Control of NF-κB dependent transcription by IκBα and p53

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DECLARATION

I, Enamul Sheikh Ahsan, hereby certify that this thesis, which is approximately 30,000 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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CONTENTS

ABBREVIATIONS

AdM	Adriamycin
AIDS	Acquired immunodeficiency syndrome
AIP	Apoptosis inducing factor
AMP	Adenosine monophosphate
ARD	Ankyrin repeat domain
ARF	Alternative reading frame
ASPP	Apoptosis stimulating protein of p53
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM-related
bp	base pairs
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
βTRCP	β-transducin repeat containing protein
cDNA	complementary DNA
CAK	Cdk-activating kinase
CBP	CREB binding protein
Cdk	Cyclin-dependant kinase
CHUK	conserved helix-loop-helix ubiquitous kinase
CREB	Cyclic AMP response element binding protein
CTD	Carboxy-terminal domain
dNTP	2'-Deoxynucleoside 5'-triphosphate
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNA-PK	Deoxyribonucleic acid - protein kinase
DTT	Dithiothreitol
E2F	Elongation factor-2
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
EST	Expressed sequence tag
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GRA	Gel retention assay
GTF	General transcription factor
HAT	Histone acetyltransferase
HBS	Hepes-buffered saline
HDAC	Histone deacetylase
Hepes	N-[2-hydroxyethyl]piperazine-N-'[2-ethanesulphonic acid]
HIV	Human immunodeficiency virus
HTLV	Human T-cell leukaemia virus

IGF-BP3	Insulin growth factor-binding protein 3
IgG	Immunoglobulin G
IκB	I kappaB
IKK	I kappaB kinase
IL1-β	Interleukin 1-beta
IRAK1	Interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
kDa	Kilodaltons
LB	Luria-Bertani broth
LMP-1	Latent membrane protein-1
LPS	Bacterial lipopolysaccharide
LSH	Loop-sheet helix
LTβ	Lymphotoxin β
mg	Milligram
ml	Millilitre
mM	Millimolar
mRNA	messenger RNA
Mdm	Murine double minute
MAP3K	Mitogen activated protein kinase kinase kinase
MEK	MAP kinase
MEKK	MAP kinase kinase
MOPS	Morpholinopropanesulphonic acid
NAK	NF-κB activating kinase
NB	Nuclear bodies
NBCS	Newborn calf serum
NEDD-8	Neuronal precursor cell-expressed developmentally down-regulated protein 8
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-kappaB
NIK	NF-κB inducing kinase
NP-40	Nonidet P-40
p.f.u	plaque forming units
P.V.D.F.	Polyvinylidene difluoride membrane
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PIG-3	p53 inducible gene-3
PKC	Protein kinase C
PKR	RNA-dependent protein kinase
PML	Promyeolocytic leukaemia
PMSF	Phenylmethanesulfonyl fluoride
rpm	revolutions per minute
RANK	Receptor activator of NF-κB
Rb	Retinoblastoma
RHD	Rel homology domain
RIP	Receptor interacting protein
RIPA	Radioimmunoprecipitation assay
RLU	Relative light unit

RNA	Ribonucleic acid
RPA	Replication protein A
SCF	Skp1, Cdc53/Cullin 1, F-Box containing protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	Saline-sodium citrate buffer
STE	Salt/Tris/EDTA
SUMO	Small ubiquitin modifier protein
SV	Simian virus
T2K	TRAF2-associated kinase
TANK	TRAF-associated NF-κB activator protein
TBE	Tris-borate EDTA
TBK	TANK binding kinase
TBP	TATA-binding protein
TE	Tris-EDTA
TES	Transformation Effector Site
TEMED	N,N,N',N'-tetramethyletheylene diamine
TNF-α	Tumour necrosis factor-α
TNFR	TNF-α receptor
TPL2	Tumour progression locus 2
TRADD	TNFR-associated death domain
TRAF	TNF-α receptor associated factor
µg	Microgram
µl	Microlitre
µM	Micromolar
UV	Ultraviolet
V	Volts
w/v	weight/volume

AMINO ACIDS and their SYMBOLS

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

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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Abstract

The NF-κB/ Rel family of transcription factors are involved in immune, inflammatory and anti-apoptotic responses. These transcription factors exist as homodimers or heterodimers containing p65, p50, p52, c-Rel or Rel B subunits. NF-κB proteins are usually found in trimeric complexes with a member of the IκB family of inhibitors. After exposure of cells to a range of stimuli, the classic NF-κB heterodimer p50/RelA is activated by signal-induced phosphorylation and ubiquitin proteasome-mediated degradation of IκBα. Although serines 32 and 36, and lysine residues 21 and 22, in mammalian IκB-α have been identified as the sites of critical post-translational modifications it was not clear which other residues were required to target their modifications. Therefore a systematic mutational analysis of this region was carried out in which individual residues were changed to alanine. Reporter assays utilising these alanine mutants confirmed the importance of serines 32,36 and lysines 21,22. Furthermore, histidine 30 and tyrosine 42 were characterised as having potential significance for the activation of NF-κB.

In 2000 a report implicated a role for NF-κB in p53-mediated apoptosis (Ryan *et al*, Nature 2000). Studies in p53-deficient cell lines, revealed that the introduction of wild-type p53 by transfection did not transcriptionally activate NF-κB. By contrast the mutant p53 H273 was noted to induce NF-κB activity. Also, it was established that adriamycin, a DNA damaging agent capable of inducing both p53 and NF-κB transcriptional activity did so by independent mechanisms. Moreover, adriamycin was shown to stimulate NF-κB transcriptional activation via the IKK2 subunit of the the IκB kinase. Also the tumour suppressor, $p14^{ARF}$ was identified to inhibit NF-κB activity.

1. INTRODUCTION

1.1 Gene Transcription

1.1.1 RNA Pol II and the basal transcription apparatus

Critical regulation of transcription is required for expression of proteins from genetic material to ensure they are expressed to the correct response, at the correct time and correct cellular location (Lee and Young 2000). Transcription is the synthesis of RNA from a DNA template. In eukaryotic cells this process is catalysed by three different types of enzymes; RNA Polymerase I, II and III respectively (Sentenac 1985; Young 1991). These enzymes are composed from 8-14 subunits with a molecular mass of approximately 500kDa. Furthermore they are each responsible for a different class of genes. RNA Pol I is responsible for synthesisng ribosomal RNA (rRNA), RNA Pol II synthesises messenger RNA(mRNA) and small nuclear RNAs (snRNA), whilst RNA Pol III transcribes transfer RNA (tRNA) and 5SRNA.

Differential gene transcription is initiated by transcription factors which are DNA-binding proteins that recognise specific sequences in the DNA (Figure 1)(Roberts 2000). They are usually triggered to function by some type of external stimuli. Two such transcription factors are NF-κB and p53, of which more is described later. Upon binding,, these transcription factors can associate with proteins from a pre-initiation complex containing the polymerase RNA Pol II at the promoters of protein-coding genes. The DNA region which contains the start site for transcription of class II genes is a sequence of roughly 100bp known as the promoter. It usually contains an AT-rich site known as the TATA box, 5' to the mRNA start point. The TATA box is the binding site for the TATA-binding protein (TBP). Certain gene promoters do not contain this TATA box but have instead an initiator element encompassing the start site for

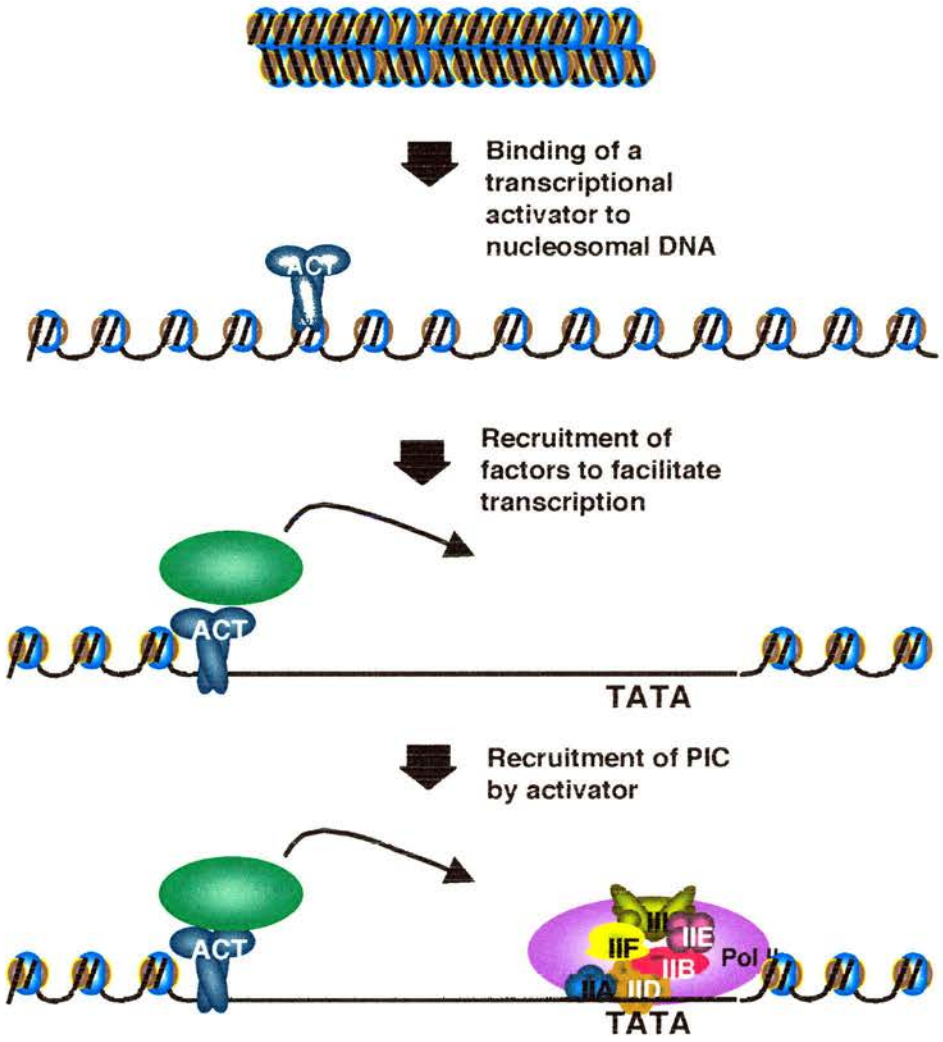


Figure 1 Chromatin modification and transcriptional regulation
Transcription is regulated at the level of chromatin structure, nucleosomal DNA inhibits access of transcription factors to the promoters. Activators bind to nucleosomal DNA disrupting the nucleosome structure allowing GTFs to gain access to the promoter and for PIC formation to occur. This figure is adapted from Roberts (2000).

transcription(Aso, Serizawa et al. 1994). This is a pyrimidine-rich sequence which binds regulatory factors that help facilitate recruitment of the basal transcription apparatus. Certain promoters that exhibit a high level of transcription have been identified to contain both these elements and are termed composite. Often viruses such as the human immunodeficiency virus exploit these promoters for their own gene expression (Garcia-Martinez, Mavankal et al. 1997). Conversely, promoters lacking both TATA-box and initiator element also exist. These contain multiple transcription start sites, but often lead to imprecise and inefficient transcriptional initiation due to poor polymerase recruitment (Lu, Lee et al. 1994).

Studies have shown that although the enzyme RNA Pol II can undergo DNA-dependant RNA synthesis *in vitro*, it requires additional factors for specific promoter recognition. Recent studies have purified a RNA Pol II holoenzyme complex that comprises the RNA Polymerase II core enzyme, general transcription factors (GTFs) and coactivators (Thompson, Koleske et al. 1993). However *in vivo,* it is not yet definitive whether a holoenzyme complex exists or that the complex forms in a stepwise manner.

The eukaryotic RNA Pol II is often regarded as a single functional unit but it actually comprises twelve subunits termed Rpb 1 to 12 (Lee and Young 2000). There is a high level of conservation between these subunits in yeast and eukaryotic RNA Pol II, so much so that they can functionally replace each other in transcription. These subunits have specific functions ranging from start-site selection, transcriptional elongation rates and interactions with coactivators. The largest of these subunits, Rpb 1 contains a carboxy-terminal domain (CTD) consisting of a seven amino-acid sequence (YSPTSPS), repeated fifty-two

times. The importance of this CTD is that its phosphorylation state represents the transcriptional activity of RNA Pol II. Non-phosphorylated RNA Pol II is involved with transcriptional initiation whereas phosphorylated RNA Pol II is associated with elongating polymerase molecules(Dahmus 1996).

The set of general transcription factors required by RNA Pol II to aid its binding to a promoter *in vitro* are TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH(Corden 1990; Orphanides, Lagrange et al. 1996; Roeder 1996). They form a transcription complex which melts promoter DNA leading to an open complex formation. Initiation continues with the formation of phosphodiester bonds before eventually undergoing promoter clearance and progressing into transcriptional elongation.

TBP is a highly conserved protein that contains the DNA binding domain in its C-terminus. In mammals, it is found in a 750kDa complex with additional polypeptides (subsequently called TAFs [TBP-associated factors]), known as TFIID(Burley and Roeder 1996; Roeder 1996). Its size enables TFIID to cover a significant region of DNA surrounding the TATA box, thus contributing to its specificity to bind the promoter. TFIIA plays a critical role in transcriptional activation by stabilising this TBP-DNA interaction and antagonising any repression caused by TAFs present in TFIID(Buratowski and Zhou 1993).

Once the initiation apparatus is bound to the TATA-box, TFIIB is involved in the selection of transcription start sites. TFIIB also forms a link between TFIID and RNA Pol II/TFIIF components of the complex(Leuther, Bushnell et al. 1996). TFIIE is suggested to further melt promoter DNA, as it can bind regions of single stranded DNA, which could be implicated in

maintaining an open promoter complex. TFIIF binds tightly to RNA Pol II, suppresses non-specific DNA binding and stabilises the preinitiation complex(Conaway and Conaway 1993). It has also been postulated that TFIIF affects DNA topology, by tightly wrapping DNA around the preinitiation complex. This probably causes torsional strain which aids in promoter melting.

TFIIH has three forms of enzymatic activity; DNA-dependant ATPase, ATP-dependant helicase and CTD kinase(Coin and Egly 1998). The actual GTF can be separated into two subcomplexes, the core and a separate kinase/cyclin complex. The core is involved in nucleotide excision repair while the cyclin/kinase complex plays a role in linking transcription with the cell cycle. The kinase activity of TFIIH is also required for elongation.

Once the initiation complex is stabilised at the promoter, the basal transcription machinery escapes from the promoter and undergoes elongation to produce the mRNA transcript. TFIIH contains the Cdk7 kinase activity which induces CTD phosphorylation of the Rpb 1 subunit of RNA Pol II generating the stable elongation complex(Flanagan, Kelleher et al. 1991). TFIIF also contributes to transcriptional elongation by counteracting repression by any negative factors and lowering the frequency of abortive transcription initiation by unknown mechanisms. Another factor that enhances the processitivity of elongation is the GTF, TFIIS which promotes synthesis of long transcripts(Reines, Ghanouni et al. 1993). Following elongation, transcription is terminated by modifying the primary transcript at both 5' and 3' ends which appears to rely on the RNA Pol II CTD. The 5' end is capped with a methylated guanosine triphosphate, whilst the 3' end is cleaved and polyadenylated(Shuman 1995; Minvielle-Sebastia and Keller 1999). The mRNA can then be further

processed before forming the mature mRNA message that can be translated to express the designated protein.

