motifs in the carboxyl terminus of the protein. Recombinant NAK has been identified to specifically activate the IKK complex purified from unstimulated Hela cells and it has also been demonstrated that its ability to activate the IKK complex is dependent on PKCε activation. Furthermore, it has been suggested that this kinase is activated in response to extracellular stimulation with platelet derived growth factor (PDGF) (Tojima *et al.*, 2000).

Upon T-cell signalling and engagement of the TCR CD3 complexes and CD28 receptors, NF-κB activity can be induced. It has been suggested that this activation is

dependent on PKCθ activation of IKKβ and thereby the IKK complex (Lin *et al.*, 2000). Generation of mice in which the gene for PKCθ has been deleted, have demonstrated that in mature T-cells crosslinked with anti-CD3 or anti-CD28 antibodies, NF-κB activation is reduced and IκBα degradation inhibited. NF-κB activation was normal upon stimulation of PKCθ -/- T-cells with TNFα or IL-1 suggesting that activation of NF-κB upon TCR stimulation results from a pathway which utilises PKCθ and which is distinct from TNFα and IL-1 signalling pathways (Sun *et al.*, 2000).

Other PKC isotypes have also been implicated in IKK activation. These include the atypical PKC isotypes (lambda/iota PKC and zeta PKC) and PKCα, a member of the conventional PKC isotypes. It has been documented that upon stimulation of cells with TNFα or PMA, the atypical PKCs or PKCα, respectively, are able to bind to the IKKS *in vitro* and *in vivo* and stimulate IKKβ activity (Lallena *et al.*, 1999). Other kinases implicated in activation of the IKK complex include the serine/threonine kinase Akt/PKB. This kinase has been demonstrated to mediate phosphorylation of IKKα on threonine 23 upon stimulation of cells with TNFα and with some growth factors. The pathway of Akt activation occurs through the upstream activation of PI(3)K (Ozes *et al.*, 1999). Another kinase called CIKS (connection to IKK and SAPK/JNK), identified as an IKKγ interacting protein in a yeast-two-hybrid screen, has also been shown to activate the IKK complex (Leonardi *et al.*, 2000).

Other molecules implicated in the activation of the IKK signalsome include viral proteins such as Tax, a transforming and transactivating protein of Human T cell leukaemia virus (HTLV) (Geleziunas *et al.*, 1998). Yin *et al* have demonstrated that IKK activity can be stimulated indirectly by Tax through binding of it to the N-terminus of MEKK1 (Yin *et al.*, 1998), although it has also been reported to stimulate IKK activity directly by interacting with IKKγ (Harhaj and Sun, 1999). Latent membrane protein 1 (LMP-1), from Eppstein Barr Virus (EBV) has been suggested to mimic a constitutively active CD40 receptor and has been implicated in activating a signalling pathway which leads to activation

of the IKK complex. LMP-1 is able to form a complex in the membrane of cells and recruits TRAF and TRADD proteins which are also critical components of the TNFR signalling pathway (Sylla *et al.*, 1998). EBV has been suggested as contributing to many different types of lymphoma (Lyons and Liebowitz, 1998) and more specifically LMP-1 has been demonstrated to transform cells *in vitro* (Wang *et al.*, 1985). A similar signalling pathway is activated upon the binding of CD40L to the CD40 receptor and it has also been demonstrated that this pathway leads to the activation of the IKK complex via NIK (Ishida *et al.*, 1996; Brady *et al.*, 2000). Recently it has been suggested that ubiquitin-proteasomal degradation of TRAF 2 and TRAF 3 occurs upon CD40 receptor stimulation, allowing the signalling pathway activated through this receptor to be regulated. However, this mode of regulation is absent when signalling through LMP-1, resulting in a constitutively active signalling pathway and constitutively active NF-κB (Brown *et al.*, 2001). Ligation of other members of the TNFR family such as CD30 and TNFR2 have also demonstrated the ability to degrade TRAF2 (Duckett and Thompson, 1997; Chan and Lenardo, 2000).

Overall, the IKK complex serves as a point at which many extracellular signals can converge. Receptor stimulation results in the activation or recruitment of receptor associated proteins that in turn activate a series of intermediate kinases. These kinases have the ability to directly or indirectly activate the IKK complex allowing activation of NF-κB. Other molecules involved in this process that could contribute to IKK activation may not be kinases, such as the viral protein Tax, or may not yet be identified. Therefore activation of the IKK complex triggers the next step in NF-κB activation by specifically phosphorylating IκBα on serines 32 and 36, and thereby setting the scene for the ubiquitination reaction to occur.

1.3 Ubiquitin-proteasome pathway

The ubiquitin-proteasome pathway is one of the many mechanisms which can regulate the control of gene expression and indeed, plays a significant role in the regulation of NF-κB activation. In addition to regulating transcription, the ubiquitin pathway has also

been shown to function in the control of a number of cellular processes. These include regulation of cell-cycle progression, receptor down-regulation, endocytosis, embryogenesis, apoptosis and immune functioning (Hershko *et al.*, 2000; Hershko and Ciechanover, 1998; Varshavsky, 1997).

Goldstein and colleagues first identified ubiquitin as a small, 76 amino acid protein present in a vast array of tissues (Goldstein *et al.*, 1975; Schlesinger *et al.*, 1975). The cellular role of ubiquitin was unknown but it had been found conjugated to histone H2A and H2B (Goldknopf and Busch, 1977). Subsequently, Hershko and co-workers purified ubiquitin, which they initially termed ATP-dependent proteolysis factor 1 (APF-1), from fractionated reticulosyte lysates (Ciehanover *et al.*, 1978) and later demonstrated that this protein could be found conjugated to protein *in vitro* (Ciechanover *et al.*, 1980). It was also proposed that this protein participated in the ATP-dependent proteolysis of proteins (Hershko *et al.*, 1980). Subsequently Wilkenson and co-workers demonstrated that APF-1 was actually ubiquitin, whose function up until then, had remained unknown (Wilkinson *et al.*, 1980). It was later demonstrated that the vast majority of abnormal and short-lived proteins were degraded in a ubiquitin-dependent pathway *in vivo* (Finley *et al.*, 1984; Ciechanover *et al.*, 1984).

Covalent attachment of ubiquitin via an isopeptide bond to the epsilon amino group of a lysine residue in the target protein is achieved through a cascade of enzymatic reactions (Hershko and Ciechanover, 1992; Ciechanover, 1994). Briefly, the C-terminal glycine residue of ubiquitin is activated in an ATP-dependent manner forming ubiquitin adenylate and inorganic pyrophosphate. Ubiquitin is attached via a thioester bond to a cysteine residue in E1, ubiquitin activating enzyme, releasing AMP. E1 transfers activated ubiquitin to an active cysteine site in one of many E2 conjugating enzymes. Ubiquitin can then be transferred to target proteins via an E3 ubiquitin ligase enzyme either by direct transfer of ubiquitin from the E3 enzyme itself, or by facilitating the transfer from E2. Figure 4. Poly-ubiquitinated proteins are formed by the addition of ubiquitin via lysine

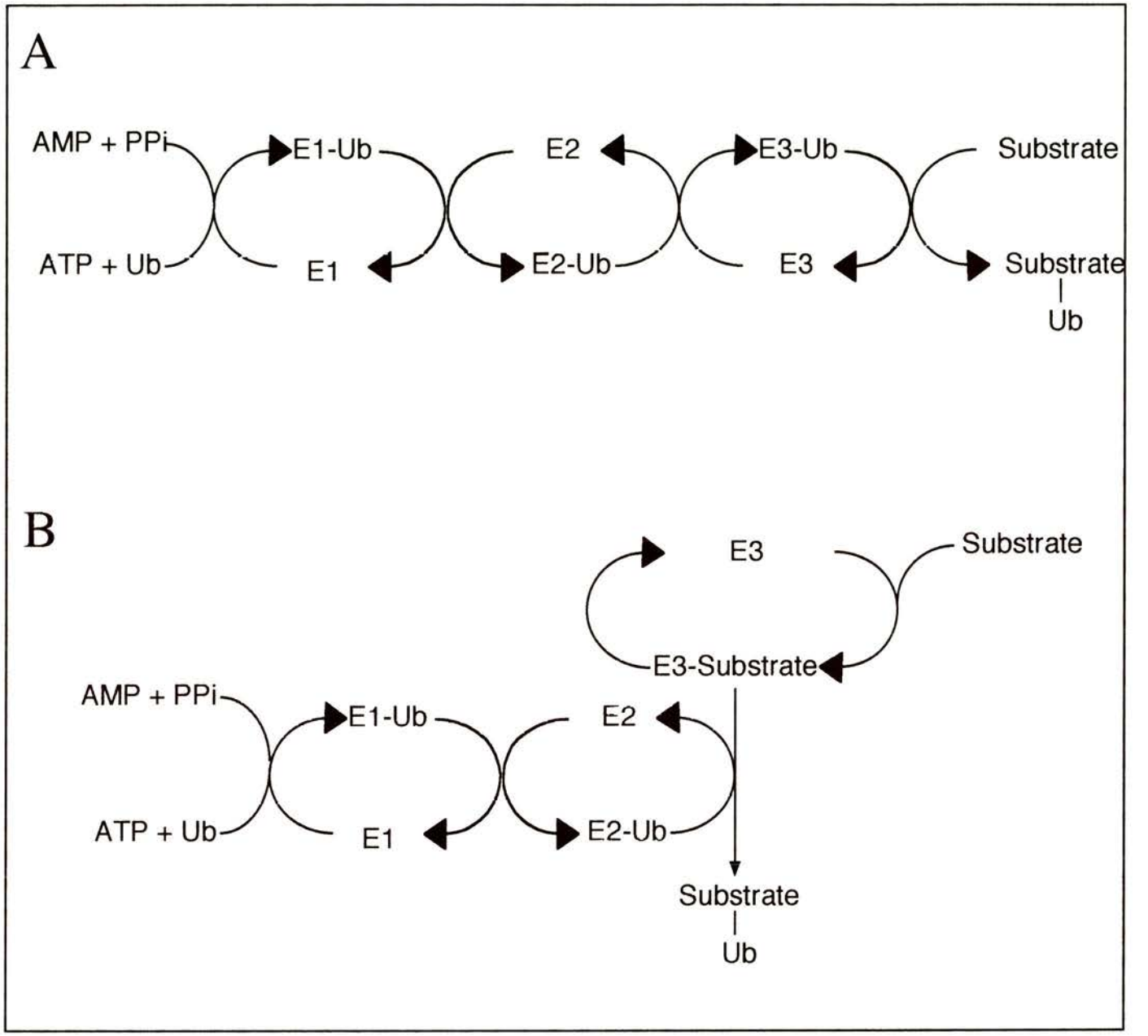


Figure 4 Ubiquitination pathway

Proteins are ubiquitinated via a cascade of enzymatic reactions involving E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligation enzyme. Ubiquitin is activated in an ATP-dependent manner and attached via a thioester linkage to a cysteine residue in E1. Ubiquitin is then transferred and attached via a thioester linkage to E2. Finally, ubiquitin is either (A) transferred and attached via a thioester linkage to E3 and then to substrate or (B) directly transferred to substrate from E2 in the presence of E3.

residues, most commonly lysine 48 (Varshavsky, 1996; Chau *et al.*, 1989) but also Lys 63 and Lys 29 (Spence *et al.*, 1995; Arnason and Ellison, 1994; Johnson *et al.*, 1995) to ubiquitin molecules that are already attached to the protein. Poly-ubiquitinated proteins are then targeted to the 26S proteasome for degradation (Coux *et al.*, 1996).

The process of ubiquitination appears hierarchal, with a single E1 enzyme transferring ubiquitin to a number of E2 enzymes that can then recognise one or more E3 enzymes. E3 enzymes can recognise one or more E2 molecules and may have multiple target substrates. Specificity within the ubiquitin system appears to be conferred by the E3 enzymes, which recognise target substrates due to unique and specific mechanisms. Overall, the ubiquitin system functions as a control mechanism for many cellular processes within the cell. By the efficient and controlled degradation of specific proteins, cellular processes can ultimately be regulated.

1.3.1 E1

A single E1 enzyme has been identified which is thought to activate ubiquitin for all subsequent modifications. This enzyme appears to contain two active sites within the molecule which allows the formation of a new ubiquitin adenylate intermediate as the previous one is transferred to the thiol site within the enzyme (Haas and Rose, 1982). Figure 5. In yeast, E1 is essential for cell viability as when the gene encoding yeast UBA1, ubiquitin-activating enzyme, is deleted from the genome, cell death occurs (McGrath *et al.*, 1991). The human E1 enzyme has been found to exist in two isoforms, E1α (117kDa) and E1β (110kDa) (Cook and Chock, 1992; Handley-Gearhart *et al.*, 1994; Cook and Chock, 1995) of which E1α, but not E1β, is phosphorylated *in vivo*. In Hela cells, it has been demonstrated that the level of E1α phosphorylation increases in G2 compared with the amount of basal phosphorylation observed during other stages of the cell cycle. The amount of ubiquitin-conjugated proteins was also demonstrated to decrease by half during this stage of the cell cycle. Although the total amount of E1 is constant

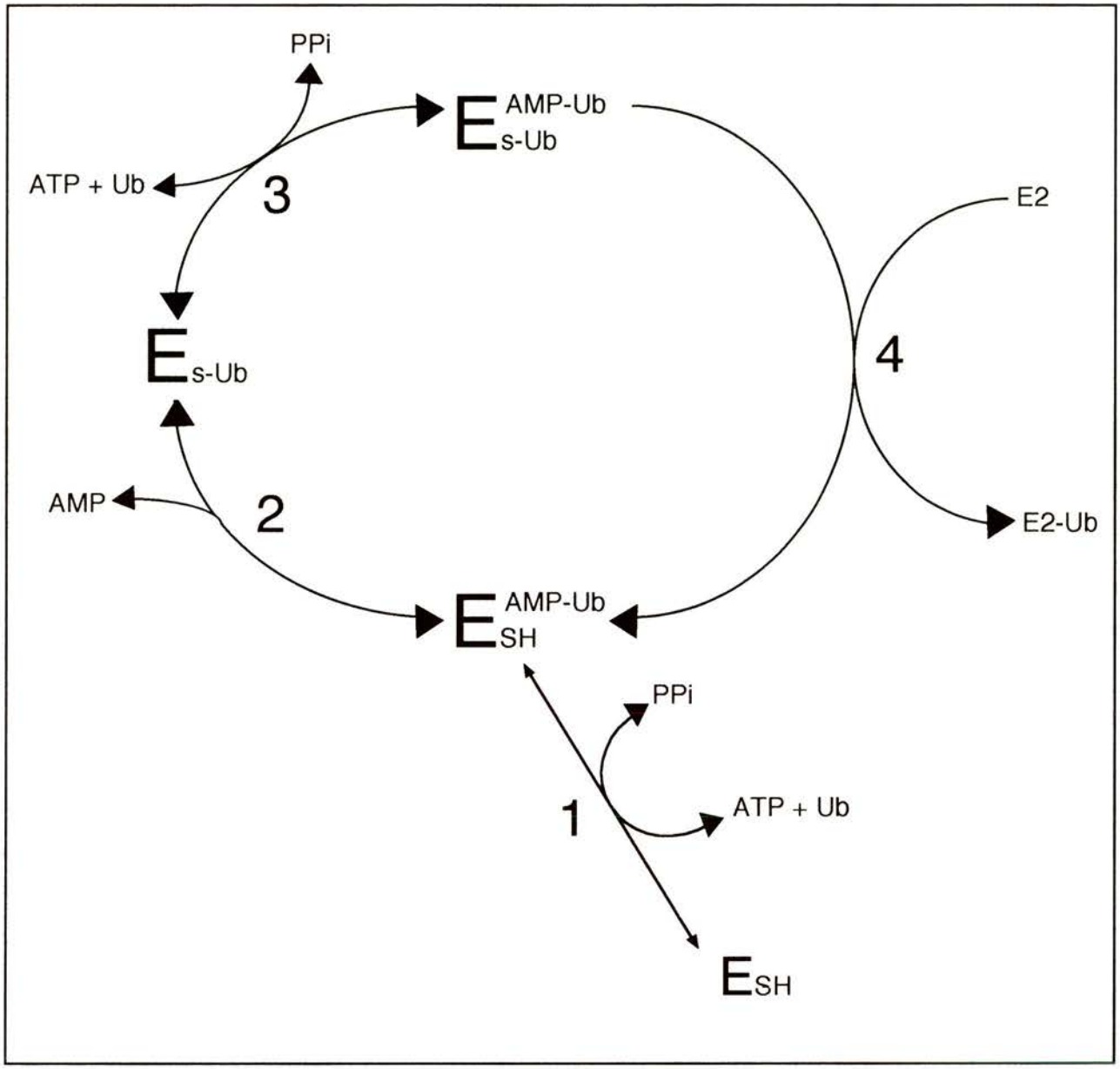


Figure 5 Mechanism of E1 ubiquitin activating enzyme

(1) Ubiquitin is attached in the presence of ATP to E1 ubiquitin activating enzyme via a ubiquitin adenylate intermediate, releasing PPi. (2) Ubiquitin is then transferred to a second site within the enzyme to form a thiolester linkage with a sulfhydral group of a cysteine residue with the subsequent release of AMP. (3) Another molecule of ubiquitin is then attached in the presence of ATP to E1 via a ubiquitin adenylate intermediate, releasing PPi. (4) Ubiquitin attached to E1 via a thiolester linkage is then transferred to a cysteine residue in an E2 ubiquitin conjugating enzyme. Subsequent rounds of ubiquitin activation can then occur in the presence of ATP and Ub.

during the different stages of the cell cycle, in G2 nuclear extracts, there is a 3-fold increase in the phosphorylated E1α isoform and an increase in the amount of this isoform in the nucleus. Phosphorylation of this isoform has been suggested to allow the localisation of the protein to the nucleus in a cell-cycle dependent manner (Stephen *et al.*, 1996).

1.3.2 E2

A number of E2 ubiquitin conjugating (UBC) enzymes have been characterised. E2 molecules belong to a family of structurally related proteins, relatively small in size, ranging from 14-25 kDa. As well as containing an N-terminal, ubiquitin-conjugating (UBC) region, encompassing the active cysteine residue involved in the ubiquitin-thiolester formation, some also have unique N- or C-terminal extensions thought to participate in recognition of substrate or of E1 ubiquitin activating enzyme (Liu *et al.*, 1999). The *Saccharomyces cerevisiae* genome has been shown to encode for thirteen E2 and E2-like proteins (Hochstrasser, 1996) and therefore it is likely that more will be found in higher eukaryotes. Analysis of the completed human genome has revealed that there are at least 27 genes encoding proteins with E2 ubiquitin-conjugating or E2-like functions. Some of these genes encode for known E2 enzymes whereas others encode for proteins of unknown function that have been predicted to behave as ubiquitin-conjugating enzymes on the basis of their structural similarity to known E2 proteins.

Various genetic studies have demonstrated that genes encoding E2 ubiquitin-conjugating enzymes are also involved in processes other than ubiquitin transfer. In yeast, the Ubc2/Rad6 protein is also involved in DNA repair as well as targeting molecules for ubiquitination via the N-end rule pathway (Jentsch *et al.*, 1987). Drosophila UbcD1 enzyme also participates in the proper detachment of telomeres during mitosis and meiosis (Cenci *et al.*, 1997). UBC3, the product of the CDC34 gene in yeast has also been shown to be essential for cell viability, with mutations in this E2 affecting G1-S cell cycle transition and DNA replication (Jentsch, 1992). It is possible to envisage that the other

cellular processes in which these enzymes participate, also involve ubiquitination of proteins within these processes.

1.3.3 E3

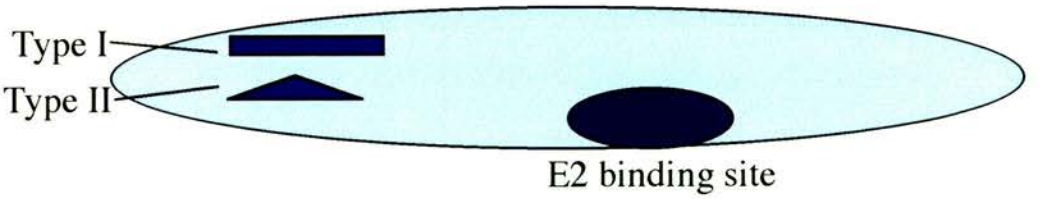
A large and still growing family of E3 ubiquitin ligases provide specificity to the ubiquitin - proteasomal degradation system. These enzymes are able to interact with E2 enzyme as well as specifically recognising their target substrates. In doing so, they are able to either directly ubiquitinate substrate themselves or are able to bring the substrate into close proximity to the E2. The discovery of a family of E3 enzymes provided the system with a unique way in which to identify target substrates. The search for members of theses families and the mechanisms and control of these enzymes are still an area of intense scientific research. Four classes of enzyme have been discovered so far, all of which involve unique mechanisms of substrate recognition and transfer of ubiquitin. These consist of the N-end rule E3, E3α (Bartel *et al.*, 1990), the HECT domain family (Huibregtse *et al.*, 1995b), the anaphase promoting complex (APC)/cyclosome (King *et al.*, 1995) and the SCF family (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). Figure 6. Due to the large family and mechanisms by which these families operate, E3 enzymes have now been defined as enzymes which bind either directly or indirectly to protein substrates. In doing so, they promote the transfer of ubiquitin directly or indirectly from a thiolester intermediate to form amide linkages with the epsilon amino groups in lysine residues of the target protein or within polyubiquitin chains.

1.3.3.1 E3α/Ubr1

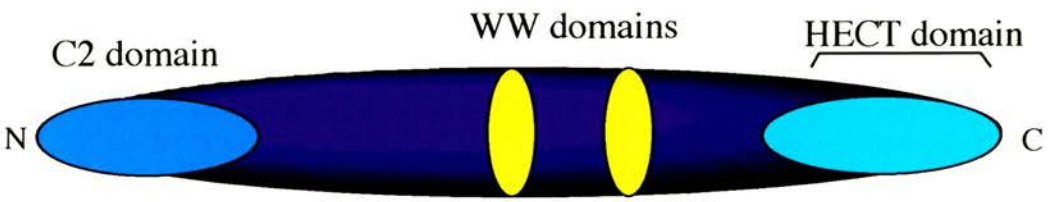
Mammalian E3α and its yeast counterpart Ubr1 are one of the best studied and characterised E3 ligases (Varshavsky, 1996). Proteins are recognised via their N-terminal amino acids. These can be basic (Type I) or hydrophobic/bulky (Type II) amino acid residues. Another related enzyme, E3β has also been discovered and has been shown to be

Figure 6 E3 ubiquitin ligase family
A family of E3 ubiquitin ligases. (A) N-end rule (E3α) is a single polypeptide which recognises TypeI, basic, or Type II, hydrophobic/bulky amino terminal residues of target substrates. (B) HECT-domain E3 ligases are single polypeptides that contain a carboxy terminal 350 amino acid domain homologous to papillomavirus E6-AP. HECT-domain E3 ligases bind ubiquitin via a thiolester linkage to a cysteine residue in the C-terminus of the protein. N-terminal variable domains C2, a domain known to mediate Ca^{2+}-dependent association with phospholipids/membranes, and WW domains involved in substrate recognition. (C) APC/Cyclosome consists of a large multi-subunit complex that can interact with ubiquitin conjugating enzymes and substrate and promote the transfer of ubiquitin from E2 to the target protein. (D) SCF complexes consist of core molecules, Skp1, Cul1/cdc53 and Rbx1 and an interchangeable F-box protein. SCF complexes interact with both the target substrate and the preferred E2 in order to facilitate the transfer of ubiquitin to the target protein.

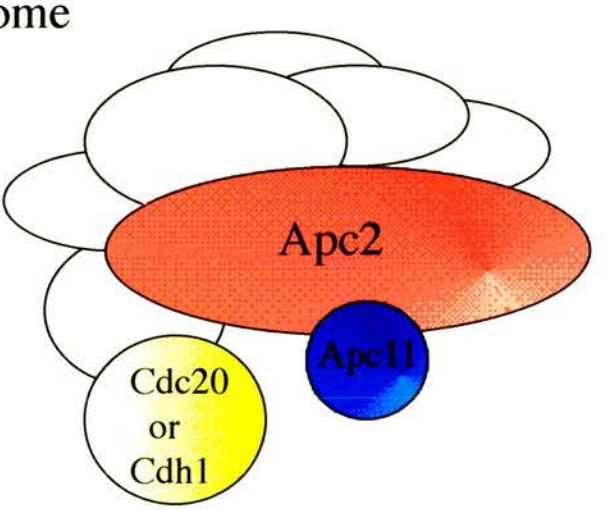
A. N-end rule E3 (E3α)



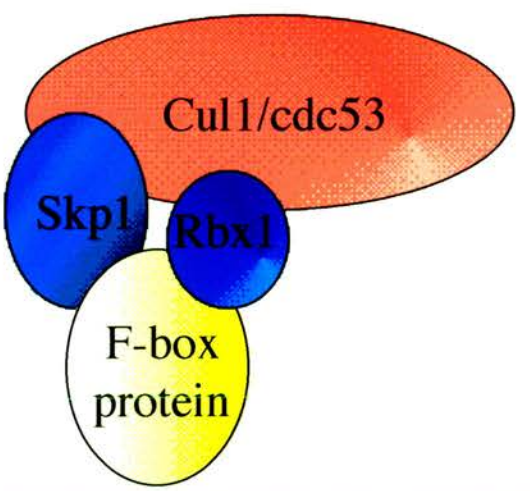
B. HECT-domain E3



C. APC/Cyclosome



D. SCF



specific for small, uncharged N-terminal amino acid residues. Substrates appear to have a destabilising amino terminal residue along with one or more internal lysine residues (Johnson *et al.*, 1990; Bachmair and Varshavsky, 1989) Although the N-end rule pathway is highly conserved among eukaryotes, many of its substrates and physiological roles remain undefined.

1.3.3.2 HECT

Hect E3 ligases contain a 350 amino acid domain that is homologous to the human papilloma viral protein E6-associated protein (E6-AP) C-terminal (HECT) domain. The first member of this family, E6-AP interacts with one of its targets, p53, indirectly through the papillomavirus oncoprotein E6 (Scheffner *et al.*, 1993). However other members of the family have been shown to interact with target proteins through a variable N-terminal domain (Huibregtse *et al.*, 1995a). In some cases recognition between E3 and substrate also involves an N-terminal protein recognition domain called the WW domain. This 30 amino acid region provides a hydrophobic binding pocket able to interact with proline-rich sequences called the PY motif as demonstrated by the epithelial sodium channel complex (Staub *et al.*, 1997). Hect domain containing proteins also contain a conserved cysteine residue in their C-terminus, which is thought to be able to accept ubiquitin from an E2 and form a thioester intermediate, before transferring ubiquitin to target substrates (Scheffner *et al.*, 1995). It has been demonstrated that regulation of p53 function is controlled within the cell by mdm2 that specifically binds to, and causes the degradation of the protein via a ubiquitin-dependent mechanism (Haupt *et al.*, 1997). Furthermore, mdm2 has also been reported to exist as an E3 ligase which bears homology in the carboxyl terminus to E6-AP (Honda and Yasuda, 2000).

1.3.3.3 APC/cyclosome

The anaphase promoting complex (APC)/cyclosome E3 ligase (King *et al.*, 1995; Sudakin *et al.*, 1995), is a large molecular weight complex involved in the ubiquitination of cell-cycle regulatory components such as mitotic cyclins, spindle-associated proteins and some anaphase inhibitors. Many of its substrates contain a nine amino acid degenerate motif ($R^{A}/_{T}ALGX^{I}/_{V}{}^{G}/_{T}N$) called the destruction box which has been shown to be essential for ubiquitination and degradation of mitotic cyclins *in vitro* and *in vivo*. The APC has been shown to be composed of at least 12 subunits in budding yeast (Zachariae *et al.*, 1998a; Zachariae *et al.*, 1996) and 10 in mammals and Xenopus laevis (Yu *et al.*, 1998). It appears to be inactive during interphase but becomes activated at metaphase and early anaphase by a mechanism thought to involve both phosphorylation and dephosphorylation of regulatory subunits. Phosphorylation of subunit Cdc20 by mitotic cyclin dependent kinases (CDKs) causes its activation and allows destruction of the budding yeast anaphase inhibitor Pds1, whereas phosphorylation of a major APC/C regulator Hct1 causes its inactivation (Morgan, 1999; Zachariae and Nasmyth, 1999). Hct1 activity can be restored by dephosphorylation of this subunit by phosphatase Cdc14, allowing Hct1 to associate with and stimulate APC/C (Visintin *et al.*, 1998). Some of the core subunits of the APC/C have also been shown to be homologous to some of the subunits found in the SCF family of E3 ligases. These include Apc2 homologous to SCF family Cdc53 and also Apc 11, homologous to the RING-finger containing protein Rbx1/Roc1 of the SCF family (Zachariae *et al.*, 1998b).

1.3.3.4 SCF - Skp1/Cdc53/cullin1/F-box family.

This multi-subunit containing family of enzymes is composed of three common proteins, Skp1, Cdc53/Cul1 and Rbx/Roc1 along with an interchangeable F-box protein. The first SCF family members were discovered and characterised in budding yeast by *in vitro* reconstitution assays (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). It was shown that the

in vitro ubiquitination of Sic1, an S phase cyclin/CDK inhibitor, could be achieved when incubated with yeast UBC cdc34 and a complex of Cdc4, Cdc53 and Skp1, expressed and purified from insect cells. Cdc4, along with other proteins were shown to share a small sequence motif, originally identified in cyclin F, termed the F-box (Bai *et al.*, 1996). Cdc53 was shown to be homologous to a family of proteins termed cullins and both Cdc4 and Cdc53 were found to co-immunoprecipitate with Cdc34 from yeast cell lysates (Mathias *et al.*, 1996). Skp1 was identified in genetic screens for a protein that could suppress the overexpression of $Cdc4^{ts}$ mutants (Bai *et al.*, 1996). A fourth subunit of the SCF complex, known as Hrt1 in yeast and Rbx1/Roc1 in mammals, was also identified through purification from HeLa cells, yeast two-hybrid screens with human Cul1 and also through immunoprecipitation experiments with Skp1 and Cdc53 (Tan *et al.*, 1999; Ohta *et al.*, 1999; Seol *et al.*, 1999).

Many SCF complexes have now been revealed in both yeast and mammals and these proteins have been shown to be involved in a vast array of cellular processes such as the cell cycle, cell signalling and innate immunity (Deshaies, 1999). SCF complexes appear to require phosphorylation of their target substrate in order for them to transfer the ubiquitin moiety from the E2 enzyme to the epsilon amino group of a lysine residue in the target protein. The interchangeable F-box proteins impart specificity to the SCF complex as they each recognise a particular set of protein substrates (Deshaies, 1999). Substrates are recognised and bind to F-box proteins via protein-protein interaction motifs, such as WD repeats or leucine rich sequences, in the C-terminus of the protein. It has been suggested that F-box proteins are able to serve as receptors for phosphorylated substrates in order to bring them into close proximity of the ubiquitination machinery and allow for their efficient ubiquitination.

Related to the SCF family of E3 ligases is a multi-subunit complex called the VBC complex that is composed of pVHL (von Hippel-Lindau disease tumour-supressor gene product), Elongin B, Elongin C along with Cullin-2 and Rbx1/Roc1. Structurally the

complexes are very similar, with the core structure of Elongin B resembling ubiquitin and Elongin C and Cullin-2 being homologous to Skp1 and Cdc53 respectively (Lonergan *et al.*, 1998; Kamura *et al.*, 1999b; Tyers and Willems, 1999).

1.3.4 26S proteasome

The major site of non-lysosomal protein degradation within eukaryotic cells occurs in structures called proteasomes. Many different proteasome complexes are found within mammalian cells both within the nucleus and cytoplasm (Coux *et al.*, 1996; Brooks *et al.*, 2000).

1.3.4.1 Proteasome structure

The 20S core proteasome was discovered by independent laboratories as a large 700kDa ATP-dependent protease complex (DeMartino and Goldberg, 1979; Wilk and Orlowski, 1983; Schmid *et al.*, 1984; Monaco and McDevitt, 1984; Arrigo *et al.*, 1988; Falkenburg *et al.*, 1988; Brown *et al.*, 1991). The archaebacterial 20S proteasome from the archaebacterium *Thermoplasma acidophilum* provided an insight into the structure of the proteasome. Electron microscopy and decoration of subunit particles by antibodies revealed that its two outer rings were composed of α subunits, with its inner rings composed of β subunits (Dahlmann *et al.*, 1989). The elucidation of the crystal structure of this archaeabacterial complex further demonstrated that the subunits were arranged in a barrel-shaped structure with the two outer rings composed of 7 α subunits and the two inner rings composed of 7 β subunits. The crystal structure of the *Saccharomyces cerevisiae* 20S proteasome has indeed demonstrated that the eukaryotic complex is also composed of 4 helical rings containing seven subunits, which are able to stack on top of each other to form a hollow cylindrical structure. Formation of the hollow cylinder gives rise to the catalytic site at which proteins can be degraded. Again, the two outer rings are composed of 7 different subunits belonging to the α family, and the two inner rings are

composed of 7 different β family member subunits giving rise to the following structure $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ (Groll *et al.*, 1997). Figure 7.

The α subunits appear to be more conserved than the β subunits and are able to self-assemble, a mechanism which is essential for β subunit formation (Zwickl *et al.*, 1994). They also function as the sites for binding the regulatory subunits 19S (PA700) and 11S (PA28) (Peters *et al.*, 1993; Gray *et al.*, 1994). The β subunits are not able to self-assemble but they form the sites of proteolytic activity as demonstrated by the ability of these sites to be blocked by certain inhibitors (Fenteany *et al.*, 1995). Indeed the crystal structure of the *Thermoplasma acidophilum* proteasome in complex with an inhibitor, first demonstrated that the hydroxyl group of the N-terminal threonine residue of β subunit particles served as the catalytic nucleophile (Lowe *et al.*, 1995). These studies were verified by the fact that the enzyme activity of this archaeabacterial proteasome was inactivated after deletion or mutation of the N-terminal threonine residue of β subunits (Seemuller *et al.*, 1995). On the basis of sequence conservation, it has been suggested that of the seven β-type subunits in an individual eukaryotic proteasome, there are only three that are proteolytically active. However, it has also been demonstrated that intersubunit contacts between active and inactive β particles is required for functional proteasome complexes (Arendt and Hochstrasser, 1997).

1.3.4.2 Proteasome function

Proteins are degraded through various proteolytic activities contained within the proteasome. These include "chymotrypsin-like" activity, cleavage of proteins after large hydrophobic residues; "trypsin-like" activity, where cleavage occurs after basic residues; and "postglutamyl" hydrolysing activity in which cleavage of proteins occurs after acidic residues (Orlowski *et al.*, 1993; Rivett, 1989). Additional peptidase activities have also been identified which consist of cleavage of the protein after branched - chain amino acids,

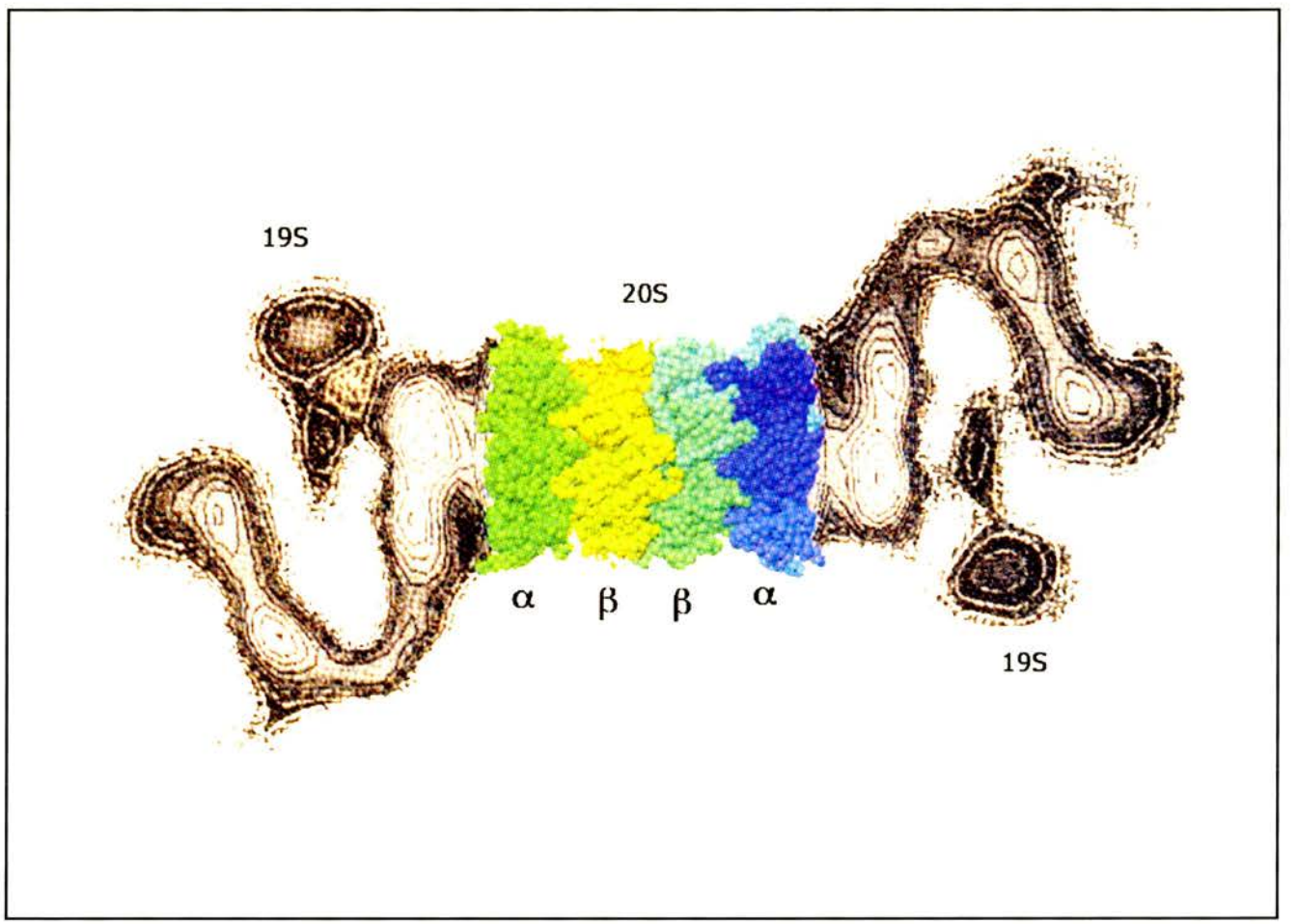


Figure 7 Structure of the 26S proteasome

X-ray crystallographic structure of the Saccharomyces cerevisiae 20S proteasome (Groll *et al.*, 1997) reveals that it is composed of four helical rings each made up of seven different members, $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The two outer rings contain subunits belonging to the α family and the two inner rings contain subunits belonging to the β subunit family. Binding of the regulatory 19S caps to either end of the 20S proteasome gives rise to the 26S proteasome. The 19S caps are shown as an image based on the results of electron microscopy (Yoshimura *et al.*, 1993). Figure taken from Stuart and Jones, 1997.

BrAAP activity, and after small neutral amino acids, SNAAP activity (Orlowski *et al.*, 1993).

1.3.4.3 Proteasome complexes

The components of the proteasome are dynamic and subpopulations may appear to exist depending on cell type and function (Brooks *et al.*, 2000). Subunit composition of the mammalian 20S proteasome can be altered upon treatment of cells with γ-interferon. It has been shown that β subunits ε (X), δ(Y) and Z can be replaced with subunits LMP7, LMP2 and MECL1 respectively. These three subunits are inducible upon γ-interferon exposure and are thought to be incorporated into proteasomes in order to participate in antigen processing and presentation of peptides in MHC class I molecules (Tanaka *et al.*, 1997). Another subunit which is up-regulated upon γ-interferon exposure is the 11S regulatory subunit. The 11S complex, also known as REG α or PA28 exists as a hexameric structure of alternating α and β subunits that bind to the α subunits of the core 20S proteasome. Binding of the 11S regulatory complex to each end of the 20S proteasome occurs in an ATP-independent manner. Proteasomes of this composition have also been implicated in the immune response and antigen processing (Lehner and Cresswell, 1996; Coux *et al.*, 1996).

The binding of the 19S regulatory subunit to each end of the core 20S proteasome gives rise to the 2MdDa 26S proteasome which is involved in degradation of proteins in a ubiquitin-dependent manner (Hershko and Ciechanover, 1998). The 19S regulatory complex is composed of approximately twenty different polypeptides ranging in size from 25 to 110kDa. Six of these subunits contain ATPase activity (Dubiel *et al.*, 1995) and are thought to function in the energy dependent step of protein degradation. Point mutations in these ATPases cause an abnormal accumulation of ubiquitinated proteins (Gordon *et al.*, 1993) and can also cause a decrease in degradation of mitotic cyclins (Ghislain *et al.*, 1993). The ATPases are also thought to mediate unfolding and translocation of the protein

into the lumen of the 20S core particle (Larsen and Finley, 1997). It has also been suggested that assembly and disassembly of the 26S proteasome occurs through phosphorylation and dephosphorylation of one of its ATPase subunits, p45 (Satoh *et al.*, 2001). The precise role of the ATPase subunits and also the non-ATPase proteins found within the 19S regulatory cap are still unknown. However it has been shown that some of these non-ATPase subunits function in identifying ubiquitinated proteins, as demonstrated by the fact that human S5a and its Arabidopsis homologue MBP1, can bind multi-ubiquitin chains (Deveraux *et al.*, 1994; van Nocker *et al.*, 1996).

In yeast, there is evidence demonstrating that ubiquitin conjugating enzymes (ubc) of the ubiquitin-conjugation pathway are able to interact with subunits of the 26S proteasome (Tongaonkar *et al.*, 2000) as well as subunits of the SCF and APC complexes, which belong to the E3 family of ubiquitin ligases (Verma *et al.*, 2000). Xie and Varshavsky also demonstrated that specific components of E3 ligases from yeast, Ubr1p and Ufd4p, directly interacted with subunits of the 19S cap (Xie and Varshavsky, 2000). Furthermore it has been demonstrated that a group of proteins in humans containing an N-terminal ubiquitin-like (Ubl) domain and a C-terminal ubiquitin-associated (Uba) domain are capable of linking the ubiquitination machinery to the proteasome. These proteins termed hPLIC-1 and hPLIC-2 (proteasome ligase interaction component) co-immunoprecipitate with proteasomal components and members of E3 ligases such as E6-AP and βTrCP. When overexpressed in HeLa cells they also interfere with the degradation of two unrelated ubiquitin-dependent proteasomal substrates, p53 and IκBα (Kleijnen *et al.*, 2000). Therefore it appears that there are many processes as yet unidentified which function in the regulation of ubiquitin-dependent degradation via the 26S proteasome.

Degradation of substrates by the 26S proteasome occurs following ubiquitin conjugation to lysine residues in the target proteins. However, it should be noted that not all substrates of the 26S proteasome need to be ubiquitinated in order to be degraded. These include the enzyme ornithine decarboxylase which functions in the synthesis of

polyamines (Coffino, 2001a; Coffino, 2001b), and also the cyclin dependent kinase (Cdk) inhibitor p21Cip1 (Sheaff *et al.*, 2000). The diverse functioning of the 26S proteasome is also demonstrated by the fact that it can selectively degrade precursor proteins, resulting in the formation of the mature active protein. Such proteins include p105, which is proteolytically processed to form p50, the active subunit of the NF-κB transcription factor (Fan and Maniatis, 1991) and also p100, which is degraded to form p52, a component of the NF-κB2 transcription factor (Thanos and Maniatis, 1995). Processing and degradation of p105 to form p50 requires ubiquitination of p105 (Palombella *et al.*, 1994). The 26S proteasome is also a major component of the immune system and functions to degrade viral or other unusual polypeptides within the cell. Degradation of these foreign bodies results in the production of antigenic peptides which can be displayed in the groove of the MHC class I molecule, a transmembrane receptor involved in presentation of antigen to T-cells (Goldberg and Rock, 1992; Heemels and Ploegh, 1995).

Overall, proteasome complexes exist within the cell to tightly control and regulate degradation of cellular proteins. Subpopulations of proteasomes can exist within the cell and within different compartments and different cell types. Not only is the proteasome involved in non-ubiquitin dependent degradation, it is also a major player in the degradation of proteins conjugated by the ubiquitin pathway. Its other functions also include proteolytical processing of precursor proteins and production of antigenic peptides for presentation within the immune system.

1.3.5 De-ubiquitinating enzymes

Removal of ubiquitin from ubiquitin-modified proteins occurs through the actions of de-ubiquitinating enzymes. More than 90 de-ubiquitinating enzymes have been identified, making them the largest family of enzymes in the ubiquitin system. De-ubiquitination is emerging as another level of regulation within the ubiquitin system, allowing generation of ubiquitin monomers from poly-ubiquitin chains and adducts as well

as playing roles in growth, oncogenesis, development, differentiation, long-term memory and transcriptional regulation (Chung and Baek, 1999). De-ubiquitinating enzymes have also been implicated in the pathogenesis of some disease states, including Parkinson's disease in which it has been found that partial loss of the catalytic activity of the deubiquitinating enzyme UCH-L1 results from a methionine to isoleucine mutation at amino acid residue 93. Loss in catalytic activity of this enzyme can lead to aberrations in the proteolytic pathway including folding, processing and degradation of proteins, which may ultimately contribute to neuronal degradation (Leroy *et al.*, 1998).

De-ubiquitinating enzymes are cysteine proteases that cleave ester, thiol ester and amido bonds to the carboxyl group of G76 of ubiquitin. DUBs can be further divided into two structurally unrelated gene families; ubiquitin C-terminal Hydrolase (UCH) family and ubiquitin-specific Processing Protease (UBP) family (Wilkinson, 1997). UCH family members are relatively small in size ranging from 20-30kDa, except BAP1 (Jensen *et al.*, 1998) and function in hydrolysing C-terminal amides and esters of ubiquitin. UBPs release ubiquitin molecules that are conjugated to proteins via amido peptide and/or epsilon amino isopeptide bonds. More than 80 UBP sequences have been isolated from a variety of organisms with each organism containing multiple isoforms.

Regulation of protein degradation through the ubiquitin-dependent pathway can therefore also be controlled by these specialised enzymes termed DUBs. DUBs can function at a number of levels. As well as participating in a number of biological processes they are able to release free ubiquitin monomers, which may be required for the subsequent modification of other proteins, from linear precursor fusion proteins or from branched-chain ubiquitin polymers. They can remove ubiquitin from inappropriately modified proteins preventing their degradation through the 26S proteasome and they can also function to clear the 26S proteasome of ubiquitin-modified peptide remnants. DUBs provide another level of regulation of protein degradation within the cell.

1.3.6 Ubiquitin-like modifications

Ubiquitin modification of proteins is a well conserved pathway. Recently it has been discovered that there are also other modifications of proteins that resemble that of ubiquitination. These include SUMO (small ubiquitin related modifier)/Sentrin (Boddy *et al.*, 1996; Matunis *et al.*, 1996; Okura *et al.*, 1996; Shen *et al.*, 1996; Lapenta *et al.*, 1997; Tsytsykova *et al.*, 1998), NEDD8 (neuronal precursor cell-expressed developmentally downregulated) (Kamitani *et al.*, 1997), UCRP (Ub cross reactive protein) (Haas *et al.*, 1987; Loeb and Haas, 1992) and Apg12 (Mizushima *et al.*, 1998b; Mizushima *et al.*, 1998a) modifications. SUMO, NEDD8 and UCRP all contain ubiquitin-like domains.

These proteins are all small molecules that are attached to target substrates via an enzyme linked cascade distinct from, but analogous to ubiquitin conjugation. Functions of these modifications are still not fully understood, but it has been shown that modification of proteins by SUMO can cause a change in cellular distribution, as demonstrated by the modification of PML (Muller *et al.*, 1998; Sternsdorf *et al.*, 1997) and also inhibition of transcription factor activity, as shown by the fact that modification of IκBα renders it resistant to proteolytical degradation, thereyby sequestering NF-κB in the cytoplasm (Desterro *et al.*, 1998). The major substrate for NEDD8 and its yeast homologue Rub1 appears to be cullin family members (Kamitani *et al.*, 1997; Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998). Cullin proteins function in a variety of cellular processes including assembly and functioning of SCF and VHL complexes. UCRP, a small 15kDa protein has also been found to conjugate to proteins in a ubiquitin-like manner upon exposure of cells to interferon (Haas *et al.*, 1987; Loeb and Haas, 1992; Narasimhan *et al.*, 1996). A fourth ubiquitin-like modification, required for autophagy in yeast, is known to occur via a ubiquitin-like conjugation system involving a protein called Apg12, which contains no homology to ubiquitin (Mizushima *et al.*, 1998b; Mizushima *et al.*, 1998a). Autophagy is a process by which cytoplasmic components are sequestered into double-membrane vesicles (autophagosomes) and delivered to the vacuole for recycling. This

process allows survival of eukaryotic cells under periods of nutritional starvation. Apg12 homologues are also found in higher eukaryotes demonstrating the fact that autophagy and modification of proteins by this protein is also likely to occur in humans.

Therefore while ubiquitin conjugation serves as a mechanism by which proteins can be tagged for specific functions, other related but different polypeptides are also able to modify proteins. Modification of proteins by these ubiquitin-like molecules occurs via similar mechanisms but results in different overall outcomes for the fates of the modified proteins. In order for a modification to occur on a particular protein, the cell must have developed a system by which to recognise target substrates. This is achieved by distinct enzyme molecules that are able to recognise both the target substrate and the protein that will modify the substrate.

1.4 Aims of project

Ubiquitination of IκBα serves as a critical step in the activation of NF-κB. Therefore the mechanisms governing the control of this process are of great importance. The main aims of this project were to develop tools that could be used to investigate the process of IκBα ubiquitination *in vitro* and thereby identify specific components involved in this process. Specific objectives included analysis of the ubiquitination machinery and its mechanism of action; the process of IκBα recognition by this ubiquitination machinery; and in particular the interaction and recognition of IκBα with βTrCP.

In order to achieve these objectives, phosphorylation of IκBα, the first step towards identifying this protein as a substrate for ubiquitination was investigated by exploring the mechanism of action of the IκBα kinase complex.

In vitro ubiquitination assays were established in order to try and identify the mechanism of action of the ubiquitination machinery and the minimal components required for IκBα ubiquitination.

In addition to identifiying the proteins involved in the ubiquitination process, the residues within IκBα were also analysed for their ability to contribute towards recognition of the protein by the ubiquitination machinery. This final objective was achieved by performing *in vitro* assays in the presence of a series of IκBα peptides.

2. MATERIALS AND METHODS

2.1. MATERIALS

In vitro TNT kit was from Promega. [γ^{32}P]ATP and Iodine 125 were purchased from Amersham. All recombinant GST-IκBα, IκBα and p65 proteins were made in-house and were gifts from Ellis Jaffray. Mini EDTA-free protease inhibitor tablets were from Roche. Adenosine trisphosphate (ATP), inorganic pyrophosphate (IPP) and ubiquitin were all purchased from Sigma. Human recombinant TNFα was obtained from the MRC AIDS Directed Programme. Human recombinant Interluekin 1β (Il-1), Creatine kinase (CK) and creatine phosphate (CP) were purchased from Sigma.

2.2 Antibodies

Anti-C terminal IKK1 antibody was generated against a peptide corresponding to the C-terminal 12 amino acids of IKK1 (SMMNLDWSWLTE) and coupled through an additional cysteine residue to Keyhole Limpet Hemocyanin (KLH). Sheep were immunised and serum collected by the Scottish Antibody Production Unit (SAPU). Serum or affinity purified antibody (section 2.2.2) was used for immunoprecipitations and affinity purified antibody used at 1μg/ml for immunodetection. Anti-N terminal IKK1 antibody was generated against a peptide corresponding to the N-terminal 12 amino acids of IKK1 (MERPPGLRPGAG) and coupled through an additional cysteine residue to KLH. Sheep were immunised and serum collected by SAPU. Affinity purified antibody was used at 1μg/ml for Western blotting. IKK1 monoclonal antibody (B8) was used at 1:100 dilution and IKK1 polyclonal antibody (H-744) used at 1:2000 dilution and both purchased from Santa Cruz. sv5 Pk Tag, 336 monoclonal antibody, recognising a 14 amino acid epitope (GKPIPNPLLGLDST) of the protein P of Simian virus 5 (sv5), was obtained from R.E. Randall, University of St. Andrews and used for detection of proteins containing the sv5

epitope tag by Western blotting (1:2000) or by immunoprecipitation (Hanke *et al.*, 1992). Anti-FLAG antibody which recognises an 8 amino acid epitope (DYKDDDDK) FLAG tag, was purchased from Kodak and used for immunoprecipitation or detection of proteins by Western blotting (1:2000 dilution) containing the FLAG tag. IκBα (C21) rabbit polyclonal antibody which recognises amino acids 297-317 corresponding to the carboxy terminus of IκBα was purchased from Santa Cruz and used in Western blotting at 1:2000 dilution. Anti-C terminal βTrCP antibody was generated against a peptide corresponding to the C-terminal 12 amino acids of βTrCP (RSPSRTYTYISR) and coupled through an additional cysteine residue to KLH. Sheep were immunised and serum collected by SAPU. Affinity purified antibody was used at 10ng/ml Western blotting. Skp 1 (C20) goat polyclonal antibody recognising an epitope at the carboxy terminus of Skp1 and Rbx1 (N16) goat polyclonal antibody recognising an epitope at the amino terminus of Rbx1 were both purchased from Santa Cruz and used in Western blotting at 1:1000 and 1:250 dilutions respectively. Cul-1 rabbit polyclonal antibody which recognises amino acids 742-752 corresponding to the carboxy terminus of Cul-1 was purchased from Neomarkers and used at 1:1000 dilution in Western blotting.

2.2.1 Coupling of peptides to Kehole Limpets Hemocyanin (KLH).

10mg/ml KLH was dissolved in PBS by extensive sonication of the sample. 20mM N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was added dropwise to KLH to a final concentration of 5mM and the sample mixed for 30 minutes at room temperature and dialysed overnight at 4°C against PBS. Activation of KLH was checked by reading the OD_{343} of the sample in the absence and presence of 30mM DTT. An increase in absorbance is caused by the release of pyridine-2-thiol, indicating that KLH is activated. 1ml (25mg) of reduced βTrCP C-terminal peptide (CRSPSRTYTYISR), 1ml (25mg) of reduced IKK1

C-terminal peptide or 1ml (25mg) of reduced IKK1 N-terminal peptide was resuspended in water and incubated for 90 minutes at room temperature with 1ml (10mg) of activated KLH and then dialysed overnight at 4°C against PBS. To check coupling, the absorbance at 343nm of 50µl peptide/KLH mix in the presence of 30mM DTT was measured at 0 and 90 minutes. 1mg aliquots were then frozen at -70°C and sent to SAPU.

2.2.2 Affinity purification of peptide antibodies

10mg of either βTrCP or IKK1 peptide was dissolved in 0.1M KH_2PO_4 buffer, pH 7.2 and OD_{280} measured. 1ml thiol sepharose 4B (Sigma), pre-swollen overnight at 4°C in PBS, was then added to the peptide solution and incubated, rotating, at 4°C overnight. Next day, supernatant was removed and absorbance at OD_{280} measured to check binding of peptide to sepharose. Beads were then washed with PBS and column poured. 5mls of peptide antisera was diluted in 45mls PBS and loaded onto the pre-washed peptide sepharose column, recirculated, washed with 10ml PBS and then 10ml of 10mM Tris HCl, pH 7.6. Antibody was eluted in 500µl aliquots with 100mM glycine, pH 2.5 into 50µl 1M Tris HCl, pH 8. Protein concentration was determined by Bradford Assay and fractions containing protein were stored in aliquots in 10% glycerol, 1mg/ml BSA, 0.01% sodium azide at -70°C. Column was washed with 0.2N NaOH, PBS and then stored in PBS/0.01% sodium azide at 4°C.

2.3 Bacterial Strains

For routine DNA preparation *E.Coli* DH5α (genotype: Φ80dlacZΔM15, *rec* A1, *end* A1, *gyr* A96, *thi*-1, *hsd* R17 (r_k-, m_k+), *sup* E44, *rel* A1, *deo*R, Δ(*lac*ZYA-*arg*F)M169) was used. For protein expression *E.Coli* B834 (genotype: F⁻, *omp*T, *hsd*S_B, (r_B-,m_B-), *dcm*, *gal*) were used. Bacteria were grown in Luria-Broth (LB) or streaked on LB-agar plates with antibiotics added when required.

2.4 Plasmids and expression vectors

pcDNA3 containing cDNA for IKK1 and IKK2 were a kind gift from Pfizer Central Research. pcDNA3 containing cDNA for NIK was a kind gift from D. Wallach, Weizmann Institute, Israel. pGEX 4T-3 was obtained from Pharmacia and pcDNA3 was purchased from Invitrogen Corporation. pcDNA3 containing sv5 Pk tag was described as in Desterro *et al.*, 1999. cDNA encoding human βTrCP was obtained from R. Benarous (Paris). Construction of an N-terminal SV5 Pk epitope tagged βTrCP was described by Vuillard and colleagues (Vuillard *et al.*, 1999). Isolation of cDNA encoding hUbc5 which was then inserted into pGEX-2T was also described by Vuillard *et al* 1999. A baculovirus containing the coding sequence for the human ubiquitin activating enzyme (E1) was obtained from M. Rolfe (Mitotix). A 6-His tagged cdc34 bacterial expression construct was obtained from R.J. Deshaies (Caltech). Baculovirus containing the coding sequence for Skp1 and Rbx1 were made in-house and were a kind gift from R. T. Hay, J. Mathews and J. Thomson.

2.4.1 DNA preparation

DNA restriction enzymes for cloning were purchased from New England Biolabs (NEB) and Promega. Vent DNA polymerase used for PCR was obtained from NEB. Gel extraction of DNA was performed using gel extraction kit from Qiagen according to the manufacturers instructions. Quality and quantity of DNA was analysed by spectrophotometric readings at 260nm and 280nm and by electrophoresis in an agarose gel in the presence of ethidium bromide, followed by U.V. (Sambrook *et al.*, 1989).

2.4.2 Small scale preparation of DNA

Plasmid DNA was extracted using either Qiagen miniprep kit following the manufacturer's instructions or by alkaline lysis where 1.5ml of bacterial culture was collected by

centrifugation at 12000*g* for 30 seconds and the pellet resuspended in 100µl of ice-cold solution I (50mM glucose, 20mM Tris HCl, pH8.0, 10mM EDTA, pH8.0). 200µl of freshly prepared solution II (0.2M NaOH, 1% SDS) was then added and the sample mixed by inversion. 150µl of ice-cold solution III (60ml of 5M potassium acetate, 11.5ml glacial acetic acid made up to 100ml with distilled water) was then added and the sample centrifuged at 12000g for 5 minutes. Supernatant was retained and DNA precipitated with 2 volumes of 100% ethanol at room temperature for 2 minutes. The sample was then centrifuged as before for 10 minutes and the DNA pellet rinsed once with 70% ethanol and allowed to air dry for approximately 30 minutes. Once dry, the DNA was resuspended in sterile water containing 20µg/ml of pancreatic Dnase free Rnase and stored at -20°C.

2.4.3 Large scale preparation of DNA

5ml bacterial cultures were grown, shaking at 37°C, from glycerol stocks in LB containing 100µg/ml ampicillin and used to innoculate larger 1 litre cultures of LB-Amp which were then grown overnight at 37°C. DNA was isolated using Qiagen Maxiprep kits following the manufacturer's instructions.

2.4.4 cDNA cloning

2.4.4.1 Preparation of electrocompetant *E.Coli* DH5α

E.Coli DH5α were streaked from a glycerol stock on L-broth (10g bacto-tryptone, 5g yeast extract, 10g (0.2M NaCl), pH 7.0 in 1 litre) plate containing 1.5% agar and grown overnight at 37°C. Next day, colonies were picked and each were grown in 10ml LB overnight at 37°C and then used the next day to inoculate 1 litre of LB. The culture was grown at 25°C until OD_{600} reached 0.5, then cells were chilled on ice for 30 minutes, centrifuged at 6000*g* for 15 minutes and resuspended in 1 litre of ice-cold, sterile 1mM HEPES, pH 7. Cells were then centrifuged again at 6000*g* for 15 minutes and

resuspended in 500ml of ice-cold 1mM HEPES, pH7. Cells were then collected again by centrifugation at 6000g for 15 minutes and washed once in 20mls of ice-cold, sterile 10% glycerol and then resuspended in a final volume of 3mls of the same buffer. Cells were then aliquoted and stored at -70°C until use. The efficiency of the cells was checked by transforming known amounts of control DNA.

2.4.4.2 Transformation in competent *E.Coli* DH5α

Electrocompetent cells were thawed and stored on ice until required. DNA from ligation reactions was precipitated with 2.5 volumes of 100% ethanol and 0.25 volumes of 3M NaAcetate, pH 5.2 and resuspended in 5µl of sterile water. DNA was then incubated on ice for 1 minute with 40µl of electrocompetant cells. After incubation the mix was transferred to cold 2mm electroporation cuvettes (Flowgen). The cells were electroporated under the following conditions: V = 2500; C = 0.025; T = 5 m. 1ml of L-broth containing 20mM glucose was then added and the cells allowed to recover at 37°C for 1 hour. Cells were then harvested by centrifugation at 12000*g* for 30-60 seconds, 800µl of supernatant removed, pellet resuspended in remaining 200µl and plated onto L-broth plates containing 1.5% agar and 100µg/ml ampicillin and incubated at 37°C overnight.

2.4.4.3 Transformation of *E. Coli* B834 cells

To increase the solubility of the GST fusion proteins, *E. Coli* B834 cells were transformed with plasmids encoding sequenced and correctly orientated inserts. *E. Coli* B834 cells were streaked from a glycerol stock onto an L-Broth plate containing 1.5% agar and grown overnight at 37°C. Next day cells were picked into an eppendorf tube containing 300µl of sterile water and washed 2-3x with 1ml of sterile water. Transformation of bacterial cells was then carried out as outlined previously.

2.4.4.4 Generation of recombinant plasmids

GST-N βTrCP

The N-terminus (amino acids 2-251) of βTrCP was subcloned from pcDNA3sv5-βTrCP into pGEX 4T3 by amplification of the DNA using the polymerase chain reaction (PCR) and primers, 5'-CGGGATCCATGGACCCGGCCGAGGCG-3' and, 5'-CGGGATCCCTATCTCCAATTAGATTCTATTGT-3'. Primers contained additional *Bam H1* restriction sites (underlined) to facilitate insertion into pGEX 4T3 such that glutathione-S-transferase-N βTrCP fusion proteins can be produced in *E.Coli*.

GST-C βTrCP

The C-terminus of βTrCP, amino acids 252-569, was subcloned from pcDNA3sv5-βTrCP into pGEX 4T3 by amplification of the DNA using the polymerase chain reaction (PCR) and primers, 5'-CGGGATCCTGTGGAAGACATAGTTTACAG-3' and 5'-CGGGATCCTTATCTGGAGATGTAGGTGTA-3'. Primers contained additional *Bam H1* restriction sites to facilitate insertion into pGEX 4T3 such that glutathione-S-transferase-C βTrCP fusion proteins can be produced in *E.Coli*.

GST-βTrCP

Full length βTrCP was subcloned from pcDNA3sv5-βTrCP into pGEX4T-3 by digestion with *Bam H1* restriction enzyme. The resultant cDNA which was produced allowed production of glutathione-S-transferase-full length βTrCP fusion proteins in *E.Coli*.

ΔF-boxβTrCP

F-box deleted mutant of βTrCP (Δ148-190) was generated by cleavage of pcDNA3 SV5 βTrCP with *HindIII* restriction enzyme. This digestion resulted in cleavage of the vector at a *HindIII* restriction site 5' of the original *BamHI* cloning site and also at base pairs 571 within the βTrCP cDNA. pcDNA3 SV5 βTrCP was then used as template to generate a fragment containing the *HindIII* restriction site of the vector, SV5 Pk tag and the N-terminus of βTrCP, amino acids 2-147 by amplification of the DNA using the polymerase chain reaction (PCR) and primers 5'-CCCAAGCTTGGTACCATGGGAAAGCCG-3' and

5'-CCCAAGCTTAGCAGTTATGAAATCTCTCTG-3'. Both primers contained *HindIII* restriction enzyme sites (underlined) which allowed facilitation of the DNA into pcDNA3 SV5 Pk tag βTrCP previously digested with *Hind III* restriction enzyme. This allowed for the generation of cDNA for βTrCP lacking the F-box domain, amino acids 148-190.

2.4.5 DNA sequencing

All constructs were used to transform *E.Coli* DH5α to ampicillin resistance. Plasmid DNA of all constructs were isolated and inserts sequenced (ABI377) by Alex Houston of the University of St. Andrews DNA sequencing facility.

2.5 Expression and purification of recombinant proteins

GST-N, C and full-length βTrCP constructs were expressed in *E.Coli* B834 cells. Induction of expression and glutathione agarose affinity chromatography was performed as described by Jaffray *et al* (Jaffray *et al.*, 1995). E1 ubiquitin activating enzyme was purified to homogeneity from baculovirus infected insect cells by affinity chromatography on ubiquitin Sepharose as described by (Desterro *et al.*, 1999). 6-His Cdc34 and GST-hUbc5 were purified as described by Vuillard *et al* (Vuillard *et al.*, 1999).

2.6 Protein Quantitation

Protein concentrations were determined using Bradford's method (Bradford, 1976). Protein samples were mixed with Bradford's reagent (Biorad) and the absorbance at 595nm measured on a spectrophotometer. Protein absorbance's were converted to mg/ml concentrations using a standard curve constructed by measuring the absorbance's of a range of bovine serum albumin (BSA) concentrations.

2.7 SDS-PAGE and Western blot analysis

Protein samples were resuspended in disruption buffer (1X: 20mM Tris HCl pH6.8, 2% SDS, 5% β-mercaptoethanol, 2.5% glycerol, 2.5% bromophenol blue), denatured by heating to 100°C for 5 minutes, and separated on a SDS-polyacrylamide gel. The percentage of gel used was dependent on the size of protein being analysed. Bio-Rad mini gel equipment was used in accordance with the manufacturers instructions. Protein molecular weight markers (NEB) were used as standards to determine the apparent molecular weights of proteins resolved on SDS-polyacrylamide gels. Separated proteins were stained with Coomassie Blue (0.2% Coomassie brilliant blue R250, 50% methanol, 10% acetic acid) for 20 minutes and then destained (20% methanol, 10% acetic acid), dried and exposed to phosphorimage screen for 10-12 hours, or transferred to a polyvinylidene difluoride membrane (Sigma) using a wet blotter (BioRad systems). Membranes were blocked in blocking buffer (PBS containing 10% skimmed milk powder, 0.1% Tween 20) and incubated with antibodies diluted in blocking buffer. Horseradish peroxidase conjugated anti-rabbit, anti-mouse IgG (Amersham) or anti-sheep IgG (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. Western blotting was performed using Enhanced Chemilumenscent (ECL) detection system. After ECL detection and when necessary Western blots were stripped as described (Roff *et al.*, 1996).

2.8 Cell Culture

Primary Human Umbilical Vein Endothelial Cells (HUVECs) prior to passage 10 were used in all experiments. HUVECS were provided by Ailsa Webster (CellTech) and maintained in EBM-2 medium supplemented with 10% FBS and bullet kit reagents™ (Biowhittaker PLC). Primary Human Coronary cells were provided by Eric Flitney (St. Andrews) and maintained as above. HeLa (Human cervical carcinoma), HeLa 57A, containing an integrated NF-κB dependent reporter gene (Rodriguez *et al.*, 1996) and Cos

7 (Monkey African Green kidney cells containing the SV40 large T antigen gene) were mainteined in exponential growth in Dulbeco's modified Eagle's medium supplemented with 10% FCS. Jurkat T-lymphocytic cells and the following Hodgkin's disease cell lines L540, L591, L428, KMH2, HDMYZ, HDLM2, were maintained in exponential growth in RPMI 1640 containing 10% fetal calf serum (FCS) apart from HDLM2 which contains 20% FCS. All cells were grown in a humidified 5% CO2 and 95% air incubator at 37°C.

2.8.1 Transfection of tissue culture cells by electroporation

HeLa 57A cells were transfected with 5-10μg of plasmid DNA by electroporation (Equibio). One hour prior to transfection the media was changed, following which the cells were trypsinized and counted for each transfection. $5x10^6$ cells were resuspended in 200μl of media supplemented with 15mM HEPES, pH 7.5. The DNA to be transfected was prepared in a total volume of 50μl containing 210mM NaCl and 30μg salmon sperm carrier DNA. DNA and cells were mixed, added to the electroporation cuvettes and electroporated at 240V, 1200mFD. Following electroporation the cells were resuspended in 5ml media containing 15mM HEPES, pH 7.5, centrifuged at 300*g* for 3 minutes, and resuspended in DMEM/10%FCS. 12 hours after transfection, NF-κB activation was determined by measuring luciferase activity.

2.8.2 Transfection of tissue culture cells by lipofectamine

Cos 7 cells were maintained as described above. 1-2μg of plasmid DNAs were transfected for 14 hours in subconfluent $75cm^3$ flasks using Lipofectamine™ according to the manufacturers instructions (Gibco). 36 hours after transfection cells were trypsinized and aliquots seeded into six-well plates and cultured for an additional 36 hours. Cells from a single transfection were stimulated with IL-1 (10ng/ml) for the times indicated. Cells were washed in PBS and cell extracts prepared by lysis in SDS sample buffer (5% SDS, 0.15M

Tris HCl, pH6.7, 30% glycerol) diluted 1:3 in RIPA buffer (25mM Tris HCl, pH 8.2, 50mM NaCl, 0.5% Nonidet P40, 0.5% Deoxycholate, 0.1% SDS, 0.1% Azide) containing complete protease inhibitor cocktail (Roche). 25µl of each lysate was fractionated by 10% or 12.5% SDS-PAGE and transferred to PVDF membrane (Sigma). Protein expression was determined by Western blotting.

2.9 Luciferase assays

12 hours after transfection of HeLa 57A cells by electroporation, the cells were washed twice in PBS and lysed in luciferase assay lysis buffer (25mM Tris phosphate pH8, 8mM MgCl2, 1mM DTT, 1% Triton X-100 and 15% glycerol). Following cell extract protein standardisation, luciferase activity was assayed in luciferase buffer (25mM luciferin, 1mM ATP, 1% BSA) diluted in lysis buffer. Luciferase activity was measured using a Berthold luminometer.

2.10 *In Vitro* Transcription/Translation

In vitro transcription/translation was performed using 1-2µg of plasmid DNAs and a TNT Coupled Rabbit Reticulosyte System (Promega) according to the manufacturer's instructions. As and when required, ^{35}S-methionine (Amersham) was used in the reactions to generate radiolabelled proteins.

2.11 Kinase assays

2.11.1 Immunoprecipitation (IP) of IKK1/IKK2 complex

In vitro transcribed/translated IKK1 and IKK2 were immunoprecipitated for 2hrs, rotating at 4°C in a total volume of 500ul containing IP buffer (40mM Tris HCl, pH8, 500mM NaCl, 0.1% NP-40, 6mM EDTA, 6mM EGTA, 10mM β-glycerophosphate, 10mM NaF,

300μM Na_3VO_4, 1mM Benzamidine, 2μM PMSF, mini-EDTA-free protease inhibitor tablet, 1mM DTT), 25μl of transcription/translation (TNT) mix, 2μg of either affinity purified anti-C-Terminal IKK1 and 15μl of protein G sepharose, or 10 μl protein G-IKK1, made previously by mixing 1ml protein G sepharose with 0.5ml IKK1 sheep serum, or 2μg of anti-FLAG Ab and 15μl of Protein A sepharose. Protein A or Protein G sepharose was first washed 3X with 1ml of PBS. After immunoprecipitation, the samples were centrifuged at 6000g for 1 min at 4°C, supernatant removed and beads washed twice with 1ml of IP buffer and once with 1ml of kinase buffer (200mM HEPES, pH7.7, 2mM $MgCl_2$, 2mM $MnCl_2$, 10mM β-glycerophosphate, 10mM NaF, 300μM Na_3VO_4, 1mM benzamidine, 2μM PMSF, mini EDTA-free protease inhibitor tablet, 1mM DTT), and resuspended in 20μl of kinase buffer.

2.11.2 *In Vitro* Kinase Assays

Kinase reactions were carried out at 30°C for 30 minutes, unless otherwise stated, in a total volume of 28μl containing kinase buffer (see above), 20μl of IP, 5μl of substrate, either (0.1-5μg) GST-IκBα amino acids 1-70, or 30 fmoles IκBα, pre-incubated with 60 fmoles p65, 10μM ATP and 3μCi [γ^{32}P]ATP. For analysis of kinase activity, reactions were stopped by addition of 15μl of 3X SDS sample buffer, separated on a 12.5% polyacrylamide gel, fixed and stained with coomassie blue, destained in 10% acetic acid, 20% methanol, dried and exposed to phosphorimage screen for 4-24hrs. For production of radiolabelled phosphorylated IκBα on serines 32 and 36, samples were centrifuged, supernatant removed and stored at 4°C until use.

2.11.3 Preparation of cell extracts for *in vitro* Kinase Assays

10^6 HUVECs were grown on glass microscope slides before being either treated with 30ng/ml TNF or subjected to laminar shear stress for varying times. Medium was

removed and cells lysed in 1ml whole cell extract (WCE) lysis buffer (20mM Tris HCl, pH 8, 0.5MNaCl, 0.25%Triton X-100, 1mM EDTA, 1mM EGTA, 10mM β-glycerophosphate, 10mM NaF, 300μM Na_3VO_4, 1mM benzamidine, 2μM PMSF, mini EDTA-free protease inhibitor tablet, 1mM DTT). Samples were clarified by centrifuging at 270000*g* for 30 minutes. Supernatant was removed and protein concentration determined by Bradford Assay. Equimolar amounts of lysate was added to 20μl protein G previously coupled to anti-C terminal IKK1 peptide serum and kinase immunoprecipitated for 30 minutes at 4°C. Beads were washed 2x in WCE lysis buffer, 2x in IP buffer and 1x in kinase buffer. Kinase reactions were carried out at 30°C in a shaking water bath in a total volume of 30μl containing kinase buffer, 10μl beads, 5μl of either GST-IκBαN7-1 GST-IκBαNS/A or GST-IκBαNS/E, 10μM ATP and 3μCi [γ-32P]-ATP. Reactions were stopped after 1 hour by the addition of 10μl 3xSDS loading buffer and analysed on a 10% polyacrylamide gel containing SDS. Gels were dried, exposed to a phosphorimage screen and quantified using a phosphorimager (Fujix BAS1500, MacBas software).

10^6 HeLa, Jurkat or Hodgkin cells (L591, L540, L428, KMH2, HDLM2 and HDMYZ) were stimulated or not with 10ng/ml TNF for 10 minutes, centrifuged at 300*g* for 1 minute, medium removed and cells washed once in 1ml ice-cold PBS. Samples were lysed in 1ml ice-cold WCE lysis buffer and clarified by centrifuging at 270000*g* for 30 minutes. Protein concentrations were determined by Bradford Assay and IKK complex immunoprecipitated from equal amounts of extract as above. Kinase assays were carried out as above.

2.12 Laminar Flow

A flow chamber was designed according to Viggers *et al* (Viggers *et al.*, 1986). The HUVECs were exposed to laminar shear stress generated by circulating tissue culture medium through a hydrostatic pump connected to the upper and lower reservoirs (Bhullar *et al.*, 1998). The medium was gassed with CO_2 prior to each run, and the temperature

was maintained at 37°C using the flow apparatus in a sterile cabinet in a temperature controlled room. The shear stress determined by the flow rate perfusing the channel and the channel dimensions, was 15 dynes/cm^2. Static control experiments were performed on HUVECs maintained on slides without being exposed to laminar shear stress.

2.13 Baculovirus cloning

The Gibco Life Technologies Bac-To-Bac Baculovirus Expression System was used to generate baculoviruses containing βTrCP, Skp1, Cul1/cdc53 and Rbx1. Briefly, βTrCP, Skp1, Cul1/cdc53 and Rbx1 were cloned into pFastBac donor plasmid using conventional cloning methods as described before. Vectors containing correctly orientated and sequenced inserts were then transformed into *DH10Bac* for transposition into the bacmid. Cells were streaked on LB-plates containing 50μg/ml kanamycin, 10μg/ml gentamycin, 10μg/ml tetracycline, 100μg/ml Bluo-gal and 40μg/ml IPTG. Colonies containing the recombinant bacmid appear white due to disruption of the *lacZα* peptide. Recombinant bacmid was then isolated and used to transfect Sf9 cells to obtain recombinant baculovirus according to the manufacturer's instructions.

2.14 Determination of virus titre - plaque assays

10^6 Sf9 cells were seeded in 6-well plates to form an even monolayer with 60% confluence. Serial log dilutions of virus were prepared using TC-100 growth medium as dilutant. Once cells have settled, media was removed and 100μl of the appropriate dilution was added dropwise to the centre of each well and plates rocked very gently every 10-15 minutes for 1 hour to ensure even coverage of the virus. Each dilution was done in triplicate. After 1 hour virus was removed and 2ml of agarose overlay (1 part 4% low-melting agarose, 2 parts TC-100 medium, heated to 37°C) was added and allowed to set before adding 1 ml TC-100 medium on top. Plates were then placed in a sandwich box

lined with moist filter paper and incubated at 28°C for 3-4 days. Medium was then removed and 1 ml diluted neutral red (1:20 dilution in PBS) was added and incubated for 2-4 hours at 28°C, removed and plates inverted and stored at room temperature overnight. Next day plaques were counted and viral titre determined. Titre (pfu/ml) = number of plaques/well x 10 x dilution.

2.15 Expression of $SCF^{\beta TrCP}$ in Sf9 cells

$75x10^6$ cells were infected with baculovirus containing Skp1, Cul1(cdc53), Rbx1 and βTrCP at an multiplicity of infection (m.o.i) of 5 for each virus. Virus for each of the four components of the SCF complex were first mixed together and incubated at 28°C for 1hour. Sf9 cells were then infected with the viruses for 1 hour at 28°C, centrifuged and resuspended in 50ml TC-100 growth medium containing 7% FCS and incubated for 68-72hours at 28oC. Cells were then divided into 4 tubes, collected by centrifugation for 3 minutes at 500*g*, washed 3x with PBS and stored at -70°C until required. SCF complex was isolated by lysis of the cell pellet in 500µl of lysis buffer (10mM HEPES, pH 7.6, 1.5mM $MgCl_2$, 10mM KCl, 5mM DTT, 1% NP-40, 10% glycerol and a mini-EDTA-free protease inhibitor tablet) and lysate clarified by centrifugation at 35000*g* for 30 minutes at 4°C. The supernatant was then incubated for two hours at 4°C with 200µl protein A sepharose beads previously linked to SV5 Pk Tag, 336 monoclonal antibody. Beads were then washed 3x in lysis buffer and 8µl beads used in subsequent reactions.

2.16 Expression of βTrCP in Sf9 ccIls

$75x10^6$ cells were infected with baculovirus containing βTrCP at multiplicity of infection (m.o.i) of 5. Sf9 cells were infected with virus for 1 hour at 28°C, centrifuged and resuspended in 50ml TC-100 growth medium containing 7% FCS and incubated for 68-72hours at 28°C. Isolation of βTrCP was then carried out as above (section 2.15).

2.17 *In vitro* Ubiquitintion Assay

30 fmoles IκBα, pre-incubated with 60 fmoles p65 was phosphorylated on serines 32 and 36 as described in section 2.11.2. 5µl of phosphorylated IκBα was then incubated for 2 hours at 37°C in a total volume of 20µl containing 50mM Tris HCl/5mM $MgCl_2$, an ATP regeneration system (2mM ATP, 10mM creatine phosphate, 3.5 units/ml creatine kinase), 0.6 units inorganic pyrophosphate, 8µl SCF^{bTrCP} beads, isolated as in section 2.14, 150ng E1 ubiquitin-activating enzyme, 150ng cdc 34 or ubc 5 and 1mg/ml ubiquitin. Reactions were then stopped by the addition of 10µl 3x SDS sample buffer, separated by 8.5% SDS-PAGE, fixed and stained, destained, gels dried and exposed to phosphorimage screen and quantified using the phosphorimager, Fujix 1500, MacBas software.