1.1.2 Chromatin

How DNA is packaged in the cell also contributes to how gene expression is regulated(Lee and Young 2000). The compaction of DNA into a highly condensed form in the cell nucleus is necessary due to the limited space available. Eukaryotic DNA is assembled into a nucleoprotein complex known as chromatin, composed of repeating units called nucleosomes. Nucleosomes are a structural arrangement involving DNA and histones which are small basic proteins (11-16kDa) highly enriched in arginine and lysine residues. There are five common histones: the four core histones H2A, H2B, H3 and H4 which contain globular domains via which DNA-histone and histone-histone interactions occur, and a fifth histone known as H1 that functions as a linker histone. The core histones form an octameric arrangement assembled from a $(H3/H4)_2$ tetramer with a H2A/H2B heterodimer on each end allowing the organisation of 160bp of DNA into two left handed superhelical turns. These nucleosomes are then separated from each other by 20-30bp of DNA associated with the linker histone H1. Linker histones then encourage coiling of nucleosomal arrays and subsequent folding into a solenoidal chromatin fibre. These fibres can then package into loops that are attached to an acidic protein scaffold to form chromosomes. Hence, this packaging of DNA renders large regions of the genome inaccessible to both RNA polymerases and transcription factors.

Accessibility to genes is generally dependant on disruption of the chromosomal structure. This occurs primarily on histones by a variety of post-translational modifications such as acetylation, methylation, phosphorylation and ubiquitination. These modifications take place on the tail domains of the histones which extend out from the chromatin and are protease-sensitive(Mutskov, Gerber et al. 1998; Puig, Belles et al. 1998). These distinct histone modifications can act sequentially or in combination to form part of a 'histone code' which can be read by other proteins to initiate downstream events(Strahl and Allis 2000).

Histone acetylation is the best characterised of these modifications. Enzymes known as histone acetyltransferases (HAT) are understood to acetylate specific lysine residues present in the N-terminal tails of histones. Conversely, the histone deacetylase (HDAC) enzyme reverses the acetylation of the lysine residues.

Lysine residues 9, 14, 18 and 23 on H3 are amongst the best characterised(Thorne, Kmiciek et al. 1990). The GCN5 family of HATs has been shown to catalyse the acetylation of lysine 14 *in vitro(Kuo, Brownell et al. 1996)*. Crystal structure data has revealed that glycine 13 and proline 16 are important for histone-binding by the GCN5 HAT(Rojas, Trievel et al. 1999). An example of acetylation of lysines being read by upstream factors involves the co-activators PCAF (p300/ CBP-associated factor (Dhalluin, Carlson et al. 1999). These molecules contain a region known as a bromodomain. This has been identified as a motif that binds specifically to acetylated lysines located in H3 and H4 tail sequences. This binding by these coactivators helps to generate a more transcriptionally active complex. Conversely Sir3 and Sir 4 are proteins in

yeast (Edmondson, Smith et al. 1996), which lead to transcriptional repression by binding these acetylated lysines.

Aside from covalent modifications, transcription can also be regulated by the chromatin remodelling of nucleosomes. This is thought to be accomplished by breaking the DNA-histone complex, and then remodelling the chromatin structure to a preferred form(Cosma, Tanaka et al. 1999). This process is ATP-dependant and has been characterised by the Swi/Snf complex involving the glucocorticoid receptor(Fryer and Archer 1998).

Hence the structure of chromatin shows another level at which gene expression has to be controlled in order to allow a response to extracellular stimuli. There are many more mechanisms that regulate how transcription is initiated of which I have not detailed. However it is evident that probably the most important role is performed by the induction of transcription factors to induce gene expression. This thesis is based on the activity of two such proteins. NF-$\varkappa$B and p53.

1.2 Transcription Factor NF-κB

1.2.1 NF-κB family of transcription factors

NF-κB was first discovered in 1986 by Sen and Baltimore as a constitutively active nuclear factor binding to a site in the kappa light chain immunoglobulin enhancer in B cells(Sen and Baltimore 1986). Since then it has been identified as a transcription factor existing in an inactive state in the cytoplasm of most cells(Liou and Baltimore 1993). NF-κB transcription is induced by various forms of stimuli including cytokines (eg TNF-α, IL-1β, LT-β), bacteria, viruses, UV irradiation and hypoxia(Baldwin 1996; Pahl 1999). This activates NF-κB to regulate the expression of numerous genes involved particularly in survival and inflammatory responses. These also include genes critical for regulating apoptosis and cell proliferation such as cIAP 1 and 2, Fas ligand. Also adhesion molecules such as VCAM and E-selectin are regulated by NF-κB(Ghosh and Karin 2002). The fact that dysregulation of NF-κB transcription can cause diseases such as rheumatoid arthritis, and cancers like Hodgkin's lymphoma has resulted in its intense study today(Finco and Baldwin 1995; Baldwin 1996).

The family of NF-κB transcription factors are a group of structurally related and evolutionarily conserved proteins that form either homodimers or heterodimers(Ghosh and Karin 2002). In vertebrates, they are composed from five subunits (Figure 2) that share a highly conserved DNA-binding and dimerisation region known as the Rel Homology Domain (RHD). These subunits can be subdivided into two groups, those that contain transcriptional activation domains and those that do not. The former include p65 (RelA), RelB

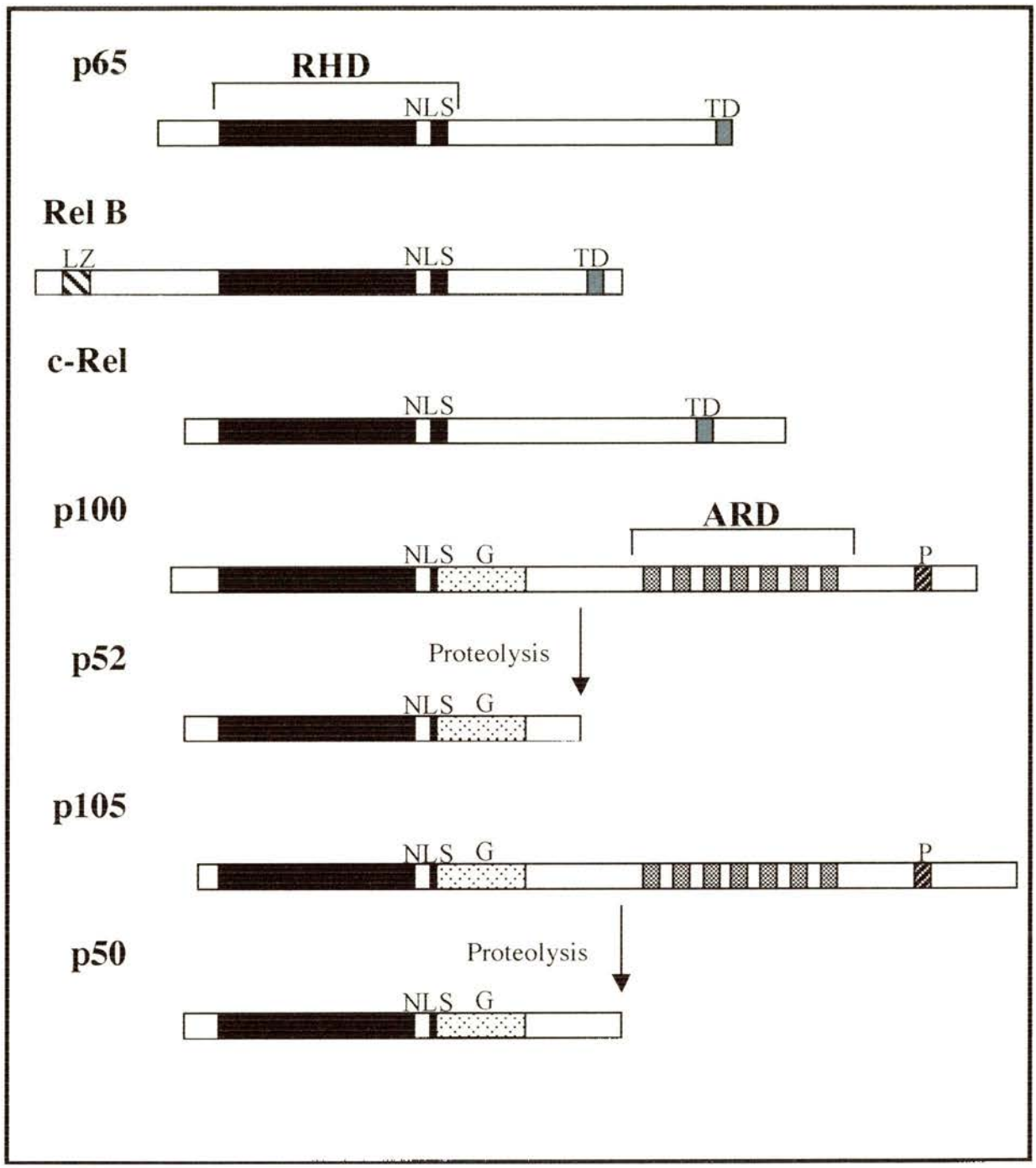


Figure 2. The mammalian NF-κB family

All NF-κB members are characterised by the presence of a Rel Homology Domain (RHD). Both p100 and p105 contain Ankyrin Repeat Domains (ARD) which are endoproteolytically cleaved to generate the subunits p52 and p50 respectively (as indicated by arrows). Abbreviations; NLS - Nuclear Localisation Signal, TD - Transactivation Domain, LZ - Leucine Zipper, G - Glycine-rich regions, P - PEST domain.

and c-Rel and each possess at least one transcriptional activation domain in their C-terminus(Ryseck, Bull et al. 1992; Blair, Bogerd et al. 1994; Schmitz, dos Santos Silva et al. 1994). The other members of the family are p100 and p105 which are precursors to the p50 and p52 subunit forms. The precursor forms both have large C-terminal regions containing ankyrin repeat domains (ARD) which result in their cytoplasmic retention. Their proteolytic processing or translational arrest result in the shorter proteins which can only function actively by forming heterodimers with subunits from the other group. In fact, the p50 homodimer form has been identified as a transcriptional repressor(Brown, Linhoff et al. 1994).

Of all NF-κB dimers, the most abundant form is the p50/RelA heterodimer which is found in virtually all cell types bound to the inhibitory protein IκBα(Baeuerle and Baltimore 1988). Following stimulation, the heterodimer is released from IκB-α and translocates rapidly to the nucleus and binds to a κB binding motif conforming to the sequence, 5'GGGRNYYCC'3(Kunsch, Ruben et al. 1992; Parry and Mackman 1994). p50 has been shown to bind the 5' end, whilst p65 binds the less conserved 3' end(Urban, Schreck et al. 1991). Other types of NF-κB dimers bind preferentially to different DNA sequence elements, thus contributing to the specificity of genes that can be regulated by this family of transcription factors.

Crystal structures of NF-κB bound to DNA have been solved for p50, p52, RelA homodimers and p50/RelA heterodimer(Ghosh, van Duyne et al. 1995; Muller, Rey et al. 1995; Chen, Huang et al. 1998). The crystal structures containing the RHDs of three Rel/NF-κB family polypeptides complexed with various DNA targets obtained indicate that the RHD has a tripartite

organisation(Ghosh, van Duyne et al. 1995; Muller, Rey et al. 1995; Chen, Huang et al. 1998). The amino-terminal 180 amino acids fold into an Ig like domain and the short, 10 amino acid, flexible linker connects this amino-terminal domain to the second Ig like domain which is approximately 100 amino acids in length. DNA recognition is primarily mediated by the amino terminal domain. The amino-terminal Ig fold acts as a scaffold for the DNA contacting flexible loops which is also employed by p53(Cho, Gorina et al. 1994). All dimerisation contacts are mediated through the carboxy terminal Ig fold. Fourteen residues from each subunit participate in the dimer interface, which is dominated by Van der Waals interactions. The Ig fold also contains the 13 amino acid Nuclear Localisation Sequence (NLS) required for nuclear translocation.

Furthermore, crystallographic data shows that p50 interacts with DNA over a complete helical turn. Each p50 subunit contacts DNA through 5 flexible loops which connect the β strands. Two of these loops are in the amino-terminal domain, two in the carboxy terminal domain and one is the flexible linker connecting the domains. The most important NF-κB-DNA interaction is between the amino-terminal of the L1 loop that connects A and B β sheets, and the bases of the major groove of target DNA. This region of the AB loop is termed the recognition loop because of its interaction with DNA determines the sequence specificity of p50 (Bell, Matthews et al. 1996). In addition partial proteolysis and chemical modification analysis suggests the carboxy-terminus of the L1 loops could also contact DNA (Hay and Nicholson 1993; Matthews, Nicholson et al. 1995). Lysine 80 residue, at the carboxy terminus of AB loop interacts with DNA through an additional lysine-phosphate backbone ionic

bond, which makes a significant contribution to the binding energy thus stabilising the protein/DNA complex (Michalopoulos and Hay 1999).

The stability of the NF-κB/DNA complex is determined by a combination of each subunit binding to the DNA, independently.

1.2.2 IκB family

In the cytoplasm of unstimulated cells, NF-κB exists in an inactive state bound to a member of the IκB family of inhibitory proteins which masks its nuclear localisation signal (NLS) (Ghosh, May et al. 1998).

The cytoplasmic removal of this inhibitor by signal induced activation has been regarded as the conventional model by which NF-κB is activated (Section 1.2.3) (Baeuerle and Baltimore 1988). This exposes the NLS of NF-κB allowing NF-κB to translocate to the nucleus to function transcriptionally. However, recently there has been some debate about this model. Firstly, it appears newly synthesised IκB molecules can remove NF-κB from binding DNA in the nucleus (Chiao, Miyamoto et al. 1994; Arenzana-Seisdedos, Thompson et al. 1995). Although IκB does not contain an apparent NLS, Turpin *et al.,* (1999) demonstrated that nuclear import of IκB is mediated by sequences present in the ankyrin repeats (Turpin, Hay et al. 1999). Moreover, it is probable that IκB utilises the NLS of another protein and enters the nucleus by a 'piggyback mechanism'. Once in the nucleus, IκB can bind NF-κB and use its own NES to export NF-κB back to the cytoplasm. Furthermore, in unstimulated cells, inhibition of the NES receptor CRM1 by leptomycin B (LMB) resulted in the nuclear distribution of NF-κB/IκB complexes by hindering the export

activity by IκBα (Ossareh-Nazari, Bachelerie et al. 1997). However, these nuclear complexes could not be activated following signal induced activation of NF-κB as IκBα degradation in the nucleus was inhibited. Thus the location for signal induced activation of this complex is most likely the cytoplasm. This is strongly validated by the fact that the kinase complex responsible for IκB phosphorylation during inducement of NF-κB activation, is positioned in the cytoplasm. However, where ubiqutin-mediated proteolysis occurs resulting in the degradation of IκB, is not yet established as a component involved in this stage of the pathway appears to be exclusively nuclear (Davis, Hatzubai et al. 2002). Therefore further investigation is required to define the different cellular compartments of this pathway.