2.18 *In vitro* Ubiquitination Assay in presence of IκBα peptides

Ubiquitination assays were performed as above in a total volume of 25µl containing 5µl of phosphorylated IκBα, 50mM Tris HCl, 5mM $MgCl_2$ an ATP regeneration system (2mM ATP, 10mM creatine phosphate, 3.5µnits/ml creatine kinase), 0.6 units inorganic pyrophosphate, 8µl SCF^{bTrCP} beads, isolated as in section 2.14, 150ng E1, 150ng cdc 34 or ubc 5, 1mg/ml ubiquitin and differing concentrations of IκBα peptide.

2.19 Interaction Assay

2.19.1 GST-WT βTrCP

30 fmoles IκBα, pre-incubated with 60 fmoles p65 was phosphorylated on serines 32 and 36 as described in section 2.11.2. 50µl of GST-WT βTrCP beads or GST beads corresponding to the same amount of protein, were then washed 2x in PBS/0.5MNaCl and 1x in kinase buffer (section 2.11.1) + 1mg/ml BSA, and incubated, rotating for 30 minutes at room temperature. Interaction assays were carried out by pre-incubating the beads for 1 hour at room temperature in 60µl of kinase buffer/1mg/ml BSA containing the desired

concentration of peptide and then adding 40µl (30 fmoles) of radiolabelled, phosphorylated IκBα and incubating for a further hour at room temperature. Beads were then washed 3x in 1ml wash buffer (10mM Tris HCl, pH8, 100mM NaCl, 1mM $MgCl_2$, 0.1% NP-40), resuspended in 10µl kinase buffer + 5µl 3x SDS sample buffer, separated on a 10% SDS-PAGE, gels dried, stained and destained and exposed to phosphorimage screen. Amount of radiolabelled IκBα pulled down was analysed using the Fujix 1500, MacBas software.

2.19.2 Baculovirus βTrCP

$75x10^6$ insect cells were infected as before with baculovirus containing βTrCP at a m.o.i of 5. See section 2.15 and 2.16. βTrCP was isolated by lysis of the cell pellet in 500µl of lysis buffer (10mM HEPES, pH 7.6, 1.5mM $MgCl_2$, 10mM KCl, 5mM DTT, 1% NP-40, 10% glycerol and a mini-EDTA-free protease inhibitor tablet) and lysate clarified by centrifugation at 35000*g* for 30 minutes at 4°C. The supernatant was then incubated for two hours at 4°C with 200µl protein A sepharose beads previously linked to SV5 Pk Tag, 336 monoclonal antibody. Protein A-336-βTrCP beads were then washed 3x in lysis buffer and 10µl incubated for 2 hours at 37oC in a total volume of 50µl, containing kinase buffer and varying concentrations of either ^{32}P-labelled IκBα or ^{125}I-labelled IκBα peptide. For ^{32}P- IκBα, beads were washed and analysed as above. For ^{125}I-labelled IκBα peptide, beads were washed as above and the amount of radioactivity measured by a Mini-assay type 6-20 (Mini-Instruments Ltd).

2.20 Iodination of IκBα peptides

IκBα peptides were radiolabelled with carrier-free Na ^{125}I (Amersham) ($6x10^4$ d.p.m/pmol) by the Chloramine-T method (Ciechanover *et al.*, 1980).

2.21 Scintillation Proximity Assays (SPA)

βTrCP infected Sf9 cell pellet was lysed in lysis buffer (10mM HEPES, pH7.6, 1.5mM $MgCl_2$, 150mM KCl, 1% Triton X-100, 10% glycerol, 0.5mM DTT) on ice and lysate clarified by centrifugation at 12000*g* for 30 minutes. Protein A PVT SPA antibody binding beads (Amersham) were resuspended in lysis buffer at a concentration of 1mg/20μl. Beads were incubated with anti-SV5, PkTag antibody at a relative mass of 1mg/μg at 4°C for 12 hours. Beads were washed 3x in lysis buffer and subsequently incubated with βTrCP infected Sf9 cell lysate at a concentration of 1mg/20μl for 3 hours at 4°C. Competitive binding assays were performed in triplicate by incubating 100μg of βTrCP conjugated beads in a total volume of 100μl of assay buffer (10mM HEPES, pH 7.6, 1.5mM $MgCl_2$, 150mM KCl, 1% Triton X-100, 10% glycerol) containing 0.05μCi ^{125}I labelled wild type IκBα peptide (LKKERLLDDRHDS(PO4)GLDS(PO4)MKDEEYE) and serial log dilutions of the competing IκBα peptide, ranging from 100μM to 0.01nM. Reactions were performed in 96 well white non-binding surface clear bottom plates (Corning 3600) for 12 hours. Radioactivity was measured on the Microbeta Trilux.

3. RESULTS

3.1 Development of an *in vitro* Kinase Assay

3.1.2 Summary

Signal-induced degradation of IκBα and subsequent activation of NF-κB has been shown to occur following phosphorylation of IκBα on serine residues 32 and 36. Identification of a large kinase complex from TNFα stimulated Hela cells by Didonato *et al* (DiDonato *et al.*, 1997), showed that this complex was capable of phosphorylating IκBα on serines 32 and 36 and also IκBβ on serines 19 and 23. Further studies identified that the complex contained two structurally and functionally similar kinases which were named IKKα (IKK1) and IKKβ (IKK2). The discovery of an IκBα kinase complex led to the development of an *in vitro* kinase assay in which IKKα (IKK1) and IKKβ (IKK2) were used to specifically phosphorylate recombinant IκBα on serine residues 32 and 36. Optimal conditions for IKK complex activity were established. The active enzyme was then used to generate IκBα phosphorylated on serine residues 32 and 36. ^{32}P-IκBα could then be used to investigate the properties of IκBα ubiquitination or of its interaction with the F-box protein βTrCP. (Sections 3.2 and 3.3). Antibodies generated against the kinase were used to investigate the activity of these kinases *in vivo*. Immunoprecipitation of the IKK1/IKK2 complex from cell lines treated with cytokines, allowed investigation of the kinase activity in a number of different cell lines and to illustrate that the kinase complex can be activated in response to TNFα. It was also demonstrated that the kinase complex was constitutively active in Hodgkin's cell lines, verifying the fact that there is a constitutively active NF-κB pathway in these cells. Furthermore, it was demonstrated that kinase activity could be stimulated in human umbilical vein endothelial cells (HUVECs) subjected to laminar flow.

Overall, the development of an IKK1/IKK2 kinase assay, allowed us to produce an investigative tool that can be used to analyse the NF-κB signalling pathway further.

3.1.3 Immunoprecipitation of *in vitro* transcribed and translated IKK 1 and IKK 2

To efficiently and specifically phosphorylate IκBα on serine residues 32 and 36, assay conditions were optimised. Initially, as there was no commercially available IKK1 or IKK2 antibodies, peptides corresponding towards the last twelve amino acids (SMMNLDWSWLTE) of IKK1, residues 734 to 745, or the first twelve amino acids (MERPPGLRPGAG) were generated. The corresponding C-terminal amino acids are also present in the C-terminus of IKK2, residues 737-745, but with an additional glutamate residue in the sequence (LDWSWLQTE). Peptide was synthesised with an addtional cysteine residue at the N-terminus to allow coupling to antigen keyhole limpet heamocyanin (KLH). These coupled peptides were then used to immunise sheep (Scottish Antibody Production Unit, SAPU) in order to generate anti-IKK antibodies. Serum was collected and either used directly or antibodies affinity purified on a thiol sepharose column coupled to the immunising peptide. IKK2 constructs contained an N-terminal FLAG epitope composed of an eight amino acid sequence DYKDDDDK. This sequence can be recognised by an anti-FLAG antibody. Positions of antibody recognition epitopes are shown in Figure 8a.

IKK1, IKK2 and NIK were next translated in rabbit reticulosytes in the presence of ^{35}S-methionine to verify that each of the kinases was translated efficiently *in vitro*. Translated products were separated by SDS-PAGE and visualised by exposing the gels to phosphorimage screen for 10 to 12 hours. Figure 8b shows that the kinases could be transcribed and translated efficiently in this *in vitro* system. To verifiy the identity of the *in vitro* translated kinases, varying amounts of ^{35}S-methionine labelled IKK1 and IKK2 were immunoprecipitated with either anti-C-terminal IKK1 antibody, or anti-FLAG antibody respectively. Antibody-antigen complexes were captured on either anti-C terminal IKK1 antibody linked Protein G or anti FLAG antibody linked Protein A. After two hours of incubation at 4°C with the antibody and beads, supernatants were removed and kept, beads

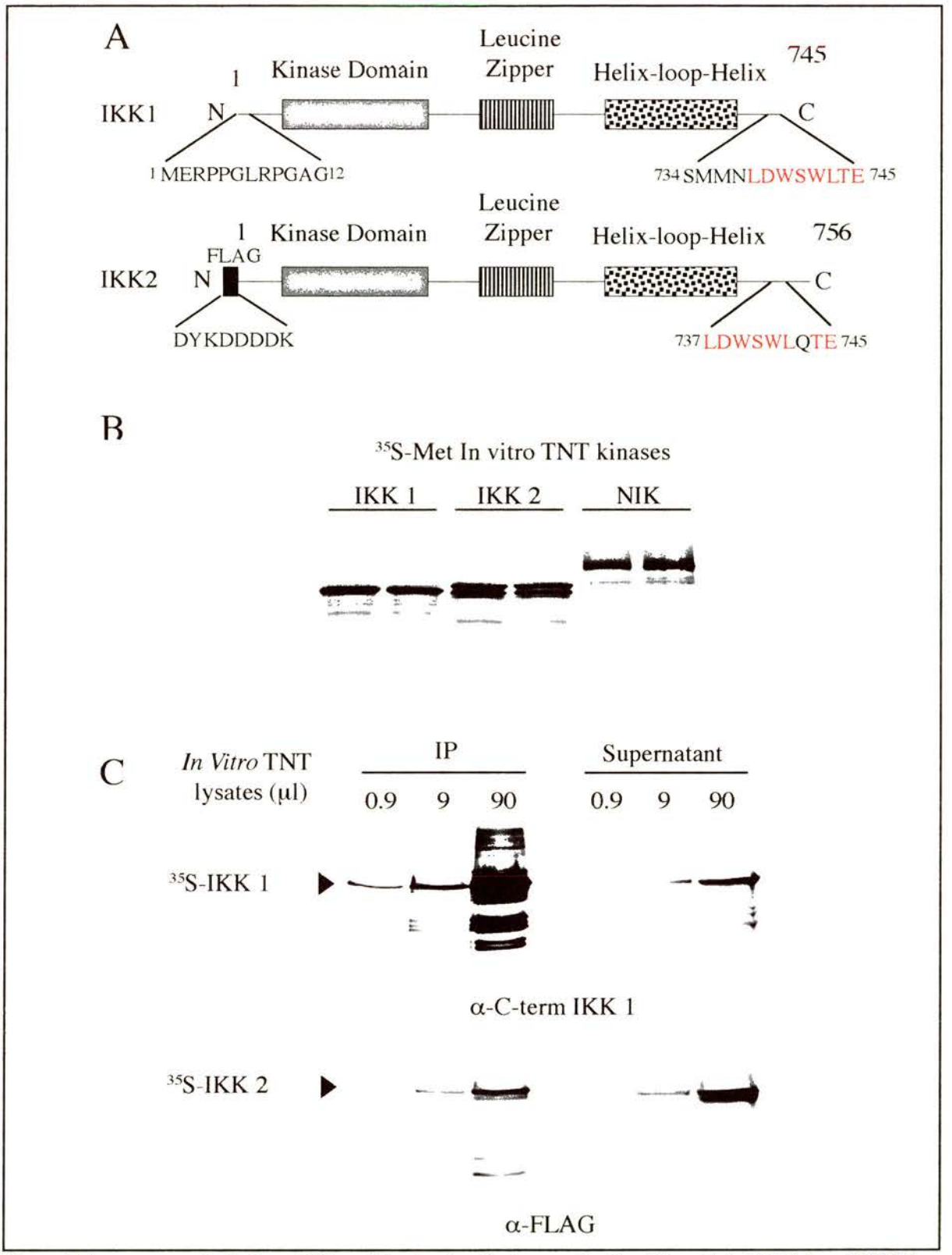


Figure 8 Immunoprecipitation of ^{35}S-methionine *in vitro* transcribed and translated IKK 1 and IKK 2.
A. Schematic representation of IKK1 and IKK2 constructs. Anti-C terminal IKK1 antibody was raised against a 12 amino acid epitope (SMMNLDWSWLTE) at the C-terminus of IKK1. Homologous C-terminal residues between IKK1 and IKK2 are highlighted in red. Anti N-terminal IKK1 antibody was raised against a 12 amino acid epitope (MERPPGLRPGAG) at the N-terminus of IKK1. IKK2 contains an N-terminal FLAG tag (DYKDDDDK). B. 1µg of pcDNA3 containing either IKK1, IKK2 or NIK were translated in rabbit reticulocytes in the presence of ^{35}s-methionine, in a total volume of 25µl according to the manufacturer's instructions. 1µl of this mix was then separated by SDS-PAGE(10%), gels stained in Coomassie blue, destained, dried and exposed to phosphoimage screen for 12 hours. C. 4µg of pcDNA3 containing IKK1 or IKK2 were translated in rabbit reticulosytes in a total volume of 100µl. IKK1 or IKK2 were then immunoprecipitated from either 0.9µl, 9µl or 90µl of in vitro transcription/translation mix in a total volume of 500µl containing IP buffer and either 10µl protein G and 2µg anti C-terminal antibodyIKK1 (IKK1) or 10µl protein A and 2µg anti-FLAG antibody (IKK2). After two hours incubation at 4°C, supernatants were removed, beads washed twice in IP buffer and then resuspended in 500µl of IP buffer. 20µl from the IP and supernatants were then separated by SDS-PAGE (10%), gels stained in Coomassie blue, destained, dried and exposed to phosphorimage screen for 12 hours.

washed and resuspended in an equivalent amount of SDS disruption buffer diluted in IP buffer. Equal amounts of samples were separated by SDS-PAGE and exposed to phosphorimage screen for 10-12 hours. As shown in Figure 8c, ^{35}S labelled kinases IKK1 and IKK2 can be efficiently immunoprecipitated with the specific antibodies. This indicates that the antibodies recognise the native IKKs and can be used to capture IKKs from various sources.

Although the proteins immunoprecipitated represented the major labelled products it was important to verify that these products were indeed IKK1 and IKK2. 25µl *in vitro* transcription/translation reactions carried out with unlabelled methionine were set up with either 1µg of pcDNA3 empty vector or 1µg pcDNA3 containing cDNA for either IKK1 or IKK2. 0.5µl of transcription/translation mix was separated by SDS-PAGE, transferred to PVDF membrane and Western blotting performed using either an anti-C-terminal IKK1 antibody, anti-N-terminal IKK1 antibody or anti-IKK1 antibody H7-44 (Santa Cruz) to detect IKK1, or an anti-FLAG antibody (Kodak) to detect IKK2. As is shown in Figure 9a, detection of IKK1 is apparent using all three antibodies. To determine that this polypeptide has the same molecular weight as protein translated *in vivo,* Hela cells were lysed in SDS-disruption buffer and 25µg of extract was also separated by SDS-PAGE and subjected to Western blotting with anti-IKK1 antibodies. A polypeptide species of the predicted molecular weight of 83kDa was detected that co-migrated with the *in vitro* translated material. *In vitro* transcribed and translated empty vector, gave no signal, confirming the specificity of the antibodies. Therefore the specificity of the antibodies allows confirmation that the *in vitro* transcribed/translated product is the same as the *in vivo* translated kinase.

As for IKK1, 0.5µl of either pcDNA3, IKK1 or IKK2 transcription/translation mix was separated by SDS-PAGE, transferred to PVDF membrane and Western blotted using an anti-FLAG antibody, which recognises a FLAG epitope fused to the N-terminus of the IKK2 construct. As shown in Figure 9b, no polypeptide of the correct molecular weight is

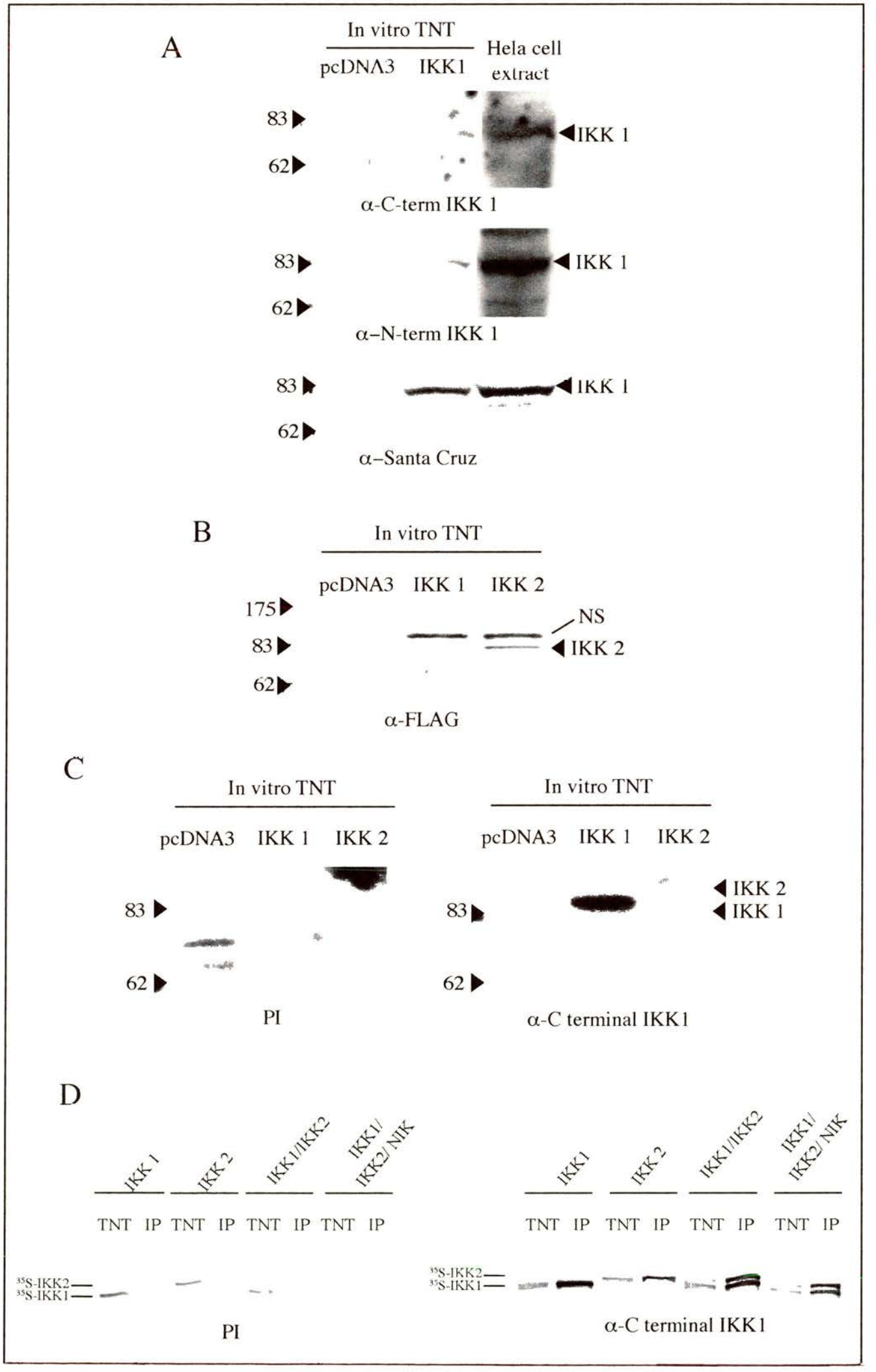
apparent in either the pcDNA3 or IKK1 lanes, whereas there is a specific polypeptide detected in the IKK2 lane. Therefore it has been show that IKK2 is being specifically immunoprecipitated by this antibody due to the fact that IKK1 or any of the proteins in the transcription/translation mix, do not contain this FLAG epitope, and that the species in the Western blot corresponds to the correct molecular weight of IKK2.

Isolated kinase was required for the establishment of an *in vitro* kinase assay. After detection of the kinases by the specific antibodies, the anti-C terminal IKK1 antibody was chosen as the antibody that was the most efficient in isolating IKK1 polypeptides. In order to verify the specificity of the antibody, Western blotting was performed using equivalent amounts of affinity purified IgG from pre-immune serum. 2µl of *in vitro* transcribed and translated empty vector, pcDNA3, IKK1 or IKK2 was separated by SDS-PAGE, transferred to PVDF membrane and Western blotting performed using either pre-immune IgG antibody or anti-C terminal IKK1 antibody. As can be shown in Figure 9c, no obvious polypeptides can be detected upon exposure of the blot to pre-immune IgG, whereas anti-C terminal IKK1 recognises polypeptides corresponding to both IKK1 and to a lesser extent, IKK2. Therefore specificity of the anti-C terminal IKK1 antibody is shown by the ability of this antibody to detect an IKK1 polypeptide. This antibody can also detect IKK2 although to a much weaker extent.

As IKK2 is weakly detected in a Western blot by anti-C terminal IKK1 antibody, it was important to verify that in the immunoprecipitated complex, both IKK1 and IKK2 are equally and efficiently isolated. Kinases were *in vitro* transcribed and translated in the presence of ^{35}S-methionine and immunoprecipitated with anti-C terminal IKK1 antibody or an equivalent amount of affinity purified pre-immune IgG. Immunoprecipitated kinases were separated by SDS-PAGE and exposed to phosphorimage screen for 10-12 hours. Figure 9d demonstrates that both IKK1 and IKK2 alone or in complex are efficiently immunoprecipitated using the anti-C terminal IKK1 antibody. 1µl of TNT mix was separated by SDS-PAGE to verify that the band that was immunoprecipitated was that of

Figure 9 Western blot and immunoprecipitation of *in vitro* transcribed/translated IKK1 and IKK2.

1μg of empty vector or vector containing cDNA for IKK1 or IKK2 were translated in rabbit reticulosytes without ^{35}S-methionine in a total volume of 25μl according to the manufacturer's instructions. 0.5μl of transcription/translation (TNT) mix was then separated by SDS-PAGE (10%), proteins transferred to PVDF and Western blotting performed using (A) an anti-C-terminal IKK1 antibody, anti-N-terminal IKK1 antibody or an anit-IKK1 rabbit polyclonal anitbody (H-744) Santa Cruz. 25μg of Hela cell extracts, prepared by lysis of 10^6 cells in 1x SDS disruption buffer was used as a positive control. (B) Detection of IKK2 polypeptides by western blotting was performed using an anti-FLAG monoclonal antibody which recognises a FLAG epitope fused to the N-terminus of IKK2. (C) Specificity of anti-C terminal IKK1 antibody and determination of cross-reactivity with IKK2 was analysed by Western blotting with pre-immune IgG (PI) and anti-C terminal IKK1 antibodies. 1μg of empty vector or vector containing cDNA for IKK1 or IKK2 were translated in rabbit reticulosytes without ^{35}S-methionine in a total volume of 25μl according to the manufacturer's instructions. 2μl of transcription/translation mix was then separated by SDS-PAGE (10%), proteins transferred to PVDF and Western blotting performed as described in Materials and Methods. (D) 1μg of vector containing cDNA for IKK1 or IKK2 were translated separately or together in rabbit reticulosytes in the presence of ^{35}S-methionine in a total volume of 25μl according to the manufacturer's instructions. 25μl of TNT mix were then immunoprecipitated with either pre-immune IgG (PI) or anti-C terminal IKK1 antibodies. Immunoprecipitated products were then washed and immunoprecipitated samples (IP) or 1 μl of TNT mix (TNT) were separated by SDS-PAGE (10%), gels dried and exposed to phosphoimage screen for 10-12 hours. Molecular weight markers in kDa are indicated, along with the specific proteins. NS, non-specific.


A
In vitro TNT
pcDNA3
IKK1
Hela cell extract
83
62
IKK 1
α-C-term IKK 1
α–N-term IKK 1
α–Santa Cruz
B
In vitro TNT
pcDNA3
IKK 1
IKK 2
175
NS
α-FLAG
C
PI
α-C terminal IKK1
D
IKK 1
IKK 2
IKK1/IKK2
IKK1/ IKK2/ NIK
TNT
IP
35S-IKK2
35S-IKK1

the *in vitro* transcribed and translated protein. Furthermore, the specificity of the antibody is demonstrated by the fact that no kinases are immunoprecipitated by the pre-immune IgG. Hence, the anti-C terminal IKK1 antibody efficiently isolates both IKK1 and IKK2.

3.1.4 *In vitro* transcribed and translated IKK1 and IKK2 exhibit kinase activity

In order to establish that the *in vitro* transcribed and translated IκBα kinases were functional, an *in vitro* kinase assay was developed. Figure 10a shows the region of IκBα that was used as substrate for the kinase assay. Amino acids 1-70 of IκBα was cloned and purified as a GST fusion protein, GST-N IκBα. This region contains serine residues 32 and 36. GST-IκBα fusion proteins were also generated which contained either aspartate or glutamate residues in these positions. IKK1 and IKK2 were either transcribed and translated separately or together in rabbit reticulocytes. IKK1 and IKK1/IKK2 complexes were immunoprecipitated with anti-C terminal IKK1 antibody and IKK2 was immunoprecipitated with anti-FLAG antibody. The immunoprecipitated kinases were either incubated with 1μg GST or 1μg GST-N IκBα in the presence of [γ32P]-ATP. Reactions were carried out at 30°C for 0, 5, 15, 30, 60 or 120 minutes and stopped by the addition of SDS-PAGE disruption buffer. Samples were separated by SDS-PAGE and the dried gels exposed to a phosphorimaging screen for 10-12 hours. As shown in Figure 10b, both IKK1 and IKK2 phosphorylate GST-N IκBα, with the extent of phosphorylation increasing with time. It appears that the activity of IKK2 is greater than that of IKK1, but comparable to the activity of the IKK1/IKK2 complex. No phosphorylation is detected when incubated with GST alone indicating that the kinases specifically phosphorylate IκBα. Thus *in vitro* transcribed and translated IκBα kinases alone or together in a complex are able to phosphorylate IκBα.

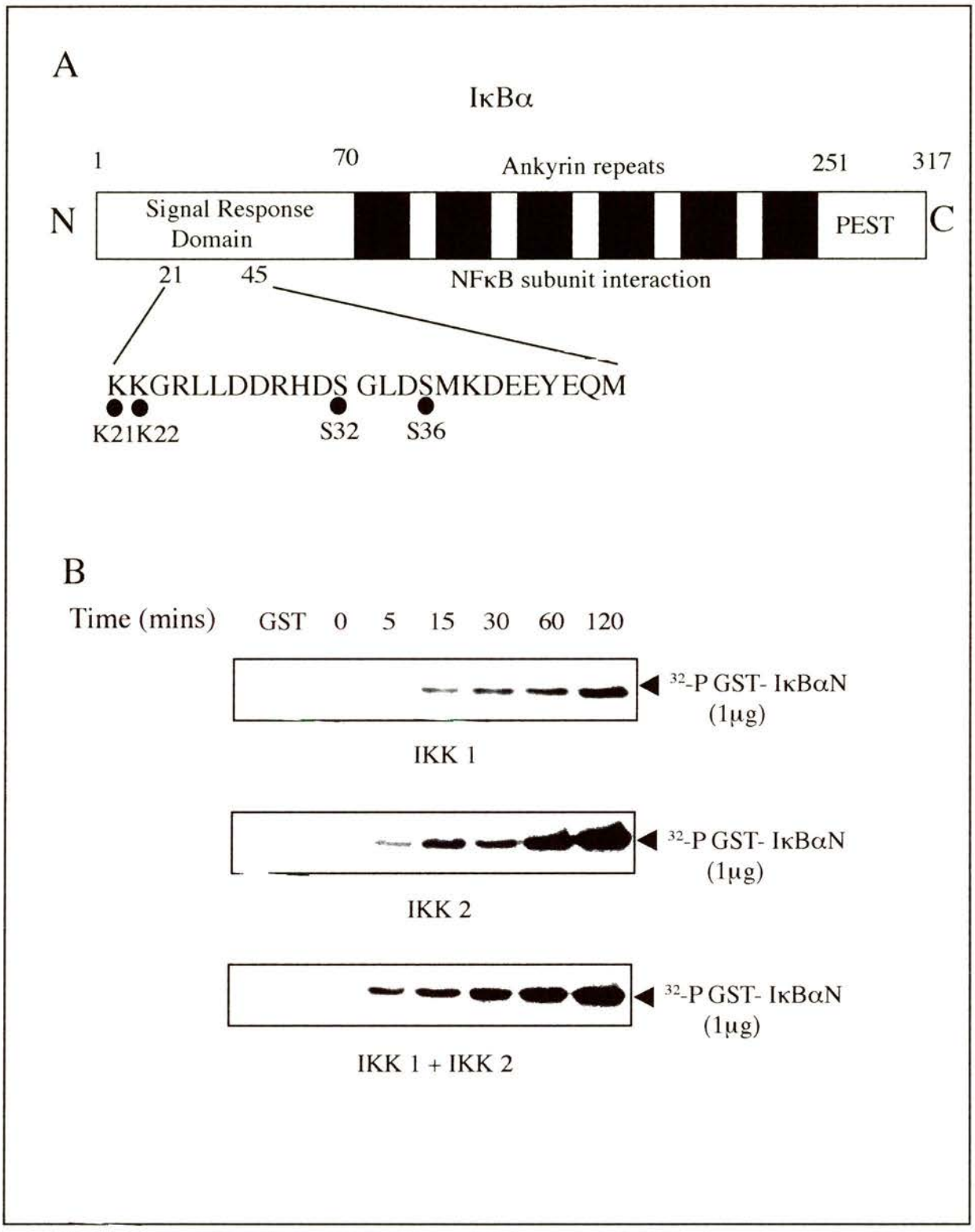


Figure 10 ***In vitro*** **transciption/translated IKK1 and IKK2 are catalytically active.**

A. Schematic representation of IκBα protein indicating the N-terminal signal response domain, NFκB subunit interaction domain and the C-terminal PEST region. Lysines residues 21 and 22 which are ubiquitinated and serines 32 and 36 which are phosphorylated in response to extracellular stimulation are shown. Amino acids 1-70 of IκBα have been produced as a GST fusion protein and used as substrate in the IKK assay. Kinase assays were carried out as described in Materials and Methods. Briefly, 25μl of *in vitro* transcription/translated kinases were immunoprecipitated with 10μl of anti-C terminal IKK1 antibody coupled to Protein G for 2 hours at 4°C in a total volume of 500μl of IP buffer. Immunoprecipitated IKK complex was then incubated at 30°C in the presence of kinase buffer containing γ^{32}P-ATP and 1μg GST-NIκBα for the indicated tmes. GST was used as a negative control. Reactions were stopped by the addition of SDS sample buffer, and samples were separated by SDS-PAGE (10%), gels stained in coomassie blue, destained and exposed to phosphoimage screen for 12 hours. Phosphorylated GST-IκBα (amino acids 1-70) is shown.

3.1.5 Phosphorylation of IκBα by an IKK complex in the presence of NIK

The mechanism by which the IKK signalsome is activated is still to be fully elucidated and the upstream signalling pathways which converge at the point of the IKK complex are still under investigation. It is thought that NIK, a MAP3K, is involved in the activation of the complex. NIK was co-translated in the presence of either IKK1 or IKK2 alone, or both together. Figure 11a shows that when NIK is translated in the presence of IKK1, IκBα phosphorylation is increased. However, as shown in Figure 11b, when NIK and IKK2 are translated together, the extent of IκBα phosphorylation decreases. Figure 11c shows that when all three kinases are translated together, the phosphorylation of IκBα increases, and even more so than when IKK1 and NIK are co-translated, suggesting that, although IKK2 seems to be inhibited by NIK, in the presence of IKK1, there is a synergistic effect. The specificity of the kinases on phosphorylating IκBα on serines 32 and 36 is demonstrated by the fact that no phosphorylation of an IκBα S32,36D mutant is observed. The observation that there is no phosphorylation of IκBα when there is no kinase present suggests the assay is specific.

Therefore it has been demonstrated that phosphorylation of recombinant IκBα can be achieved *in vitro* using IKK1, IKK2 and NIK co-translated in rabbit reticulosytes. Assay conditions were investigated and specificity of phosphorylation of IκBα on serine residues 32 and 36 was demonstrated.

3.1.6 IKK activity can be stimulated by TNFα *in vivo*

Upon extracellular stimuli nuclear NF-κB can be detected within 30 minutes. This activation process occurs via a series of intracellular signalling pathways, which involve the IKK signalsome. To investigate this process we employed the developed IKK assay to determine the activity of IKKs *in vivo*. Kinase activity was evaluated in two transformed

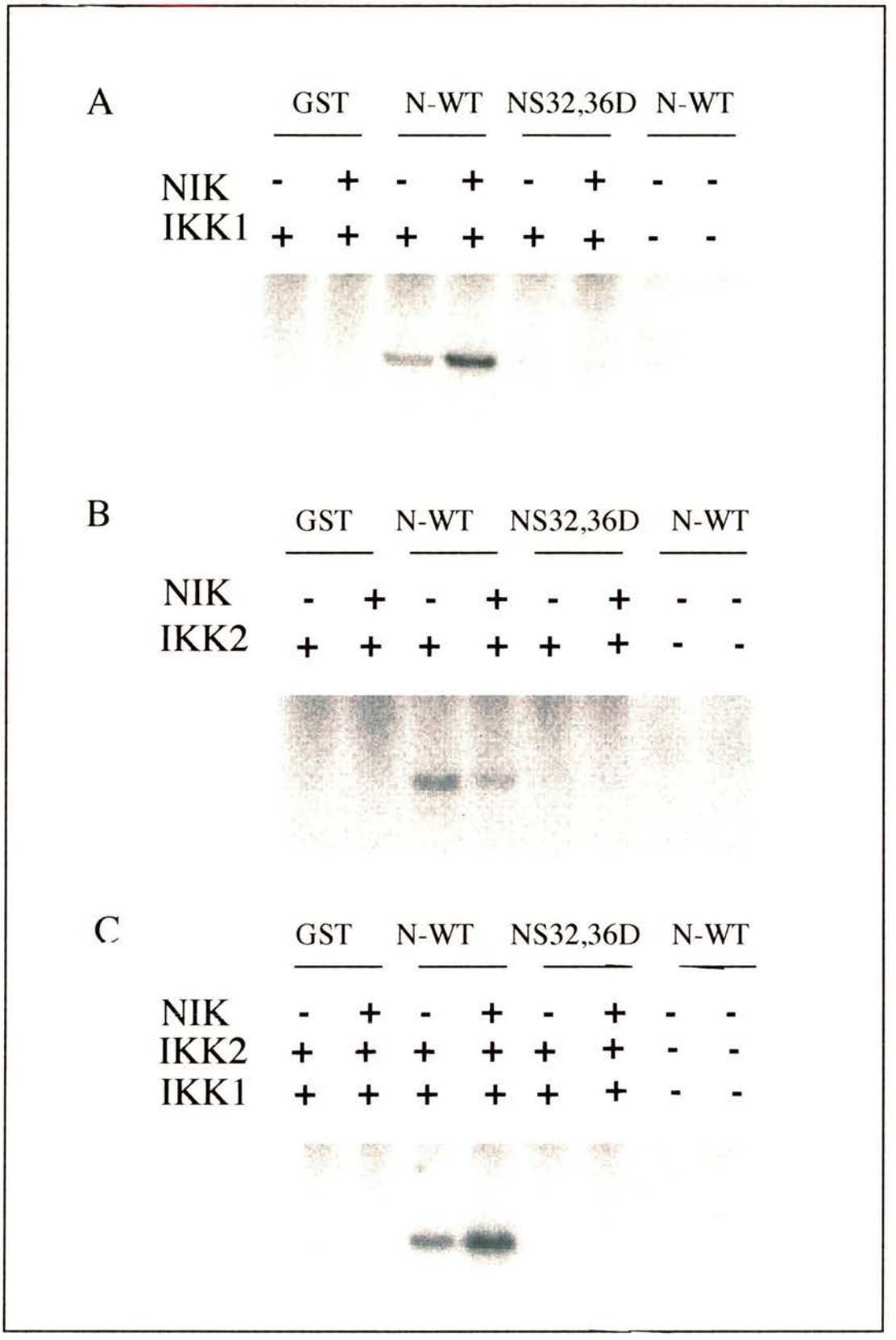


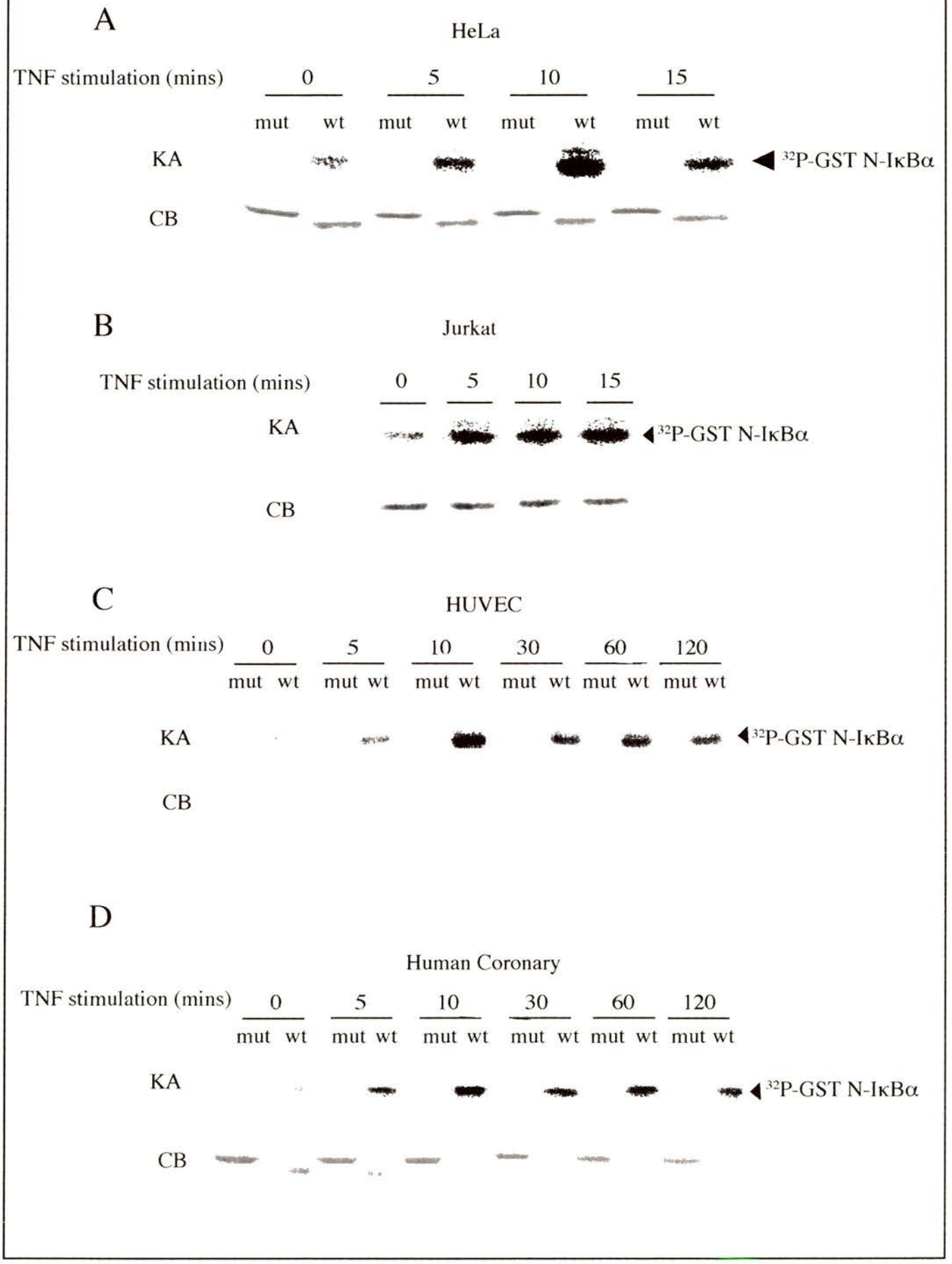
Figure 11 Phosphorylation of IκBα by an IKK1/2 complex.
Kinase assays were carried out as described in Materials and Methods. IKK1 (A), IKK2 (B) or IKK1+IKK2 (C) were translated in rabbit reticulosytes in total volume of 25μl in the absence or presence of NIK. Kinase was then immunoprecipitated with 10μl of protein G-IKK1 beads for 2 hours at 4°C in a total volume of 500μl of IP buffer. Immunoprecipitated IKK complex was then incubated at 30°C for 1 hour in the presence of kinase buffer containing γ^{32}P-ATP and 1μg of either GST, GST-NIκBα or GST-N IκBα S32/36D. Translation of empty vector, pcDNA3, served as a no kinase, negative control. Reactions were stopped by the addition of SDS disruption buffer, samples separated by SDS-PAGE (10%), gels stained in coomasssie blue, destained, dried and exposed to phosphoimage screen for 12 hours.

cell lines, Hela and Jurkat, and in two primary cell lines, Human umbilical vein endothelial cells (HUVEC) and human coronary cells. Hela and Jurkat cell lines were stimulated for varying times with 10ng/ml TNFα and HUVEC and Human coronary cell lines were stimulated for varying times with 30ng/ml TNFα. At each time point cells were lysed by the addition of hypotonic lysis buffer and lysates clarified by centifugation at 270000*g* for 30 minutes. Supernatants were removed and protein concentrations were determined for each sample by Bradford Assay. IKK complex was immunoprecipitated from equimolar amounts of protein extract by the addition of Protein G-sepharose beads previously coupled to anti-C terminal IKK1 peptide serum. Beads were washed and incubated with 1μg of GST-N IκBα or 1μg GST-N IκBα S32,36E for 60 minutes at 30°C in the presence of [γ^{32}P]-ATP. Reactions were stopped by the addition of SDS disruption buffer, the products separated by SDS-PAGE and the dried gels exposed to a phosphorimaging screen. Activation of the kinase complex after TNFα stimulation is indicated by an increase in phosphorylation of GST-N IκBα, in each cell line tested. Figure12. For Hela, HUVEC and Human coronary cells, maximal IKK activation is observed after 10 minutes. Jurkat cells show activation after 5 minutes, which is sustained for 15 minutes. The specificity of the kinase complex is shown by the lack of phosphorylation of the IκBα molecule containing mutations in the phosphoacceptor serine residues 32 and 36. Coomassie blue staining of the gel reveals that equal amounts of substrate were added to each assay. Therefore IKK activity can be detected *in vivo* upon extracellular stimulation of cells with TNFα.