To explain the shuttling movement, in the case of the p50/p65/IκB-α, IκB-α masks the NLS of p65 but not that of p50 (Malek, Chen et al. 2001). Thus the complex is directed to the nucleus, but is most likely immediately expelled out, because of the nuclear export signals present on p65 and IκBα. This suggests that these complexes are shuttling between the nucleus and cytoplasm continuously during unstressful situations but the significance of this is not yet been established. (Johnson, Van Antwerp et al. 1999; Rodriguez, Thompson et al. 1999; Huang, Kudo et al. 2000) .

Presently, seven forms of IκB proteins have been identified; IκBα, IκBβ, IκBδ, IκBγ, IκBε, IκBR and bcl-3 (Figure 3) (Ghosh, May et al. 1998). IκBδ and IκBε are actually the C-terminal regions of the p50 and p52 precursors, p100 and p105, respectively. All IκB proteins are structurally similar in that they share a conserved domain containing six to eight repeats of the ankyrin repeat which are involved in protein-protein interactions with NF-κB

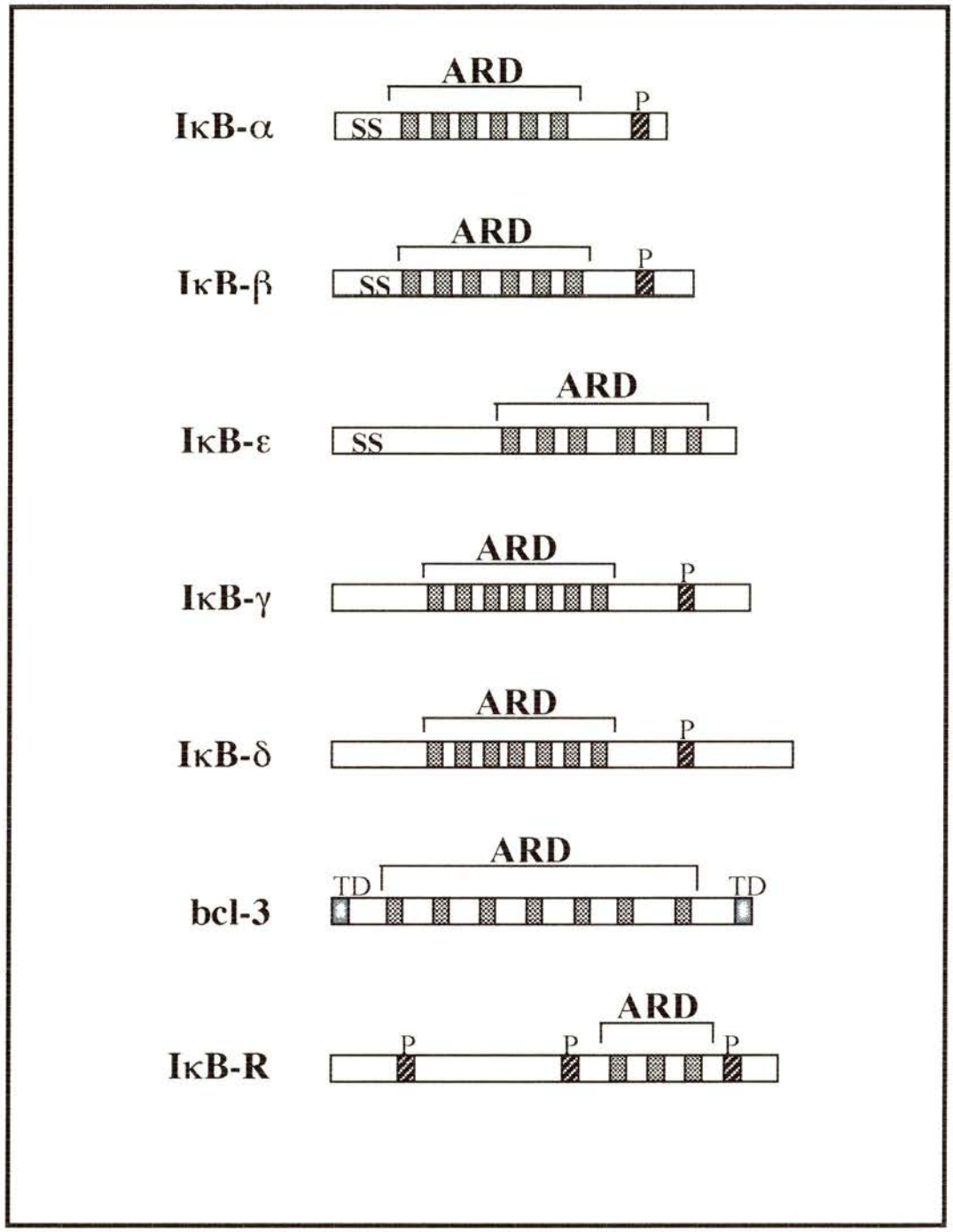


Figure 3. The mammalian IκB family

Ankyrin repeat domains (ARD) are the defining feature of the IκB family. IκB-γ and IκB-δ are the cleaved C-terminii of the precursors, p105 and p100, respectively. The pair of serine residues phosphorylated in response to signalling on IκB-α, IκB-β and IκB-ε are represented by SS. Also displayed are P (PEST domain) and TD (Transactivation Domain).

(Lux, John et al. 1990). Their differences appear in which forms of NF-κB they prefer to bind. For example, IκBα and IκBβ inhibit complexes containing RelA and c-Rel whilst IκBγ inhibit p50 and p52 homodimers (Thompson, Phillips et al. 1995; Whiteside, Epinat et al. 1997). The precursors p100 and p105 retain complexes with p50, RelA and c-Rel in the cytoplasm. However the IκB member, bcl-3 is different in that it is primarily a nuclear IκB protein and has been shown to interact with the p52 homodimer and cause its κB dependant transcription (Bours, Franzoso et al. 1993).

Presently, of the seven IκB proteins, only IκBα, IκBβ and IκBε are known to be involved in the signal induced activation of NF-κB by differing stimuli. In particular, the most extensively studied form is IκBα. IκBα is rapidly degraded in response to most NF-κB inducers and is also positively regulated by NF-κB. It is a 37kDa protein that can be divided into three parts; a 70 amino acid N-terminus containing a signal response domain, a central protease-resistant 250 amino acid domain consisting of six ankyrin repeat domains (ARD) and a final 42 amino acid C-terminus comprising of PEST (proline, glutamatic acid, serine and threonine) rich sequences that contribute to IκBα turnover (Lin, Beauparlant et al. 1996; Brown, Franzoso et al. 1997; Kroll, Conconi et al. 1997). The ankyrin repeats of IκBα which bind regions covering the NLS of NF-κB (in particular the RelA subunit), inhibit NF-κB translocation by impeding access to nuclear import machinery. This was shown by a crystal structure of IκBα (lacking the both N and C terminii) bound to a dimer comprising RelA and part of the p50 subunit. This masking of the NLS has also been shown by IκBγ upon binding p50 (Huxford, Huang et al. 1998; Jacobs and Harrison 1998). As explained above, IκBα also contains a nuclear export

sequence (NES) which is recognised by the nuclear export protein CRM1(Malek, Chen et al. 2001). This enables it to inhibit NF-κB activity in the nucleus by facilitating NF-κB's transportation back to the cytoplasm (Arenzana-Seisdedos, Thompson et al. 1995; Arenzana-Seisdedos, Turpin et al. 1997). Furthermore, NF-κB activates the transcription of IκB mRNA essentially expressing the inhibitor to control its own NF-κB activity. This form of negative feedback control is illustrated in Figure 4A. Figure 4B shows the transcription factor p53 (discussed in Section 1.3) which also utilises this mechanism with its inhibitor Mdm2 (murine double minute 2) to control its own levels of activity.

The other inhibitors affected by stimuli, IκBβ and IκBε degrade more slowly and are not as potent as IκBα (Thompson, Phillips et al. 1995; McKinsey, Brockman et al. 1996). However, experiments involving knock-in mice where IκBβ gene has been expressed under control of the IκBα promoter, have surprisingly shown that IκBβ can regenerate the functionality of IκBα protein (Cheng, Ryseck et al. 1998).

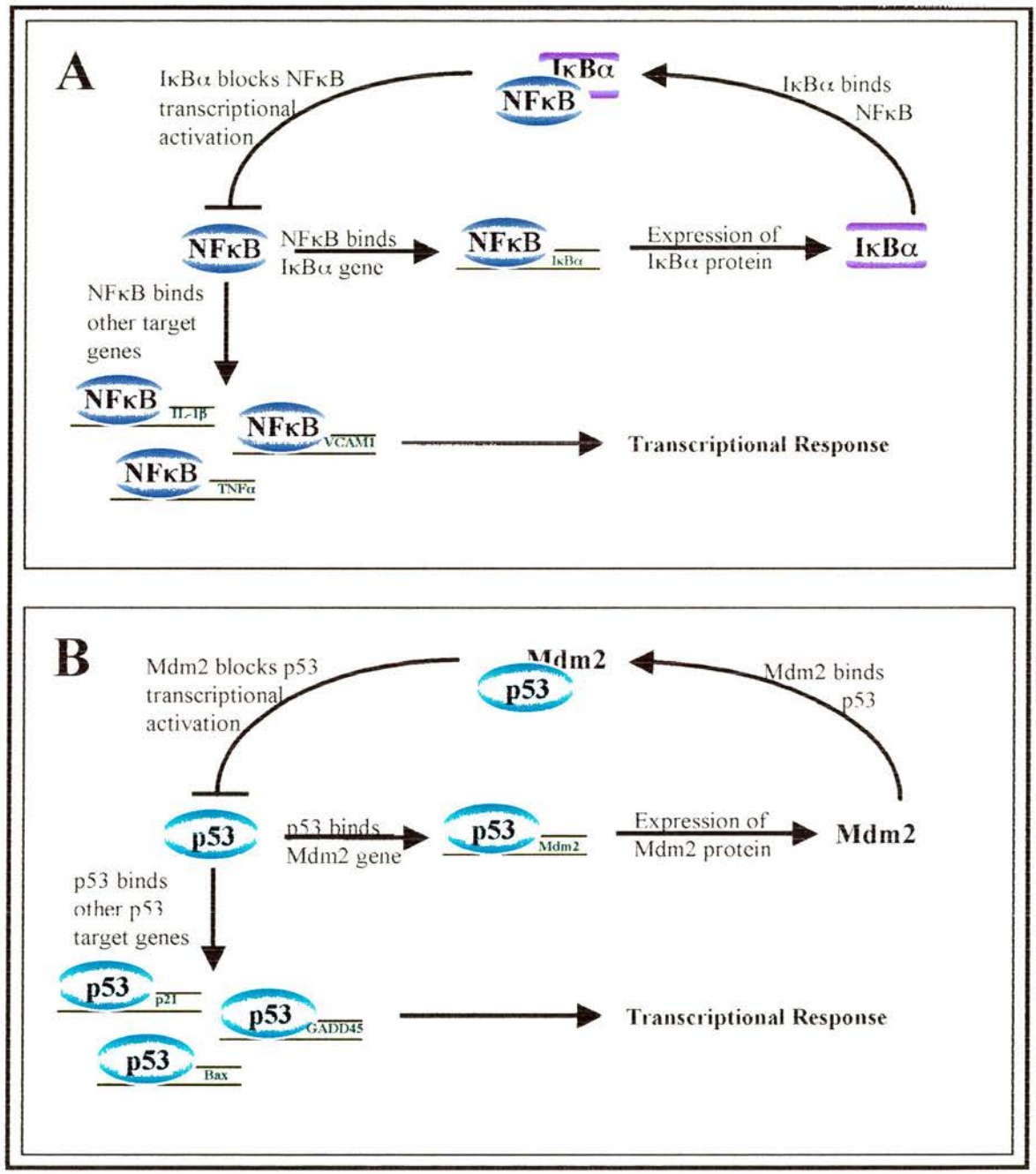


Figure 4. Autoregulation of NFκB and p53

A. NFκB binds to IκBα gene and stimulates its transcription. The resulting IκBα protein then binds to NFκB and inhibits its transcriptional activity. B. p53 performs the same type of negative feedback control as NFκB with its inhibitory protein Mdm2.

1.2.3 Signal-induced activation of NF-κB

NF-κB is the central mediator of genes involved in survival and inflammatory responses. The rapid inducibility in upregulating NF-κB target genes in answer to a diverse range of stimuli contributes to this role. *In vivo* analysis showed that activation by inducers such as TNF-α (Figure 5) and IL1-β correlated with hyperphosphorylation of IκBα, its subsequent degradation and the nuclear translocation of NF-κB (Verma, Stevenson et al. 1995).

Previous reports had shown the C-terminus of IκBα to be phosphorylated in unstimulated cells, however this form of signal induced phosphorylation by TNF-α and IL1-β was identified to occur at the N-terminal domain (Brockman, Scherer et al. 1995; Brown, Gerstberger et al. 1995; Traenckner, Pahl et al. 1995; DiDonato, Mercurio et al. 1996; Roff, Thompson et al. 1996). Hyperphosphorylated IκBαwas shown to accumulate upon inhibition of the 26S proteasome, bound to NF-κB, thereby blocking NF-κB activation. Using mutational analysis to locate the phosphorylation sites, 2 serine residues (S32 and S36) were identified as such in the N-terminus of IκBα. In addition to the hyperphosphorylated forms of IκBα, higher molecular forms of IκBα were also detected. This was attributed to the covalent attachment of ubiquitin molecules to IκBα. As ubiquitin conjugation occurs at lysine residues, mutagenesis identified K21 and K22 as the amino acids responsible for this modification (Scherer, Brockman et al. 1995; Baldi, Brown et al. 1996; Rodriguez, Wright et al. 1996). The ubiquitinated forms of IκBα were shown to rely on prior phosphorylation as the presence of a S32A/S36A mutation prevents phosphorylation or ubiquitination whilst the K21R/K22R