3.1.7 IKK complex is constitutively active in Hodgkin's cells

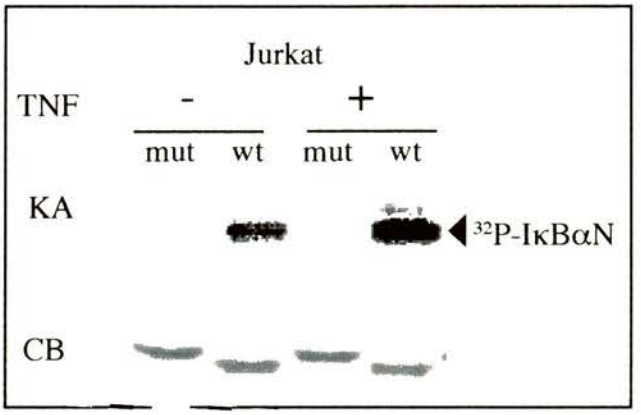
Hodgkin's disease is characterised by the presence of mononucleated Hodgkin and multinucleated Reed-Sternberg cells surrounded by a mixture of reactive cells comprising normal lymphocytes, eosinophils, plasma cells and fibroblasts (Kuppers and Rajewsky, 1998). It has been shown that NF-κB is constitutively active in Hodgkin's disease patients

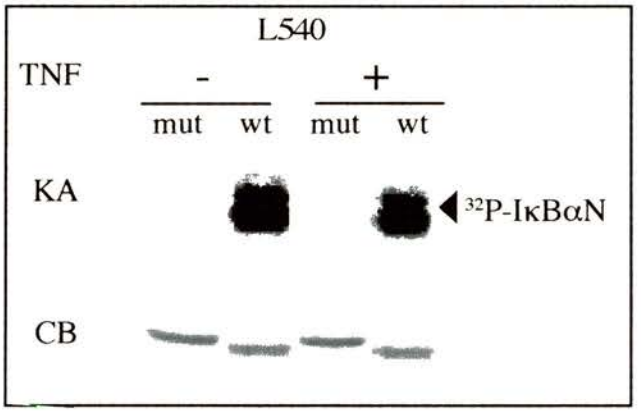
Figure 12 IKK acitivity can be stimulated *in vivo*.
Cells were maintained in growth medium as described in Materials and Methods. A. $5x10^6$ Hela cells were stimulated with 10ng/ml TNF for the indicated periods of time. Cells were then lysed in 1ml WCE lysis buffer and cleared by centrifugation at 279000*g* for 30 minutes. Supernatants were removed, protein concentration determined by Bradford Assay and equal amounts of protein used to isolate IKK complex by immunoprecipitation with 20µl protein G-IKK1 beads. After incubation for 2 hours at 4°C, beads were collected and washed once in WCE lysis buffer, twice in IP buffer and once in KB. Kinase assays were carried out at 30°C for 1 hour in a total volume of 30µl containing KB, 10µl protein G-IKK complex, 1µg GST-NIκBα or GST N-IκBα S32,36E and γ^{32}P-ATP. Reactions were stopped by the addition of 3x SDS disruption buffer and samples separated by SDS-PAGE (10%), gels were stained in Coomassie blue, destained, dried and exposed to phosphoimage screen for 12 hours. Amount of input substrate proteins are also shown in a coomassie blue stained gel. B. $5x10^6$ Jurkat cells were maintained in growth medium as described in Materials and Methods, and stimulated with 10ng/ml TNF for the indicated times. Cells were lysed as above and IKK complex isolated by immunoprecipitation with 10µl protein G-IKK 1 beads, as above. Kinase assays were carried out as described in A, in 30µl KB containing 10µl protein G-IKK complex, 1µg GST-NIκBα and γ^{32}P-ATP. C,D. HUVECs and human coronary cells were maintained in growth medium as described in Materials and Methods. 10^6 cells were stimulated with 10ng/ml TNF for the indicated times and subsequently lysed in 1ml WCE lysis buffer. Immunoprecipitations and kinase assays were carried out as described in A. (CB - coomassie blue, KA- kinase assay).

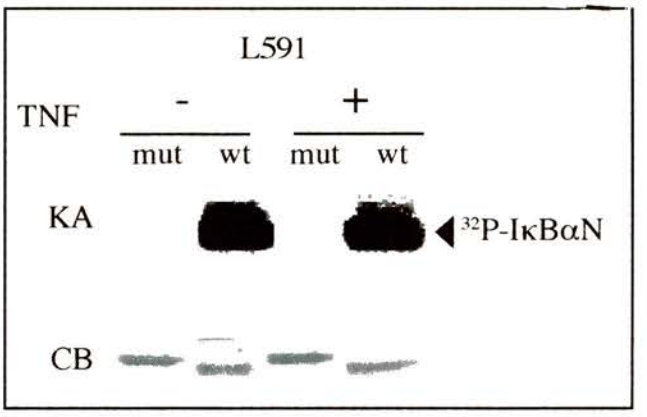

A
HeLa
TNF stimulation (mins)
0
5
10
15
mut wt mut wt mut wt mut wt
KA
CB
32P-GST N-IκBα
B
Jurkat
TNF stimulation (mins)
0
5
10
15
KA
CB
32P-GST N-IκBα
C
HUVEC
TNF stimulation (mins)
0
5
10
30
60
120
mut wt mut wt mut wt mut wt mut wt mut wt
KA
CB
32P-GST N-IκBα
D
Human Coronary
TNF stimulation (mins)
0
5
10
30
60
120
mut wt mut wt mut wt mut wt mut wt mut wt
KA
CB
32P-GST N-IκBα

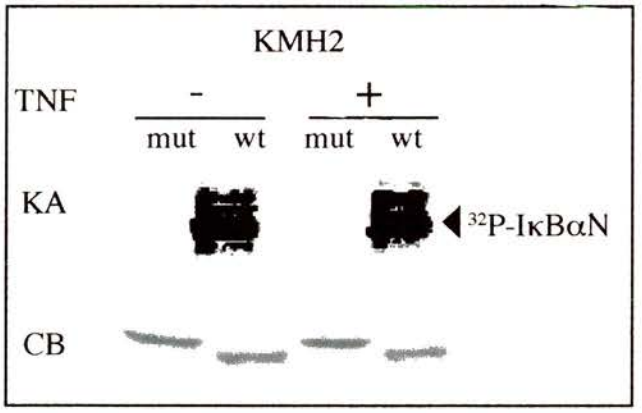
(Bargou *et al.*, 1996; Wood *et al.*, 1998; Krappmann *et al.*, 1999). The point in the signalling pathway that may contribute to the overall activation of NF-κB in these cell lines, is not clearly understood. The role of the IKK complex in the activation of NF-κB was investigated in order to determine if the activity of the kinases might contribute to constitutively active NF-κB. 10^7 cells from each Hodgkin's disease cell lines, L540, L591, KMH2, L428, HDMYZ and HDLM2 and a from non-Hodgkin's disease cell line, Jurkat, were either stimulated or not with 10ng/ml TNFα for 10 minutes at 37°C. Cells were collected by centrifugation and lysed in 1ml of lysis buffer. Protein concentrations were determined by Bradford Assay and equal amounts of protein from each cell line was used to isolate the IKK complex by immunoprecipitation with anti-C terminal IKK1 antibodies linked to Protein G. The activity of the IKK complexes were determined by their ability to phosphorylate 1μg GST-N IκBα or GST N IκBα S/E in the presence of [γ^{32}P-ATP]. Figure 13. By utilising the developed kinase assay it was demonstrated that there is a constitutively active kinase complex within Hodgkin's disease cell lines, as the activity is high in the unstimulated state and is not further increased by TNFα. The fact that you can stimulate IKK activity in response to TNFα in Jurkat, a cell line which is not of Hodgkin's disease origin, highlights the fact that the constitutive activation of the kinases is specific to Hodgkin's disease derived cells. Specificity of the assay is displayed by the fact that an IκBα mutant in which the serine residues 32 and 36 have been mutated to glutamate, does not undergo phosphorylation. The amount of input substrate, either GST N IκBα or GST N IκBα S32/36E, is shown in a Coomassie blue stained gel of the same kinase assay. Hence it can be revealed that not only is NF-κB constitutively active in Hodgkin's disease, but the kinase complex which acts to phosphorylate and target the inhibitor protein of NF-κB, IκBα, is also constitutively active. This may contribute to the overall pathology of the disease.

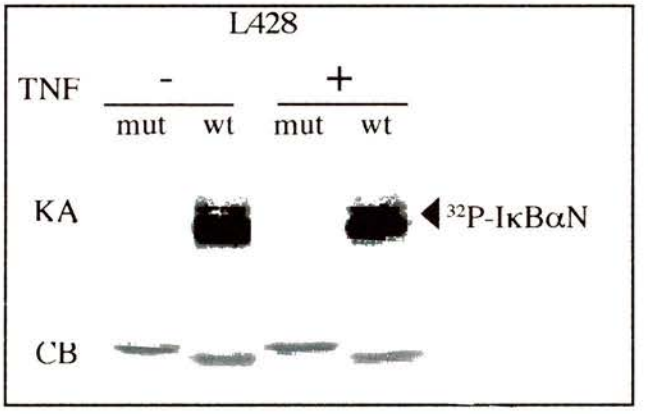
Figure 13 IKK is constitutively active in Hodgkins disease cell lines.
Jurkat and Hodgkin cell lines were maintained in growth medium as described in Materials and Methods. 10^7 cells were stimulated or not with 10ng/ml TNF for 10 minutes at 37°C. Cells were then lysed in 1ml WCE lysis buffer and cleared by centrifugation at 279000*g* for 30 minutes. Supernatants were removed, protein concentration determined by Bradford Assay and equal amounts of protein used to isolate IKK complex by immunoprecipitation with 20µl protein G-IKK1 beads. After incubation for 2 hours at 4°C, beads were collected and washed once in WCE lysis buffer, twice in IP buffer and once in KB. Kinase assays were carried out at 30°C for 1 hour in a total volume of 30µl containing KB, 10µl protein G-IKK complex, 1µg GST-NIκBα or GST N-IκBα S32,36E and γ32P-ATP. Reactions were stopped by the addition of 3x SDS disruption buffer and samples separated by SDS-PAGE (10%), gels stained in Coomassie blue, destained, dried and exposed to phosphoimage screen for 12 hours. Amount of input substrate proteins are also shown. CB-coomassie blue, KA- kinase assay.

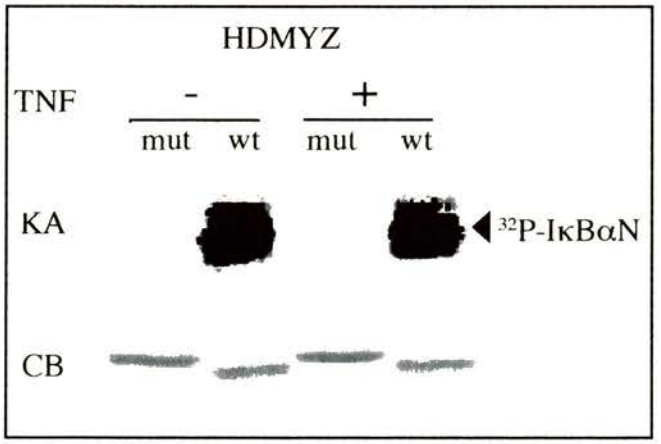

Jurkat
TNF
-
+
mut
wt
mut
wt
KA
32P-IκBαN
CB

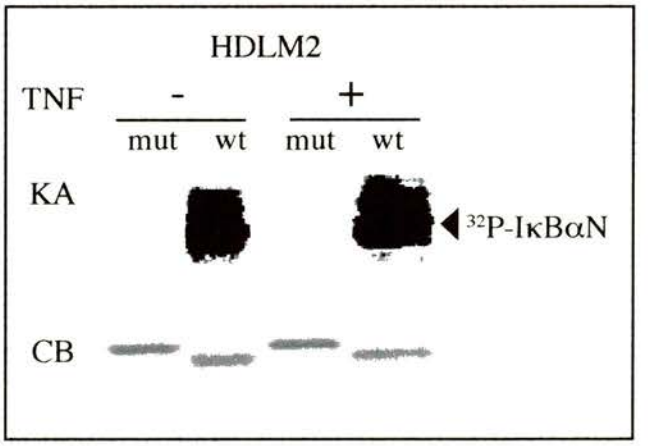

L540
TNF
-
+
mut
wt
mut
wt
KA
32P-IκBαN
CB


L591
TNF
-
+
mut
wt
mut
wt
KA
32P-IκBαN
CB


KMH2
TNF
-
+
mut
wt
mut
wt
KA
32P-IκBαN
CB


L428
TNF
-
+
mut
wt
mut
wt
KA
32P-IκBαN
CB


HDMYZ
TNF
-
+
mut
wt
mut
wt
KA
32P-IκBαN
CB


HDLM2
TNF
-
+
mut
wt
mut
wt
KA
32P-IκBαN
CB

3.1.8 IKK activity can be stimulated upon exposure of Human umbilical vein endothelial cells to shear stress

Arteriosclerosis is a chronic disease that is manifested by the deposition of fatty plaques in the lining of blood vessel walls, particularly coronary arteries. It is however a geometrically focal disease and plaques are preferentially deposited on the outer edges of vessel bifurcations and curved regions of the arterial tree (Nerem, 1993). Endothelial cells, which line the blood vessel walls are constantly subjected to fluid shear stress. *In vitro* and *in vivo* experiments using cultured endothelial cells and flow chambers have demonstrated that these hemodynamic forces can activate several intracellular signalling pathways. Ultimately these pathways lead to the induction and expression of various genes. *In vitro* and *in vivo* experiments using cultured bovine aortic endothelial cells (BAECs) has demonstrated that NF-κB activation occurs following stimulation with fluid shear stress (Bhullar *et al.*, 1998). Since NF-κB activation can occur through the activation of the IKK signalsome, the *in vitro* kinase assay was employed to investigate kinase activity in HUVECs in response to fluid shear stress.

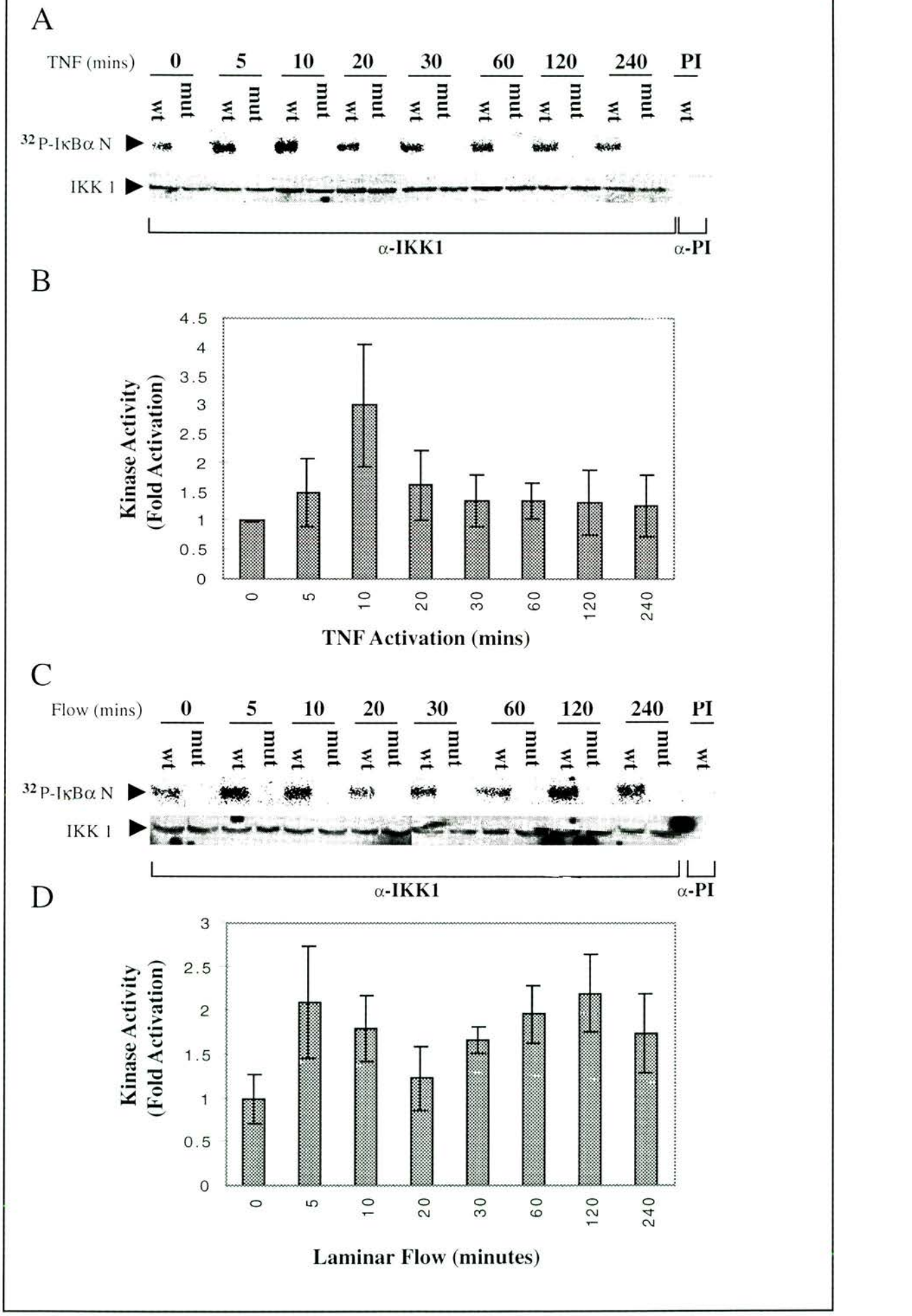
HUVECs form a boundary between recirculating blood and the vessel wall and are constantly subjected to fluid shear stress. Therefore these primary cell lines provide a model for the study of kinase activity in endothelial cells in response to shear flow. Initially, kinase activity was investigated in response to a known activator of the kinase complex, TNFα. 10^6 HUVECs were stimulated with 30ng/ml TNFα for the indicated time points. Cells were lysed in 1ml hyptonic lysis buffer and lysates clarified by centrifugation at 270000*g* for 30 minutes at 4°C. Supernatants were removed and protein concentration determined by Bradford Assay. IKK complex was isolated from equal amounts of cell extracts by immunoprecipitation with anti-C-terminal IKK1 peptide serum antibody linked to protein G. Immunoprecipitated IKK complex was washed and incubated with 1μg GST IκBα N or GST IκBα S32,36E for 60 minutes in the presence of [γ^{32}P]ATP. Reactions were stopped by the addition of SDS disruption buffer, proteins separated by SDS-PAGE

and the dried gel exposed to a phosphorimaging screen for 10-12 hours. Autoradiography of the kinase assay is shown in Figure 14a. Kinase activity was quantified from the autoradiographs of the kinase assay using the FujixBas 1500, MacBas software and are shown in Figure 14b. Results represent the percentage of GST IκBα N phosphorylation in response to TNFα compared with the control that was not subjected to TNFα stimulation. It was demonstrated that an increase in kinase activity could be detected after 5 minutes and reached a maximal level of activity after 10 minutes of stimulation with TNFα. To verify that equal amounts of kinase was immunoprecipitated at each point, an equivalent fraction of the immunoprecipitated complex was subjected to Western blotting. Immunoprecipitated complex was resuspended in kinase buffer and SDS disruption buffer, resolved by SDS-PAGE (8.5%), transferred to PVDF membrane and immunoblotted with a monoclonal anti-IKK1 antibody B8 (Santa Cruz), 1:100 dilution, which recognises the C-terminal of IKK1. An equal amount of IKK complex is present at each point, indicating that the increase in phosphorylation of GST- IκBα N is due to increased activity of the kinases. Specificity of the assay is demonstrated as there is no phosphorylation of GST-IκBα S32,S36E. Thus, HUVECs contain functionally active IκBα kinase that can be activated in reponse to stimulation with TNFα.

Subsequently, investigation of the kinase activity in response to shear flow was investigated. 10^6 HUVECs were exposed to fluid shear stress for the indicated times and subsequently lysed in 1ml of hypotonic lysis buffer containing protease inhibitors. Kinase assays were carried out as above and kinase activity determined in response to laminer shear, 15 dynes/cm^2. Results are shown in Figure 14c and 14d and indicate that the kinase is activated in a biphasic manner with maximal kinase activity evident after 5 minutes which decreases and reappears after 120 minutes. Quantification of the kinase activity was measured as for TNFα stimulation described previously. Results represent the percentage of GST IκBα N phosphorylation in response to laminar flow compared with the static control that was not subjected to laminar flow. As above, verification that equal amounts

Figure 14 Activation of kinase activity in HUVECs in response to TNFα stimulation and laminar flow.

10^6 HUVECS were grown on glass coverslips before being subjected to treatment with (A,B) 30ng/ml TNFα or (C,D) laminar flow for the times indicated. Cells were then lysed in whole cell extract (WCE) lysis buffer and protein concentration determined by Bradford Assay. (A) IKK complex was immunoprecipitated from equal amounts of cell lysate stimulated with TNFα using anti-C terminal IKK1 antibody coupled to Protein G and assessed for its ability to phosphorylate GST-IκBα N (wt) or GST-IκBα N S32,36E (mut) in the presence of γ-[^{32}P]ATP. As a control, cell lysates from 10 minute stimulation with TNF were immunoprecipitated with pre-immune IgG (PI) antibodies. Products of the kinase reaction were separated by SDS-PAGE (10%), gels dried and exposed to phosphoimage screen for 10-12 hours (top panel). Equivalent amounts of immunoprecipitated IKK complex were separated by SDS-PAGE (10%), transferred to PVDF and western blotting performed with monoclonal IKK1 antibody (B8) purchased from Santa Cruz, or pre-immune IgG (PI) (bottom panel). (B) Kinase activity was quantified using Fujix 1500 and MacBas software. The amount of phosphorylated substrate in the absence of stimuli was used as a baseline measurement and all other points compared to this. The data represents the average of three separate experiments. (C) IKK complex was immunoprecipitated from equal amounts of cell lysate subjected to laminar flow using anti-C terminal IKK1 antibody coupled to Protein G and assessed for its ability to phosphorylate GST-IκBα N (wt) or GST-IκBα N S32,36E (mut) in the presence of γ-[^{32}P]ATP. As a control, cell lysates subjected to 5 minute stimulation with laminar flow were immunoprecipitated with pre-immune IgG (PI) antibodies. Products of the kinase reaction were separated by SDS-PAGE (10%), gels dried and exposed to phosphoimage screen for 10-12 hours (top panel). Equivalent amounts of immunoprecipitated IKK complex were separated by SDS-PAGE (10%), transferred to PVDF and western blotting performed with monoclonal IKK1 antibody (B8) purchased from Santa Cruz, or pre-immune IgG (PI) (bottom panel). (D) Kinase activity was quantified using Fujix 1500 and MacBas software. The amount of phosphorylated substrate in the absence of stimuli was used as a baseline measurement and all other points compared to this. The data represents the average of three separate experiments.


A
TNF (mins)
0
5
10
20
30
60
120
240
PI
wt
mut
32 P-IκBα N
IKK 1
α-IKK1
α-PI
B
Kinase Activity (Fold Activation)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
TNF Activation (mins)
C
Flow (mins)
D
Laminar Flow (minutes)

of kinase was used in the assay was determined by Western blotting. Immunoprecipitated complex was resuspended in kinase buffer and SDS disruption buffer, resolved by SDS-PAGE (8.5%), transferred to PVDF membrane and immunoblotted with anti-IKK1 antibody B8 (Santa Cruz), 1:100 dilution. Equal amounts of IKK complex present at each flow point indicates that the increase in phosphorylation of GST-IκBαN is due to increased activity of the kinases. Specificity of the assay is shown by the fact that there is no phosphorylation of GST-IκBαS32,S36E. These results demonstrate that kinase activity can be increased in HUVECs in response to shear stress and that the response is biphasic. This suggests that NF-κB activation in these cells is dependent on the activation of the IKK signalsome.

3.1.9 Discussion

The discovery of a large kinase complex that could specifically phosphorylate members of the IκBα family, provided a clue as to how the many different stimuli which activate NF-κB could converge. The complexity of the NF-κB pathway is exemplified by the many activators of this transcription factor. However, not all of the activators of NF-κB are known and it is not clear if all activators signal through the IKK complex. By using an antibody that is able to specifically isolate both IKK1 and IKK2, a simple and specific kinase assay was developed. The development of tools, such as an *in vitro* kinase assay would allow the investigation of the upstream and downstream signalling events prior to or in effect of IKK activation to be undertaken. It has also provided a tool with which to investigate the activity of the complex *in vivo* in different cell lines, in response to TNFα and laminar flow.

Initially optimal conditions were established for kinase activity and it was demonstrated that *in vitro* transcribed/translated kinases were capable of phosphorylating IκBα on serine residues 32 and 36. Activity of the kinases *in vitro* has shown that both are capable of phosphorylating recombinant IκBα, but that IKK2 seems to be more active than

IKK1. This is also evident *in vivo*, and it is thought that IKK2 is able to control the regulation and activity of IKK1 and also of the IKK1 signalsome, through phosphorylation of IKK1 and of itself (Li *et al.*, 1999c). IKK2 has also been shown to regulate itself through phosphorylating serine residues in its carboxy terminus thereby reducing kinase activity presumably by causing a conformational change between the HLH domain and the kinase domain (Delhase *et al.*, 1999).

It is interesting to note that when IKK1 and IKK2 are co-translated together, there is a synergistic effect, indicating that the complex achieves its maximal activity *in vitro* when both components are present. This was also demonstrated *in vivo* when recombinant IKK1/IKK2 heterodimers were expressed in insect cells and their activity compared with IKK1 or IKK2 homodimers. Results suggested that the IKK1/IKK2 heterodimers were the preferred IκBα peptide substrate than either homodimer (Huynh *et al.*, 2000). This suggests that both kinases are required for optimal phosphorylation of IκBα.

Phosphorylation and thus activation of the kinases is thought to be achieved by upstream signalling molecules, as illustrated by the fact that upon stimulation of cells with TNFα, phosphorylation of all three components of the IKK complex is observed (Delhase *et al.*, 1999). Some of the molecules responsible for this phosphorylation have also been identified. One of these belongs to the MAP kinase kinase kinase (MAP3K) family of proteins, NIK. The role of NIK in NF-κB activation remains controversial, but it has been shown *in vivo* to phosphorylate IKK1 on serines 176 and 180, and IKK2 on serines 177 and 181, residues which lie within the activation loop of the kinases (Delhase *et al.*, 1999). However, phosphorylation of IKK2 *in vivo* is not solely dependent on the expression of NIK but is also dependent on an IKK2 intact kinase domain as catalytically inactive IKK2 mutants fail to be phosphorylated. It has been shown earlier that NIK is able to interact with IKK1 and, although to a much lesser extent, IKK2 (Woronicz *et al.*, 1997) and that co-expression of NIK and IKK1 also increases IKK1 activity (Regnier *et al.*, 1997), but not IKK2 activity (Woronicz *et al.*, 1997). NIK has also been shown *in vitro* to

preferentially phosphorylate IKK1 over IKK2 (Ling *et al.*, 1998). Consequently it has been implicated that NIK can activate and interact with IKK1 but does not affect IKK2 activation. Results also show *in vitro*, that co-translation of IKK1 and NIK, increases the activity of this kinase (Figure 4) and also of the IKK1/2 complex. However, co-translation of NIK in the presence of IKK2 alone appears inhibitory. This may explain why the IKK complex *in vivo* appears to consist of IKK1/IKK2 heterodimers rather than IKK1 or IKK2 homodimers.

Generation of mice in which the genes for either IKK1 or IKK2, or both, have been deleted have suggested that IKK2 is the predominantly active kinase *in vivo* in response to cytokine stimulation. It has also been suggested that IKK1 is more active in response to other types of stimuli that are possibly activated through different as yet unidentified signalling pathways. Matsushima A et al, using embryonic fibroblasts from alymphoplasic mice, in which there is a point mutation in the gene encoding NIK or from IKK1 -/- mice, has suggested that IKK1 (and NIK) function in signalling through the LTβR and not through the TNFR (Matsushima *et al.*, 2001). Similarly, recent studies on mouse embryonic fibroblasts from NIK deficient mice have suggested that NIK functions in the LTβR signalling pathway and not the TNFα-activated NF-κB pathway (Yin *et al.*, 2001). However the above studies remain somewhat controversial and experiments have shown that IKK1 is important in NF-κB activation by pro-inflammatory signals. A kinase inactive form of IKK1 (K44M) blocks NF-κB activation by various stimuli including TNF and IL-1 (DiDonato *et al.*, 1997; Fischer *et al.*, 1999; Regnier *et al.*, 1997). A mutant form of NIK(T559A), in which substitution of threonine residue 559 in its activation loop by alanine, abolishes its kinase activity and phosphorylation of IKK1, and also abolishes NF-κB activation by TNFα. Furthermore, deletions in the C-terminus of NIK, which disrupt NIK-IKK1 interactions, also abolish TNFα-induced NF-κB activation (Lin *et al.*, 1998b). Therefore the involvement of NIK and IKK1 appears to be important in NF-κB activation through TNFα.

Recently it has been demonstrated that IKK1 is required for regulation and activation of IKK2 *in vivo* and that activation of the IKK signalsome by various stimuli including TNFα, NIK, HTLV Tax, Cot/Tpl2 and MEKK1 is dependent on kinase proficient IKK1 (O'Mahony *et al.*, 2000). Therefore, IKK1 may exist as a regulating kinase whereas IKK2 is the functional kinase within the complex. Deletion of the regulating kinase can be compensated for within the cell, but deletion of the functional kinase cannot be tolerated, leading to embryonic lethality. The identification of functional IKK1 isoforms that lack either a HLH domain or both leucine zipper and HLH domains, suggest that other kinases may also compensate for the loss of IKK1 activity in IKK-/- knockout mice. These specific isoforms are generated by alternative splicing of the IKK1 mRNA and exhibit tissue specific expression. Both these isoforms can potentiate NF-κB activation in response to TNFα (McKenzie *et al.*, 2000). Therefore, the roles of IKK1 and IKK2 in the activation of NF-κB by pro-inflammatory stimuli remains controversial with further experiments required to fully understand the nature of these kinases.

Activation of the IKK complex was examined in some typical cell lines found within the laboratory, Hela and Jurkat. Hela cells represent cervical epithelial cells and Jurkat represent T-lymphocytes. In both cell lines, activation of IKKs occurs upon stimulation of the cells with TNFα, a typical NF-κB inducer. This indicates that there is a functional TNFα signalling cascade present in these types of cells, which converge in the activation of the IKK complex. Whether both types of cells use the same pathway and the same proteins is unknown. Both cell lines, however, are derived from transformed cells and thus are able to grow in culture irrespective of growth control checks. Analysis of the activation of the IKK complex within two primary cell lines currently being used in the laboratory, human umbilical vein endothelial cells (HUVECs) and Human coronary cells, was investigated. These cells have been derived from non-transformed primary cells and can only grow in culture for a limited number of passages. Again, stimulation of these cells with TNFα, shows activation of the kinase complex indicating that there is a

functional TNFα signalling pathway in these cells. The importance of the IKK complex in the TNFα signalling pathway, is therefore demonstrated by the fact that in a number of different types of cells, the pathway converges on the activation of the IKKs. However, it is true to note, that there could be other points of convergence or other mechanisms by which TNFα may activate NF-κB. In fact, PAK1, a serine/threonine kinase which is involved in the activation of the small G-proteins Rac1 and Cdc42hs, has been shown to activate NF-κB in the absence of IKK activation (Frost *et al.*, 2000) and it has also been shown that treatment of cells with pervanadate or exposure to UV-C can stimulate NF-κB activity in the absence of IKK activation (Li and Karin, 1998; Bender *et al.*, 1998; Imbert *et al.*, 1996). Overall, it was demonstrated that there is an inducible kinase complex in Hela, Jurkat, HUVECs and Human coronary cells which can be isolated and used to phosphorylate IκBα on serine residues 32 and 36.

This assay was also used to determine the status of NF-κB signalling pathways in cells derived from patients with Hodgkin's disease. Cell lines derived from the cells of tumours from patients with Hodgkin's disease provide a model of the disease itself. Six different cell lines, L540, L591, KMH2, L428, HDMYZ and HDLM2, which all have their own unique characteristics that contribute to Hodgkin's disease, were tested for IKK activity. In these cells, it was shown that the IKKs were constitutively active as shown by the fact that immunoprecipitated complex could phosphorylate IκBα in the absence of exogenous stimuli. This is consistent with several Hodgkin's disease cell lines showing constitutive NF-κB DNA binding activity (Bargou *et al.*, 1996; Wood *et al.*, 1998). Activation of the IκBα kinases in Hodgkin's disease cell lines has been demonstrated by others (Krappmann *et al.*, 1999). Therefore, in addition to other factors that could contribute to NF-κB activation in these cells, activation of the IKK complex within these cells is also apparent.

In vitro kinase assays were also employed as a tool to investigate the activation of the IKK complex in endothelial cells in response to shear flow. Biochemical and

biomechanical stimuli affects vascular cell walls. It has been demonstrated that stimulation of the endothelial cells lining the vascular walls by such hemodyanimic forces such as laminar flow results in the activation of a number of intracellular signalling pathways. Ultimately, these pathways result in the transactivation of a number of genes that are necessary and efficient for the proper functioning of endothelial cells and vascular structure. The importance of proper functioning of endothelial cells has been noted by the pathological situations which arise from endothelial malfunctions (Luscher, 1993). In artheriosclerosis deposition of fatty plaques in vessel bifurcations, leads to a decrease in shear flow at these points. Disruption of flow at sites of arteriosclerotic plaques can therefore disrupt the normal signal transduction pathways that may be required for the efficient functioning of the blood vessel wall. Activation of NF-κB in response to shear stress has been demonstrated *in vitro* and *in vivo* (Khachigian *et al.*, 1995; Lan *et al.*, 1994; Shyy *et al.*, 1995; Resnick *et al.*, 1993). Activation of the IKK complex in response to shear stress has also been demonstrated in BAECs (Bhullar *et al.*, 1998). *In vitro* kinase assays were used to determine the activity of the IKK complex in HUVECs in response to shear flow. IKK activity was apparent after 5 minutes of stimulation of the cells, which decreased after 20 minutes. The IKK response appeared to be biphasic with IKK activity being stimulated again after 120 minutes. Using the *in vitro* kinase assay, demonstration of kinase activity in response to shear flow was determined. Kinase activity appeared to be biphasic in response to flow stimulus, whereas in response to TNF, kinase activity is only transiently activated. This suggests that these stimuli activate the IKK complex by different signalling pathways that may utilise different downstream signalling molecules. Although these pathways might be distinct from one another, both converge at the point of activation of the IKK complex. Therefore the involvement of IKK activation in response to such diverse stimuli, enhances the importance of this complex in NF-κB signalling within the cell.

Overall, development of an *in vitro* kinase assay provides us with a biological tool in which to investigate the pathway of activation of NF-κB. The assay can be used in a number of ways. Firstly it can be utilised to investigate the NF-κB signalling pathway *in vivo* in a number of cell types and also in response to a number of different stimuli including TNFα and laminar flow. Secondly, the assay can be used to produce phosphorylated substrate (P-IκBα) for ubiquitination assays, allowing us to probe the molecular determinants of IκBα ubiquitination, degradation and subsequent NF-κB activation.

3.2 *In vitro* ubiquitination of phosphorylated IκBα and interaction with βTrCP

3.2.1 Summary

Ubiquitination of IκBα occurs *in vivo* through a serious of enzyme reactions, involving an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin ligation enzyme. It has been established that there is a large, and still growing family of E3 enzymes, which are involved in conferring specificity to ubiquitination of target substrates. Currently there are four known families of E3, and it has been shown that the E3 responsible for ubiquitination of IκBα belongs to the SCF family of enzymes. This complex consists of Skp1, Cul1/cdc53, an interchangeable F-box protein and also Rbx1. Although this complex has been identified as the E3 responsible for ubiquitination and thus degradation of IκBα, it was not known if these were the only proteins present, or if they were solely responsible and capable of transferring ubiquitin to IκBα. An *in vitro* ubiquitination assay, using all purified components, was used to identify the minimal proteins involved in IκBα ubiquitination. Using recombinant E1, cdc 34 and $SCF^{\beta TrCP}$ expressed and purified from insect cells, reconstitution of ubiquitination of phosphorylated IκBα was demonstrated. This reaction was dependent on E1, an E2, either cdc34 or ubc5, an E3 ligase ($SCF^{\beta TrCP}$) and ubiquitin. The interaction between IκBα and βTrCP facillitates the mechanism by which IκBα is brought into close proximity of the ubiquitin machinery. The interaction between these two proteins is highly significant. Investigation of the properties of the specific interaction between IκBα and βTrCP was also evaluated. An F-box deleted mutant of βTrCP was capable of reducing NF-κB activation *in vivo* and also capable of inhibiting degradation of IκBα, confirming the importance of βTrCP in NF-κB signalling. By using peptides corresponding to amino acids 28-39, or 20-43 of IκBα it was demonstrated that βTrCP can only interact with the phosphorylated form of IκBα and that this interaction was dependent on the presence of phosphate groups at serine residues 32 and 36 on the target protein.