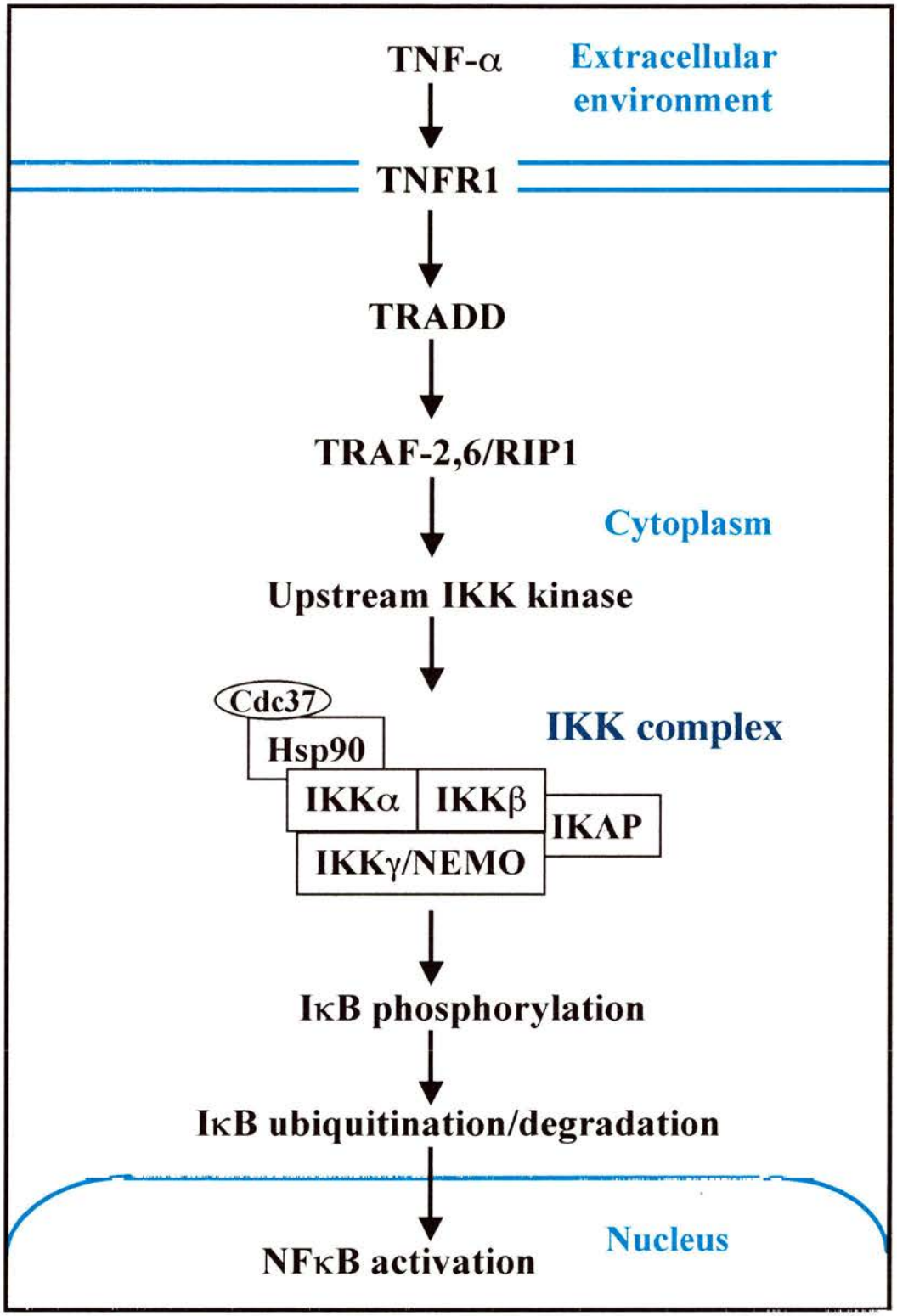


Figure 5. Activation of NFκB by TNF-α

TNF-α induces a signalling pathway which can ultimately lead to the degradation of IκB and subsequent activation of NF-κB. This figure was adapted from Hay *et al.*, 1999. See text for further details. Abbreviations of signalling components: TNF - tumour necrosis factor; TNFR1 - TNF receptor subtype 1, TRADD - TNFR1 associated death domain protein, TRAF - TNFR associated factor, RIP - ring finger interacting protein, Cdc - cell division cycle, Hsp - heat shock protein IKK - IκB kinase, IKAP - IKK complex-associated protein, NEMO - NFκB essential factor.

mutant was able to undergo signal induced phosphorylation but not ubiquitination. As ubiquitination targets a protein specifically for proteasomal degradation, neither of these mutants could be degraded, thus preventing any NF-κB activation. This also explains why ubiquitinated forms of IκBα were present when the 26S proteasome was inhibited.

Therefore signal-induced activation of NF-κB by TNF-α and IL1-β targets the phosphorylation of IκBα at serines 32 and 36. This triggers the ubiquitination of IκBα at lysines 21 and 22, which leads to degradation by the 26S proteasome, allowing NF-κB translocation to the nucleus where it can regulate transcription of the appropriate genes.

1.2.4 The IκB kinase

The search for a protein kinase responsible for IκB-α phosphorylation on residues S32 and S36 has provoked much interest because of its implications on the NF-κB pathway. Chen *et al.* were the first to identify a potential large, multisubunit complex (purified from unstimulated cytosolic HeLa extracts) that had kinase activity (Chen, Parent et al. 1996). However, it was noted that this 700 kDa complex could only function in an ubiquitinated state. Experiments performed in this paper suggested that a non-polyubiquitinated complex prevented IκB-α phosphorylation. The problem however with this complex, was that no associated kinase subunit was located. Since then, the same laboratory have also demonstrated that MEKK-1 (mitogen-activated protein kinase/ ERK kinase kinase 1) a TNF-α inducible kinase, could activate the complex *in vitro*, allowing it to phosphorylate IκB-α at S32 and S36 *in vivo*.

In 1997 five papers were published that used molecular cloning techniques to purify a novel larger cytokine-responsive IκB-kinase complex that was not dependant on ubiquitination to achieve IκB phosphorylation (DiDonato, Hayakawa et al. 1997; Mercurio, Zhu et al. 1997; Regnier, Song et al. 1997; Woronicz, Gao et al. 1997; Zandi, Rothwarf et al. 1997). Also one of the papers, in particular provided evidence that the complex was not activated in response to MEKK-1 Three of these papers described a 900 kDa complex that was capable, upon TNF-α stimulation of specifically phosphorylating IκB-α at S32 and S36, as well as IκB-β at S19 and S23 (DiDonato, Hayakawa et al. 1997; Mercurio, Zhu et al. 1997; Zandi, Rothwarf et al. 1997). This complex contained two polypeptides (85 kDa and 87kDa in size) associated with kinase activities which were eluted on affinity columns(DiDonato, Hayakawa et al. 1997).

DiDonato *et al.* subjected the 85 kDa polypeptide to microsequencing and showed it to contain related sequences to a human serine/threonine kinase named CHUK (conserved helix-loop-helix ubiquitous kinase). Although described a couple of years earlier its function was unknown, but with the identification now as an IκB-kinase, CHUK was renamed as IKK(Connelly and Marcu 1995). The other two papers showed the 87 kDa polypeptide, more efficient at phosphorylating IκB-β, also to be similar to CHUK, sharing 50% homology with the 85 kDa polypeptide(Mercurio, Zhu et al. 1997; Zandi, Rothwarf et al. 1997). Therefore, the two polypeptides were designated as subtypes of IKK, where the 85 kDa polypeptide now corresponded to IKK-α and the 87 kDa polypeptide as IKK-β. Another laboratory also identified IKK-α, but this time using a different technique Reigner *et al.* applied a yeast two-hybrid screen for NIK-interacting proteins. NIK (NF-κB inducing kinase) was

utilised, as it was shown to activate NF-κB in response to the cytokines, TNF-α and IL-1β, whilst inactive NIK mutants inhibited NF-κB activation. So NIK was identified as the probable converging point of the signal transduction pathways used by the cytokines TNF-α and IL-1β. Therefore using NIK in the 2-hybrid screen, this group also characterised CHUK. Further investigation of CHUK's association with IκB-α uncovered its ability to specifically phosphorylate IκB-α on residues S32 and S36. Again using inactive mutants, this time of CHUK, TNF-α and IL-1-mediated activation of NF-κB was inhibited. The fifth paper discovered IKK-β(Woronicz, Gao et al. 1997).

From these papers, the kinase that phosphorylated IκB was located in a large complex with other, as yet, unidentified proteins. As results differed on whether the kinase was in a ubiquitinated state or not, prior to phosphorylation, it was probable that the differences between the papers arose from purification procedures.

Following on from this, one of the unidentified proteins from the complex was discovered as the regulatory subunit IKKγ. Using a monoclonal antibody to IKK-α, a kinase complex was pulled out containing both catalytic IKK subunits and two extra polypeptides of 50 and 52kDa. These were microsequenced and termed IKKγ1 and IKKγ2 respectively, as they were discovered to be different forms of the same protein (Rothwarf, Zandi et al. 1998). IKKγ was also discovered as another protein, NEMO (NF-κB essential factor), this time by complementation cloning of cDNAs whose products restored NF-κB activation into cell lines defective in this condition (Yamaoka, Courtois et al. 1998). IKKγ/NEMO is a glutamine rich protein composed of 419 amino acids which contain no catalytic domain. It contains coiled-coil motifs via

which it can bind IKK1/IKK2 heterodimers. The significance of this regulatory subunit was further confirmed when removal of its C-terminus, prevented signal-induced activation by various stimuli (Rothwarf, Zandi et al. 1998). These experiments helped establish that the IKK complex was the IκB kinase that was part of the NF-κB activation pathway. The complex was found to also contain a scaffold protein known as the IKK complex-associated protein (Cohen, Henzel et al. 1998). This is a 150kDa protein that appeared to assemble the kinase complex with both NIK and NF-κB/IκB, but not actively feature in signal transduction pathway of NF-κB. More recently, a chaperone protein Hsp90 and its kinase specific targeting subunit Cdc37, have also been identified as members of the complex involved in recruiting the kinase to the type 1 TNF-α receptor, in response to TNF-α (Chen, Cao et al. 2002).

Activation of the kinase complex depends on the phosphorylation of two conserved serines found in the activation loops of either catalytic subunits; S176 and S180 in IKKα and S177 and S181 in IKKβ (Mercurio, Zhu et al. 1997). Although cytokines such as TNF-α and IL-1β result in phosphorylation of both IKKα and IKKβ, gene targeting experiments have shown that IKKβ and not IKKα is necessary for NF-κB activation by most proinflammatory stimuli (Li, Van Antwerp et al. 1999; Tanaka, Fuentes et al. 1999). Moreover, IKKα is required for IKK activation in response to RANK protein and the B-lymphocyte stimulator (Blys/BAFF) which do not require IKKβ (Cao, Bonizzi et al. 2001). Therefore, although certain signals can activate the kinase complex via IKKα, the majority of NF-κB inducers target the IKKβ subunit. In addition, the IKK complex targets phosphorylation of both IκB-α and IκB-β but the IKKα subunit preferentially targets IκB-β (Zandi, Rothwarf et al. 1997).

Presently, how various stimuli converge on the IKK complex is not yet clearly established. MEKK1, MEKK2, MEKK3 and NIK, members of the MAP3K (mitogen activated protein kinase kinase kinase) family, have all been shown *in vitro* to phosphorylate IKK (Mercurio, Zhu et al. 1997; Nakano, Shindo et al. 1998). However to date, none of these kinases have been identified as the direct IKK kinase *in vivo*. MEKK1 (mitogen activated protein kinase/ERK kinase kinase1) which displays an affinity for IKK-β, is also part of the c-Jun N-terminal kinase (JNK) pathway, that is involved in the stress response pathway activated by small GTP-binding proteins such as Cdc42 and rac1 (Nakano, Shindo et al. 1998). In contrast, NIK (NF-κB inducing kinase), a serine/threonine kinase involved in the proteolysis of p100 to the p52 subunit, prefers to activate IKK-α. However, NIK interacts with a TNFα pathway component, TNF receptor associated factor type 2 (TRAF2), when activating the IKK (Ling, Cao et al. 1998). Furthermore, it is not clear how NIK functions as NIK -/- deficient mice respond to TNF-α and IL1β, but not lymphotoxin-β (LTβ) (Yin, Wu et al. 2001). Nevertheless, when comparing NIK -/- mouse embryo fibroblasts with wild-type, in response to each stimulus there were no differences in NF-κB DNA binding activity. This evidence suggests NIK possibly mediates NF-κB transcriptional activity in a receptor-selective manner via the IKK-α subunit. Other kinases known to activate the IKK complex include TBK/NAK/T2K (TANK binding kinase-1/ NF-κB activating kinase/ TRAF2-associated kinase) in response to PDGF (platelet derived growth factor) and the kinase TPL2 (tumour progression locus 2) in response to T-cell signalling (Lin, O'Mahony et al. 2000; Tojima, Fujimoto et al. 2000). TPL2 also performs a major role in p105 processing to p50 (Belich, Salmeron et al. 1999).

Aside from protein kinases, the IKK complex can also be activated by other molecules. Latent membrane protein 1 (LMP1), a transmembrane protein expressed in Epstein-Barr Virus (EBV) associated carcinomas results in NF-$\varkappa$B activation by utilising proteins in the TNF-α signalling pathway (Sylla, Hung et al. 1998). It activates IKK by interacting with TRAF2 via its carboxy-terminal domain, known as the TES1 (Transformation Effector Site 1) domain and with RIP and TRADD (TNFR-associated death domain) via a second site known as TES2. Another protein, Tax involved with the Human T-cell leukaemia virus (HTLV) has been suggested to activate the IKK complex by directly binding to IKKγ/NEMO (Geleziunas, Ferrell et al. 1998).

Since proteins other than kinases can also activate the IKK complex directly, it has been postulated that protein-protein interaction rather than kinase activity induces IKK phosphorylation. For example, TNF-α stimulation uses TRADD and two TRAF proteins TRAF2 and TRAF5 to recruit the kinase complex to the type 1 TNF-α receptor, where the protein kinase RIP1 can activate IKK1 (Kelliher, Grimm et al. 1998; Devin, Cook et al. 2000). However, IKK activation has been shown not to require RIP1 kinase activity (Devin, Cook et al. 2000) IL-1 stimulation which utilises IRAK1 (the equivalent to RIP1 in IL-1 activation) in IKK activation also dispenses with its kinase activity (Li, Commane et al. 1999). Another kinase that appears to activate IKK via protein-protein interactions is PKR which responds to dsRNA, viral infection and LPS from Gram-negative bacteria (Chu, Ostertag et al. 1999).

Thus, it is probable that a protein-protein interaction facilitates transautophosphorylation within the IKK complex subsequently activating it to phosphorylate I$\varkappa$Bα.

1.2.5 Ubiquitination of IκBα

Once the IKK complex has phosphorylated IκBα at serines 32 and 36, the inhibitor is targeted for ubiquitination-dependant degradation by the 26S proteasome, thus allowing NF-κB to enter the nucleus to turn on its target genes.

Ubiquitination of IκBα involves the covalent attachment of a small 76 amino acid protein called ubiquitin via its C-terminal glycine residue to the epsilon amino group of lysine residues 21 and 22 (Scherer, Brockman et al. 1995; Baldi, Brown et al. 1996; Rodriguez, Wright et al. 1996). The ubiquitination process is mediated by a cascade of three enzymes (Ciechanover 1994). Firstly, ubiquitin is activated by a unique E1 enzyme that adenylates its C-terminal glycine. This allows formation of a thioester bond between ubiquitin and a cysteine residue in the E1 enzyme along with the subsequent release of AMP. Then via a transesterification reaction, ubiquitin is transferred from the ubiquitin-activating enzyme to E2 ubiquitin-conjugating enzymes such as Ubc5 and cdc34. In many ubiquitination reactions, ubiquitin is directly transferred to the target protein via the E2 enzyme. However, IκBα ubiquitination involves the E3 ubiquitin ligase, the $SCF^{\beta trcp}$ complex, which recognises phosphorylated IκBα and brings it into the vicinity of Ubc5 to catalyse the addition of ubiquitin to IκBα. The E3 family consists of at least four different classes of proteins, of which one class is the $SCF^{\beta trcp}$ complex composed from Skp1, Cdc53/Cullin 1, F-Box containing protein (SCF) and a β-transducin repeat containing protein (βTRCP) (Feldman, Correll et al. 1997; Skowyra, Craig et al. 1997). Skp1 and the F-box protein interact through a F-box motif, and connect to the E2 via the Cdc53/cullin 1 whilst the specificity of identifying the phosphorylated IκBα to

be ubiquitinated is established through the F-box/Wd40 repeat protein, βTRCP. This subunit recognises the motif DSG**S (where * represents other amino acids) containing phosphorylated serines (i.e. S32 and S36 in IκBα) (Yaron, Hatzubai et al. 1998). The HIV1 protein, vpu and β-catenin, which is involved in the Wnt/wingless signalling cascade, also contain this consensus motif and undergo the same ubiquitination using the E3, $SCF^{\beta trcp}$ complex. Once the ubiquitin is transferred onto IκBα, formation of long multi-ubiquitin chains occur via lysine residues in ubiquitin, generating a degradation signal for proteolysis by the 26S proteasome.