3.2.2 Expression and purification of $SCF^{\beta TrCP}$ from SF9 cells

The four components of the E3 ligase for IκBα, βTrCP, Skp1, Cul1/cdc53 and Rbx1 were cloned into baculovirus expression vectors and used to infect $75x10^6$ Sf9 cells at an m.o.i of 5 for each virus. Figure 15 shows a schematic overview of the infection process. By simultaneously infecting the insect cells with each of the four viruses, hetero-oligomeric protein complexes can be formed and expressed. Another advantage to using this expression system compared with a bacterial expression system is that these complexes represent the complex found naturally; in that they are assembled in the correct ratios and have acquired any post-translational modificatons that may contribute to their overall structure and function. After 68-72 hours, depending on infection and condition of cells, the insect cells were collected by centrifugation, divided into four aliquots and stored at -70°C until required. For isolation of the SCF complex, cells were thawed and lysed on ice in hypotonic buffer containing glycerol, and lysates clarified by centrifugation. Since the βTrCP construct has been cloned, fused at the N-terminus to an epitope from Simian virus 5 (sv5), it can be immunoprecipitated using an antibody that specifically recognises this epitope, sv5 Pk Tag, 336 (Hanke *et al.*, 1992). βTrCP interacts through its F-box motif with Skp1 (Margottin *et al.*, 1998; Hattori *et al.*, 1999). Skp1 interacts, not only with βTrCP, but also with Cul1/cdc53 (Hattori *et al.*, 1999; Wu *et al.*, 2000) which in turn interacts with Rbx1 (Kamura *et al.*, 1999b; Skowyra *et al.*, 1999) and cdc34 (Patton *et al.*, 1998). Rbx1 is also thought to interact with cdc 34 (Kamura *et al.*, 1999b; Ohta *et al.*, 1999; Skowyra *et al.*, 1999; Seol *et al.*, 1999; Chen *et al.*, 2000). Therefore, by utilising their physical association the $SCF^{\beta TrCP}$ complex can be isolated by immunoprecipitation of βTrCP.

To verify that each of the four proteins were immunoprecipitated, the beads were separated by SDS PAGE and immunoblotted with the appropriate antibodies. Since no commercially available antibodies to βTrCP were known, a peptide corresponding to the last C-terminal twelve amino acids of βTrCP was generated with an additional cysteine

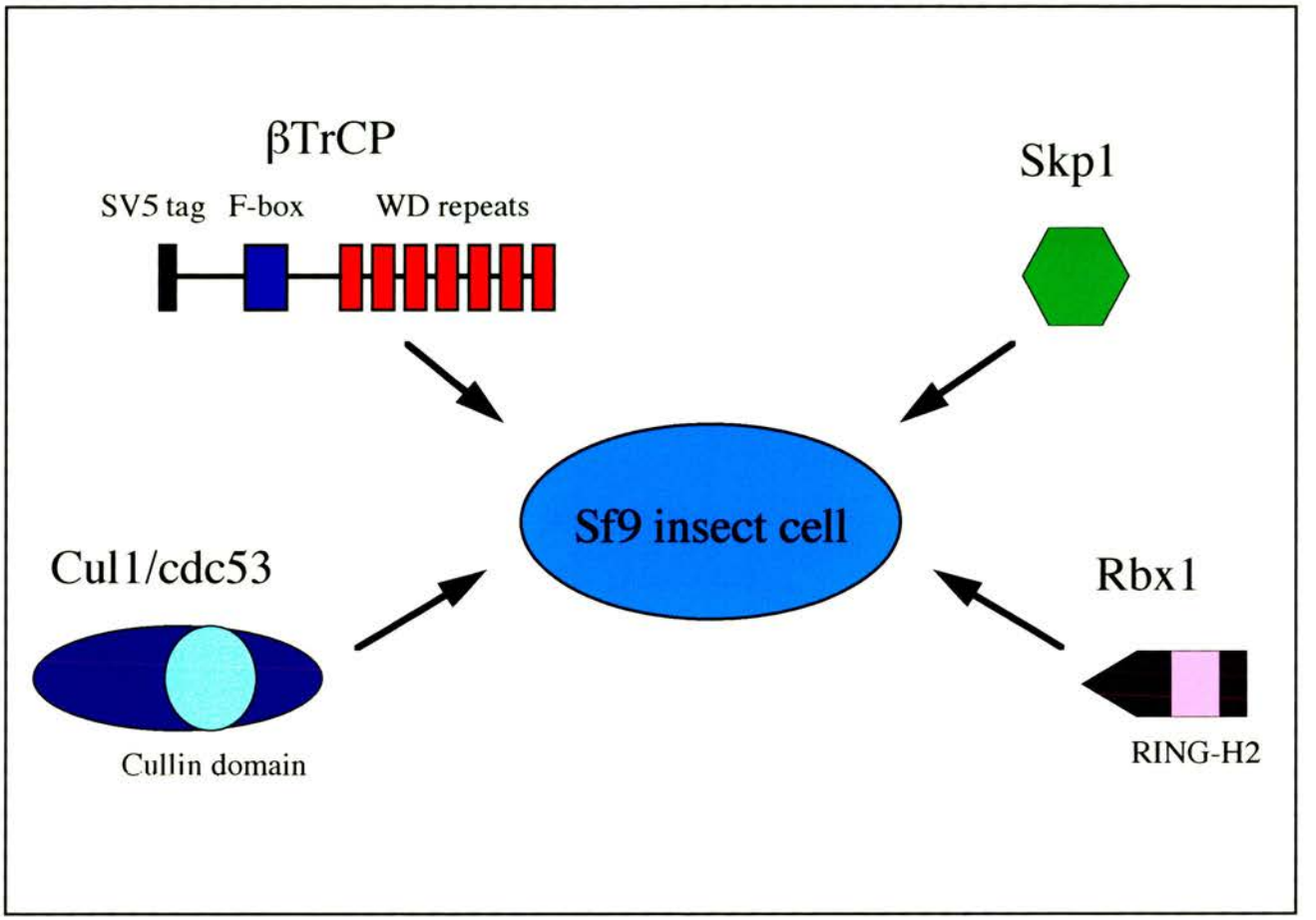


Figure 15 Expression of $SCF^{\beta TrCP}$ in Sf9 insect cells.
Simplified diagram showing the mechanism by which Sf9 insect cells are infected with each of the four baculovirus containing the proteins to assemble the $SCF^{\beta TrCP}$ complex. After infection by baculovirus, the cells are harvested when they are at the late stage of protein expression, therefore allowing maximal levels of recombinant protein to be obtained. Infection of one insect cell by four viruses expressing different proteins, allows for heteroligomeric complexes to be formed. βTrCP contains an amino terminal F-box domain and carboxyl terminal WD repeat domains and is constructed with an N-terminal tag, sv5 PkTag. Cul1/cdc53 contains a cullin homology domain thought to be involved in the interaction and recognition of E2. Skp1 contains an N-terminal region involved in binding Cul1/cdc53 and a C-terminal domain involved in the interaction with F-box containing proteins. Rbx1 contains a carboxyl terminal RING-H2 finger domain comprised of a cysteine-rich fold that encases two structurally essential zinc ions.

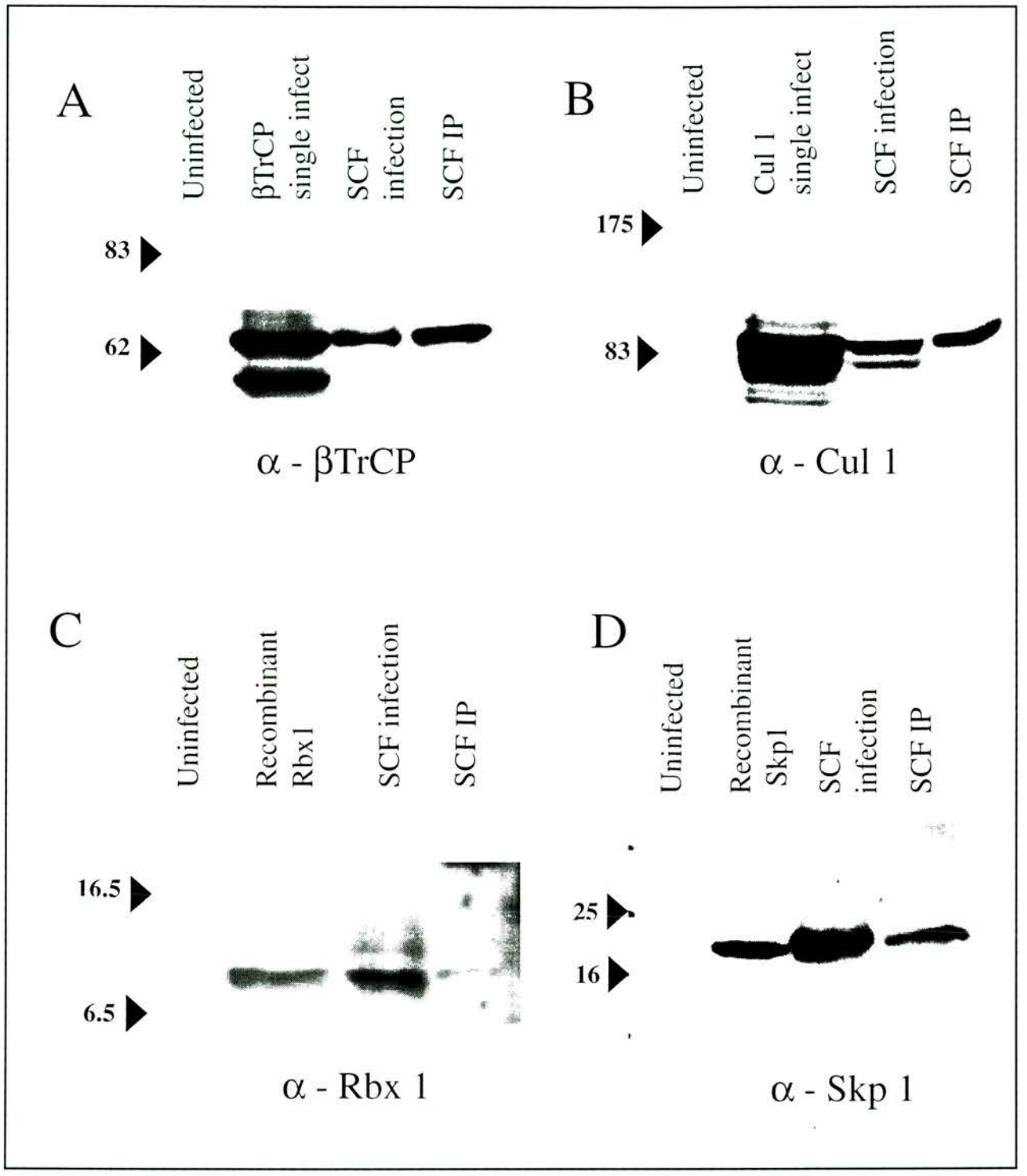


Figure 16 $SCF^{\beta TrCP}$ can be expressed in Sf9 cells.

Sf9 cells were infected with baculovirus expressing βTrCP, Skp1, Cul1/cdc53 and Rbx1 at an m.o.i of 5 for each virus. After 68-72 hours, insect cells were collected and $SCF^{\beta TrCP}$ complex isolated by lysing the cells in lysis buffer and immunopurified on protein A-sv5Pk Tag, 336 beads, as described in Materials and Methods. Samples were separated by SDS-PAGE and detection of proteins analysed by western blotting using anti-C terminal βTrCP antibody (16A), Cullin1 Ab-1 (16B), Rbx1 (N-16) (16C) or Skp1 p19 (C-20) (16D). Uninfected Sf9 cells were used as a negative control and single infection (βTrCP, Cul1/cdc53) or 500ng of recombinant protein (Rbx1, Skp1) were used as a positve control. Expression of proteins in the SCF complex (SCF infection), and the detection of each of the proteins within the complex (SCF IP) are shown. Position of prestained markers in kDa are as indicated.

residue to allow coupling to KLH. Coupled peptide was then used as antigen for immunisation of sheep (SAPU). Serum was collected and antibody affinity purified on thiol sepharose conjugated with immunising peptide. This antibody was used for detection of βTrCP immunoprecipitated with anti-SV5 Pk Tag antibody. As shown in Figure 16, each of the four proteins were expressed in the infected cells (SCF infection) and can also be found in a complex with βTrCP (SCP IP). Thus an $SCF^{\beta TrCP}$ E3 ligase can be expressed and purified from insect cells.

3.2.3 *In vitro* ubiquitination of IκBα

It has been demonstrated *in vivo* that ubiquitination of IκBα depends on the actions of an E1 activating enzyme, an E2 conjugating enzyme, either ubc5 or cdc 34 and an E3 ligase, $SCF^{\beta TrCP}$. It has also been demonstrated that IκBα must be phosphorylated on serines 32 and 36 before ubiqutination of lysine residues 21 and 22 can occur (Vuillard *et al.*, 1999; Rodriguez *et al.*, 1996; Spencer *et al.*, 1999; Suzuki *et al.*, 1999; Hatakeyama *et al.*, 1999; Winston *et al.*, 1999). However, it is still unclear as to whether the components of the E3 ligase for IκBα, identified so far, are able to target phosphorylated IκBα for ubiquitination by themselves or if they require any other additional, yet unidentified proteins. Using all purified components, an *in vitro* assay was set up to determine the minimal requirements of IκBα ubiquitination. IκBα was phosphorylated in the presence of p65 by an *in vitro* kinase assay using IKK1 and IKK2 co-translated in the presence of NIK. As shown in Figure 17 when ^{32}P-IκBα was incubated with recombinant E1, cdc 34 and $SCF^{\beta TrCP}$ in the presence of ubiquitin and an ATP regeneration system, ubiquitination of IκBα occurred. This was shown to be dependent on E1, cdc 34, $SCF^{\beta TrCP}$ and ubiquitin as ubiquitination was abrogated when either of these components was omitted from the assay. Thus ubiquitination of ^{32}P-IκBα could be achieved *in vitro* using an SCF expressed and purified in insect cells. Therefore this demonstrates that the proteins identified in the E3 ligase

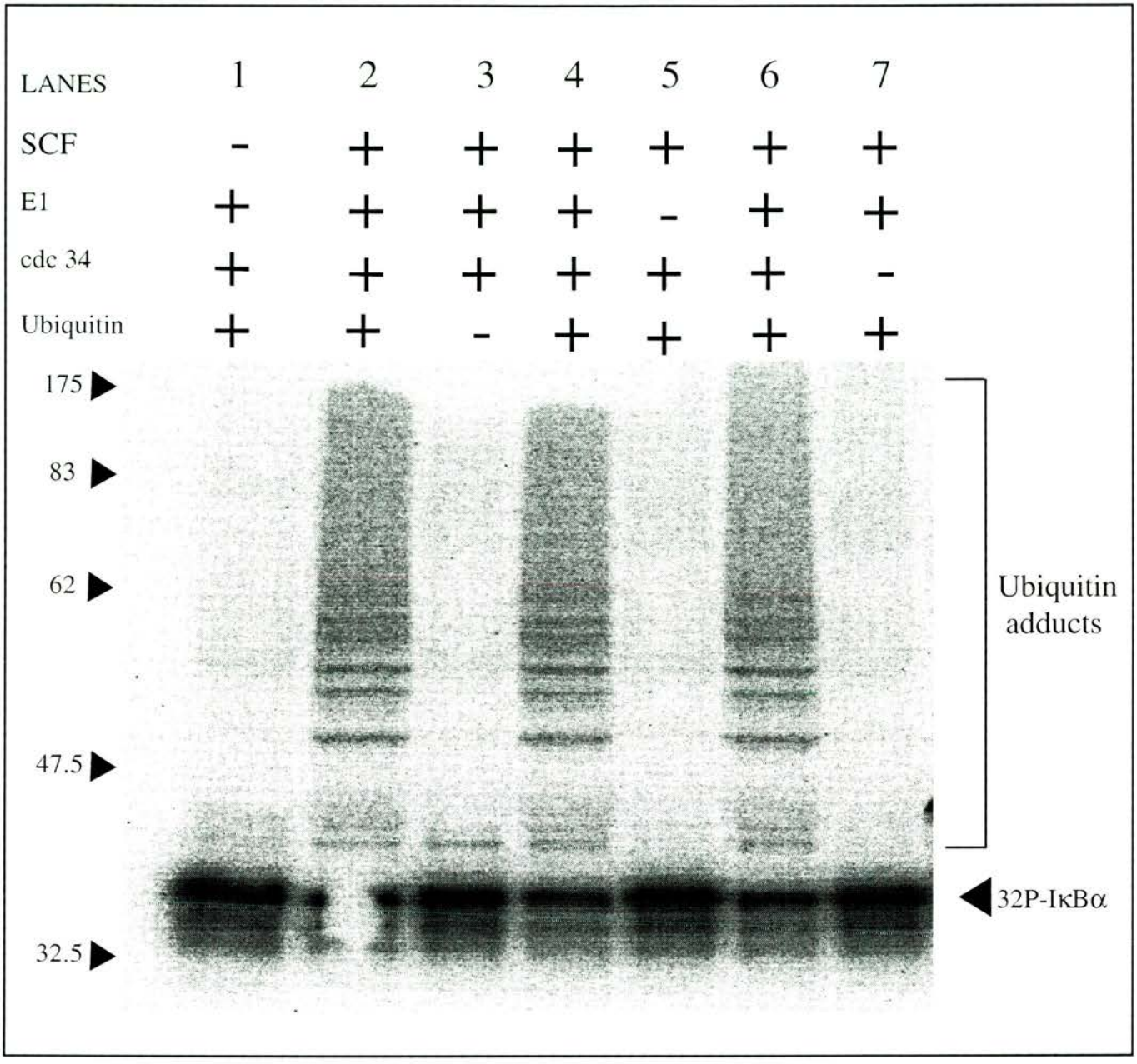
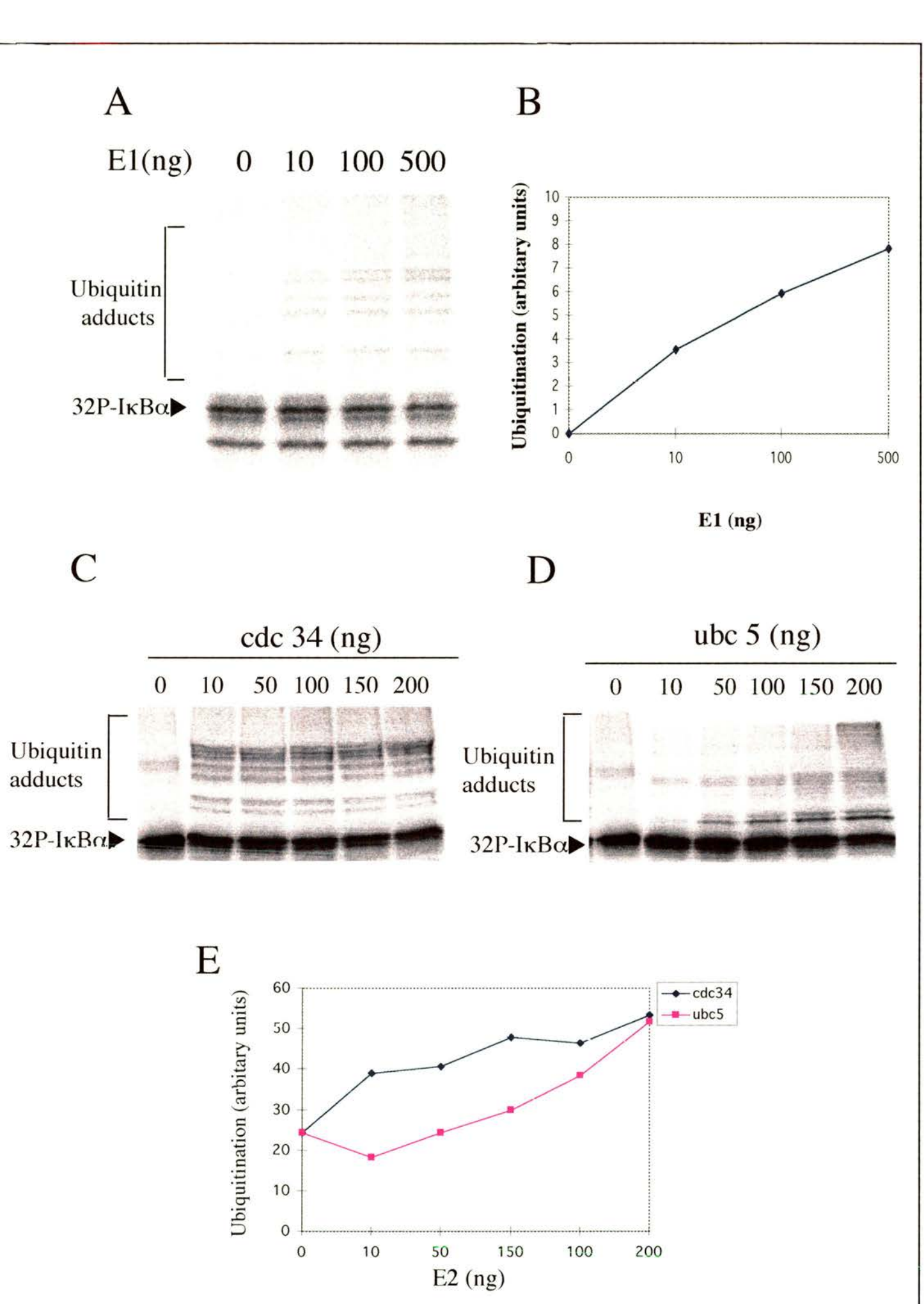


Figure 17 ^{32}P-IκBα is ubiquitinated *in vitro* and is dependent on E1, cdc34, SCF$^{\beta TrCP}$ and ubiquitin.
IκBα was phosphorylated *in vitro* by IKK1 and 2 and subjected to an *in vitro* ubiquitination assay as described in Materials and Methods. ^{32}P-IκBα was incubated in a total volume of 20µl containing an ATP regeneration system, ubiquitin, E1, cdc34 and SCF$^{\beta TrCP}$ (lanes 2, 4, and 6). Ubiquitination assays were also carried out in the absence of SCF$^{\beta TrCP}$ (lane 1), ubiquitin (lane 3), E1 (lane 5) or cdc 34 (lane 7). Reactions were stopped by the addition of SDS disruption buffer, separated by SDS-PAGE (8.5%), stained with Coomassie Blue, destained, dried and exposed to phosphoimagescreen for 10-12 hours. Molecular weight markers are shown on the left.

complex are capable of IκBα ubiquitination and that no other proteins are required for this reaction.

Optimal conditions for ubiquitination of ^{32}P-IκBα were also investigated. As shown in Figure 17, ubiquitination of phosphorylated IκBα is dependent on E1. Assay conditions were investigated to identify the minimal amount of E1 required for ubiquitination to occur. *In vitro* ubiquitination assays were set up as described previously but varying the amounts of E1. As shown in Figure 18a and 18b, increasing the amount of E1 in the reaction, increases the ubiquitination of ^{32}P-IκBα. Therefore this indicates that the ubiquitination reaction is dependent on the presence of E1 ubiquitin activating enzyme and an increase in the amount of ubiquitination correlated well with an increase in the concentration of E1 enzyme used in the assay. As with E1, ubiquitination of IκBα is also dependent on the presence of an E2 ubiquitin conjugating enzyme. The E2 responsible for ubiquitination of IκBα remains controversial, with both cdc 34 and UbcH5 (Yaron *et al.*, 1998; Vuillard *et al.*, 1999; Wu *et al.*, 2000; Chen *et al.*, 1996; Gonen *et al.*, 1999; Ohta *et al.*, 1999; Spencer *et al.*, 1999; Tan *et al.*, 1999; Strack *et al.*, 2000) being shown to be involved. Ubiquitination assays were set up with varying concentrations of either cdc34 or ubcH5 as the source of E2. ^{32}P-IκBα was incubated with varying concentrations of E2 as indicated, 100ng E1, $SCF^{\beta TrCP}$ expressed and purified from insect cells as above, in the presence of ubiquitin and an ATP regeneration system. From Figure 18c, 18d and 18e it was demonstrated that both enzymes are able to participate in IκBα ubiquitination, although the rate and pattern of ubiquitination seems to differ for each enzyme. However, when 200ng of either enzyme is used in the assay, the same level of ubiquitination is achieved. Therefore this suggests that both cdc34 and ubcH5 enzymes are capable of ubiquitinating ^{32}P-IκBα. However, the ubiquitination pattern is different for each enzyme indicating that the mechanism by which these two enzymes work may differ.

Figure 18. Ubiquitination of ^{32}P-IκBα in the presence of E1 or cdc34 or ubc5
Ubiquitination assays were carried out as described in Materials and Methods with varying amounts of E1 (18A), cdc34 (18C) or ubc5 (18D). Samples were then separated by SDS-PAGE (8.5%), stained in Coomassie Blue, destained, dried and exposed to phosphoimaging screens for 10-12 hours. The amount of polyubiquitinated IκBα was quantified by phosphorimager both for E1 (18B), cdc34 and ubc5 (18E).


A
E1(ng) 0 10 100 500
Ubiquitin adducts
32P-IκBα
B
Ubiquitination (arbitary units)
0 1 2 3 4 5 6 7 8 9 10
0 10 100 500
E1 (ng)
C
cdc 34 (ng)
0 10 50 100 150 200
Ubiquitin adducts
32P-IκBα
D
ubc 5 (ng)
0 10 50 100 150 200
Ubiquitin adducts
32P-IκBα
E
Ubiquitination (arbitary units)
0 10 20 30 40 50 60
0 10 50 150 100 200
E2 (ng)
cdc34
ubc5

3.2.4 Ubiquitination of phosporylated IκBα *in vitro* requires all four components of the SCF complex

To fully identify the minimal requirements of the E3 ligase responsible for ubiquitination of phosphorylated IκBα, assays were performed in the absence of 3 of the components of the $SCF^{\beta TrCP}$ complex, Skp1, cul1/cdc53 or Rbx1. Sf9 cells were co infected with baculovirus containing βTrCP and a combination of either Skp1, Cul1/cdc53 or Rbx1. Complexes were isolated as before by immunoprecipitation with protein A sepharose beads linked to SV5 Pk Tag, 336 monoclonal antibody which recognises an N-terminal epitope fused to βTrCP. $SCF^{\beta TrCP}$, $SCF^{\beta TrCP}$ minus Skp1, $SCF^{\beta TrCP}$ minus Cul1/cdc53 and $SCF^{\beta TrCP}$ minus Rbx1 were used in an *in vitro* ubiquitination assay as described in materials and methods. As can be shown in Figure 19, when either Skp1, Cul1/cdc 53 or Rbx 1 are omitted from the complex, ubiquitination of IκBα is reduced. Ubiquitination is not completely abolished when either Skp1, Cul1/cdc 53 or Rbx 1 are absent from the complex, but it is significantly reduced compared to the intact $SCF^{\beta TrCP}$ complex. Since the $SCF^{\beta TrCP}$ has been expressed and purified from insect cells, it is possible that the ubiquitination that does occur could be due to substitution of the missing components by insect cell homologues. It should be noted that the high molecular weight material, probably representing multi-ubiquitinated species, is almost completely abolished in the absence of either Skp1, Cul1/cdc 53 or Rbx 1. The level of unmodified ^{32}P-IκBα remaining upon ubiquitination in the absence of either Skp1, Cul1/cdc 53 or Rbx 1 is also elevated compared with ubiquitination in the presence of an intact $SCF^{\beta TrCP}$ complex, suggesting that the ubiquitination process is down-regulated when the $SCF^{\beta TrCP}$ complex is deficient in either of these components. These results suggest that all four components of the $SCF^{\beta TrCP}$ complex are required for efficient ubiquitination of ^{32}P-IκBα.

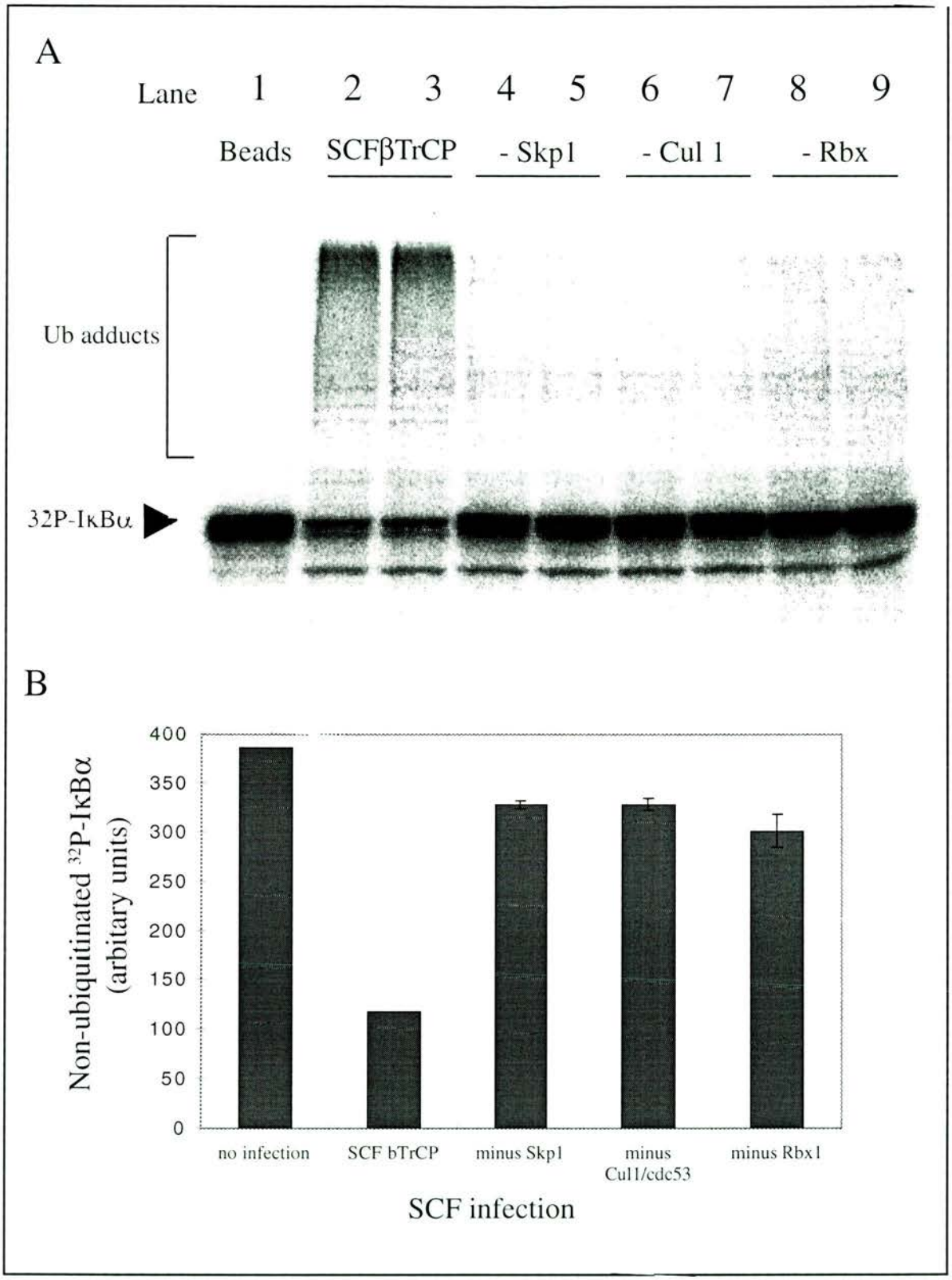


Figure 19 Ubiquitination of ^{32}P-IκBα is dependent on an intact SCFβTrCP complex.
Sf9 cells were infected with baculovirus expressing βTrCP and a combination of either Skp1, Cul1/cdc53 and/or Rbx1. SCF complexes were isolated as described in Materials and Methods and used as source of E3 ligase in ubiquitination assays with purified E1, cdc34, an ATP regeneration system and ^{32}P-labelled IκBα, phosphorylated *in vitro* by IKK1 and IKK2. (A) Samples were separated by SDS-PAGE (8.5%), gels stained in Coomassie blue, destained,dried and exposed to phosphoimage screen for 12 hours. Lane 1 shows beads alone, lane 2 and 3 contain SCF$^{\beta TrCP}$, lanes 4 and 5 contain SCF$^{\beta TrCP}$ minus Skp1, lanes 6 and 7 contain SCF$^{\beta TrCP}$ minus Cul1/cdc53 and lanes 8 and 9 contain SCF$^{\beta TrCP}$ minus Rbx1. (B) Amount of non-ubiquitinated ^{32}P-IκBα remaining was quantified by phosphoimager.

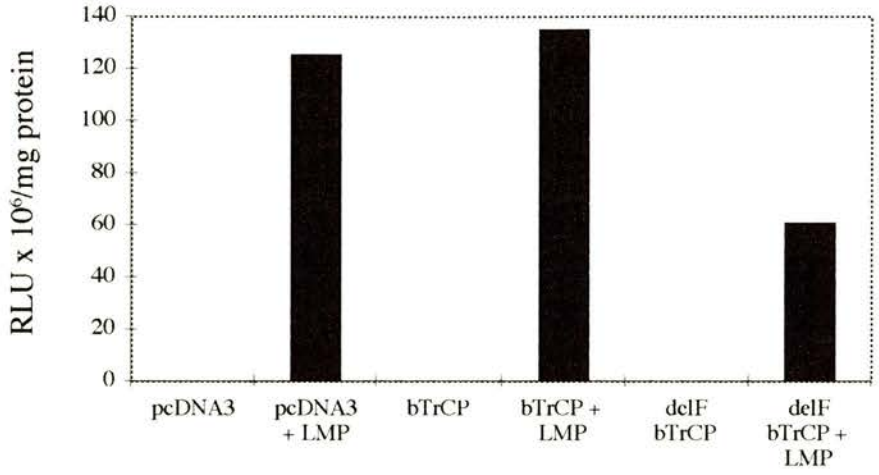
3.2.5 Deletion of the F-box domain of βTrCP inhibits NF-κB activation and IκBα degradation

The discovery that the F-box containing protein termed βTrCP within the E3 ligase was responsible for recognising IκBα, provided further clues as to the mechanism by which IκBα was targeted for ubiquitination and subsequent degradation by the E3 ligase. The F-box domain of βTrCP has been shown to be involved in the binding of Skp1, another protein within the E3 ligase, and deletion of this region results in a dominant negative mutant which can stabilise IκBα *in vivo* (Yaron *et al.*, 1998; Wu and Ghosh, 1999; Spencer *et al.*, 1999; Margottin *et al.*, 1998; Hattori *et al.*, 1999; Hatakeyama *et al.*, 1999; Kroll *et al.*, 1999; Fuchs *et al.*, 1999). Dominant negative mutants of βTrCP that lacked the F-box domain (amino acids 148-189) were constructed and used to investigate the role of this protein in NF-κB signalling pathways. HeLa 57A cells were transfected with βTrCP dominant negative mutants in the absence or presence of co-transfection with Eppstein Barr Virus (EBV) Latent Membrane Protein-1 (LMP-1). LMP-1 constitutively activates the NF κB pathway, and therefore the effect that the transfected plasmids have on NF-κB activation can be assessed, rather than having to add an extracellular stimulus which can affect those cells which are not transfected and which would give a false reading. By co-transfecting with LMP-1, it is assumed that if one construct is transfected into cells, then the other one is as well. Therefore NF-κB activation should only be evident in transfected cells. HeLa 57A cells are stably transformed cells that contain an NF-κB dependent luciferase reporter. Upon binding of NF-κB to these sites in the promoter of the luciferase gene, luciferase is expressed which can be assayed and used to calculate the amount of NF-κB activity (measured in relative light units (RLU)/mg protein). Figure 20A shows that NF-κB activity was reduced by approximately 50% upon co-transfection of the reporter cell line, HeLa 57A, with LMP-1 and ΔF-box βTrCP, compared with LMP and WT-βTrCP. The effect this mutant had on IκBα levels within the cell was also investigated. Cos 7 cells were transfected with either empty vector, pcDNA3 WT βTrCP or pcDNA3ΔF-box βTrCP.

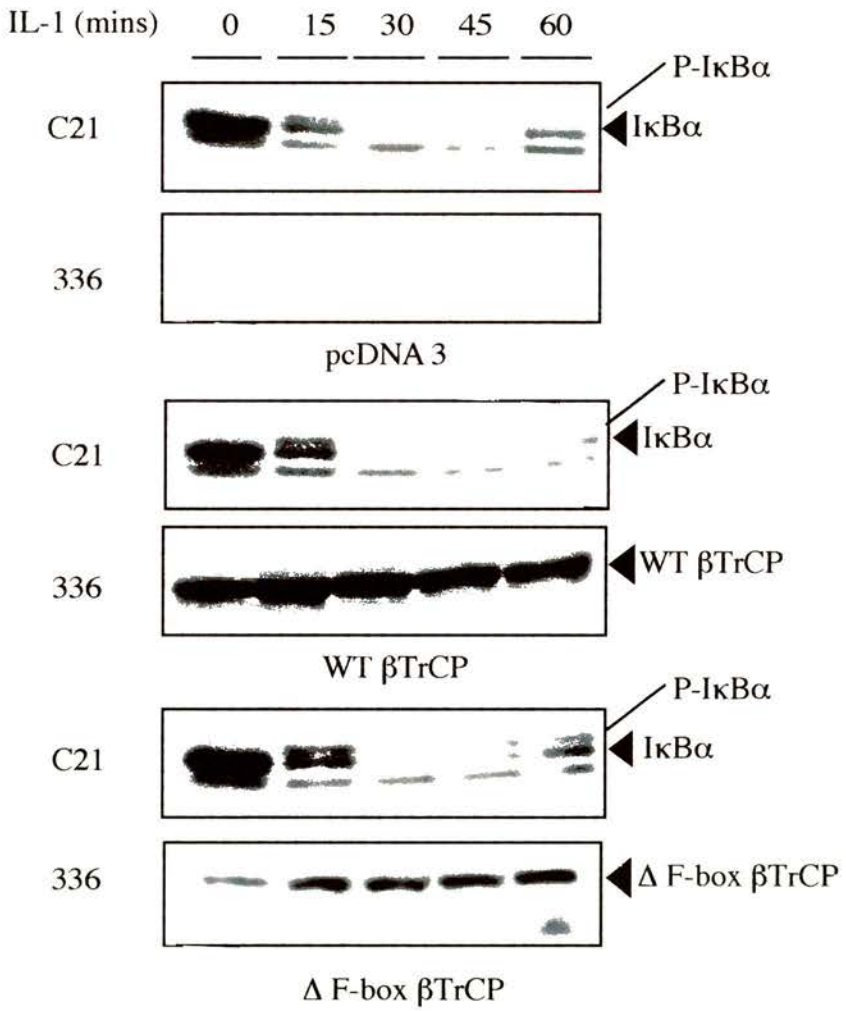
Figure 20 A dominant negative mutant of βTrCP inhibits NFκB activation and IκBα degradation in vivo.

A. 10^6 57A Hela cells were transfected by electroporation with either empty vector, pcDNA3,or βTrCP or the dominant negative mutant, ΔF-box βTrCP in the absence or presence of co-transfection with LMP. 24 hours after transfection, cells were lysed and assayed for luciferase activity. Luciferase activity is expressed as relative light units (RLU) per mg of protein. B. Cos-7 were transfected by lipofectamine with either empty vector, pcDNA3, wild-type βTrCP, or ΔF-box βTrCP. 24 hours after transfection cells were split into 5 wells of a 6 well plate and stimulated with 20ng/ml Il-1 for the indicated times. Cells were then lysed and 25μl of extracts were separated by SDS-PAGE (10%), transferred to PVDF membrane and probed with a polyclonal antibody which recognises IκBα (C21). The same blots were stripped and re-probed with a monoclonal antibody which recognises the N-terminal tag of βTrCP, sv5, 336 Tag.(336).

A


RLU x 10⁶/mg protein
140
120
100
80
60
40
20
0
pcDNA3
pcDNA3 + LMP
bTrCP
bTrCP + LMP
delF bTrCP
delF bTrCP + LMP

B


IL-1 (mins)
0
15
30
45
60
C21
P-IκBα
IκBα
336
pcDNA 3
C21
P-IκBα
IκBα
336
WT βTrCP
WT βTrCP
C21
P-IκBα
IκBα
336
Δ F-box βTrCP
Δ F-box βTrCP

24 hours after transfection, cells were divided into 6 well plates and stimulated with 20ng/ml of IL-1 for 0, 15, 30, 45 or 60 minutes. After stimulation, cells were lysed, and extracts were separated by SDS-PAGE, transferred to PVDF membrane and probed with either C21, a polyclonal antibody which recognises IκBα, or 336 monoclonal antibody, which recognises the sv5 Pk Tag of βTrCP constructs. As shown in Figure 20b, IκBα is degraded in a pattern which is normal to this cell line; in cells transfected with empty vector; there is a reduction in the amount of IκBα after 15 minutes of stimulation with IL-1, consistent with activation of the signalling pathway and IKK signalsome. Levels of IκBα are reduced further after 30 minutes of IL-1 stimulation and by 45 minutes, completely abolished. NF-κB activation and translocation into the nucleus, resulting from degradation of IκBα, allows synthesis of NF-κB responsive genes, one of which is IκBα, and appearance of newly re-synthesised IκBα protein is evident after 60 minutes of IL-1 stimulation. The same pattern of IκBα degradation occurs when WT βTrCP is expressed in Cos 7 cells. However, when the dominant negative mutant of βTrCP, lacking the F-box is expressed (ΔF-box βTrCP), there is an accumulation of the hyper-phosphorylated form of IκBα, suggesting that the protein is being phosphorylated efficiently by the IKK signalsome, but is not able to be degraded. Considering transfection of cells by this method results in only a fraction of the cells expressing the protein of interest, the overall effect the ΔF-box βTrCP has within the cell is underestimated. It should be noted that there are different levels of expression of the WT βTrCP compared with ΔF-box βTrCP. This could be due ΔF-box βTrCP protein itself being unstable when not within the $SCF^{\beta TrCP}$ complex or that the mutant protein could be folded in a way which might affect its stability. Overall, our findings suggest a role for βTrCP in NF-κB activation and correlate well with the observations in the current literature.

3.2.6 βTrCP interacts with phosphorylated IκBα but not non-phosphorylated

βTrCP is therefore involved in the degradation of IκBα *in vivo*. Since the F-box proteins within the $SCF^{\beta TrCP}$ complex are important in conferring specificity to the ubiquitination and hence degradation process, it is interesting to investigate the mechanism or molecular requirements by which these proteins actually recognise their substrate. Identification of some of the key elements involved in the recognition of IκBα by βTrCP was investigated. Previously Vuillard *et al*, by transfecting HeLa cells with tagged βTrCP and immunoprecipitating the tagged protein and therefore the SCF complex, showed that a peptide corresponding to amino acids 20-43 of IκBα could be ubiquitinated only when serine residues 32 and 36 were phosphorylated (Vuillard *et al.*, 1999). As shown in Figure 21 by using βTrCP expressed and purified from insect cells it was demonstrated that it could only interact with a phosphorylated IκBα peptide. Baculovirus containing tagged βTrCP was used to infect insect cells at an m.o.i of 5, purified by immunoprecipitation with protein A-sepharose beads linked to sv5 Pk tag, 336, monoclonal antibody, and incubated with either P-IκBα peptide or non-P IκBα peptide which had been labelled with ^{125}Iodine at tyrosine residue 40. As shown in Figure 21a, there was a 3.5 fold increase in binding of the phosphorylated peptide to the βTrCP-beads compared with the non-phosphorylated peptide, indicating that phosphorylation of serine residues 32 and 36 were important for binding. This could also be demonstrated by incubating βTrCP-beads with increasing amounts of ^{125}Iodine-labelled phosphorylated peptide (Figure 21c). Figure 21b shows that baculovirus expressed βTrCP could be immunoprecipitated by sv5, Pk tag, 336-Protein A beads. Therefore initial experiments indicated that phosphorylation of IκBα at serine residues 32 and 36 was important for the interaction between IκBα and βTrCP.

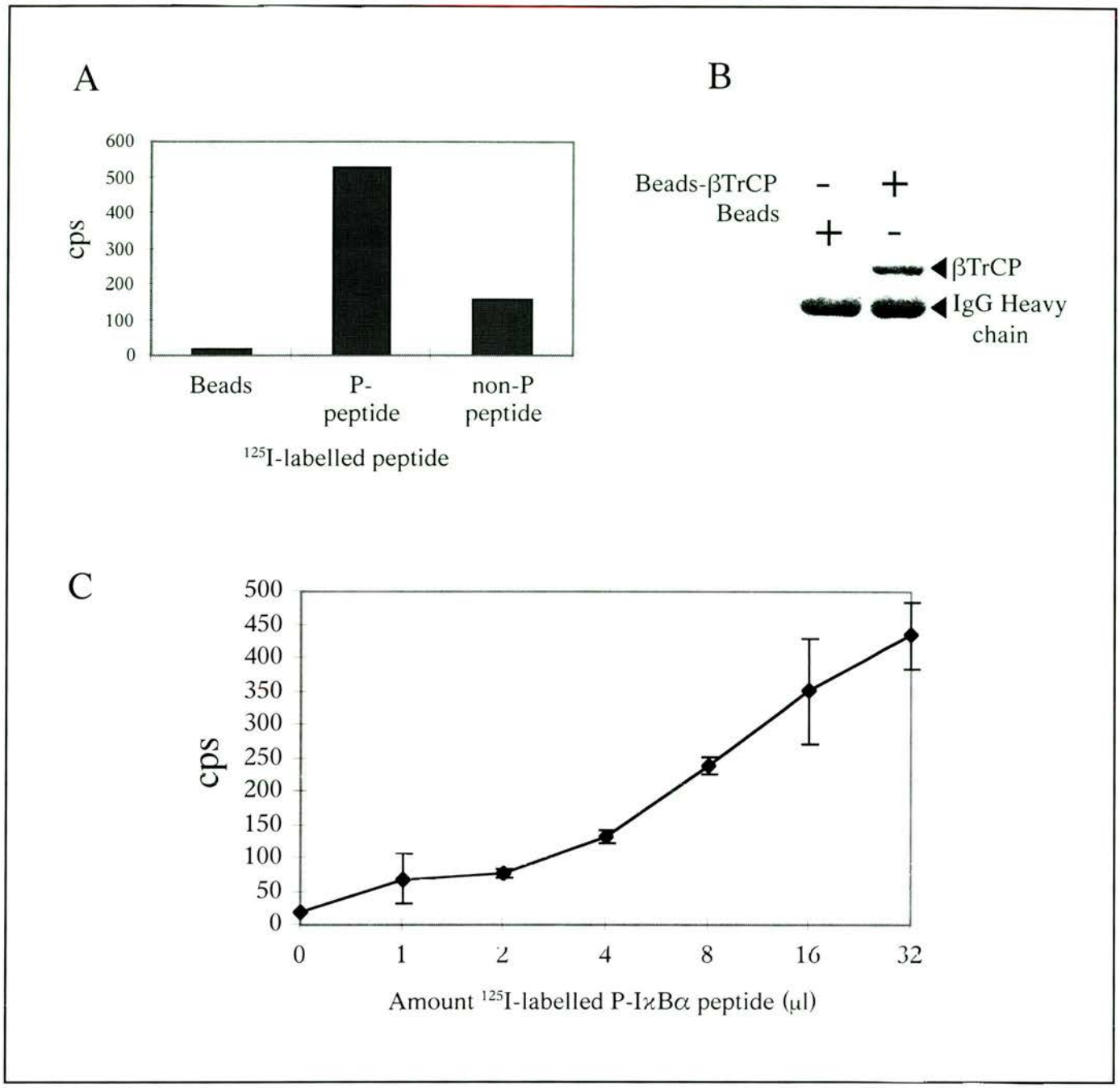


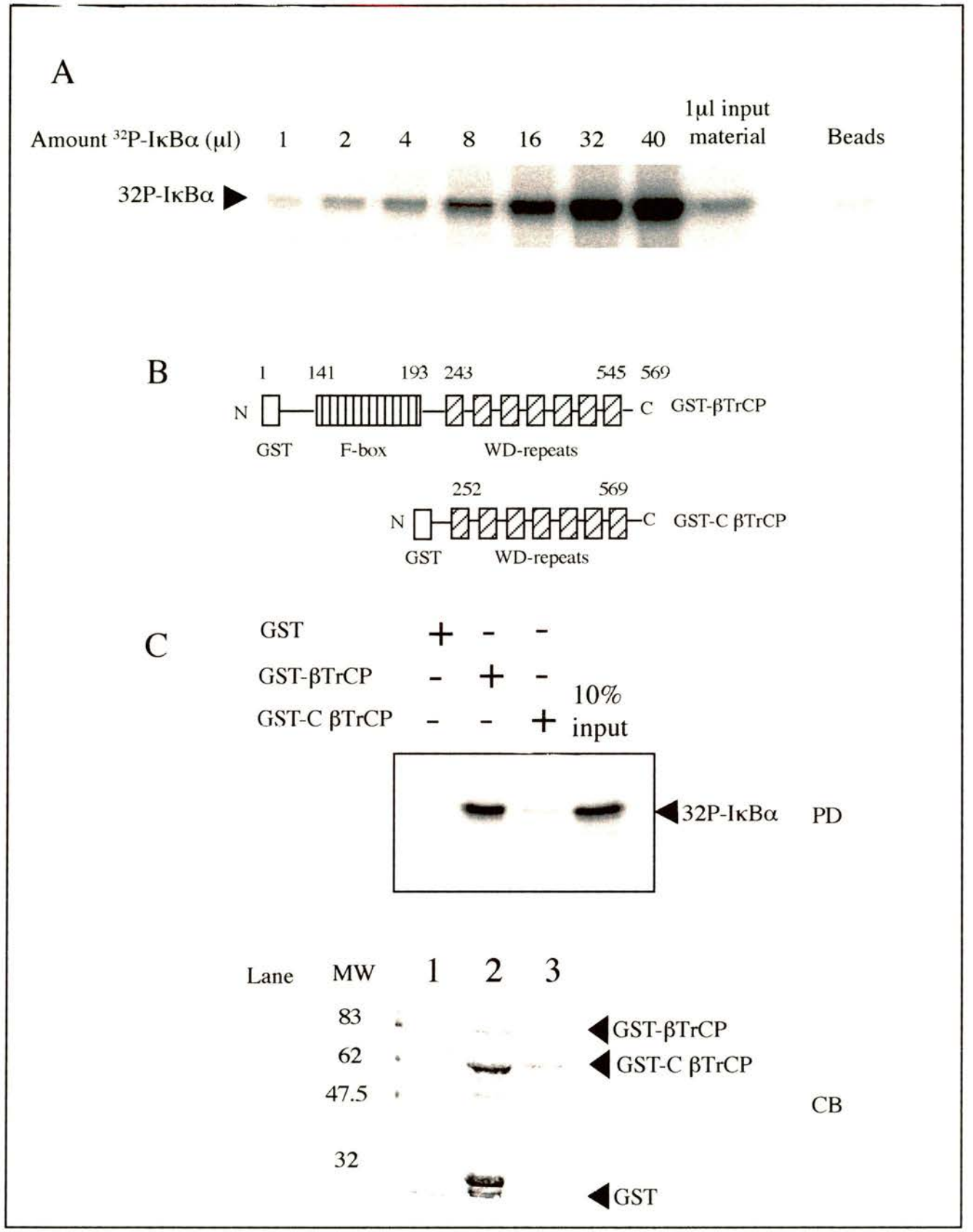
Figure 21 βTrCP interacts only with a phosphorylated IκBα peptide.

βTrCP was expressed and purified from insect cells by immunoprecipitation with Protein A-sv5, 336 beads. A. 10μl of beads were incubated for 1 hour at room temperaturein a total reaction volume of 50μl containing 10mM Tris HCl, pH 8, 4mM$MgCl_2$, 1mg/ml BSA and 1nmole iodine labelled IκBα peptide, phosphorylated or not on serine residues 32 and 36. Beads were then washed 3x in 1ml of 10mM Tris HCl, pH 8, 100mM NaCl and 1mM $MgCl_2$, and the amount of iodine labelled peptide associating with the beads was measured in a scintillation counter, Mini-assay type 6-20 (Mini-Instruments Ltd). B. SDS-Page (8.5%) showing the binding of βTrCP, expressed from insect cells, to Protein A-sv5, 336 beads. C. 10μl βTrCP-protein A, sv5, 336 beads were also incubated for 1 hour at room temperature in a total reaction volume of 50μl containing 10mM Tris HCl, pH 8, 4mM$MgCl_2$, 1mg/ml BSA and increasing amounts of iodine labelled peptide phosphorylated at serines 32 and 36. Beads were then washed as before and amount of iodine labelled peptide associated with the beads measured as above.

3.2.7 Phosphorylated IκBα physically associates with βTrCP

Finding that βTrCP interacted with only the phosphorylated IκBα peptide, the interaction with phosphorylated, full-length protein was investigated. Incubation of βTrCP expressed and purified fron insect cells, as above, with recombinant IκBα which had been radiolabelled using [γ-^{32}P]-ATP on serines 32 and 36 in an *in vitro* kinase assay with IKK1 and IKK2, demonstrated that phosphorylated IκBα was able to associate with βTrCP *in vitro*. As can be shown in Figure 22a, the amount of ^{32}P-IκBα which associates with βTrCP increased in proportion to the amount added in the assay. βTrCP cloned and expressed as a GST fusion protein in bacteria was also used to investigate the interaction between this protein and phosphorylated IκBα. Several βTrCP constructs were made as GST fusion proteins; full-length or wild-type βTrCP (1-569), N-terminal βTrCP (2-251) and C-terminal βTrCP (252-569). A schematic overview is shown in Figure 22b. Unfortunately, the N-terminal construct was unable to be expressed in bacteria and therefore could not be used in the interaction assays. This may be because the structure of βTrCP may contribute to its stability and when a large and major part is removed, such as the WD repeats, the protein is no longer stable. The full length protein displayed a number of degradation products indicating that this protein by itself, and when expressed in bacteria, is not stable. By incubating ^{32}P-IκBα with the WT and C-terminal fusion proteins the interaction between ^{32}P-IκBα and βTrCP could be demonstrated (Figure 22c). It has been reported that βTrCP interacts with P-IκBα through its WD repeats (Margottin *et al.*, 1998), however results here demonstrate that not only are the WD repeats important for the binding of P-IκBα to βTrCP but that there must also be some regions within the N-terminus that contribute to the interaction, since the C-terminal fusion protein, by itself, is not able to sustain binding to ^{32}P-IκBα. Overall, it was demonstrated that βTrCP interacts with IκBα only when IκBα is phosphorylated.

Figure 22. βTrCP interacts directly with IKK1/2 phosphorylated IκBα.
Recombinant IκBα was phosphorylated *in vitro* by IKK1/2 and used as source of substrate for interaction with βTrCP. A. Increasing amounts of phosphorylated IκBα was incubated with 10μl βTrCP-Protein A, sv5, 336 beads in interaction buffer (10mM Tris HCl, pH 8, 4mM $MgCl_2$, 1mg/ml BSA) for 1 hour at room temperature. Beads were washed 3x in 1ml wash buffer (10mM Tris HCl, pH8, 100mM NaCl, 10mM $MgCl_2$), resuspended in 10μl of interaction buffer and 3x SDS disruption buffer, separated by SDS-PAGE (10%), gels stained in Coomassie blue, destained, dried and exposed to phosphorimage screen for 10-12 hours. B. GST-βTrCP constructs showing the F-box and WD repreat regions. C. GST, full length βTrCP or C-terminal, residues 252-569, expressed as a GST fusion and bound to glutathione agarose beads, were incubated for 1 hour at room temperature in a total reaction volume of 100μl containing kinase buffer, 1mg/ml BSA and 1.1μg IκBα previously phosphorylated *in vitro* by IKK1/2. Beads were then washed 3x with 1ml wash buffer (10mM Tris HCl, 100mM NaCl, 1mM $MgCl_2$, 0.1% NP-40), resuspended in 10μl of kinase buffer + 5μl 3x SDS disruption buffer, separated by SDS-PAGE (10%), stained in Coomassie blue, destained, dried and exposed to phosphorimage screen as above. CB = coomassie blue and indicates coomassie blue stained gel of input GST proteins.


A
Amount 32P-IκBα (μl)
1
2
4
8
16
32
40
1μl input material
Beads
32P-IκBα
B
1
141
193
243
545
569
N
C
GST-βTrCP
GST
F-box
WD-repeats
252
569
N
C
GST-C βTrCP
GST
WD-repeats
C
GST
GST-βTrCP
GST-C βTrCP
10% input
32P-IκBα
PD
Lane
MW
1
2
3
83
62
47.5
32
GST-βTrCP
GST-C βTrCP
CB
GST

3.2.8 Discussion

Ubiquitination of IκBα has been previously demonstrated *in vitro* using partially purified components where one or more of the $SCF^{\beta TrCP}$ components was transfected into cells and then immunopurified (Hatakeyama *et al.*, 1999; Spencer *et al.*, 1999; Vuillard *et al.*, 1999) or by using crude cell lysates (Winston *et al.*, 1999). However it is possible that other proteins may be present within this immunopurified complex that have not yet been identified. Ubiquitination of IκBα *in vitro* using purified components was investigated. These consist of an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, either cdc34 or ubc5, and an E3 ubiquitin ligase, in the presence of ubiquitin and an ATP regeneration system. *In vitro* ubiquitination assays using these purified components demonstrated that these proteins are both sufficient and necessary for the ubiquitination of IκBα. It is true, however, that there may also be other proteins associated with the E3 ligase not yet identified, or that there may be modifications of the proteins within the $SCF^{\beta TrCP}$ that are necessary for optimal functioning of the complex. In fact, it has been shown that conjugation of NEDD 8 (a small ubiquitin-like protein), at lysine residue 720 of Cul 1 is important for the functioning of $SCF^{\beta TrCP}$ in ubiquitination of IκBα, and that mutation of this lysine residue to arginine, results in decreased ubiquitination of substrates when used in ubiquitination assays (Read *et al.*, 2000). Results here demonstrate that uniquitination of IκBα occurs in the presence of an $SCF^{\beta TrCP}$ complex expressed and purified from insect cells. The fact that this complex is active and that its activity seems to be dependent on modification of Cul1 by NEDD8, it is reasonable to believe that insect cell NEDD8 is able to modify Cul1 in the overexpressed $SCF^{\beta TrCP}$ complex.

There is a single E1 ubiquitin activating enzyme known, but there have been several E2 ubiquitin conjugating enzymes identified. Identification of the E2 for IκBα has remained somewhat controversial with both cdc 34 and ubc5 being shown to be involved. Comparison of IκBα ubiquitination in the presence of either enzymes was therefore investigated. As shown in Figures 18c, 18d and 18e, ubiquitination of ^{32}P- IκBα occurs in

the presence of both enzymes. However, the pattern of ubiquitination appears to be different for each enzyme. Vuillard et al initially demonstrated that cdc34 appears to favour the formation of polyubiquitinated products compared with the mono- and di-ubiquitinated products observed when ubc 5 is used as the source of E2 (Vuillard *et al.*, 1999; Wu *et al.*, 2000). The differences and functioning of these two different forms of ubiquitinated species is still not clearly understood. It is well known that polyubiquitin chains linked through lysine 48 are the principle signal for targeting of ubiquitinated substrates to the 26S proteasome (Chau *et al.*, 1989; Finley *et al.*, 1994), however it has been discovered that a minimum of 4 ubiquitins are required to serve as a targeting signal (Thrower *et al.*, 2000). This raises the questions as to why do mono- and di-ubiquitinated forms of IκBα exist and are both targeted as efficiently to the 26S proteasome? Alternatively, it has been suggested that ubc4/5 is the preferred enzyme *in vivo* as it has been found to be more abundant within the cell and observed to be more efficient in the conjugation of ubiquitin to substrate (Wu *et al.*, 2000; Ohta *et al.*, 1999; Strack *et al.*, 2000). From the data presented here, it appears that both cdc34 and ubc5 are capable of functioning in the ubiquitination of P-IκBα. It seems redundant for the cell to have two enzymes carrying out the same function and hence it is possible to imagine that under certain circumstances one E2 is favoured over another. Could it be possible that the cellular distribution of the two enzymes differ or that one enzyme is more abundant in one tissue than another? Given these results it would also be interesting to identify if the pattern of ubiquitinated product has any influence on its degradation via the 26S proteasome and what purpose this would serve in the functioning of the cell. For example, are poly-ubiquitintated species more efficiently recognised and degraded by the 26S proteasome, compared with mono-and di-ubiquitinated species and if so, would they serve as a more efficient method in rapidly degrading IκBα?

The discovery of a family of E3 enzymes led to the elucidation of the mechanism by which IκBα was ubiquitinated and targeted for degradtion. The four proteins identified at present which comprise the E3 for IκBα (βTrCP, Skp1, Cul1/cdc53 and Rbx1) were

demonstrated as being efficient and capable of ubiquitinating ^{32}P- IκBα in an *in vitro* system. Figure 19. The specificity by which phosphorylated IκBα is recognised depends on the properties of an F-box protein, βTrCP. However as shown from experiments presented here, each protein within the SCF complex is required and is necessary for efficient ubiquitination of ^{32}P-IκBα. The diversity of the complex is exemplified by the fact that the F-box proteins are interchangeable, with each F-box protein recognising a number of other types of substrate (Skowyra *et al.*, 1997; Feldman *et al.*, 1997; Bai *et al.*, 1996; Li and Johnston, 1997). It will also be interesting to find out how the SCF complexes are themselves regulated. Investigations into the regulation of these E3 ligases have shown that some of the F-box proteins (Grr1p, Cdc4p Met30p) are unstable and are themselves ubiquitinated and degraded in a proteasome-dependent manner (Galan and Peter, 1999; Zhou and Howley, 1998). In addition the expression and activity of βTrCP is upregulated by a post-transcriptional mechanism and induced upon signalling through the β-catenin/Tcf pathway, β-catenin acting as a substrate of the $SCF^{\beta TrCP}$ complex (Spiegelman *et al.*, 2000). This not only creates a negative feedback loop which in turn allows regulation of the TCF signalling pathway, it also suggests a mechanism by which the $SCF^{\beta TrCP}$ complex can be regulated and controlled. Other mechanisms of regulation have also been identified within the SCF complex. These include the discovery that Rbx1 is able to recruit the Rub1 conjugating enzyme Ubc12 and thereby contribute to the NEDD 8/Rub1 conjugation of, cdc53/cul1. Mutations in essential residues within the RING H2 structure of Rbx1 diminishes this conjugation and also the ubiquitination activity of SCF^{Grr1}, suggesting that Rub1 conjugation of cdc53/Cul1 is essential for activity of the complex (Kamura *et al.*, 1999a).

Ultimately, in order for a protein to be recognised as a substrate for ubiquitination, there must be some element of recognition between the protein itself, and the preferred SCF complex. The F-box protein within the SCF complex serves to recognise and recruit substrate in order for it to be targeted for modification by ubiquitin. The F-box domain of

βTrCP has been identified as the region that connects the protein to Skp1 and therefore the rest of the E3 ligase. The fact that NF-κB activity could be reduced in a reporter cell line, when the F-box deleted protein was co-transfected with LMP, indicates that this protein is involved in the NF-κB signalling pathway. As shown in Figure 20, it was also demonstrated that the point at which the pathway was blocked was at the stage of IκBα degradation. The F-box motif is the common element amongst the inter-changeable F-box proteins associated with the core components of the SCF complex. This therefore suggests that the F-box domain within βTrCP is important for the overall degradation of the target protein. Although the F-box participates in the interaction with Skp1, is it also possible that specific residues or structural features within this domain serves as a signal for ubiquitination and degradation via the 26S proteasome. Overall, these results illustrate the importance of this protein in NF-κB activation.

Investigation of the interaction between IκBα and βTrCP was determined and it was established that they could associate *in vitro*. Using βTrCP expressed in insect cells and purified by immunoprecipitating with Protein A-sv5, Pk Tag 336 beads, which recognise the sv5 epitope fused to the N-terminus of βTrCP, it was demonstrated that an IκBα peptide can only interact with βTrCP when it is phosphorylated on serine residues 32 and 36. This was confirmed by the fact that a non-phosphorylated peptide binds poorly. Figure 21. Therefore it was demonstrated that the recognition of IκBα by βTrCP is dependent on on phosphorylation at serine residues 32 and 36 of IκBα.

These studies were carried out using peptides corresponding to small regions of IκBα. The interaction between IκBα and βTrCP was further investigated using IκBα protein in complex with NF-κB, the natural physiological substrate. These studies verified the fact that IκBα must also be phosphorylated before it can interact with βTrCP. Recombinant IκBα, which had been allowed to interact with recombinant p65 in a ratio of one molecule of IκBα to two molecules of p65 was phosphorylated *in vitro* using an IKK1/IKK2 kinase complex and [γ^{32}P]-ATP. This substrate closely represents the

physiological form of IκBα that is targeted for ubiquitination and degradation. Interaction of ^{32}P-labelled IκBα with βTrCP expressed and immunoprecipitated from insect cells was demonstrated, along with interaction of the labelled IκBα with βTrCP expressed as a GST fusion protein in *E. Coli*. Figure 22. It has been demonstrated that phosphorylation of IκBα is a pre-requisite for ubiquitination and degradation of IκBα and the identification of the kinases responsible for IκBα phosphorylation has allowed further elucidation of this degradation process. By phosphorylating IκBα *in vitro* using the IκBα kinases, IKK1 and IKK2, determination of the mechanism by which IKK phosphorylated IκBα interacts with βTrCP was investigated. Overall, the classical and most typical pathway of activation of NF-κB occurs through the IKK signalsome and we were able to show that IκBα phosphorylated by this IKK complex in turn allows for the association of the complex with βTrCP, and hence the E3 ubiquitin ligase.

Expression of βTrCP constructs in bacteria proved problematic with no expression of the GST-N βTrCP construct, amino acids 2-251, and very little expression of GST-βTrCP and GST-C βTrCP. The instability of these proteins in bacteria might arise from it being expressed in a prokaryotic expression system and therefore they do not undergo all the post-translational modifications which are inherent in eukaryotes. The actual structure of the protein itself may also contribute to its overall stability or instability, and it may only be stable when associated with other proteins, for example, Skp1. The fact that other F-box proteins are themselves degraded in an ubiquitin-dependent manner suggests that proteins belonging to this family could also be regulated in this way.

Identification of the regions by which βTrCP interacts with phosphorylated IκBα was investigated by incubating ^{32}P- IκBα with either GST, GST-βTrCP or GST-C βTrCP in which reisdues 1-251 are absent. Figure 22b and 22c. It was demonstrated that phosphorylated IκBα was able to bind to GST-βTrCP but not to GST alone or GST-C βTrCP. This therefore indicates that not only are the WD repeats of βTrCP required for interaction with IκBα, but that there must also be some elements required in the N-

terminus, amino acid residues 1-251 important for binding. Indeed, Margottin *et al* was able to show that it is the WD repeats in βTrCP which are required for the interaction of this protein with vpu, particularly the first WD repeat (Margottin *et al.*, 1998), but others have also demonstrated that the F-box deleted version of βTrCP, which is only devoid of residues 141-193, can also bind phosphorylated IκBα, and in some cases, even better than the wild-type protein (Yaron *et al.*, 1998; Hatakeyama *et al.*, 1999; Kroll *et al.*, 1999; Spencer *et al.*, 1999; Winston *et al.*, 1999). This suggests that either residues within 1-140, 193-252 or both are also important in this interaction.

Overall, *in vitro* ubiquitination assays using purified components of the ubiquitin conjugating system allowed the investigation of the process by which IκBα is ubiquitinated. The mechanism of interaction between IκBα and βTrCP, the protein that confers specifcity to the ubiquitination process was also investigated. Analysis of this interaction reveals the requirement of phosphorylated residues within the target protein. These initial experiments provide a basis for examining the mechanism of interaction and ubiquitination of IκBα in further detail.

3.3 Investigation of the residues in IκBα important for ubiquitination and interaction with βTrCP

3.3.1 Summary

The discovery of E3 ubiquitin ligases identified the pathway by which specific proteins are targeted for ubiquitination and degradation via the 26S proteasome. In the case of IκBα, specificity of this degradation process is conferred by recognition of this protein by an F-box protein termed βTrCP, which belongs to the SCF family of E3 ligases. To investigate the basis of substrate recognition by βTrCP a series of peptides corresponding to amino acids 28-39, or 20-43 of IκBα were synthesised and their ability to interact with βTrCP and inhibit ubiquitination of IκBα was determined. Ubiquitination of IκBα and interaction with βTrCP was dependent on the presence of phosphate groups at serine residues 32 and 36 of IκBα. Further studies demonstrated that the residues within and around the consensus motif, DSGXXS, in βTrCP substrates could also contribute to the ubiquitination of IκBα and its interaction with βTrCP. Aspartate 31 was demonstrated to be of particular importance in both these steps. Inhibitor concentrations and binding affinities of each of the peptides used in the assays was also investigated. Overall, the mechanism by which IκBα is recognised by the E3 ligase and the importance of phosphorylation at serines 32 and 36 of IκBα was established. It was also demonstrated that residues within IκBα other than phosphorylated serine residues 32 and 36 contribute to this interaction and hence contribute to the interaction of the SCF ligase and its target protein.

3.3.2 IκBα consensus motif residues involved for recognition by βTrCP are involved in the ubiquitination of IκBα

It has been demonstrated that βTrCP is the protein within the SCF complex which confers specificity and targeting of substrates for the attachment of ubiquitin and hence degradation through the 26S proteasome (Yaron *et al.*, 1998). βTrCP not only recognises phosphorylated IκBα but also a number of other known proteins including β–catenin (Hatakeyama *et al.*, 1999; Kitagawa *et al.*, 1999) and the HIV1 protein, vpu (Margottin *et al.*, 1998). Furthermore, it has been demonstrated that all these proteins contain a consensus motif, DSGXXS. Figure 23 shows a sequence alignment of the consensus motif found in IκBα, β-catenin and vpu proteins. Residues within and surrounding this consensus motif were assessed for their ability to participate in ubiquitination of IκBα. A series of peptides were generated corresponding to either amino acids 28-39, or 20-40 of IκBα and are displayed in Figure 24. These represented the wild-type protein, non-P, (1), wild-type protein phosphorylated on serine residues 32 and 36, P-S32,36, (2), wild-type protein phosphorylated only on serine 32, P-S32, (3), wild-type protein phosphorylated only on serine 36, P-S36, (4), then a series of mutations, S32,36E (5), H30A (6), D31A (7), G33A (8), L34A (9), D35A (10), M37A (11), D31K (12) and Δ35D/E40D (13). These peptides were used to investigate inhibition of ubiquitination of recombinant ^{32}P-IκBα, by incubating the peptides, in excess, in an *in vitro* ubiquitination assay. Products of the ubiquitination assay were resolved by SDS-PAGE, gels dried, exposed to phosphorimage screen and quantified using a Fujix1500 and MacBas software. Hence residues that may be important for the overall recognition, ubiquitination and degradation of IκBα could be identified. As shown in Figure 25, both serines 32 and 36 are required to be phosphorylated before they can inhibit ubiquitination of radiolabelled ^{32}P-IκBα, as non-phosphorylated peptide has no effect on ubiquitination of IκBα whereas p-S32,36 reduces ubiquitination by 40%. Mutation of serines 32 and 36 to glutamic acid had no effect either and single phosphorylation on either serine 32 or 36 is also not able to block ubiquitination,

Figure 23. Alignment of the consensus motif found in βTrCP substrates.
Amino acid alignment of the consensus motif and surrounding residues within 3 known βTrCP substrates. Consensus motif DSGXXS is highlighted in red.

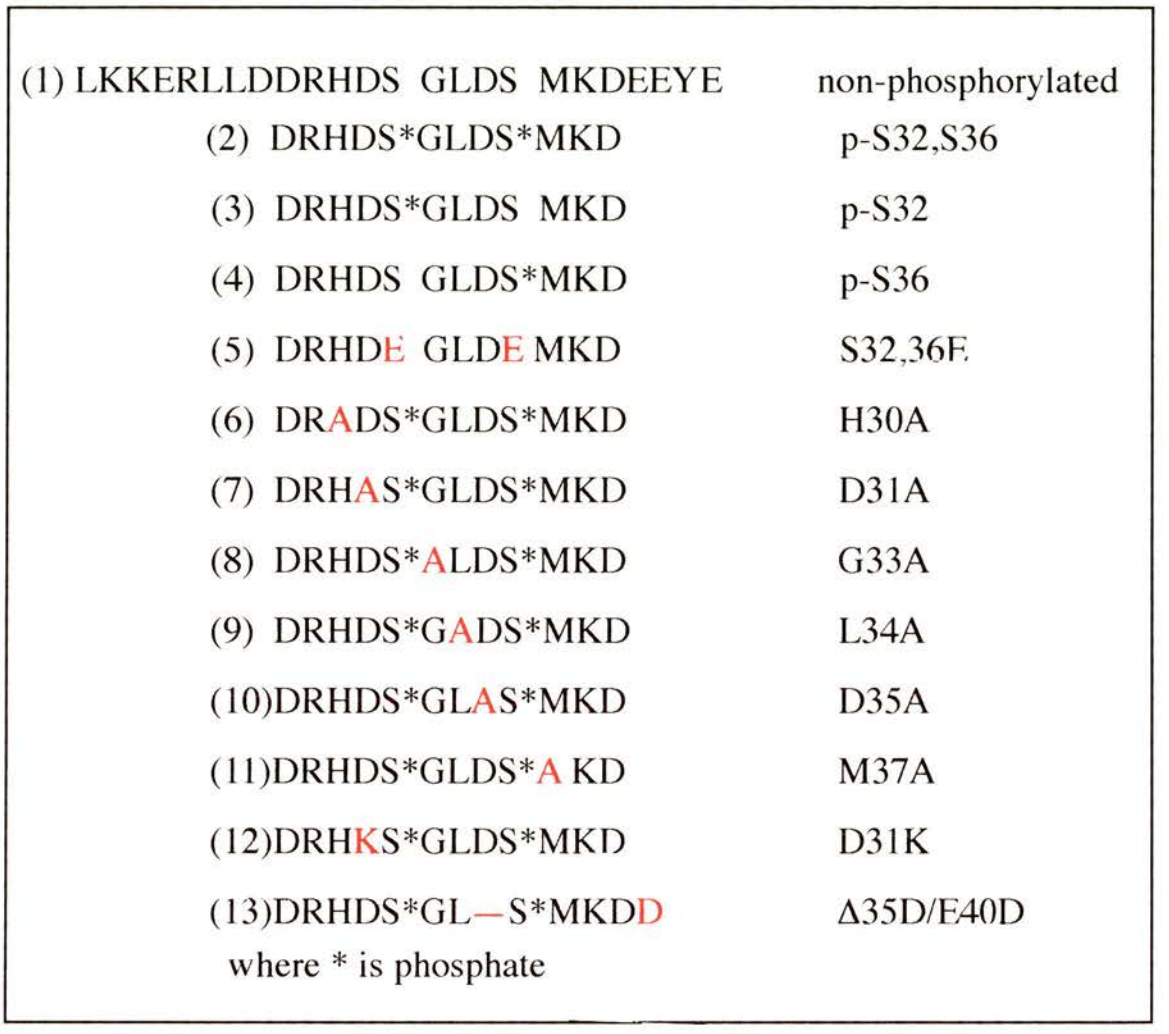


Figure 24 IκBα competing peptides.
A series of peptides were generated corresponding to amino acids 28 to 39, and one corresponding to amino acids 28 to 40, of IκBα in which a number of mutations and or modifications have been introduced. Mutations are outlined in red. * indicates phosphate group.

indicating the importance of the phosphate group at these sites. A reduction in the percentage of IκBα ubiquitination was also demonstrated when H30, L34, D35 and M37 are mutated to alanine. These amino acids all lie within and surrounding the phosphorylation sites. Mutation of these residues to alanine inhibits ubiquitination comparable to that of the p-S32,36 peptide, indicating that these amino acids are of no importance in the actual ubiquitination process. Peptides D31A, D31K and G33A do not seem to interfere with the ubiquitination process dramatically. This would indicate that aspartate 31 and glycine 33 are important, or play a part in the overall recognition of IκBα by the E3 complex and ubiquitination of IκBα.

3.3.3 IκBα consensus motif residues are involved in the interaction of IκBα with βTrCP

The residues that were important for IκBα ubiquitination, were also analysed for their role in the interaction of P-IκBα with βTrCP. Interaction assays were set up by incubating GST-WT βTrCP with radiolabelled ^{32}P- IκBα in the presence of a series of un-labelled IκBα peptides as described in Figure 24. The IκBα peptides are added in excess and compete with ^{32}P-IκBα for binding to βTrCP. Samples were resolved by SDS-PAGE, gels dried, exposed to phosphorimage screen and quantified using a Fujix1500 and MacBas software. As shown in Figure 26, those peptides with residues that may be important in the recognition of ^{32}P-IκBα by βTrCP, can be identified due to the lack of inhibition of binding of ^{32}P-labelled IκBα to βTrCP. p-S32,36 competes for the binding of βTrCP and can reduce binding of ^{32}P-IκBα to βTrCP by 50%. In contrast non-phosphorylated peptide does not compete, indicating that phosphorylated serine residues are important in the recognition of IκBα by βTrCP, and again confirming that βTrCP does not interact with non-phosphorylated substrate. A number of other peptides also compete, although with varying degrees of antagonism. These include p-S32, H30A, G33A, L34A,

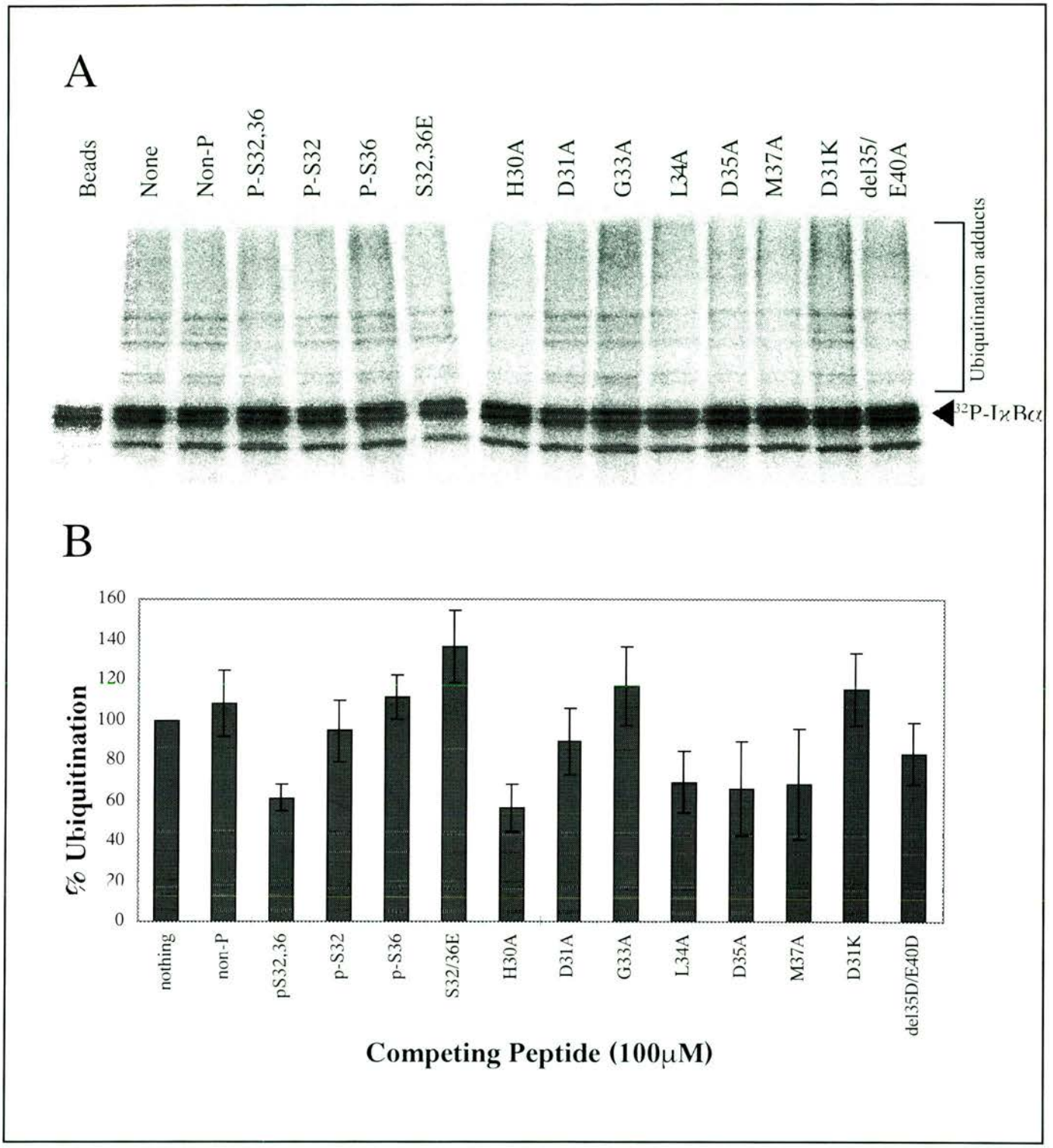


Figure 25. Inhibition of ubiquitination of ^{32}P-IκBα by competing IκBα peptides.
Ubiquitination assays were carried out as described in Materials and Methods in the presence of 100μM unlabelled IκBα peptide. (A) Samples were separated by SDS-PAGE (8.5%), gels stained in Coomassie Blue, destained, dried and exposed to phosphoimage screen for 12 hours. (B) Ubiquitination of ^{32}P-IκBα was quantified on the phosphoimager. Amount of ubiquitination observed in the absence of any competing peptide was calculated as being 100% and ubiquitination in the presence of peptides was quantified and compared to this value. Values represent the average of 3 assays with error bars representing one standard deviation.