However, this ubiquitin-mediated degradation of IκB-α can be inhibited by SUMO-1 modification (Matunis, Coutavas et al. 1996; Shen, Pardington-Purtymun et al. 1996; Mahajan, Delphin et al. 1997). SUMO-1 (small ubiquitin-like modifier) is a protein that has similar secondary structure to ubiquitin but shares only 18% homology in protein sequence. SUMO-1 conjugates IκB-α at the same lysine residues that ubiquitin binds using a similar pathway but with distinct features. Although SUMO-1 conjugation is inhibited by IκB-α phosphorylation at S32 and S36, SUMO-1 modified IκB-α cannot be ubiquitinated and is therefore unable to be targeted for degradation by the 26S proteasome (Desterro, Thomson et al. 1997). Therefore SUMO-1 modification generates IκB-α resistant to degradation, which results in inhibition of NF-κB activation.

1.2.6 Proteasome

The proteasome is a multisubunit proteolytic complex, which is the major site of non-lysosomal protein degradation in both nuclear and cytoplasmic

regions of the eukaryotic cell (Baumeister, Walz et al. 1998). In particular, the proteasome targets degradation of molecules that have been polyubiquitinated such as IκBα. The 26S proteasome is a large 2.5 Mda complex consisting of the core catalytic 20S proteasome and two regulatory subunits termed 19S complex (also known as PA700). The crystal structure of the *Saccharomyces cerevisiae* 20S proteasome demonstrated the complex to be composed from two copies, each of fourteen different subunits which divide into the α-type and β-type subunit family structures (Groll, Ditzel et al. 1997). These subunits form a hollow cylindrical structure composed from four helical heptameric rings stacked together. The α-subtype appear in the outer rings whilst the β-subtype form the inner central rings (α_7,β_7,α_7,β_7). Here the β-subtype expose their active sites to perform proteolytic activity. The entrance to the cylindrical structure is narrow such that attachment of PA700 via the α-type subunits is required to widen the channel so that substrates can access the proteasome (Murakami, Matsufuji et al. 1999). PA700 is also suggested to unfold substrates so that they can be threaded into the 20S proteasome.

The crystal structure of the archaebacterial *Thermoplasma acidophilum* proteasome identified the N-terminal threonine of the β-type subunit to be the catalytic nucleophile involved in peptide bond hydrolysis by the proteasome (Lowe, Stock et al. 1995). Upon mutation of this residue, enzymatic activity by the proteasome was lost. Furthermore, in eukaryotic cells, of the seven β-type subunits, only three subunits are associated with active N-terminal threonine activity. These are the β1, β2 and β5 subtypes which are associated with chymotrypsin-, trypsin- and peptidylglutamyl hydrolysing- like activities, respectively (Orlowski, Cardozo et al. 1993). These three activities cleave the

substrates after hydrophobic, basic and acidic residues, respectively. Although only three of the subtypes are active, it is important that these active forms interact with the inactive subunits as this is required to preserve the functionality of the proteasome (Arendt and Hochstrasser 1997).

In mammals, active subunits of the 20S proteasome are interchangeable when cells are stimulated by the immunomodulatory cytokine IFNγ (γ-interferon) (Fruh and Yang 1999). They are replaced by β1i, β2i and β5i which lead to the formation of an immunoproteasome, which can process antigens and ligands involved with class I molecules of the major histocompatability (MHC) complex (Rock and Goldberg 1999). IFNγ also induces formation of a regulatory subunit 11S (also known as PA28) which binds the 20S proteasome via the α-type subunits (DeMartino and Slaughter 1999). PA28 activates various peptidases in the 20S proteasome via an ATP-independant manner but cannot hydrolyse large protein substrates even if they are ubiquitinated.

However the regulatory subunit, PA700 can activate the proteasome by ATP-hydrolysis. It is composed from 18 subunits ranging in size from 25 to 110kDa (Holzl, Kapelari et al. 2000). ATPase activity is found in six of these subunits and is required to initiate ubiquitin-dependant degradation by the 26S proteasome. Mutations resulting in loss of ATPase activity has resulted in an accumulation of ubiquitinated proteins ready to be degraded. Certain non-ATPase subunits are known to be involved with identifying ubiquitinated proteins but the others are not well categorised. In yeast, the PA700 subunit has been linked to interacting with E3 ligases involved in protein ubiquitination (Kleijnen, Shih et al. 2000; Tongaonkar, Chen et al. 2000). Also in humans, the proteasome can link to E3 ligases via the proteasome ligase interaction

components 1 and 2 (PLIC-1 and -2). Thus it appears the ubiquitination process can occur within the cellular vicinity of the proteasome.

The 26S proteasome does not solely target proteins such as IκB-α for degradation. Moreover, it is involved with the proteolytic processing of NF-κB precursor proteins, p100 and p105 to form the mature NF-κB subunits, p52 and p50 (Fan and Maniatis 1991; Thanos and Maniatis 1995). Hence, the proteasome performs a critical role in activation pathway of NF-κB.

1.2.7 Alternative pathways of NF-κB activation

The TNF-α mediated signal-induced activation is the best characterised pathway of NF-κB activation. However, other pathways have also been implicated. One such mechanism involves $pp90^{RSK}$, (90kDa ribosomal S6 kinase) which is activated by the MAP kinase pathway (Ghoda, Lin et al. 1997). It has been shown to specifically target serine 32, but not serine 36. However, NF-κB activity cannot be initiated unless the second serine is phosphorylated, but this is performed by an independent mechanism. Furthermore, this kinase has recently been implicated in the p53 dependent activation of NF-κB (Ryan, Ernst et al. 2000).

Another pathway involved in activating NF-κB utilises the phosphorylation of IκBα at tyrosine 42 by pervanadate (Mukhopadhyay, Manna et al. 2000). This does not involve the serine residues at all, and also appeared to have no requirement for IκBα ubiquitination. However, it did result in the degradation of IκBα prior to ensuing NF-κB activity.

Hence, it is probable there are multiple pathways that target NF-κB activity. But it is apparent that in all situations the removal of IκB is paramount to any NF-κB dependent transcriptional behaviour..

1.2.8 NF-κB and Apoptosis

Apoptosis is required to rid multicellular organisms of individual cells without damaging the organism. This involves initiating a caspase cascade that results in membrane blebbing, nuclear condensation and DNA fragmentation of the targeted cell. TNF-α is an initiator of this pathway, which can also negatively control its own ability to induce apoptosis. It mediates this by activating NF-κB which inhibits apoptosis.

TNF-α initiates apoptosis by inducing TRADD (a component in the NF-κB activation pathway) binding to FADD via its carboxy-terminal death domain (Hsu, Xiong et al. 1995). Following this the amino-terminal death effector domain of FADD interacts with the prodomain of caspase 8 (also known as FLICE) which initiates the apoptotic protease cascades leading to cell death. NF-κB suppresses this apoptosis by upregulating anti-apoptotic genes such as cIAP-1 and -2 (cellular inhibitors of apoptosis 1 and 2) and FLIP (a caspase inhibitor) (Rothe, Sarma et al. 1995).

Very recently the protein E2F, involved in p53-dependant growth arrest, has also been discovered to also induce apoptosis by inhibiting NF-κB activity. Overexpression of E2F resulted in accumulation of reactive oxygen species, which enhanced serum-deprived apoptosis. NF-κB regulates the expression of manganese superoxide dismutase (MnSOD), which functions in a redox pathway that ultimately leads to elimination of ROS (Tanaka, Matsumura et al.

2002). Tanaka *et al.* showed that E2F can bind to p65 subunit of NF-κB, thus inhibiting NF-κB activity. Hence, ROS is free to cause apoptosis.

1.2.9 NF-κB and Disease

Activation of NF-κB, has been implicated in the pathogenesis of chronic inflammatory diseases such as asthma, rheumatoid arthritis, bowel disease (Barnes and Karin 1997)and various cancers isuch as Hodgkin's lymphoma and breast cancer (Wood, Roff et al. 1998). Asthma and rheumatoid arthritis are diseases which are characterised by both chronic activation of NF-κB and the subsequent inflammatory process. Chronic stimulation of tissues can ultimately lead to NF-κB damage that in turn causes severe pain and swelling in the affected tissues of the individual. In Hodgkin's lymphoma mutations in IκBα result in the constitutive activity of NF-κB (Cabannes, Khan et al. 1999). In other types of cancers, dysregulation of NF-κB activity results in the loss of the anti-apoptotic response by NF-κB (Gilmore, Koedood et al. 1996).

Thus a better understanding of the regulation of this transcription factor may enable development of treatments towards the above diseases and cancers.

1.3 Transcription Factor p53

1.3.1 Role of p53

p53 was first identified as a protein that immunoprecipitated with the SV40 large T antigen, a product of the viral SV40 genome implicated in SV40-

mediated oncogenesis (Potter, Potter et al. 1970; Lane and Crawford 1979). Later studies showed p53 was also found to associate with E1B-55kDa of adenovirus (Sarnow, Ho et al. 1982). In addition, p53 expression levels in certain tumour cells were found to be elevated in comparison to that of primary cell lines (Crawford, Pim et al. 1981). This suggested a role for p53 in tumourigenesis. The transfection of wild-type p53, causing cells to exhibit growth arrest or apoptosis revealed that p53 actually functioned as a tumour suppressor (Finlay, Hinds et al. 1989). For example, transcriptional activation of p53 in response to DNA damage enabled growth arrest to occur, allowing DNA repair so that damaged DNA was not replicated (Vousden 2000). The p53 transcriptional response is also initiated by abnormal growth stimuli inducing programmed cell death. Loss of these functions would be undesirable as firstly it would cause genetic instability, increasing the susceptibility of cells to become carcinogenic and secondly remove the barrier to uncontrolled growth of cancerous cells. In fact, mutations in the p53 gene, resulting in loss of p53 activity have been identified in half of all known human cancers (Hollstein, Sidransky et al. 1991).

p53 exists as a sequence-specific transcription factor (Kern, Kinzler et al. 1991) which can be activated by stress signals such as DNA damage and hypoxia or tumourigenic symptoms such as abnormal proliferation (Pietenpol, Tokino et al. 1994). It responds appropriately by regulating the gene expression of proteins which can be involved in growth arrest, DNA repair or apoptosis (Farmer, Bargonetti et al. 1992; Zambetti, Bargonetti et al. 1992). These include p21, GADD45, Bax and also Mdm2 (murine double minute 2) which controls the regulation and stability of p53 (Kastan, Zhan et al. 1992; Barak, Juven et al.

1993; el-Deiry, Tokino et al. 1993; Miyashita and Reed 1995). p53 also controls itself by a negative feedback mechanism, in a similar manner to NF-κB (Kubbutat, Jones et al. 1997). p53 is a family member of related transcription factors, also including p63 and p73.

The human p53 gene spans approximately 20kb, containing 11 exons and encoding a 393 amino-acid polypeptide (Figure 6) (Levine 1997). The protein can be divided into three distinct domains; an acidic N-terminal transactivation domain, a central DNA-binding domain and a basic C-terminal oligomerisation domain (Iwabuchi, Li et al. 1993; Unger, Mietz et al. 1993). Each domain plays an important role in the functionality of p53.

The transactivation domain interacts with the basal transcription apparatus, thus regulating gene expression (Fields and Jang 1990; Lu and Levine 1995; Thut, Chen et al. 1995). The amino acids, phenylalanine 19, leucine 22 and tryptophan 23 have been shown to make contacts with the TAF subunits of TFIID. Mdm2 also binds via the transactivation domain (Momand, Zambetti et al. 1992). Furthermore, the N-terminus of p53 contains a proline-rich sub-domain (amino-acids 64-92) which is required for PIG3 (p53-inducible gene 3) activation (Venot, Maratrat et al. 1998).

The DNA-binding domain encompasses the amino-acids 102 to 293 where the majority of mutations occur, disrupting p53's ability to bind DNA (Kern, Kinzler et al. 1991). It is a protease-resistant region (Bargonetti, Wang et al. 1993), containing a zinc ion required for its sequence-specific binding to DNA (Hainaut and Milner 1993). p53 usually binds to two copies of the sequence 5'RRRC(A/T)(T/A)GYYY'3 separated by 0-13bp (Tokino, Thiagalingam et al. 1994) which has been shown to occur at least 200 times in

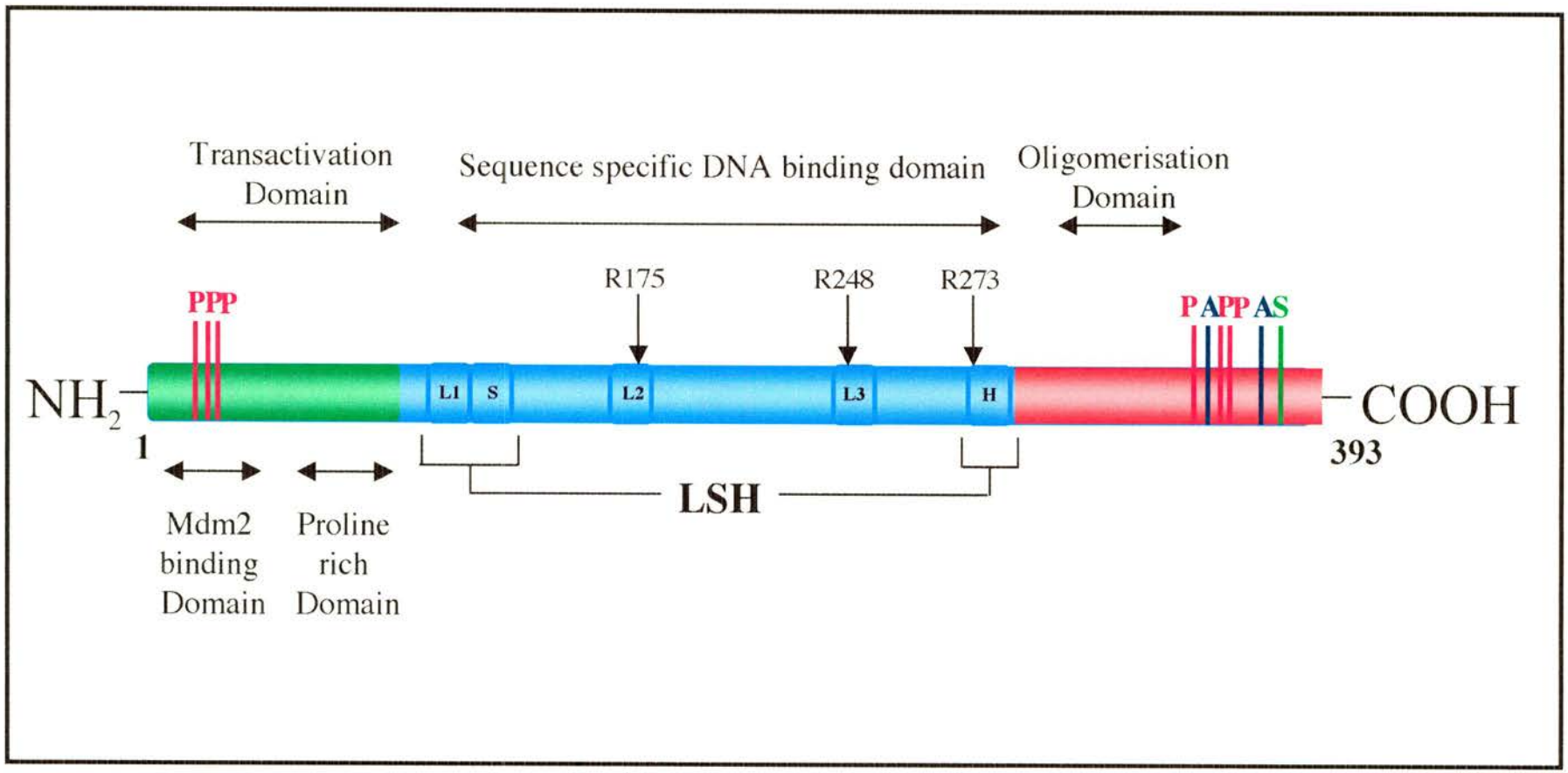


Figure 6. Schematic representation of p53

The positions of domains are indicated above and below p53. The green and red regions represent the N- and C-terminii respectively, while the blue region is the central core domain. The regions involved in forming the leucine-sheet helix (LSH) are also displayed (L1,2 & 3 - Loops 1,2 & 3; S - Sheet and H - Helix). Certain sites of modification are denoted as follows; P - Phosphorylation, A - Acetylation and S - Sumoylation. The most commonly mutated sites, arginines 175, 248 and 273 are also labelled. References for p53 domains and modifications are present in the text.