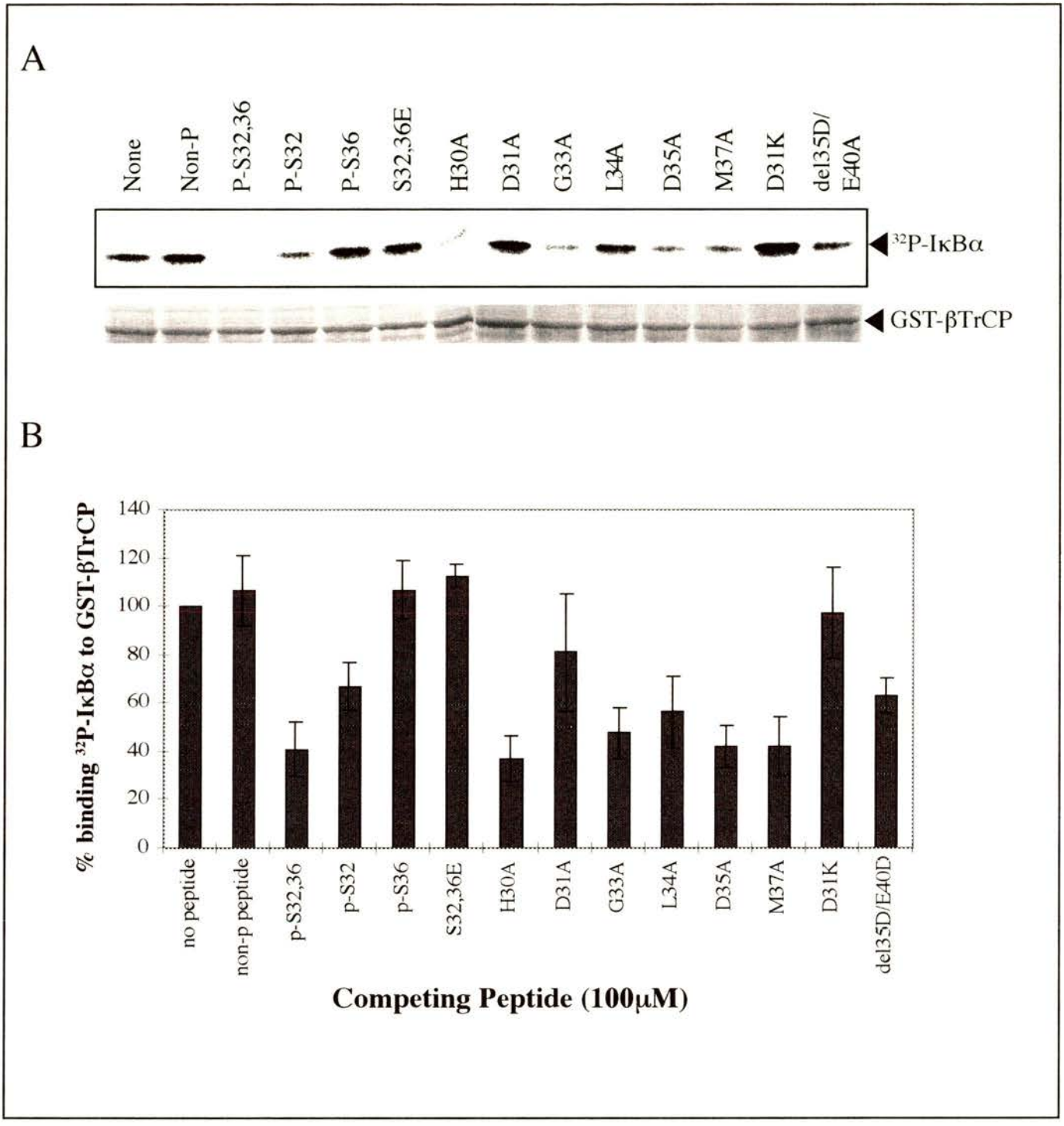


Figure 26 Inhibition of binding of ^{32}P-IκBα to βTrCP by competition with IκBα peptides.
Interaction assays were carried out as described in Material and Methods. A. GST or GST-βTrCP bound to glutathione agarose beads were incubated for 1 hour at room temperature in a total reaction volume of 100μl, containing kinase buffer, 1mg/ml BSA, 1.1μg IκBα, which had been previously labelled by IKK1/2 on serines 32 and 36 wtih 32phosphate and 100μM of competing peptide. Beads were then washed 3x with 1ml wash buffer, transferred to clean eppendorfs and reusupended in kinase buffer + 3X SDS disruption buffer, separated by SDS-PAGE (10%), stained with coomassie blue, destained, dried and exposed to phosphorimage screen for 10-12 hours. B. The amount of ^{32}P-IκBα associated with GST-βTrCP was quantified by phosphorimager and the amount of binding of ^{32}P-IκBα to βTrCP in the absence of any competing peptide was used as 100% binding. Each point is the mean from 5 assays with error bars representing one standard deviation.

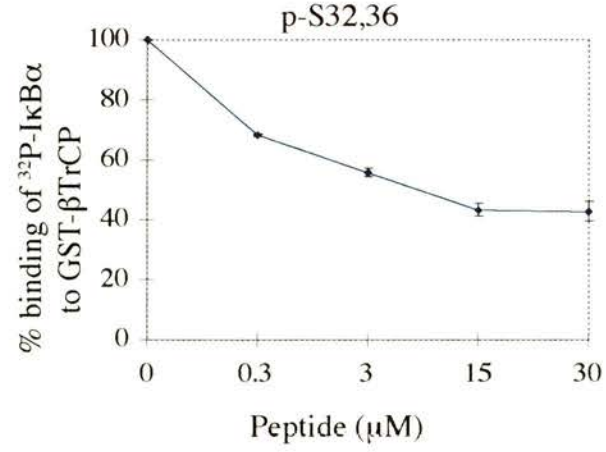
D35A, M37A and Δ35D/E40D. These peptides, with the exception of p-S32, indicate that these residues are not important for the recognition of IκBα by βTrCP. Most importantly, the peptides which do not compete indicate residues which are potentially important in the interaction of IκBα with βTrCP. Results show that peptides p-S36, S32,36E, D31A and D31K do not interfere with binding of ^{32}P-IκBα to βTrCP at all or only partially demonstrating that these residues are important in the overall recognition process of IκBα by βTrCP. There appears to be varying degrees of inhibition with peptides G33A, L34A, D35A, M37A and Δ35D/E40D. It also indicates that aspartate at residue 35 is dispensable and that glutamate 40 does not participate in the overall recognition site. From these initial observations, these peptides appear to compete for binding of βTrCP comparable to that of the p-S32,36 peptide, indicating that these residues are not important for the interaction between P-IκBα and βTrCP.

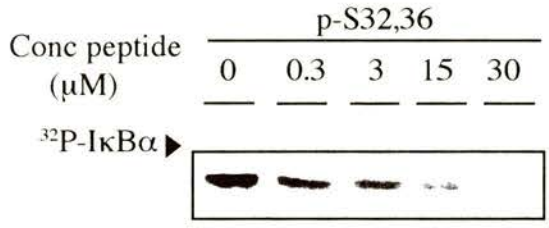
3.3.4 Different peptides have different potencies of inhibition

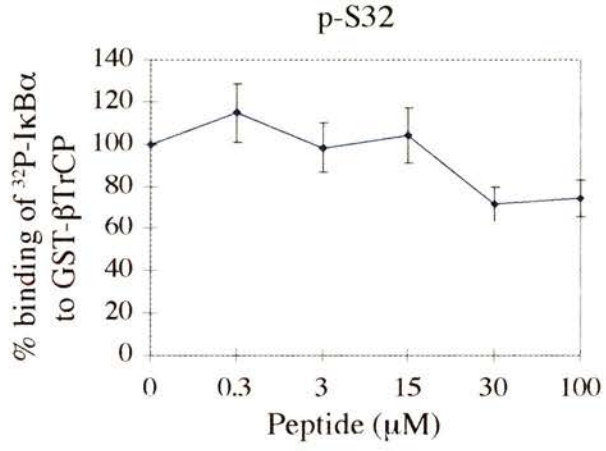
From the above results, identification of residues in IκBα which contribute to the recognition of this protein by βTrCP were determined. However, since the peptides used in the above assay were in vast excess compared with ^{32}P-labelled recombinant IκBα, the potency of the peptides were investigated. Interaction assays were carried out as before, in the presence of a range of concentrations of each of the peptides that showed inhibition at 100μM. Figures 27 and 28. Binding of ^{32}P-IκBα to GST-βTrCP in the absence of any competing peptides was used as the maximum, 100%, binding of IκBα to βTrCP, and each value was compared to this. The amount of ^{32}P-IκBα associated with GST-βTrCP was quantified using the phosphorimager (Fujix BAS1500 and MacBAS software). Data was exported into Microsoft Excel and used to construct graphs of inhibitor concentration against percentage binding of ^{32}P-IκBα to GST-βTrCP. Logarithmic trendlines were created for each graph by calculating the least squares fit through points using the equation $y = c \ln x + b$ where c and b are constants, and ln is the natural logarithmic function. A

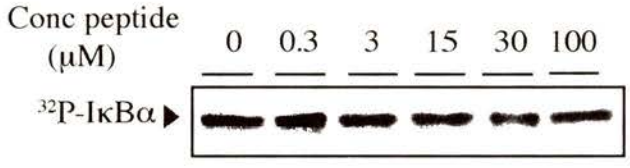
Figure 27 Inhibition of ^{32}P-IκBα binding to GST-βTrcP by IκBα peptides, p-S32,36, p-S32, H30A and G33A.

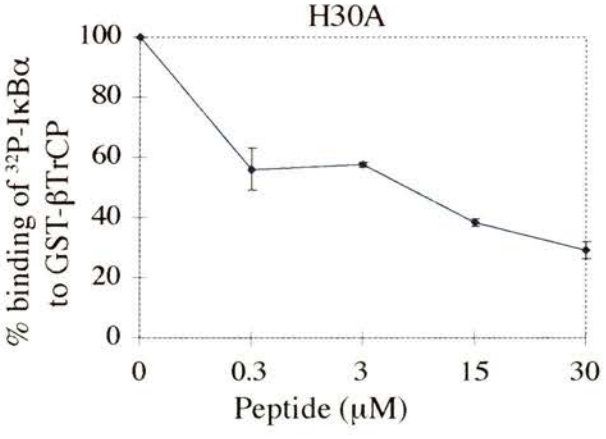
Interaction assays were carried out as described in Materials and Methods in which GST-βTrCP -glutathione agarose beads were incubated in a total reaction volume of 100μl containing kinase buffer, 1mg/ml BSA 1.1μg ^{32}P-labelled IκBα and varying concentrations of IκBα inhibitor peptide. After 1 hour of incubation at room temperature, beads were then washed 3x with 1ml wash buffer, transferred to clean eppendorfs and reusupended in kinase buffer + 3X SDS disruption buffer, separated by SDS-PAGE (10%), stained with coomassie blue, destained, dried and exposed to phosphorimage screen for 10-12 hours. The amount of ^{32}P-IκBα associated with GST-βTrCP was quantified by phosphorimager and the amount of binding of ^{32}P-IκBα to βTrCP in the absence of any competing peptide was used as 100% binding. Graphs were plotted in Microsoft Excel and trendlines were calculated according to the equation $y = c \ln x + b$, for each competing peptide. The amount of ^{32}P-IκBα which associates with βTrCP in the presence of each of the competing peptides is also shown.

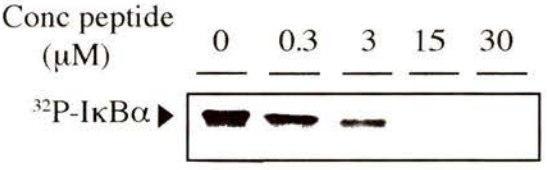

p-S32,36
% binding of ^{32}P-IκBα to GST-βTrCP
100
80
60
40
20
0
0
0.3
3
15
30
Peptide (μM)

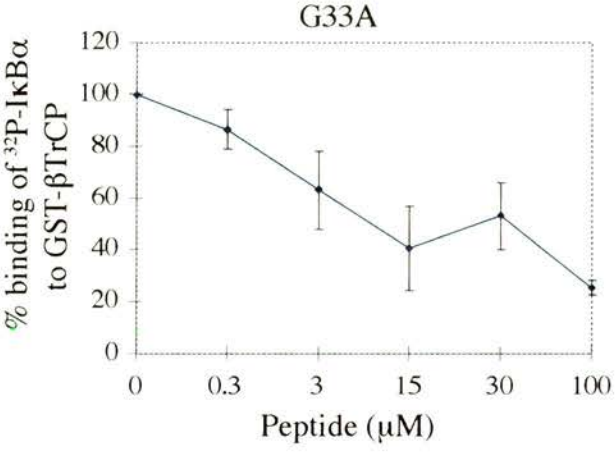

p-S32,36
Conc peptide (μM)
0
0.3
3
15
30
^{32}P-IκBα


p-S32
% binding of ^{32}P-IκBα to GST-βTrCP
140
120
100
80
60
40
20
0
0
0.3
3
15
30
100
Peptide (μM)


Conc peptide (μM)
0
0.3
3
15
30
100
^{32}P-IκBα


H30A
% binding of ^{32}P-IκBα to GST-βTrCP
100
80
60
40
20
0
0
0.3
3
15
30
Peptide (μM)


Conc peptide (μM)
0
0.3
3
15
30
^{32}P-IκBα


G33A
% binding of ^{32}P-IκBα to GST-βTrCP
120
100
80
60
40
20
0
0
0.3
3
15
30
100
Peptide (μM)

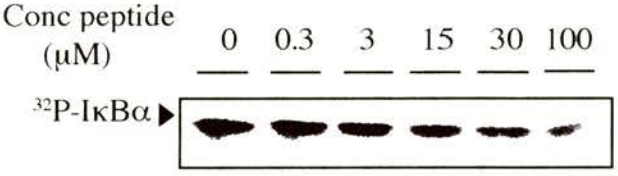
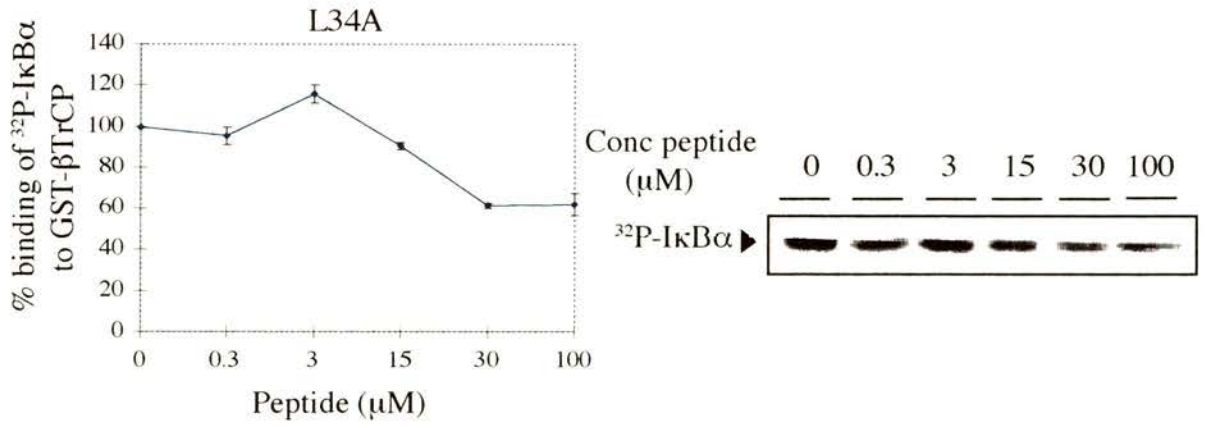
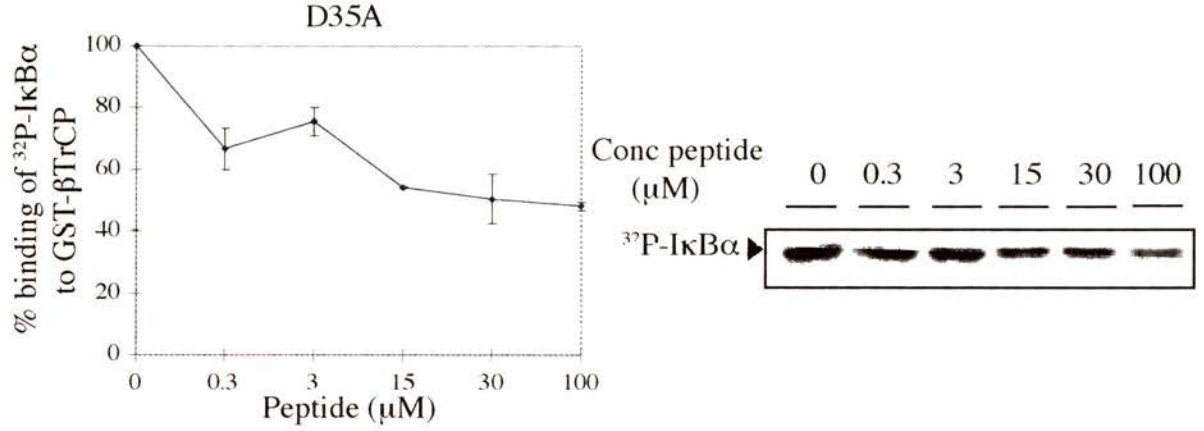
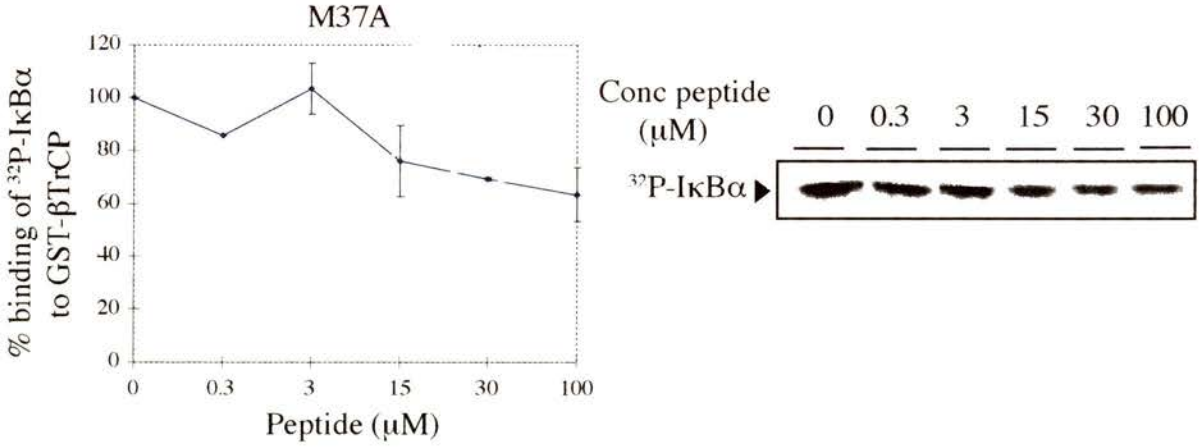
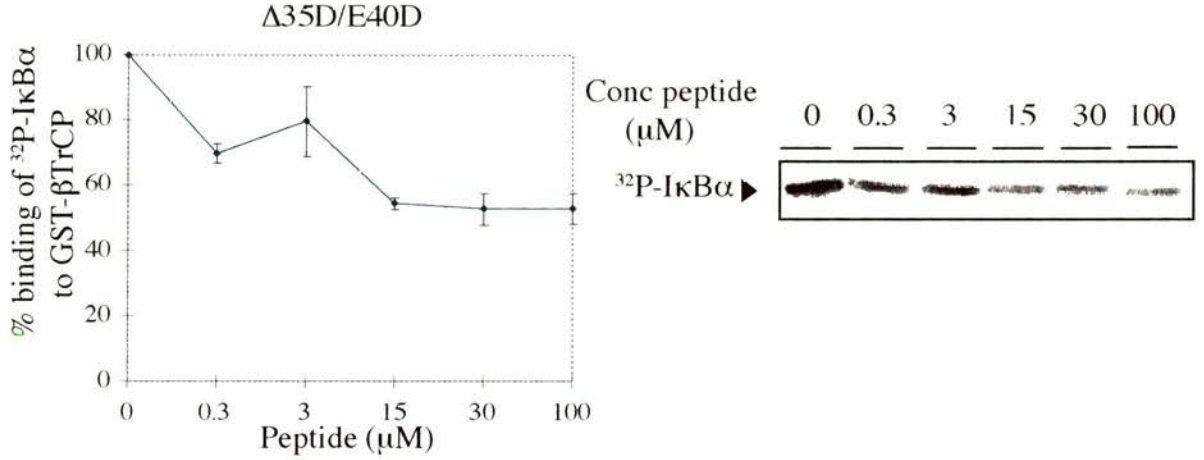

Conc peptide (μM)
0
0.3
3
15
30
100
^{32}P-IκBα

Figure 28 Inhibition of ^{32}P-IκBα binding to GST-βTrcP by IκBα peptides, L34A, D35A, M37A and Δ35D/E40D.
Interaction assays were carried out as described in Figure 27.


L34A
% binding of 32P-IκBα to GST-βTrCP
140
120
100
80
60
40
20
0
0
0.3
3
15
30
100
Peptide (μM)
Conc peptide (μM)
0
0.3
3
15
30
100
32P-IκBα


D35A
% binding of 32P-IκBα to GST-βTrCP
100
80
60
40
20
0
0
0.3
3
15
30
100
Peptide (μM)
Conc peptide (μM)
0
0.3
3
15
30
100
32P-IκBα


M37A
% binding of 32P-IκBα to GST-βTrCP
120
100
80
60
40
20
0
0
0.3
3
15
30
100
Peptide (μM)
Conc peptide (μM)
0
0.3
3
15
30
100
32P-IκBα


Δ35D/E40D
% binding of 32P-IκBα to GST-βTrCP
100
80
60
40
20
0
0
0.3
3
15
30
100
Peptide (μM)
Conc peptide (μM)
0
0.3
3
15
30
100
32P-IκBα

trendline allows you to show the trend, or the direction of the data in a series. These were then used to determine the concentration of peptide that inhibited binding of ^{32}P-IκBα to GST-βTrCP by 50%. A summary of the results are shown in Table 1.

As can be shown from Figures 27 and 28 and Table 1, peptides p-S32,36 and H30A inhibit at a concentration of 19μM and 13 μM respectively. Peptides G33A, D35A and Δ35D/E40D are able to inhibit at concentrations of 25 μM, 50 μM and 60 μM respectively, with peptides p-S32, L34A and M37A inhibiting at concentrations greater than 100 μM. Non-phosphorylated peptide, p-S36, S32,36E, D31A and D31K cannot be measured in the scope of the assay. Therefore, from these results it is apparent that IκBα must be phosphorylated in order to be recognised by βTrCP and that an acidic residue at position 31 is also required. It was also demonstrated that other residues which lie within and around the phosphorylated serine residues contribute to the recognition of IκBα by βTrCP, although to varying degrees.

Table 1 IC_{50} of IκBα peptides

PEPTIDE	IC50 (μM)
non-phosphorylated	-
P-S32,36	19
P-S32	>100
P-S36	-
S32,36E	-
H30A	13
D31A	-
G33A	25
L34A	>100
D35A	50
M37A	>100
D31K	-
deletion 35D/E40D	60

where - indicates values could not be measured under these conditions

3.3.5 IκBα peptide binding constants

Competition studies provided evidence that residues within and around the consensus motif contribute to the interaction of IκBα with βTrCP. In collaboration with colleagues from Pfizer Pharmaceuticals, determination of the binding constant for each peptide was investigated. $75x10^6$ Sf9 cells were infected with baculovirus containing βTrCP cDNA at an m.o.i equal to 5. 68-72 hours post-infection, cells were collected by centrifugation, washed 3x in PBS and stored at -70°C until use. Protein A PVT scintillation proximity assay (SPA) beads were resuspended in lysis buffer and incubated with anti-sv5, Pk tag antibody overnight at 4°C. Sf9 cells were lysed in hypotonic lysis buffer containing glycerol and βTrCP was conjugated to protein A PVT SPA anti-SV5 Pk beads for three hours at 4°C. The affinity of IκBα peptides for βTrCP was measured by scintillation proximity assay. Serially diluted peptides from 100μM to 1nM were incubated with 100μg βTrCP conjugated protein A PVT SPA beads in the presence of I^{125}-labelled wild type IκBα peptide (LKKERLLDDRHDS(PO4)GLDS(PO4)MKDEEYE). Reactions were performed in 96 well white non-binding surface clear bottom plates and amount of iodine labelled peptide bound to βTrCP recorded using a Microbeta Trilux counter.

Determination of specific binding of iodine labelled wild-type IκBα peptide to βTrCP in the presence of competing peptide was calculated through subtracting non-specific binding from total binding for each point. The construction of Scatchard Plots allowed the calculation of binding constants for each of the peptides. As shown in Figure 29, wild-type IκBα peptides (LKKERLLDDRHDS(PO4)GLDS(PO4)MKDEEYE and DRHDS(PO4)GLDS(PO4)KKD) phosphorylated on both serine residues 32 and 36 exhibited a high affinity for βTrCP, with Kd values of 6nM and 43nM respectively. H30A, D35A, M37A and ΔD35/E40D conferred intermediate binding constants of 111, 36, 213 and 555nM respectively. Peptides p-S32, G33A and L34A exhibited binding constants of 4μM, 3μM and 2μM respectively whereas peptides p-S36, S32,36E, D31A and D31K exhibited no binding to βTrCP at the concentrations used in the assay. These

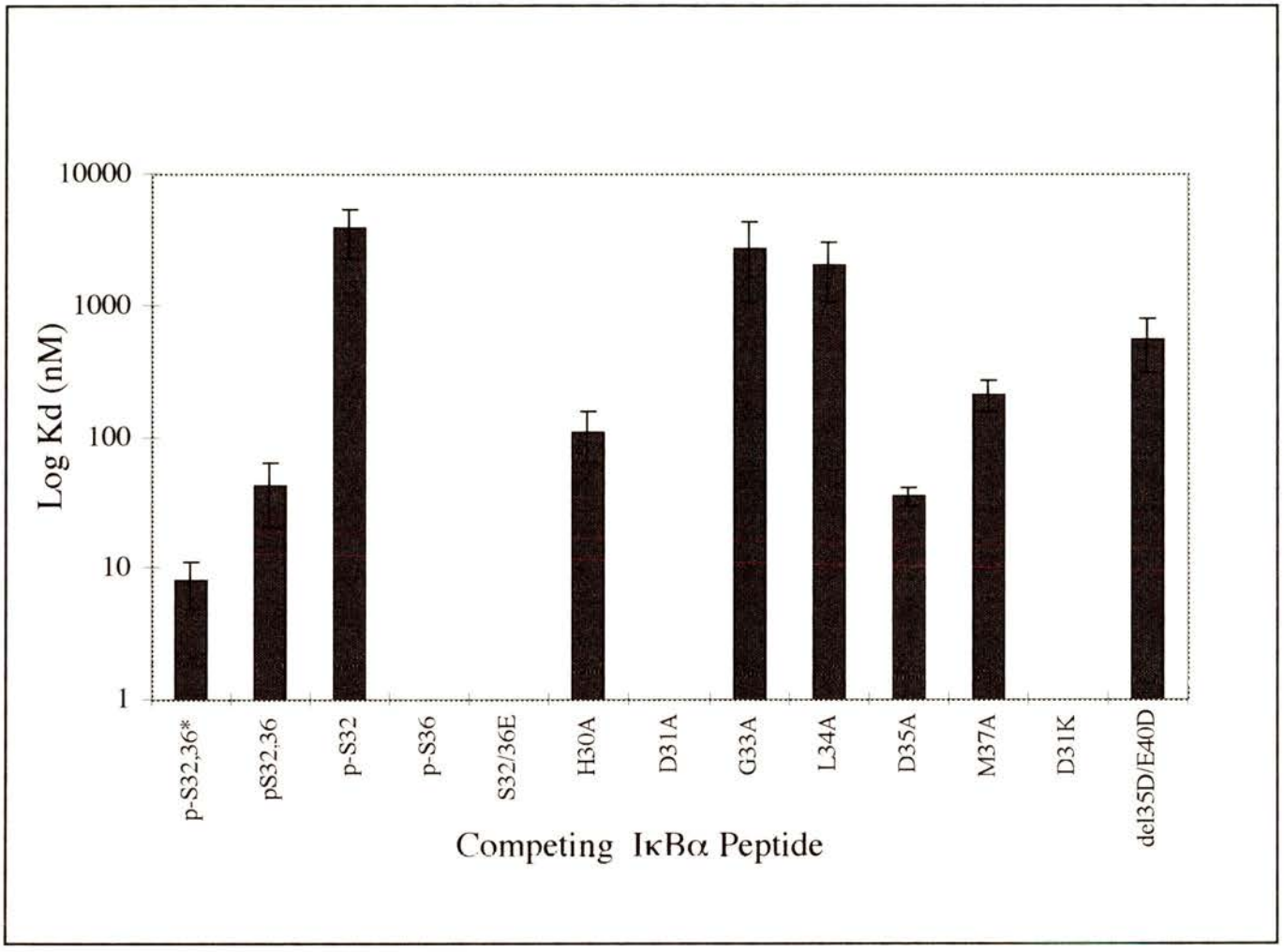


Figure 29 Summary of Kd values for IκBα peptides.
Binding constants were determined for IκBα peptides by Scintillation Proximity Assays (SPA). SPA PVT antibody binding beads were coupled to sv5 Pk Tag antibody. Sf9 cells infected with baculovirus containing cDNA for βTrCP were lysed in lysis buffer (10mM HEPES, pH 7.6, 1.5mM $MgCl_2$, 150mM KCl, 1% NP-40, 10% glycerol, 0.5mM DTT and a protease inhibitor tablet) and incubated with SPA PVT sv5 Pk Tag beads for 2 hours at 4°C. Beads were then washed and binding assays performed as described in Materials and Methods. Each point represents the average from 5 separate experiments with error bars representing one standard deviation.

results indicate that different residues are involved in the binding of IκBα to βTrCP and that some residues are highly significant in this interaction.

3.3.6 Discussion

The recognition of proteins to be targeted for ubiquitination and degradation by the 26S proteasome is a cellular process that requires controlled and timed execution. The fact that the ubiquitin system is involved in a number of different pathways within the cell, such as the degradation of cyclins involved in the cell cycle, degradation of transcription factors, tumour suppressors and oncoproteins as well as degradation of damaged or mis-folded proteins, indicates the importance of this system in the functioning of the cell (Hershko and Ciechanover, 1998; Hershko *et al.*, 2000; Varshavsky, 1997). Therefore inappropriately identified proteins or untimely degradation of proteins could have serious consequences for the cell and it is possible to think that aberations in this pathway could lead to a number of disease states. Indeed a number of genetic diseases such as cystic fibrosis and Angelman's syndrome have been found to be linked to alterations in the ubiquitin system (Ward *et al.*, 1995; Kishino *et al.*, 1997). Therefore it is sensible to envisage that there must be a precise and specific mechanism by which target proteins are recognised by the degradation machinery. The identification of a family of E3 ubiquitin ligases, led to the discovery that IκBα is targeted for ubiquitination by an $SCF^{\beta TrCP}$ complex. The F-box protein within the complex, βTrCP, was also identified as the protein that specifically recognises IκBα. Previously it was demonstrated that IκBα could be ubiquitinated in an *in vitro* ubiquitination assay using all purified components. Demonstration of the interaction between IκBα and βTrCP was also investigated. Discovery of the conserved recognition motif for βTrCP substrates prompted the investigation into the role the residues within and surrounding this motif have on the interaction between IκBα and βTrCP.

Yaron *et al*, using a series of peptides demonstrated that there was a ubiquitin ligase recognition motif composed of six amino acids, DS(PO3)GXXS(PO3), found within IκBα and conserved within all the IκBs (α, β, ε and cactus; Yaron *et al.*, 1998). This was also found in other βTrCP substrates including β-catenin (Aberle *et al.*, 1997) and vpu (Margottin *et al.*, 1998). Figure 23. Using this knowledge, a series of peptides were generated corresponding to amino acids 28-39 or 20-43 of IκBα, which encompass this recognition motif as well as unique residues which belong to IκBα. Figure 24. These peptides were used to compete for binding to βTrCP and hence block the ability of ^{32}P-IκBα to be ubiquitinated. As shown in Figure 25, non-phosphorylated peptide has no effect on IκBα ubiquitination, whereas a peptide phosphorylated on serines 32 and 36, reduce ubiquitination. Mutation of residues 34, 35 and 37 to alanine can also inhibit ubiquitination, although not so dramatically as P-S32,36 alone. Not surprisingly aspartate 31 and glycine 33, which are both part of the conserved recognition motif, appear to be important in the ubiquitination procedure, as mutation of these residues results in loss of competition of ubiquitination of ^{32}P-IκBα. Inhibition of ubiquitination of a D31A IκBα mutant protein has also been demonstrated *in vivo* by Hattori *et al*, confirming that this residue is important in the ubiquitination of IκBα *in vivo* (Hattori *et al.*, 1999) They also demonstrated that there is a requirement for an acidic residue at this position as replacement of aspartate by glutamate, still allowed ubiquitination to occur. Using an *in vitro* ubiquitination assay, the requirement of an acidic residue at this position was also demonstrated. Peptides in which aspartate is substituted by a basic residue, lysine, are still not able to inhibit ubiquitination of wild-type phosphorylated IκBα. This indicates that not only must there be a charged amino acid at this position, but that it must also be a negatively charged residue. Hattori and co-workers also showed that a G33A IκBα mutant could be ubiquitinated *in vivo*, although to a lesser extent than wild-type IκBα protein. Results here, however, demonstrate that when glycine 33 is replaced by alanine, no inhibition of ubiquitination *in vitro* can be seen. These observations remain somewhat

contradictory and might only be resolved with future investigation into the ubiquitination process.

There is never complete inhibition of ubiquitination *in vitro*. This could be due to experimental conditions during the assay. The peptides could be precipitating and losing their activity. It is also not known exactly how the IκBα /$SCF^{\beta TrCP}$ interaction occurs, or if there are distinct structural motifs within the SCF complex which may enhance binding of IκBα, in addition to the recognition of IκBα and βTrCP through the DSGXXS interaction motif. Therefore it is possible that there are other elements required for recognition of substrate by the E3 ligase. The fact that the same SCF complex recognises many different substrates, suggests that there must be some element of specificity which will make that substrate unique. This element could be other interaction domains or motifs within βTrCP or the substrate which are as yet, undiscovered. It could be due to localisation of the substrate within the cell, the mechanism or signalling pathway which is activated and used to phosphorylate the substrate. It could even be dependent on which stage of the cell cycle is in progress. Overall, there is still much more to be learned in the identification, targeting and ubiquitination of substrates by the SCF complexes.

Identification of residues within IκBα that were important in the ubiquitination of this protein by the SCF complex, prompted investigation into the importance these residues had in the interaction of IκBα with βTrCP. By using the same series of peptides employed in the *in vitro* ubiquitination of IκBα it was also demonstrated that phosphorylation of serine residues 32 and 36 were important in the recognition of IκBα by βTrCP. This was further highlighted by the lack of inhibition of binding in the presence of a non-phosphorylated peptide. The importance of the phosphate group at this site is also highlighted by the fact that replacement of these residues by glutamic acid, which is thought to mimic phosphorylation, still has no effect. A peptide, singly phosphorylated on serine residue 32, reduced binding of ^{32}P-IκBα to βTrCP by 30% whereas a peptide singly phosphorylated at serine residue 36 cannot compete for binding of βTrCP in the presence of

recombinant ^{32}P-labelled IκBα. It is likely that phosphorylation of serine 32 is more important for binding than serine 36.

Overall the same pattern of inhibition of ubiquitination and interaction of ^{32}P-IκBα with βTrCP can be demonstrated. An acidic residue at position 31 appears important for both the ubiquitination and interaction of ^{32}P-IκBα with βTrCP, as mutation of this residue to either alanine or a basic residue, lysine, results in a loss of its inhibitory properties. Histidine 30, leucine 34, aspartate 35 and methionine 37 appear to have little effect in either the recognition of ^{32}P-IκBα by βTrCP or of its ubiquitination. Interestingly, when the structure of the peptide is altered, due to deletion of aspartate 35, and substitution of glutamate at position 40 with aspartate, inhibition of binding still occurs. This, again suggests that p-S36 is not important in the overall recognition of IκBα with βTrCP, as by deleting aspartate 35, we inherently move serine 36 into the 35 position, and change the structure of the recognition site. It also indicates that aspartate at residue 35 is dispensable and that glutamate 40 does not participate in the overall recognition site. Contradictory to the results obtained in the ubiquitination assay, it appears that glycine residue at position 33, although demonstrated to be important in IκBα ubiquitination, does not seem to be important for the binding of IκBα to βTrCP, as when this residue is mutated to alanine, it is still able to compete. These differences may arise from the assay system itself considering that the ubiquitination assays were carried out using SCF expressed in insect cells and that the interaction assays were carried out using βTrCP expressed alone in bacteria.

Within the consensus motif, aspartic acid 31 of IκBα appears to play a crucial role in both the ubiquitination and interaction of IκBα with βTrCP. Although residues which are not conserved in the recognition motif, histidine 30, lysine 34, aspartate 35 and methionine 37 are still able to compete for binding of IκBα to βTrCP when they are mutated to alanine, the initial experiments were carried out with the peptides in vast excess. Therefore the concentration at which these peptides could inhibit IκBα binding to βTrCP by 50% was investigated. As shown in Figures 27, 28 and Table 1, H30A is able to compete

as well as the wild-type peptide, p-S32,36 and inhibits at a concentration of 13μM. D35A competes at a concentration of 50μM, and L34A and M37A compete at concentrations greater than 100 μM. Peptides L34A, D35A and M37A, although they are still able to bind βTrCP, they do so at concentratons that are greater than the wild-type peptide. The altered peptide, Δ35D/E40D, can also inhibit binding of IκBα to hTrCP by 50% at a concentration of 63μM. These studies indicate that not only is phosphorylation of IκBα at positions 32 and 36 important for the recognition of this substrate by βTrCP, but that other residues within and around these sites also play a role in the recognition of this protein by the F-box protein.