the human genome. Moreover, this sequence can be divided into four pairs of inverted repeats of the 5'RRRC(A/T)'3 sub-sequence to which p53 binds the DNA as a tetramer. Crystal studies of a core p53/DNA crystal at 2.2Å resolution have revealed this domain to consist of two anti-parallel β-sheets that serve to organise both a loop-sheet helix motif (LSH) and two large loops (L2 and L3 in place) into place (Cho, Gorina et al. 1994). The LSH binds the major groove of DNA, while L2 and L3 bind the minor groove, allowing the residue arginine 248, present in L3, to form multiple hydrogen bonds with the A/T residue in the consensus site (Cho, Gorina et al. 1994). Arginine 248 is the most commonly mutated residue in p53 and generally a mutation here directly hinders p53's ability to bind DNA (Hollstein, Rice et al. 1994). Disruption of the DNA binding ability also occurs when histidine 273 is mutated. Mutations also target residues that affect the ability of p53 to organise its core domain. When the arginine 175, which stabilises L2 and L3 via the zinc atom, is mutated it disrupts the tetramer structure. This also indirectly impedes p53's DNA binding ability. Hence, it is apparent that any change in the central domain of p53 results in a destabilising of the interaction between p53 and DNA and thus its loss of function as a transcription factor.

The carboxy-terminus spans the amino-acids 293-393 and contains the oligomerisation domain (Pavletich, Chambers et al. 1993). The structure of this region is an amphipathic helix and it controls the dimerisation/tetramerisation of the transcriptionally active p53 molecule (Sturzbecher, Brain et al. 1992). Aside from this domain the C-terminus also contain sites that can be covalently modified to induce latent p53 (Meek 1999). Furthermore, both the nuclear

export and localisation signal of p53 are also positioned here (Shaulsky, Goldfinger et al. 1991).

1.3.2 Activation of p53

p53 is usually found at low levels in the nucleus of normal cells where its regulation is controlled by Mdm2 (human homologue, Hdm2). Mdm2 functions as an E3 ubiquitin protein ligase for p53, similar to the $SCF^{\beta trcp}$ complex involved in IκBα degradation (Kubbutat, Jones et al. 1997). Mdm2 binds to the N-terminal transactivation domain of p53, targeting p53 to the cytoplasm for ubiquitin-mediated proteolysis via the 26S proteasome. This helps to maintain p53 with a half life of approximately 20 minutes in unstressed cells (Maltzman and Czyzyk 1984). In turn, p53 also transcriptionally activates the Mdm2 gene, forming a negative feedback control for itself (Figure 4B). Mdm2 is also known to regulate its own stability by auto-ubiquitination (Jones, Roe et al. 1995). Recently, MdmX a protein with 44% homology to Mdm2 was shown to bind p53 protecting it from degradation by Mdm2 but still maintaining suppression of p53 dependant-transactivation . It was also shown to dimerise with Mdm2 and thus inhibit degradation of Mdm2.

Activation of p53 as a transcription factor often involves disrupting the p53/Mdm2 relationship. Phosphorylation of p53 at any of the three residues encompassing the Mdm2 binding site (serine 15, threonine 18 or serine 20) leads to stabilisation of p53 (Craig, Burch et al. 1999). It is probable that these modifications interfere with Mdm2's function as a negative regulator of p53. Phosphorylation of the three residues can be mediated by members of the phospho-inositide kinase (PI-3K) family such as ATM (Ataxia Telangiectasia

Mutated) and ATR (ATM-related) kinases which are activated in response to genotoxic damage (Banin, Moyal et al. 1998; Tibbetts, Brumbaugh et al. 1999). Protein Kinase C (PKC) has also been shown to phosphorylate p53. However, it targets the three serine phosphorylation sites 371, 376 and 378, present in the carboxy-terminus of p53, therefore not actually affecting the p53/Mdm2 complex (Baudier, Delphin et al. 1992). *In vitro*, these C-terminal phosphorylations have stabilised p53 and it's binding to DNA, but *in vivo* the significance of such modifications has not been determined (Takenaka, Morin et al. 1995).

Conversely, phosphorylation of Mdm2 is an alternative route that can lead to the stabilisation of p53. The DNA protein kinase (DNA-PK) has been shown to phosphorylate Mdm2 at serine 17, which inhibits formation of the p53/Mdm2 complex, thus liberating p53 and allowing it to function as a transcription factor (Appella and Anderson 2001). Also, the kinase ATM which phosphorylates p53 has been implicated in Mdm2 phosphorylation (Maya, Balass et al. 2001).

Other types of modifications can enhance p53's transcriptional activity. For example, acetylation of the C-terminus of p53, increases p53 binding to DNA (Gu and Roeder 1997). Acetylation of lysine residues 373 and 382 in this region is performed by the coactivators p300/CBP, both of which possess HAT activity (Lill, Grossman et al. 1997). This usually occurs following ionising radiation induced phosphorylation of p53 at serine 15 (Lambert, Kashanchi et al. 1998). Interestingly, Mdm2 can abrogate this recruitment of p300/CBP to acetylate p53, thus inhibiting p53 stability through a mechanism other than

ubiquitination (Ito, Lai et al. 2001). Although not well defined, the enhancement of p53 binding to DNA has also been associated with glycosylation.

Moreover, SUMO-1 has also been shown to modify p53. Following exposure of cells to UV irradiation, SUMO-1 binds covalently to p53 at lysine residue 386 (Rodriguez, Dargemont et al. 2001). *In vitro*, overexpression of SUMO-1 led to an increase in p53 transcriptional activity. This was dependant on SUMO-1 modification rather than overexpression, as there were no increase on the transcriptional activity of the p53 mutant K386R. SUMO-1 modification has also been implicated in the regulation of Mdm2.

Besides these covalent modifications on p53, protein-protein interactions can also affect p53 transcription. Alternative reading frame product, $p14^{ARF}$ one of the proteins encoded by the *INK4a/ARF* locus, can cause growth arrest or apoptosis of cells when induced by inappropriate mitogenic stimuli such as elevated levels of myc and ras (de Stanchina, McCurrach et al. 1998; Zindy, Eischen et al. 1998). $p14^{ARF}$ binds to Mdm2 thus sequestering it to the nucleolus and thereby inhibiting Mdm2 interaction with p53 (Kamijo, Weber et al. 1998). Furthermore, $p14^{ARF}$ also associates with MdmX. Therefore it would appear that these combined effects could result in an increase in p53 transcriptional activity. However, the binding of $p14^{ARF}$ to MdmX, lowers the proportion of $p14^{ARF}$/Mdm2 complexes, liberating Mdm2 to perform the degradation of p53 and thus lowering the p53-dependant transactivation . Alongside these interactions with Mdm2 and MdmX, recent evidence has also implicated $p14^{ARF}$ with the elongation factor-2 (E2F) (Mason, Loughran et al. 2002). This could be an important finding as E2F is critical in p53-dependant growth arrest.

Other proteins that can control p53 transcriptional activity are ASPP (apoptosis stimulating protein of p53) and PML (promyeolocytic leukaemia protein). These proteins appear to selectively target p53 to specific promoters. ASPP interacts via the DNA binding domain and targets p53 to bind promoters associated with apoptotic genes such as *Bax (Samuels-Lev, O'Connor et al. 2001)*. PML also binds p53 at its core domain, targeting it to nuclear bodies (NB) where it upregulates transcription, also of promoters associated with pro-apoptotic genes (Fogal, Gostissa et al. 2000).

1.3.3 Growth arrest by p53

When a cell undergoes DNA damage, it is important that it is repaired before being allowed to proceed through the cell cycle, otherwise this can lead to the amplification of mutations which will ultimately result in abnormal cell growth. p53 has been identified as playing a critical role during transitions in the mitotic cell division to help prevent tumourigenesis (Paulovich, Toczyski et al. 1997).

During the G1/S checkpoint, cell cycle arrest can be initiated by p53. If the cell undergoes DNA damage following exposure to genotoxic stress, p53 is activated to transcriptionally upregulate the protein p21, an inhibitor of the cyclin dependant kinases (Cdk) (el-Deiry, Tokino et al. 1993). Cdk are a family of kinases that combine with cyclins to aid progression of the cell cycle. At the G1/S phase, this is accomplished by hyperphosphorylating the Retinoblastoma protein (pRb) which allows the release of bound E2F. In a free state, E2F can activate genes that initiate S-phase entry. Consequently, p21 hinders this advance by inhibiting the cyclin-Cdk, leaving pRb in its hypophosphorylated

state bound to E2F. Therefore if the cell is to enter S-phase the damage must be repaired, but if the damage is too severe, the cells will undergo apoptosis (el-Deiry, Tokino et al. 1993).

DNA damage can also initiate arrest at the G2/M checkpoint (Figure 7) (Stewart and Pietenpol 2001). The target at this stage is to inhibit the activation of Cdc2 which controls entry into mitosis (Hwang and Muschel 1998). Cdc2 is activated by both binding to Cyclin B and undergoing phosphorylation at threonine 161 by the Cdk-activating kinase (CAK) (Pines 1995). However, at the G2 stage, this complex is inactive as threonine 14 and tyrosine 15 have been phosphorylated by kinases Myt1 and Wee1 (Booher, Holman et al. 1997; Liu, Stanton et al. 1997). To regenerate an active complex to initiate mitotic entry, these two sites are dephosphorylated by the phosphatase Cdc25 (Draetta and Eckstein 1997). Thus, in response to stress, the PI-3K members ATM and ATR activate the serine kinases Chk1 and Chk2 respectively (Matsuoka, Huang et al. 1998; Guo, Kumagai et al. 2000; Liu, Guntuku et al. 2000; Melchionna, Chen et al. 2000). The kinases then phosphorylate Cdc25, creating a binding site for members of the 14-3-3 family (Furnari, Rhind et al. 1997; Matsuoka, Huang et al. 1998). These family members can then bind Cdc25 and prevent it from activating Cdc2. Although this inhibitory pathway is p53-independent, ATM and ATR kinases also induce p53 phosphorylation and its ensuing activation (Canman, Lim et al. 1998; Hall-Jackson, Cross et al. 1999; Tibbetts, Brumbaugh et al. 1999). p53 can cause G2/M arrest by upregulating various genes. Firstly it can activate p21 and subsequently inhibit E2F release again, thereby preventing the biosynthesis of Cdc2 and cyclin B (Innocente, Abrahamson et al. 1999). Secondly, it can transcribe 14-3-3σ (a member of the 14-3-3 family), which can

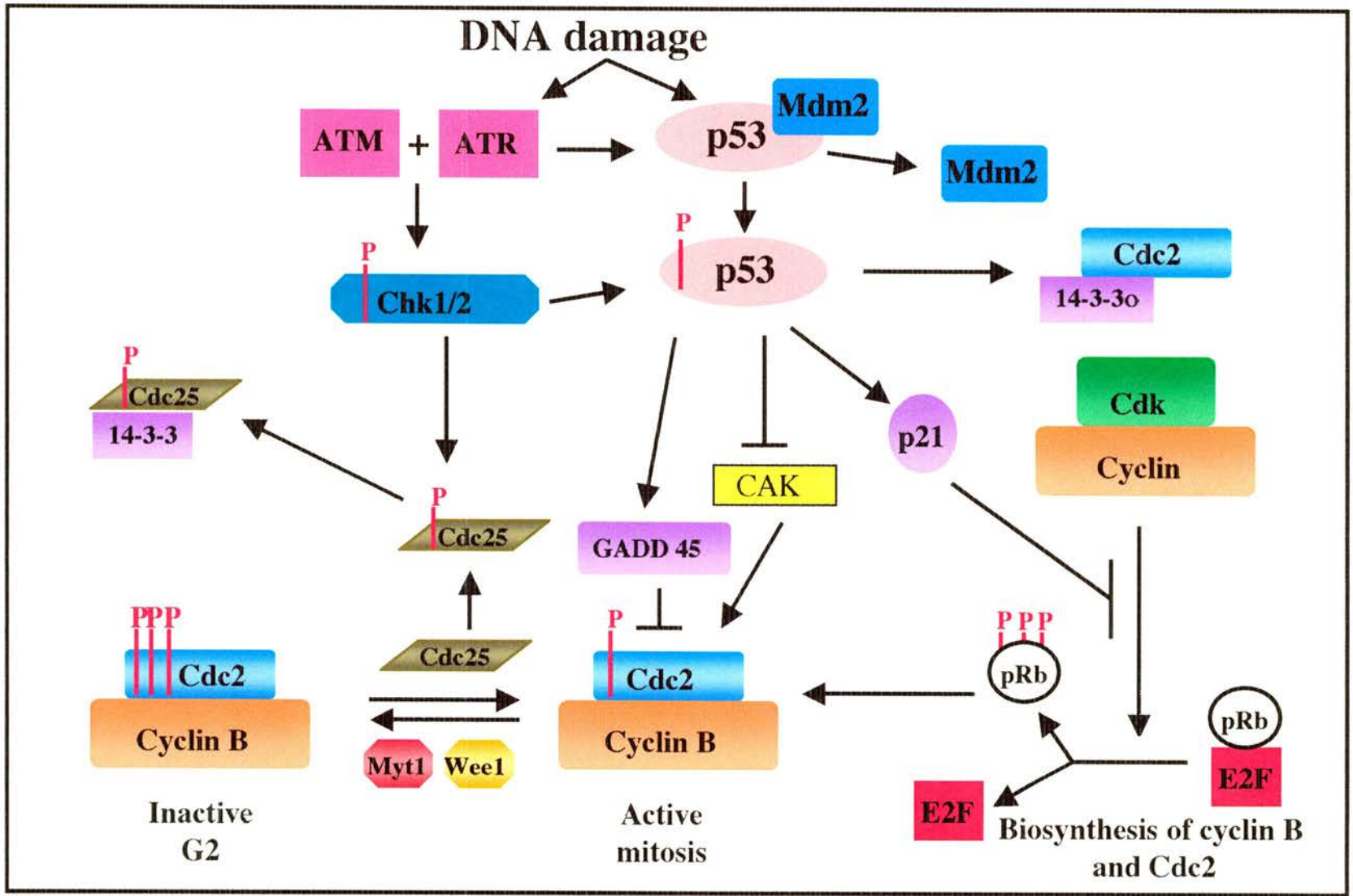


Figure 7 Growth arrest at the G2/M checkpoint

During G2 arrest by p53, regulation of active cyclinB1/ Cdc2 complex is required to initiate cells into mitosis. See text for details. This figure is adapted from (Stewart *et al.*, 2001; Taylor *et al.*, 2001). Abbreviations; ATM - Ataxia Telangiectasia Mutated, ATR - ATM related kinase, Mdm2 - Murine double minute 2, Cdc - cell division cycle, P - Site of phosphorylation, Cdk - cyclin dependant kinase , CAK - Cdk activating kinase , pRb - Retinoblastoma protein , E2F - elongation factor 2.

bind to the PI-3K induced form of phosphorylated Cdc25 (Chan, Hermeking et al. 1999). Thirdly, expression levels of GADD45 protein which directly inhibits cyclin B/Cdc2, are elevated (Wang, Zhan et al. 1999). Besides activating these genes to induce arrest, p53 can also bind to CAK and inhibit Cdc2 phosphorylation (Schneider, Montenarh et al. 1998).

There are suggestions that the exit from mitosis and entry into G1 is regulated by p53 (Lanni and Jacks 1998; Stewart, Leach et al. 1999). This is to ensure that the cells do not exit mitosis abnormally and enter S-phase. This cell cycle transition is known as the spindle checkpoint and it monitors spindle structure, chromosome alignment, attachment and segregation. During this stage, cells can undergo endoreduplication, a defective condition that results in cells entering S-phase with a tetraploid DNA content, rather than the normal diploid content (Sorger, Dobles et al. 1997). Evidence suggests that p53 controls this event by activating p21 inhibition of Cdk2 activity, which halts entry into G1/S-phase (Stewart, Leach et al. 1999).

1.3.4 DNA Repair by p53

Once p53 has initiated growth arrest, it can mediate DNA repair by different mechanisms. These include regulation of downstream genes involved with DNA repair, interacting with constituents of the repair machinery or direct binding to damaged DNA (Steinmeyer and Deppert 1988; Waga and Stillman 1998; Janus, Albrechtsen et al. 1999). p21 which is activated by p53 to inhibit the cell cycle at various checkpoints, can also hinder the elongation step in DNA replication during S-phase. p21 achieves this by binding to the proliferating cell nuclear antigen (PCNA) which is involved in recognising the DNA

primer/template complex. PCNA performs both DNA replication and repair, but only the former step is inhibited by p21 (Waga and Stillman 1998). Another protein activated by p53 transcription, GADD45 is also capable of interacting with PCNA (Smith, Chen et al. 1994). However, its major function is to directly stimulate DNA excision repair (Smith, Kontny et al. 1996).

p53 can also interact with proteins involved with DNA repair. A trimeric complex consisting of p53, Cockayne syndrome B repair helicase and replication protein A (RPA) is involved in both DNA replication and repair (Abramova, Russell et al. 1997). To perform the repair function, RPA is phosphorylated upon DNA damage, releasing RPA/p53 dimers from the complex. This allows the RPA subunit to participate in nucleotide excision repair (NER).

It has become apparent that another way in which p53 may initiate DNA repair is by directly binding damaged DNA via its carboxy-terminus (Lee, Elenbaas et al. 1995; Reed, Woelker et al. 1995). p53 has been identified to bind short strands of single stranded DNA and anneal them together. Short single strands can be an indication of DNA damage. and have been shown to induce p53 binding to its promoter, *in vitro (Jayaraman and Prives 1995)*.

1.3.5 Apoptosis by p53

Consequently, p53 performs an important role in repairing damaged DNA following growth arrest, but if required p53 can induce the apoptotic pathway leading to programmed cell death. p53 can activate apoptosis by

expressing pro-apoptotic genes such as Bax, insulin growth factor-binding protein 3 (IGF-BP3) and Fas/CD95 ligand (Miyashita and Reed 1995; Friesen, Herr et al. 1996; Sionov and Haupt 1999). These target genes can be subdivided into two groups; those regulating apoptotic effector proteins or those involved in a receptor-mediated pathway.

Bax is a target gene associated with the former group. It is a member of the Bcl-2 family, and is upregulated during p53-dependant apoptosis (Miyashita and Reed 1995). Bax helps release apoptosis-inducing factor (AIP) and cytochrome C from the mitochondria which targets the cell to advance into the caspase cascade (Sionov and Haupt 1999), which is similar to the NF-$\varkappa$B pathway involving TNF-α. Simultaneously, p53 represses the anti-apoptotic protein, bcl-2 (Thomas, Giesler et al. 2000). Another way that apoptotic signalling by the mitochondria is initiated involves p53-inducible gene 8 (PIG-8) being activated upon oxidative stress (Polyak, Xia et al. 1997). . However, the pathway linking this gene and apoptosis has not been established yet. The other form of apoptosis can be attained by IGF-BP3 (Sionov and Haupt 1999). This entails IGF-BP3 disrupting cell survival by blocking IGF-1 signalling at the IGF-1 receptor.

Recently NF-$\varkappa$B has been implicated in p53-mediated programmed cell death. Ryan *et al.* suggested that induction of p53 resulted in NF-$\varkappa$B activation which led to p53-dependant apoptosis (Ryan, Ernst et al. 2000). This activation of NF-$\varkappa$B by p53 was not via the TNF-α pathway, but that involving the $pp90^{RSK}$. This possible link between NF-$\varkappa$B and p53 will assist in comprehending how dysregulation of transcription factors can lead to tumourigenesis.

1.4 AIMS/OBJECTIVES

To identify an IκBα recognition motif required for phosphorylation by the IκB kinase complex.

To establish a role for NF-κB in response to p53.

2. MATERIALS & METHODS

2.1 Materials

Human recombinant TNF-α and human recombinant IL1-β were both purchased from Insight Biotechnology Ltd. Mini EDTA-protease free inhibitor tablets were obtained from Roche. (α^{32}P)-dCTP, (γ^{32}P)-ATP and dNTPs were purchased from Amersham Pharmacia. All enzymes were obtained from New England BioLabs. The primers for cDNA cloning were provided by Pfizer. All other materials were purchased from Sigma, unless stated.

2.2 Antibodies

The polyclonal p65 antibody was purchased from Santa Cruz and used at 1:200 dilution in immunofluorescence. Monoclonal antibody β-actin was purchased from Sigma and used for western blotting at 1:3000 dilution. Straight p65 and p50 antisera that were used for EMSA were also provided by Ellis Jaffray. SV5 Pk tag monoclonal antibody (Hanke, Szawlowski et al. 1992) was obtained from R.E. Randall, University of St. Andrews and used at 1:2000 dilution in western blotting. Monoclonal antibody DO-1 (Vojtesek, Dolezalova et al. 1995) recognises the N-terminus of p53 and was used at 1:1000 dilution in western blotting and 1:150 in immunofluorescence. This was obtained from D.P. Lane, University of Dundee. The secondary immunofluorescence antibodies; goat anti-mouse FITC conjugate, and goat anti-rabbit Texas Red conjugate were purchased from Oxford Biotechnology Ltd. and used at a concentration of 1:200. The horseradish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG (Amersham) were both used in western blotting at 1:2000 dilution.

2.3 Viruses

Adenovirus expressing the human p53 gene was a kind gift from D.P. Lane (University of Dundee). The adenovirus expressing the βgalactosidase was provided by John Nicholson at the University of St. Andrews.

2.4 Plasmids

pcDNA3 was purchased from Invitrogen. pRc/591.1 encoding the cDNA for IκBα was a kind gift from Eric Cabannes (University of St. Andrews). Jill Thomson (University of St. Andrews) provided the reporter plasmid encoding for the β-galactosidase gene while the luciferase reporter vectors 3EnhConALuc and ConALuc were a kind gift from F.Arenzana-Seisdedos (Institut Pasteur, France).

Plasmids expressing wild-type p53 and ARF and the p53 mutant plasmids for H175 and H273, as well as the p53 dependent luciferase reporter vector, PG13-Luc were provided by D.P. Lane, University of Dundee. LMP plasmid was obtained from P. Farrell at Ludwig Institute for Cancer Research, London.

2.5 Bacterial strain

For routine DNA preparation, *E. Coli* DH5α was used. Bacteria were grown in Luria-Bertani broth (LB: 1% w/v bacto-tryptone {Melford Laboratories}, 0.5% w/v yeast extract {Melford Laboratories} and 1% w/v NaCl; pH 7.5; made up in dH_2O), or streaked onto LB-agar plates (LB with 1.5% agar {Melford Laboratories}) with antibiotics when required.

2.6 cDNA cloning

2.6.1 Preparation of IκBα template for mutagenesis

To release the IκBα cDNA, 1μg of pRc/591.1 was digested with 10u HindIII and 8u XbaI restriction enzymes in a 4 hour reaction (containing 1x Buffer 2 {10mM Tris-HCl pH 7.9, 10mM $MgCl_2$, 50mM NaCl and 1mM DTT} and 0.1μg/μl BSA) at 37°C. On addition of 1x DNA loading buffer (5x buffer; 40% sucrose, 0.05% bromophenol blue), the reaction was run on a 1% agarose gel at 70V (containing 10ml 10x TBE {0.89M Tris base, 0.89M boric acid and 20mM EDTA; pH 8.3} and 0.05μg/ml ethidium bromide) in 1x TBE running buffer. DNA markers (Invitrogen) were run alongside to enable identification of the correct fragment. After 30 minutes, the gel was visualised using ultraviolet light (Kozutsumi, Normington et al. 1989) and the band containing IκBα was excised. The band was then purified and eluted into dH_2O using the QIAGEN gel extraction kit. 1μg of pcDNA3 (plasmid map in Appendix) was also restriction digested, electrophoresed and purified as above, to provide an expression vector for ligation of the IκBα insert. Following the ligation reaction and subsequent transformation, colonies were miniprepped and sequenced (described in further detail below). 10ng of this plasmid was then used in a 100μl polymerase chain reaction (PCR) containing 20μM dNTPs, 4u Vent® DNA Polymerase, 1x ThermoPol buffer (10mM KCl, 20mM Tris-HCl pH 8.8, 10mM $(NH_4)_2SO_4$, dH_2O, 2mM $MgSO_4$ and 0.1% Triton-X-100) and 1μM of the following upstream and downstream primers containing the BamHI and EcoRI restriction sites respectively.

Upstream: 5' -GTACTAGGATCCATGTTCCAGGCGGCCGAG- 3'

Downstream: 5' -GCCGCGGAATTCTAACGTCAGACGCTGGCCTCC- 3'

The reaction was performed in a PCR machine (Perkin-Elmer) using the following conditions; 5 minutes at 94°C, then 30 cycles each consisting of 94°C for 90 seconds, 58°C for 90 seconds and 72°C for 120 seconds before a final stage for 7 minutes at 72°C. On cooling down to room temperature, 40µl of the reaction was separated by agarose gel electrophoresis and visualised by ultraviolet light as explained before (Kozutsumi, Normington et al. 1989). The newly generated IκBα was then excised and gel extracted. Thereafter, both the IκBα insert and pcDNA3/SV-5 vector were digested using 10u BamHI and 12u EcoRI restriction enzymes before being ligated together. The novel plasmid was transformed, colonies miniprepped and sequenced as described below. This plasmid was termed pcDNA3/IκBα/SV-5 (map in Appendix) and was used as the template to generate the mutant IκBα plasmids.

2.6.2 Preparation of pcDNA3/SV-5 vector

pcDNA3/SV-5 vector was prepared by cloning the SV-5 tag (Hanke, Szawlowski et al. 1992) via EcoRI and XhoI restriction sites. The SV-5 tag had been generated by hybridising 200ng of the following two oligonucleotides (ordered from Oswel DNA Service) in 1x STE buffer (100mM NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA) using these conditions; 2 minutes at 88°C, 10 minutes at 65°C, 10 minutes at 37°C and a final 5 minutes at room temperature. Before hybridisation, each of the oligonucleotides had already been subjected to these conditions separately.

Upstream: 5' -AATTCGGAAAGCCGATCCCAAACCCTTTGCTGGGATTGGA

CTCCACCTAGTGAC- 3'

Downstream: 5' -

TCGAGTCACTAGGTGGAGTCCAATCCCAGCAAAGGGTT

TGGGATCGGCTTTCCG- 3'

The hybridised SV-5 tag was then ligated into EcoRI/XhoI digested pcDNA3, transformed, miniprepped and sequenced as detailed below. A plasmid map of pcDNA3/SV-5 vector is shown in the Appendix.

2.6.3 Generation of pcDNA3/E14A-IκBα/SV-5 plasmid

A PCR mutagenesis technique (Landt, Grunert et al. 1990) was utilised to create the E14A-IκBα mutant. This involved 3 separate PCRs (PCR-1, PCR-2 and PCR-3).

PCR-1 was set-up in a 100µl reaction containing 10ng pcDNA3/IκBα/SV-5, 20µM dNTPs, 4u Vent® DNA Polymerase, 1x ThermoPol buffer, dH_2O, 1µM of the following external upstream primer including the BamHI site and 1µM of the internal downstream primer with the mutation E14A (glutamic acid 14 changed to alanine).

Upstream: 5' -GTACTAGGATCCATGTTCCAGGCGGCCGAG- 3'

Downstream: 5' -GCGGGGGCCCGCCATGGCCCA- 3'

This reaction was performed in the PCR machine using the following conditions; 5 minutes at 94°C, then 30 cycles each consisting of 94°C for 90 seconds, 55°C for 90 seconds and 72°C for 20 seconds before a final stage for 7 minutes at 72°C. The small PCR product was then purified by agarose gel electrophoresis and gel extracted as detailed before (Kozutsumi, Normington et al. 1989)

PCR-2 was similar to PCR-1 except the internal upstream primer with the mutation E14A and the external downstream primer including the EcoRI site were used.

Upstream: 5' -TGGGCCATGGCGGGCCCCCGC- 3'

Downstream: 5' -GCCGCGGAATTCTAACGTCAGACGCTGGCCTCC- 3'

Also the elongation step at 72°C in the 30 cycles was performed for 90 seconds, to allow for generation of a larger DNA fragment.

The final PCR-3 was the same as the PCR-2 but with a few differences in the 100μl reaction. Instead of using pcDNA3/IκBα/SV-5 as template, 5ng of both DNA products purified from PCR-1 and -2 were used in addition to the two external primers containing the BamHI and EcoRI restriction sites.

Upstream: 5' -GTACTAGGATCCATGTTCCAGGCGGCCGAG- 3'

Downstream: 5' -GCCGCGGAATTCTAACGTCAGACGCTGGCCTCC- 3'

On completion of PCR-3, the new product encoding for the full length IκBα DNA insert with mutation E14A was purified as described in PCR-1.

E14A-IκBα insert and pcDNA3/SV-5 vector were then digested using 10u BamHI and 12u EcoRI restriction enzymes, before being ligated together. The ligation was then transformed, miniprepped and sequenced to generate the mutant expression plasmid pcDNA3/ E14A-IκBα/SV-5 (as detailed below).