Scintillation proximity assays (SPA) were performed to determine the concentration at which the IκBα peptides interacted with βTrCP. Iodine-labelled wild-type IκBα peptide was incubated with PVT-sv5 Pk Tag SPA beads, coupled to βTrCP expressed in insect cells, in the presence of unlabelled competing IκBα peptides. Binding affinities for each peptide were calculated from Scatchard plots constructed from the binding data. From these results it was demonstrated that some peptides exhibit higher binding affinities for βTrCP than others. Wild-type phosphorylated peptides LKKERLLDDRHDS(PO4)GLDS(PO4)MKDEEYE and DRHDS(PO4)GLDS(PO4)KKD exhibit binding constants of 6 and 43nM respectively. This enhances and verifies the importance of phosphorylation of the wild-type peptides. The discovery of the consensus motif found in substrates for βTrCP suggested the importance of the residues aspartate and glycine within the DSGXXS sequence. An IκBα peptide encompassing not only these residues of IκBα but also lysines 21 and 22 which are the targets of ubiquitination, exhibits a higher affinity for βTrCP than the smaller IκBα peptide which does not contain these residues. This indicates therefore that residues outwith the consensus motif are involved in the recognition or the interaction with βTrCP. It would be interesting to identify if the ubiquitinated peptide has a decreased affinity for binding βTrCP. This could be the mechanism by which the molecule is released and allowed to interact with the 26S

proteasome for degradation. However, it has been suggested that components of the E3 ligase and also E2 family members are able to interact with the 26S proteasome directly (Tongaonkar *et al.*, 2000; Xie and Varshavsky, 2000). It is possible that once IκBα is ubiquitinated, it remains attached to βTrCP which could then interact with the 26S proteasome. The finding that some F-box proteins are themselves degraded in a ubiquitin-proteasome dependent manner (Galan and Peter, 1999) prompts the intriguing question that the F-box protein and its target protein are degraded at the same time. This mechanism would also allow regulation of the E3 ligase by releasing the core enzyme (Skp1, cdc53/Cul1 and Rbx1), which could then interact with other types of F-box protein if required. Furthermore, this would also allow for the timed and controlled expression of the F-box protein. It could be that once it has been degraded along with its substrate, it is immediately transcribed by the action of the downstream transcription factors that it has ultimately activated. However since βTrCP is involved in the degradation of a number of proteins which are a part of distinct signalling pathways it would mean that each of the transcription factors it activates would be able to transcribe βTrCP independently, or is it possible that the pathways in which βTrCP participates are linked in some way? It has been demonstrated that activation of JNK, a typical kinase activated in the response to stress stimuli, is able to increase the level of βTrCP mRNA levels within the cell and that this, along with the simultaneous stress-induced activation of the IKK complex, culminates in the activation of NF-κB (Spiegelman *et al.*, 2001). These studies provide evidence that a multitude of levels exist in the control of the activation of NF-κB.

Competition studies using GST-βTrCP and ^{32}P -labelled IκBα suggests that residues H30, G33, L34, D35, M37 and Δ35D/E40D are not important in the overall recognition of IκBα by βTrCP. This observation was confirmed by competition studies using SPA technology. Table 2 displays a comparison between IC_{50} and Kd values. Although, the binding affinities and inhibitor concentrations are higher than the wild-type peptide, the fact that they are still able to compete for binding, suggests that their mutation

to alanine does not have a profound effect on binding. The differences in concentrations of inhibition between the two assays may be due to the technology used and the source of βTrCP. For the IC_{50} data, βTrCP was expressed and purified from bacteria, whereas the scintillation proximity assays were carried out using βTrCP expressed in insect cells. It is possible that insect cell homologues of the other three components of the SCF complex are present in the βTrCP expressed and immunoprecipitated from the insect cell system. The presence of these components could therefore contribute to the binding of IκBα with βTrCP and enhance the overall affinity of the IκBα peptide for βTrCP. The insect cell βTrCP expressed material may also contain post-translational modifications that could effect the functioning of the protein such that it may bind the IκBα peptides with higher affinity. Bacterially expressed βTrCP would not contain any post-translational modifications and although can still bind IκBα, may not bind with maximal efficiency.

Table 2 Comparison of IC_{50} and Kd values for IκBα peptides

PEPTIDE	IC50 (μM)	Kd(nM)
non-phosphorylated	-	-
P-S32,36	19	43
P-S32	>100	3850
P-S36	-	-
S32,36E	-	-
H30A	13	111
D31A	-	-
G33A	25	2746
L34A	>100	2065
D35A	50	36
M37A	>100	213
D31K	-	-
deletion 35/E40D	60	555

where - indicates those peptides which cannot be measured using this technology

Peptides that do not display binding constants or inhibitor concentrations suggest the importance of these residues in the interaction of IκBα with βTrCP. These peptides

bind so weakly that binding cannot be measured by the technology used. As can be shown from the results phosphorylation of both serine residues are important for this interaction to occur. The fact that p-S32 peptide displays binding to βTrCP whereas p-S36 does not, indicates that phosphorylation at this site appears to be more important than phosphorylation at serine 36. However, both serines must be phosphorylated in order to compete efficiently for binding to βTrCP. The importance of phosphorylation at these sites is enhanced by the fact that a peptide in which the serine residues are replaced by glutamic acid, which in some circumstances can mimic phosphorylation, does not compete for binding.

The importance of an acidic residue at 31 is exemplified by the fact that peptides in which this residue has been mutated to alanine or lysine are not able to compete for binding of βTrCP. As before this suggests that this residue is highly significant, not only in the interaction of IκBα with βTrCP, but also in the ubiquitination reaction. The above findings also indicate that glycine at position 33, although part of the consensus motif, is not important in the interaction of IκBα with βTrCP. G33A peptides are still able to compete for binding of βTrCP.

The data obtained for both the interaction of IκBα peptides with GST-βTrCP and with baculovirus βTrCP exhibit similar results, such that aspartate 31 but not glycine 33 is important in the recognition of IκBα with βTrCP. Since the interaction of IκBα with βTrCP precedes ubiquitination, it is probable that once IκBα is bound to the E3 ligase, ubiquitination must proceed. Therefore it is hard to imagine that glycine 33 in IκBα is dispensable for binding βTrCP but is a requirement for its ubiquitination. However, future work would be needed to resolve this issue.

Overall, this data suggests that IκBα must be phosphorylated on both serine residues 32 and 36 in order to be recognised by βTrCP and to be targeted for ubiquitination. Residues that surround the consensus motif are involved in this recognition procedure but to varying degrees. It appears, however, that an aspartic acid at residue 31 is

absolutely required for both binding and interaction of IκBα with βTrCP. This residue is also important in the ubiquitination process. Therefore, it appears that not only is phosphorylation of serine residues 32 and 36 important in the control of IκBα degradation but that aspartic acid at position 31 is of equal significance.

4. CONCLUSIONS

Eukaryotic gene transcription is a tightly controlled and regulated process. The control of the transcription factor NF-κB exemplifies the complexity and specificity involved in this process. Sequestration, phosphorylation and protein degradation are just some of the tools employed by the cell in order to control the activity and functioning of NF-κB. These processes were investigated in order to identify key elements important for the control of this transcription factor.

Firstly, NF-κB is maintained in the cytoplasm of unstimulated cells, by the inhibitor protein IκB. The discovery that upon extracellular stimulus of cells, IκBα was phosphorylated and degraded in a ubiquitin-dependent manner, prompted the search for the kinase responsible for IκBα degradation. Many kinases have been nominated as the "IκBα kinase", but it was not until the discovery of the IKK signalsome, that the emergence of the complexity of NF-κB regulation was revealed. Isolation of IKK1 and IKK2 prompted investigation into the process of IκBα phosphorylation by these two enzymes. *In vitro* kinase assays were established and used to probe the mechanism of action of the kinases *in vivo*. The assay also provided a tool with which to produce phosphorylated IκBα on serine residues 32 and 36. Although the IKKs have been identified solely as components of the NF-κB signalling pathway, it would be interesting to establish if they indeed functioned in other signalling pathways or if they were responsible for phosphorylation and activation or deactivation of some of the other components of the NF-κB pathway. In fact, it has been demonstrated that upon stimulation of cells with TNFα, IKK is activated upon recruitment, via TRAF-2, to the TNFRI (Devin *et al.*, 2001; Devin *et al.*, 2000). It is interesting to note that TRAF-2 is degraded in a ubiquitin-dependent manner upon ligation of the CD40 receptor and its ligand (Brown *et al.*, 2001). Therefore could the IKK complex not only activate NF-κB but also function in switching off the activation signal by phosphorylating and degrading TRAF-2, a component of the activation pathway. It would also be interesting to identify which E3 was responsible for TRAF-2 degradation, and whether phosphorylation was required.

Phosphorylated IκBα was found to be ubiquitinated and degraded by the 26S proteasome, allowing for the release of NF-κB and translocation of this protein into the nucleus. This process of ubiquitination and degradation provided a further mechanism by which to control the functioning of the transcription factor. The discovery of a family of E3 ubiquitin ligases revealed that IκBα was specifically recognised by a complex containing Skp1, Cul1, F-box protein (βTrCP) and Rbx1, collectively termed $SCF^{\beta TrCP}$. *In vitro* ubiquitination assays were established in order to identify the minimal components required for ubiquitination of phosphorylated IκBα. It could be demonstrated that phosphorylated IκBα was ubiquitinated by $SCF^{\beta TrCP}$ expressed in insect cells and that both cdc34 and ubc5 were capable of acting as an E2 enzyme in the reaction. It was also demonstrated that each of the four components of the $SCF^{\beta TrCP}$ complex must be present for efficient ubiquitination of phosphorylated IκBα to occur. Furthermore, it was revealed that not only was phosphorylation of serine residues 32 and 36 important for the ubiquitination of IκBα, but that there was also a requirement for an acidic residue, aspartic acid, at position 31.

The mechanism by which the E3 ligase acts is still unknown. Interactions between each of the proteins in the complex have been studied and have revealed which of these proteins interact with each other. However detailed analysis of the mechanism by which ubiquitin is added to substrates might only be revealed by studies into the 3-dimensional structure of the complex. In fact, analysis of the Skp1-Skp2 interaction by Schulman and colleagues (Schulman *et al.*, 2000), reveals that not only does the F-box protein (Skp2) recruit substrate but that it may also position it at an optimal site for the ubiquitination reaction. Figure 30. Therefore it is possible to imagine that, similar to Skp2, βTrCP also functions in this manner.

The interaction of IκBα with βTrCP was investigated using a series of IκBα peptides. The consensus motif discovered by Yaron et al, DSGXXS, indicated the importance of the phosphorylated serine residues and also aspartic acid and glycine

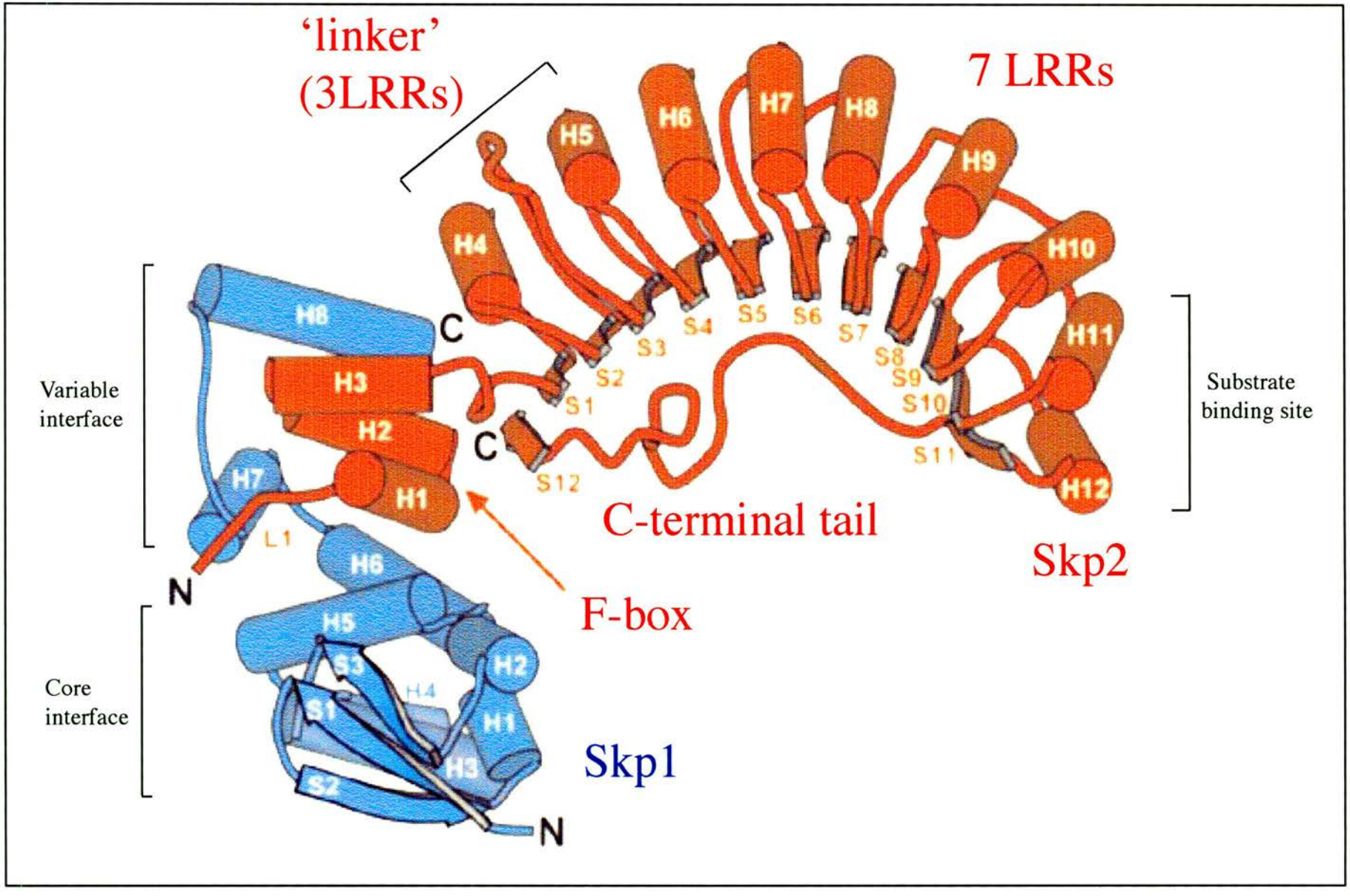


Figure 30 Crystal structure of the Skp1-Skp2 complex at 2.8 Angstrom.
Skp1 interacts with Skp2 through the F-box motif in the amino terminus of Skp2. Substate binding is thought to occur through the interaction with helix 11 and 12 (H11, H12) of Skp2 providing an interface for substrate recognition. LRR, leucine rich repeat. Figure taken from Schulmann *et al.*, 2001.

residues within this sequence. Indeed it was demonstrated that phosphorylated serines 32 and 36 are important as well as aspartic acid. However, it was revealed that glycine does not contribute to the overall interaction of the two proteins suggesting that this residue is not of critical importance in the recognition of IκBα by βTrCP.

It is interesting to note, however, that an IκBα peptide encompassing the putative ubiquitinated lysine residues, exhibits a higher affinity for βTrCP than a smaller peptide without these residues. Therefore, is it possible that within βTrCP there is a second site that acts as a binding pocket for substrate. Analysis of the residues of βTrCP involved in binding substrate would reveal if these amino acids were used to bind all of its target substrates or whether some are more specific for one substrate than another. Indeed it has been demonstrated that βTrCP interacts with and induces ubiquitination of target proteins independent of the DSGXXS motif. IκBε, contains both the DSGXXS motif and a number of SXXXS motifs in its amino terminal. It has been demonstrated that ubiquitination of IκBε is dependent on phosphorylation of serine residues 18 and 22 in the DSGXXS motif as mutation of these residues reduces the amount of ubiquitination. However, it is apparent that ubiquitination can still occur and that βTrCP can still interact with an IκBε S18,22A mutant. This suggests that sites other than the DSGXXS motif within IκBε are important in the recognition of this protein by βTrCP (Shirane *et al.*, 1999).

In addition to IκBε, the mechanism by which p105 is processed and degraded is somewhat controversial. Earlier studies demonstrated that p105 processing was mediated by the ubiquitin system (Fan and Maniatis, 1991; Palombella *et al.*, 1994) and some have suggested that p105 processing occurs co-translationally (Lin *et al.*, 1998a). However further studies have revealed that p105 is post-translationally ubiquitinated and proteolytically processed to form p50 (Orian *et al.*, 1995; Coux and Goldberg, 1998; Orian *et al.*, 1999). This mechanism has been shown to occur through IKK mediated phosphorylation of the carboxy terminus of p105 and subsequent interaction with βTrCP and the $SCF^{\beta TrCP}$ complex (Orian *et al.*, 2000). However, the role of the $SCF^{\beta TrCP}$ complex

in p105 ubiquitination and subsequent processing is contentious and it has been proposed that activation of the IKK signalling pathway and ubiquitination of p105 by the $SCF^{\beta TrCP}$ results in the complete degradation of the protein and that the processing of p105 to p50 is independent of IKK signalling (Heissmeyer *et al.*, 2001). Further work has demonstrated that the serine residues in the carborxyl terminus of p105 are the sites of phosphorylation by the IKK complex and that mutation of these residues to alanine, abrogates βTrCP mediated p105 ubiquitination (Heissmeyer *et al.*, 2001; Orian *et al.*, 2000). Furthermore, it has been demonstrated that serine 923 and in particular serine 927 are important in conferring ubiquitination of the protein by the $SCF^{\beta TrCP}$ complex. Interestingly, these serines are located within a DSXXXS motif, similar to the DSGXXS motif found in substrates for βTrCP. Alignment of this region with other known βTrCP substrates reveals that it lies upstream of the DSGXXS motif located in these proteins. However, in addition to this upstream site, p105 also contains a DSGXXXS motif that aligns with the other known βTrCP substrates, albeit for a threonine residue in place of the carboxyl serine residue . Figure 31. It would therefore be interesting to investigate if both motifs are necessary for recognition of p105 by βTrCP or if only one site is critical. This upstream motif SXXXS can also be found in IκBε, ESQYDS and β-catenin, QSYLDS where the aspartic acid of DSXXXS is replaced by glutamic acid in IκBε and glutamine in β-catenin. Could it be possible that these sites serve as additional recognition sites and are thereby the mechanism by which βTrCP is able to recognise distinct substrates? Or could they have a different specificity of interaction with βTrCP, and provide a mechanism by which this protein is able to preferentially target one substrate over another? Such actions may be required in response to a particular cellular environment that is produced upon activation of signalling pathways by a specific subset of extracellular stimuli. This upstream site in p105 and IκBε bears extended sequence similarity to the DSGXXS motif due to the presence of an acidic amino acid residue at the start of the motif. From results shown here, the importance of the presence of this residue in the DSGXXS motif was

p105	922 **D S V C D S** G V E T S F 933
IκBε	13 **E S Q Y D S** G I E S L R 24
IκBα	27 D D R H D S G L D S M K 38
IκBβ	14 D E W C D S G L G S L G 25
vpu	46 E R A E D S G N E S E G 57
β-catenin	28 **Q S Y L D S** G I H S G A 40

Figure 31 Amino acid alignment of βTrCP substrates.
Consensus motif for recognition of substrates by βTrCP is highlighted in red, DSGXXS. Additional possible sites of interaction with βTrCP are highlighted in bold with upstream serine residues depicted in green.

demonstrated. Therefore it further enhances the idea that this upstream motif in p105 and IκBε may be possible targets for recognition by βTrCP.

The role of the ubiquitin system in the control of eukaryotic gene expression is highlighted by the dynamic and fundamental nature of this process. The transcription factor HIFα (hypoxia-inducible factor alpha) is ubiquitinated and degraded in a manner which is anologous but distinct from the mechanism by which IκBα is ubiquitinated and degraded. HIF transcription factor, composed of a heterodimer of α and β subunits, is activated in response to hypoxia and is involved in the expression of a number of genes that control angiogenesis and glucose metabolism (Semenza, 2001; Semenza, 2000). In a similar manner to IκBα, HIF1α is targeted for ubiquitination by a large multisubunit E3 ligase called the VBC (pVHL-Elongin B-Elongin-C) complex. Ubiquitination and degradation occurs continuously in cells exposed to physiological concentrations of oxgen, controlling the amount of HIF present within the cell. However, under hypoxic conditions, HIFα is no longer able to be degraded and in turn dimerises with HIFβ to form a fully functional transcription factor that is able to bind to and activate transcription of its responsive genes. Modification of IκBα by phosphorylation of serine residues 32 and 36 by the IKK complex, results in the subsequent interaction of IκBα with the E3 ligase. Similarly, it has been demonstrated that HIFα is modified by

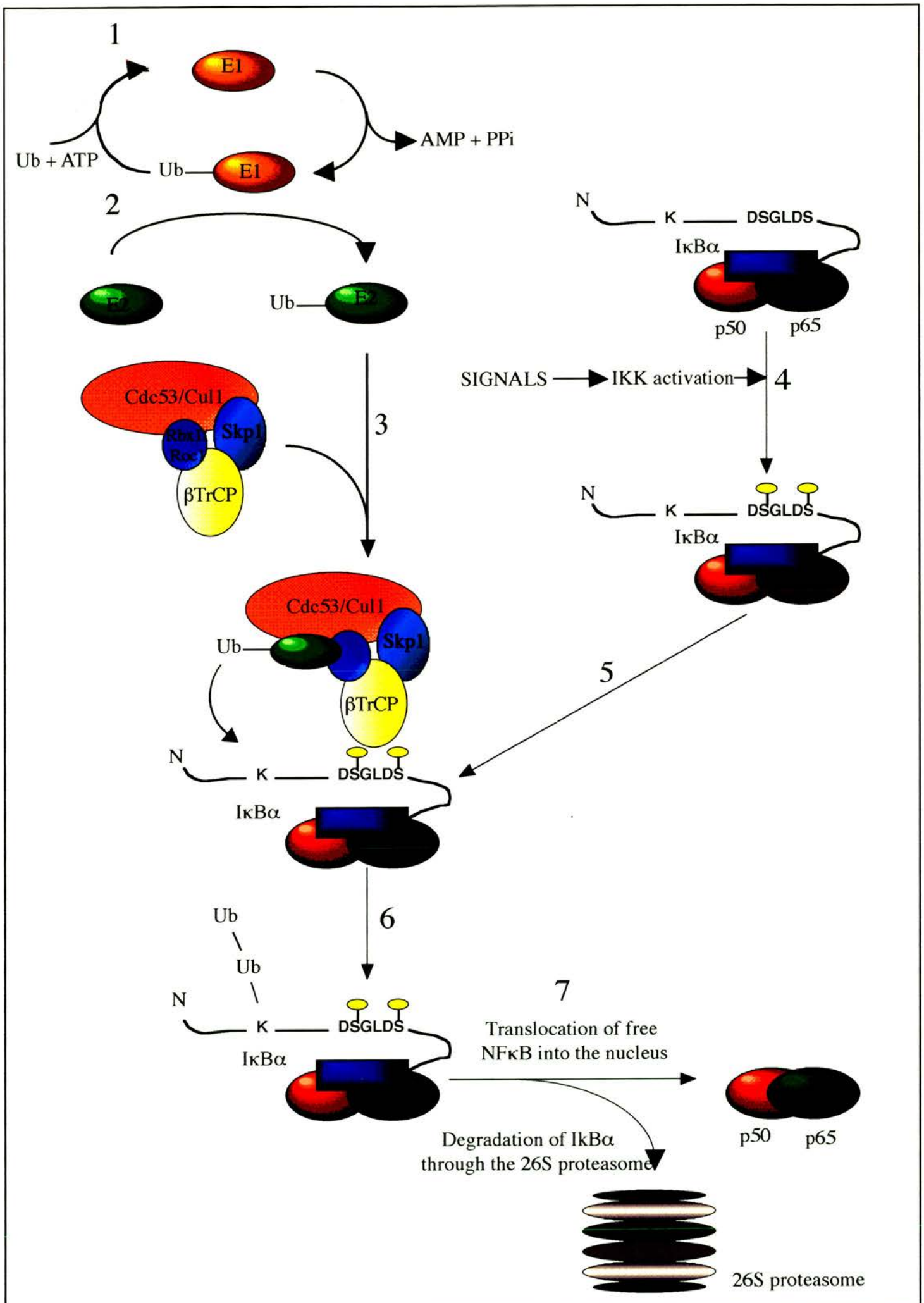
hydroxylation on proline residue 564 by a specific HIFα prolyl hydroxylase. Modification of this residue allows for the subsequent interaction of the protein with the VBC complex. The enzyme responsible for proline hydroxylation has been demonstrated as being oxygen and Fe^{2+} dependent. Therefore when oxygen levels decrease the enzyme cannot function and HIFα is no longer modified. Unmodified HIFα is not recognised by the VBC E3 ligase and ultimately is able to associate with HIFβ to form a fully functional transcription factor that can activate the expression of HIF dependent genes (Zhu and Bunn, 2001; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001).

The elegance of this system highlights the role of the ubiquitin-proteasomal pathway in the control of transcription factor functioning. Specificity is determined by the presence of a family of E3 ligases which recognise their target substrates through unique and simplistic mechanisms such as serine phosphorylation or prolyl hydroxylation. Specificity is further acheived by the action of specific enzymes that recognise their target substrates. These enzymes themselves are also subject to a variety of controls and regulation. Overall, the ubiquitin-proteasomal pathway is a powerful tool within the cell that not only functions in control of a variety of processes, but provides strict and regulated control of transcription factors and consequently eukaryotic transcription.

Overall, some of the mechanisms that have evolved within the cell in order to control transcription factor functioning, are highlighted in the control of NF-κB. The crucial steps in its activation are highlighted in Figure 32. IκBα acts as a powerful and potent inhibitor of NF-κB, retaining the protein in the cytoplasm of unstimulated cells. Only after degradation of IκBα and subsequent release of NF-κB, is the transcription factor able to exert its effect within the cell. The elegance of NF-κB control, however is exemplified by the fact that the degradation of the inhibitor protein, itself, is also under strict control. Firstly, IκBα must be specifically phosphorylated on serine residues 32 and 36. This is achieved by the activation of the IKK signalsome whose activation is also dependent on the presence of extracellular stimuli and activation of upstream molecules.

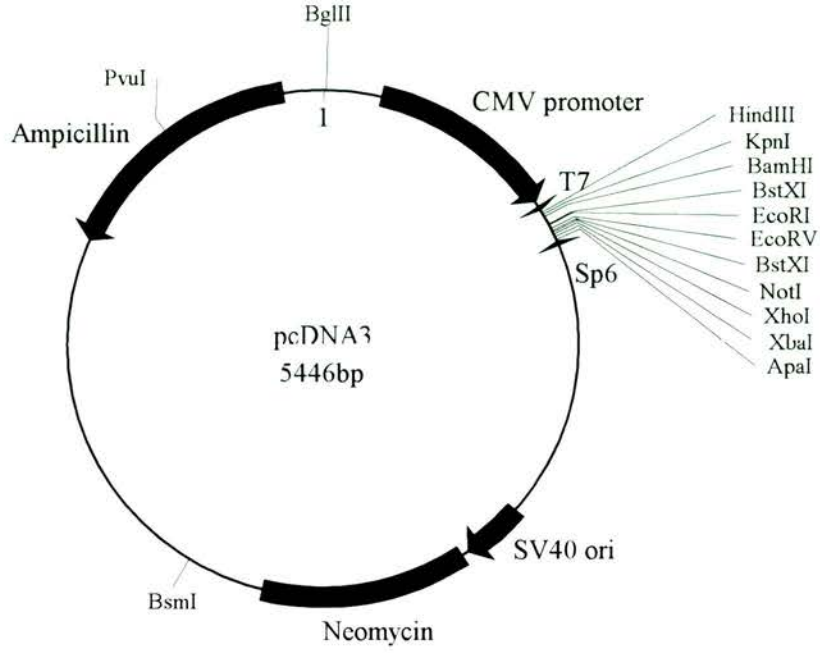
Figure 32 Signal-Induced Ubiquitination of IκBα

Schematic representation of the mechanism of IκBα ubiquitination. 1. Ubiquitin is activated in an ATP-dependent manner by E1 ubiquitin activating enzyme. 2. Ubiquitin is then transferred via a thiolester intermediate to an E2 ubiquitin conjugating enzyme (cdc34 or ubc5). 3. E2 is then able to associate with the $SCF^{\beta TrCP}$ complex. 4. Upon extracellular stimuli, the IKK signalsome is activated which subsequently phosphorylates IκBα on serine residues 32 and 36. Phosphorylated residues are exhibited as filled yellow circles. 5. Phosphorylated IκBα is now recognised by $SCF^{\beta TrCP}$ and interacts with the complex through association with the F-box protein βTrCP. 6. Interaction of phosphorylated IκBα with the $SCF^{\beta TrCP}$ complex allows the transfer of ubiquitin from the E2 ubiquitin conjugating enzyme to an epsilon amino group of lysine residues 21 and 22. (One lysine residue is depicted for simplicity). 7. Poly-ubiquitination of IκBα signals for the degradation of IκBα through the 26S proteasome allowing for the subsequent release of NFκB and translocation to the nucleus.


1
E1
Ub + ATP
Ub
E1
AMP + PPi
2
Ub
3
Cdc53/Cul1
Rbx1
Roc1
Skp1
βTrCP
N
K
DSGLDS
IκBα
p50
p65
SIGNALS
IKK activation
4
5
Ub
6
Ub
Ub
7
Translocation of free
NFκB into the nucleus
Degradation of IkBα
through the 26S proteasome
26S proteasome

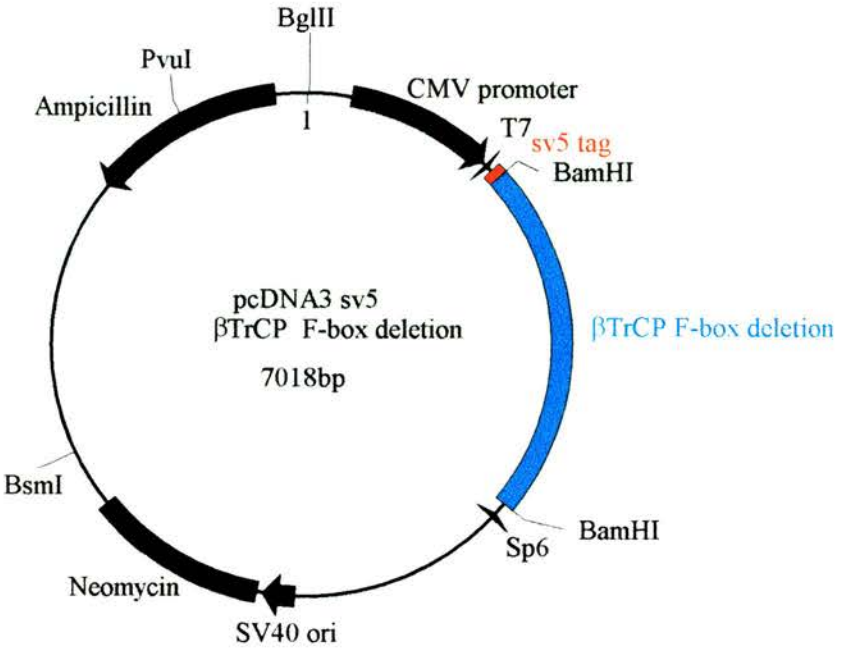
Once phosphorylated, IκBα is recognised by βTrCP that exists in a complex containing Skp1, Cul1 and Rbx1, all of which must be present in order for efficient ubiquitination of IκBα to occur. Not only must serine residues 32 and 36 of IκBα be phosphorylated, aspartic acid at position 31 must be also be present. Once all of these factors have been adhered to, the degradation of IκBα can occur. Therefore NF-κB activation is controlled at a multitude of levels that culminate in the timed and controlled regulation of gene expression by this protein complex.

5. VECTOR MAPS



Comments for pcDNA3 (5446 nucleotides)
Supplier: Invitrogen

CMV promotor: bases 209-863
T7 promotor: bases 864-882
Polylinker: bases 889-994
Sp6 promotor: bases 999-1016
BGH ploy A: bases 1018-1249
SV40 promotor: bases 1790-2115
SV40 origin or replication: 1984-2069
Neo^R ORF: 2151-2932
SV40 poly A: 3120-3250
PUC19 backbone: 3272-5446
Amp^R ORF: 4450-5310

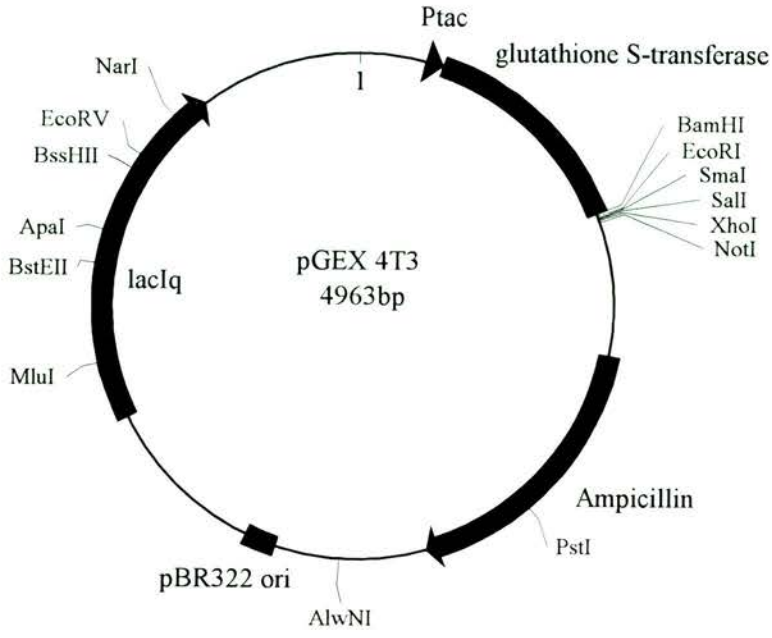


Cloning primers:
Upstream (HindIII): 5'-CCCAAGCTTGGTACCATGGGAAAGCCG-3'
Downstream (HindIII): 5'-CCCAAGCTTAGCAGTTATGAAATCTCTCTG-3'

βTrCP F-box deletion:
Organism – Homo sapiens
Coding region – 1572bp
F-box domain deletion – 148-190 amino acids

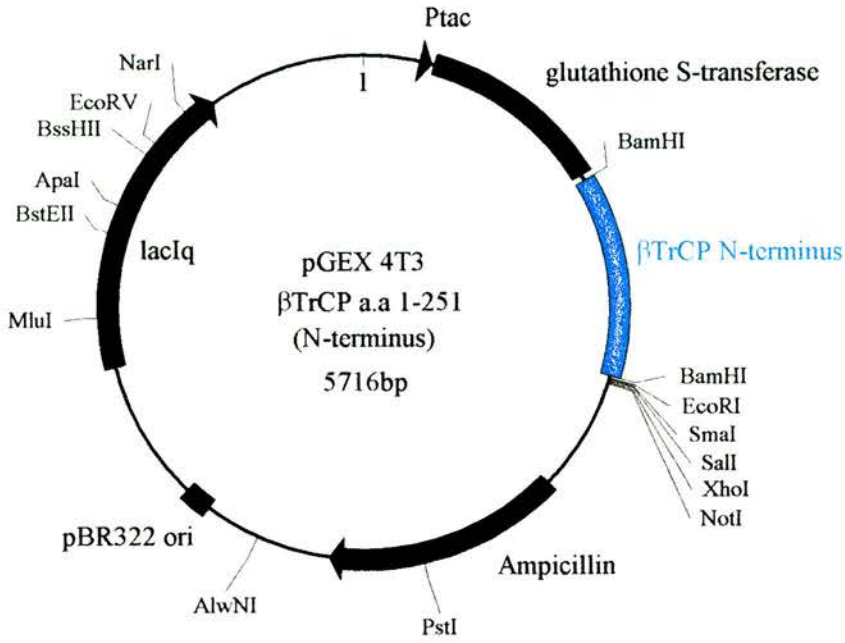
Antibodies:
Mouse 336 monoclonal antibody against PK-sv5 peptide (IPNPLLGLE)

Sheep anti-βTrCP polyclonal antibody against βTrCP c-terminal peptide (CRSPSRTYTYISR).



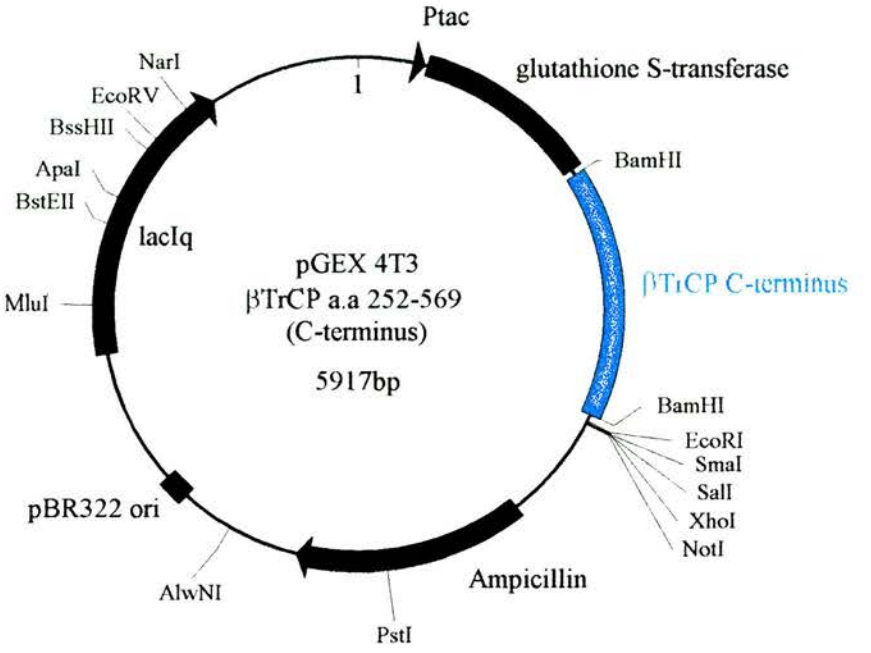
Comments for pGEX 4T3 (4963nucleotides)
Supplier: Pharmacia

tac promotor-10: bases 205-211
tac promotor-35: bases 183-188
lac operator: bases217-237
Start codon for GST: bases 258
Amp^R ORF: bases 1376-2234
LacIq ORF: bases 3317-4397
Site of replication initiation: bases 2994
Multiple Cloning Site: bases 930-965



Cloning primers:
Upstream (BamHI): 5'-CGGGATCCATGGACCCGGCCGAGGCG-3'
Downstream (BamHI): 5'-CGGGATCCCTATCTCCAATTAGATTCTATTGT-3'

N-terminus βTrCP:
Organism – Homo sapiens
Coding region – 753bp
Amino acids (a.a) 1-251

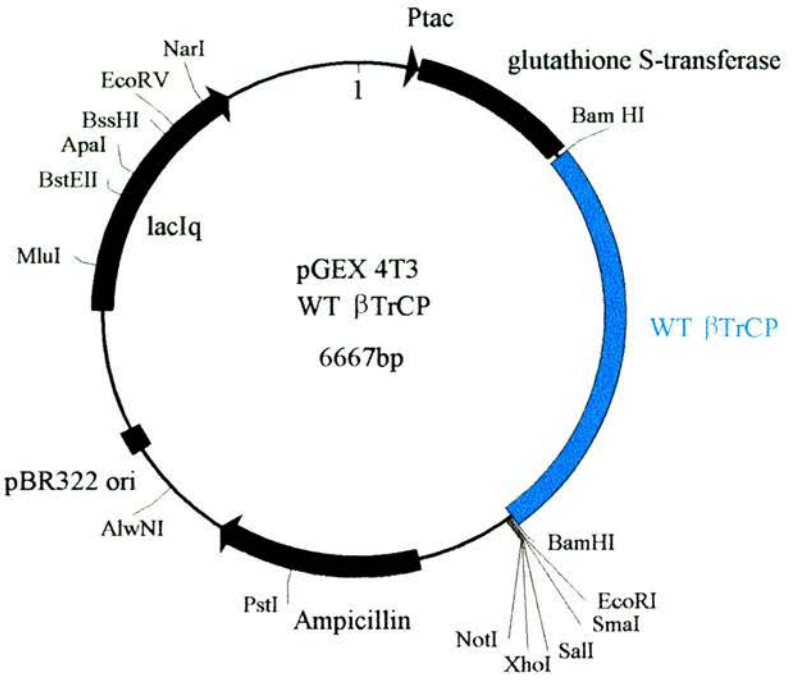


Cloning primers:
Upstream (BamHI): 5'-CGGGATCCTGTGGAAGACATAGTTTACAG-3'
Downstream (BamHI): 5'-CGGGATCCTTATCTGGAGATGTAGGTGTA-3'

C-terminus βTrCP:
Organism Homo sapiens
Coding region – 954bp
Amino acids (a.a) 252-569

Antibodies:
Sheep anti-βTrCP polyclonal antibody against βTrCP c-terminal peptide (CRSPSRTYTYISR).

Bacterial Expression:
Expressed as a GST fusion protein in *E. Coli* B834



Cloning strategy:
Full length βTrCP was subcloned from pcDNA3sv5-βTrCP into pGEX4T-3 by digestion with *Bam H1* restriction enzyme.

βTrCP:
Organism – Homo sapiens
Coding region – 1704bp

Antibodies:
Sheep anti-βTrCP polyclonal antibody against βTrCP c-terminal peptide (CRSPSRTYTYISR).

Bacterial Expression:
Expressed as a GST fusion protein in *E. Coli* B834

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