2.6.4 Generation of other mutant IκBα plasmids

All alanine mutants of IκBα encompassing amino acids 15 to 42 and the double alanine mutant S32A/S36A were generated in an identical way to pcDNA3/ E14A-IκBα /SV-5. The only differences were the internal primers used in PCR-

1 and PCR-2 and the PCR conditions were optimised as required. All primers used to generate the different mutants are stated in the Appendix.

2.6.5 DNA ligations

Various molar ratios of vector:insert DNA fragments from 1:1 to 1:5 were added to 20µl reaction mixes composed of 2µl 10x Ligase Buffer (300mM Tris-HCl pH 7.8, 100mM $MgCl_2$, 100mM DTT and 10mM ATP) and dH_2O. 0.1-10u T4 DNA Ligase was then added to the ligation reaction and incubated at 16°C for 12-16 hours. Half of the ligation mixture was used for subsequent transformation of chemically competent cells.

2.6.6 Transformation of chemically competent bacteria

80µl of chemically competent cells were incubated with either plasmid DNA or ligation mixtures for 30 minutes on ice. The transformation mix was heat-shocked for 1-2 minutes in a water bath at 42°C, before being cooled on ice for 5 minutes. 1ml of LB medium was added to the mix and then incubated at 37°C for 20 minutes to allow the cells to recover. Cells were harvested by centrifugation at 13,000rpm for 15 seconds. 800µl of the supernatant was removed before resuspending pellet in the remaining 200µl. This was then spread onto LB-agar plates (containing 100µg/ml ampicillin) and left overnight in 37°C incubator. Colonies found on the plate were then used to prepare plasmid DNA.

2.6.7 Preparation of chemically competent bacteria (DH5α)

E. Coli DH5α was used for routine DNA preparation. A 10ml overnight culture was grown in LB at 37°C. This was used to inoculate one litre of LB at 37°C with shaking at a speed of 120rpm for approximately 2 hours, by which time an OD_{600} of approximately 0.5 was attained. The cells were then chilled on ice for 30 minutes before being centrifuged for 5 minutes at 2,800rpm at 4°C. The supernatant was removed and the pellet was resuspended in a pre-chilled mix containing 12.5ml of 100mM $CaCl_2$ and 12.5ml of $MgSO_4$. The cells were again chilled on ice for 30 minutes and centrifuged for 5 minutes at 2,800rpm at 4°C. The pellet was resuspended this time in a pre-chilled mix containing 2.5ml of 100mM $CaCl_2$ and 2.5ml of $MgSO_4$. Cold glycerol was added to a final concentration of 10% before the bacterial cells were aliquoted into 400µl volumes, frozen in dry ice and stored at -70 °C. All solutions used were previously autoclaved and kept at 4 °C.

2.6.8 Preparation of plasmid DNA

All DNA preparations (minipreps and maxipreps) used for both cloning and transfections were prepared with QIAGEN kits as in accordance with manufacturer's instructions. Quality and quantity of DNA was analysed by spectrophotometric readings at 260nm and 280nm and also by visualisation under UV light, following electrophoresis in an agarose gel in the presence of ethidium bromide (Kozutsumi *et al.,* 1989).

2.6.9 DNA sequencing

Plasmid DNA of all constructs were insert sequenced using an automated DNA sequencer (ABI PRISM 377™, Applied Biosystems) by Alex Houston at the University of St. Andrews.

2.7 Cell Culture

Cos 7(African green monkey kidney cell) and HeLa (human cervical carcinoma) cell lines were all grown in lab-based DMEM (Dulbecco's modified Eagle's medium {which contains antibiotics penicillin (12μg/ml) and streptomycin (50μg/ml)}, supplied by Ian Armitt, University of St. Andrews), with 10% FCS (foetal calf serum). The following types of mouse embryo fibroblasts (MEF); wild-type (wt), IKK1 knockout ($IKK1^-$), IKK2 knockout ($IKK2^-$) and p65 knockout ($p65^-$) cell lines were also grown in the same media and serum.

HFF (wild-type p53 cell line {human foreskin fibroblasts}), SAOS2 (p53 null cell line {human primary osteogenic}), and $p53^{-/-}mdm2^{-/-}$ MEF(p53 double knockout {mouse embryo fibroblasts}) were grown in DMEM ({containing no antibiotics} purchased from Invitrogen) supplemented with 10% FBS (foetal bovine serum). Two tetracycline inducible SAOS2 cell lines expressing wild-type p53 and mutant p53 H169 were also grown in DMEM (Invitrogen) but were supplemented with 10% Tet-system approved FBS (Clontech) and the antibiotics geneticin (1mg/ml) and hygromycin B (150μg/ml, {Boehringer Mannheim}). These cell lines with p53 characteristics were a kind gift from D.P. Lane (University of Dundee).

The above cells were all maintained in exponential growth as monolayers in a humidified 95% air /5% CO_2 incubator at 37°C.

293 spinners (human embryo kidney cells) were grown in Joklik's media ({ICN-Flow Laboratories}, supplied by Ian Armitt, University of St. Andrews) with 10% NBCS ({newborn calf serum}, Invitrogen). They were grown at 37°C in a flask with a magnetic stirrer to keep the culture spinning.

2.8 Transfection of tissue culture cells

2.8.1 Transfection of tissue culture cells by Lipofectamine™

Cells were passed into $25cm^2$ flasks to obtain 70-80% confluency the following day. 1-5μg plasmid DNA (including reporter plasmids and 100ng β-galactosidase plasmid) was transfected into cells using Lipofectamine™ (Invitrogen) in accordance with manufacturer's instructions. For transfections assaying NF-κB transcriptional activity, 500ng of NF-κB dependent or independent luciferase reporter plasmids were cotransfected, but for assaying of p53 transcriptional activity 200ng of p53 dependent luciferase reporter plasmid was used. After 6-14 hours transfection in a 37°C CO_2 incubator, cells were trypsinised, and seeded into 6-well plates. They were left to incubate. Transfections involving TNF-α (10ng/ml), Il-1β (2ng/ml), Adriamycin (4ng/ml) were stimulated for the times indicated with normal media used as a control. On completion of transfection, cells were subjected to luciferase assay, nuclear extraction or western blot analysis as stated.

2.8.2 Transfection of tetracycline inducible cell lines

The tetracycline inducible SAOS2 cell lines expressing wild-type p53 and mutant p53 H169 were transfected by the lipofectamine method as detailed

above. The cells were then induced with 1μg/ml doxycyline for the times as indicated before being subjected to luciferase assay or western blot analysis.

2.9 Reporter Assays

2.9.1 Luciferase Assay

Transfections to be assayed for luciferase activity were stopped by washing the cells twice in ice-cold phosphate-buffered saline (PBS). Cells were then lysed in 100-200μl buffer (25mM Tris-phosphate pH 7.8, 8mM $MgCl_2$, 1mM DTT, 1% Triton X-100 and 15% glycerol; made up in dH_2O). 50μl of the lysate was injected with luciferase assay buffer (0.25mM luciferin, 1mM ATP and 1% BSA diluted in the lysis buffer) using the luminometer (Lumat LB 901, Berthold) which also measured the luciferase activity. The activity was stated in relative light units (RLU).

2.9.2 β-galactosidase assay

5μl of the samples lysed for luciferase activity which had been transfected with β-galactosidase plasmid were subjected to the Galacto-Light Plus Chemilluminescent Reporter Assay (Tropix) to detect the RLU produced by β-galactosidase. This was performed in accordance with manufacturers instructions.

2.9.3 Luciferase : β-galactosidase ratio

NF-κB units of activity in transfection experiments were calculated by luciferase : β-galactosidase ratio. The results presented were representative of 3 sets of data, each performed in triplicate. Data obtained was processed using the Microsoft Excel program and the standard error was measured as a function of the standard deviation. In certain transfections, NF-κB units of activity could not be calculated, so the results were expressed as RLU/μg. This was determined by dividing the luciferase assay readings by the amount of protein in the sample. The protein had been determined by a Bradford Assay (see below). In calculating either NF-κB units of activity or RLU/μg, data was corrected for background reading with a cell extract from non-transfected cells. Fold of induction was reported as in comparison to control media.

2.10 Protein quantitation

Protein concentrations were determined using the Bradford Assay (Bradford 1976) or the BCA protein assay system (Pierce). For the Bradford Assay, protein samples were mixed with Bradford's reagent (Biorad) and left for 5 minutes at room temperature. The absorbance at 595nm was then measured on a spectrophotometer. Protein absorbances were converted to mg/ml concentrations using a standard curve of a range of bovine serum albumin (BSA) concentrations that had been incubated with Bradford's reagent. Quantitation using the BCA Assay was as recommended in the manufacturer's instructions.

2.11 SDS-PAGE and western blot analysis

Cells from transfections were washed in PBS. Cell extracts were then prepared by lysis in SDS sample buffer (5% SDS, 0.15M Tris-HCl pH 6.7, 30% glycerol)

diluted 1:3 in RIPA buffer (25mM Tris-HCl pH 8.2, 50mM NaCl, 0.5% NP-40, 0.5% Deoxycholate, 0.1% Azide) containing protease inhibitor tablets. The lysates were then denatured at 100°C for 5 minutes before loading onto a 10% SDS-polyacrylamide gel. Biorad mini-gel equipment was used in accordance with the manufacturer's instructions. Protein molecular weight markers (Invitrogen) were used as standards to establish the apparent molecular weight of proteins resolved on SDS-polyacrylamide gels. Separated polypeptides were transferred to a polyvinylidene difluoride membrane (P.V.D.F. (Sigma)) using a wet blotter (Biorad Systems). The membranes were then blocked for 1 hour, with PBS containing 10% skimmed milk powder and 0.1% Tween 20, subjected to three 5 minute washes in PBS/0.1% Tween 20, before being incubated for 1 hour with monoclonal or polyclonal antibodies diluted in the blocking buffer. After another three 5 minute washes in PBS/0.1% Tween 20, the corresponding horseradish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG diluted in blocking buffer were used as secondary antibodies for a 30 minute incubation. The membranes were then washed 3 times in PBS before detecting the antibodies using the Enhanced ChemiLuminesence (ECL) technique (Amersham). A 1:1 ratio mixture of the reagents (prepared by Catherine Botting, University of St. Andrews) was made and placed on the membrane for 1 minute. The membrane was then exposed to X-ray film for different periods of time depending on the strength of the signal. After ECL detection and when necessary, the P.V.D.F. membranes were stripped as described below.

2.12 Stripping of P.V.D.F. membranes

After ECL, the membranes were washed in PBS for 20 minutes before being transferred into stripping buffer (142µl β-mercaptoethanol, 2ml 20% SDS, 1.25ml Tris pH 6.7 and 16.6ml dH_2O) and incubated for 30 minutes in a hybridisation oven at 72°C. The membranes were then washed twice in 250ml PBS containing O.1% Tween 20. The membranes were blocked for 1 hour in PBS containing 5% skimmed milk powder and 0.1% Tween 20 and then probed as in the Western Blot.

2.13 Electrophoretic mobility shift assay (EMSA)

5µg of the cellular extract to be gel-shifted was added to an assay mix composed of 22µl binding buffer (3.5mM Spermidine, 25mM Hepes pH 7.5, 6.0mM $MgCl_2$, 1mM EDTA, 100mM NaCl, 0.15% NP-40, 10% glycerol), 1µl 100mM DTT, 1µl 10mg/ml BSA, 1µl 0.5µg/µl poly dGC:dAT and 1µl 20ng/µl NFIII (made up in STE buffer). This was left at room temperature for 15 minutes. Certain binding assays (as indicated in relevant figures) were also incubated with 1µl p50 antisera, 1µl p65 antisera, 20ng unlabelled κB oligonucleotide or 20ng recombinant IκBα(all provided by Ellis Jaffray, University of St. Andrews) during this time. 1µl of the radioactively labelled κB probe was then added and the assay incubated for a further 15 minutes at room temperature. Upon addition of 4µl GRA dyes (25mM Hepes pH 7.5, 10mM DTT, 50% glycerol and 0.1% bromophenol blue), the samples were run on a 6% non-denaturing PAGE in 1x TBE for 2 hours at 200V. The gel was then dried onto chromatography paper (Whatman) and exposed to a phosphorimager screen for

1-2 hours. The protein/DNA interactions were then analysed by using the MacBas v2.5 program on the phosphorimager (Fuji Bas 1500).

2.14 Preparation of a radioactively labelled double-stranded DNA probe for EMSA

The 16-mer oligonucleotide (5' -CTGGGGACTTTCCACC- 3') representing the κB motif in the HIV-1 enhancer ({provided by Ellis Jaffray, University of St. Andrews}, Arenzana-Seisdedos *et al.,* 1995), was radiolabelled at the 5' end using T4 Polynucleotide Kinase. 5 picomoles of the bottom strand were added to a reaction mix containing 10u T4 Polynucleotide Kinase, 1μl 10x Polynucleotide Kinase buffer (200mM Tris-HCl pH 7.6, 0.1 $MgCl_2$, 10mM DTT, 1mM EDTA pH 8.0), 50μCi (γ^{32}P)-ATP and 9μl dH_2O. The reaction was incubated in a lead pot for 30 minutes at 37°C, before addition of 5 picomoles of the top strand and 0.5μl NaCl. The reaction was heated to 100°C for 2 minutes in a water bath before being allowed to cool down to room temperature. Upon addition of 5μl of DNA loading buffer (50mM EDTA, 50% glycerol and 0.25% bromophenol blue), the sample was run for 90 minutes at 150V on a 12% polyacrylamide gel to separate the radiolabelled probe from unlabelled probe and free isotope. The gel was exposed to X-ray film for 3 minutes to detect the radiolabelled probe. The correct band was then excised and eluted overnight into 400μl STE buffer at room temperature.

2.15 Preparation of nuclear extracts

After appropriate transfection experiment, cells were harvested by cell scraping into the media before a 3 minute centrifugation at 1,200rpm. Upon aspiration of the supernatant, the pellet was washed with PBS and spun at 14,000rpm for 30 seconds. 15 minutes prior to the next step, 5ml of subsequent buffers to be used were treated with a protease inhibitor tablet and 100µl 100mM PMSF. On removal of PBS, the pellet was resuspended in 400µl Buffer A (50mM NaCl, 0.5M sucrose, 1mM EDTA, O.5mM spermidine, 0.15mM spermine, 0.2% Triton-X-100 and 10mM Hepes pH 8.0; made up in dH_2O) and left on ice for 6 minutes. The lysate was then spun at 6,500rpm for 3 minutes at 4°C. The supernatant containing the cytoplasmic extract was removed, quantitated for protein and stored at -20°C. The pellet was subjected to a wash with 400µl Buffer B (50mM NaCl, 0.1mM EDTA, O.5mM spermidine, 0.15mM spermine, 25% glycerol and 10mM Hepes pH 8.0; made up in dH_2O), before another 3 minute spin at 6,500rpm, 4°C. Following this, the supernatant was aspirated off and the pellet resuspended in 200µl Buffer C (350mM NaCl, 0.1mM EDTA, O.5mM spermidine, 0.15mM spermine, 25% glycerol and 10mM Hepes pH 8.0; made up in dH_2O). This was then left on ice and mixed regularly over a period of 30 minutes. The lysate was then spun at 6,500rpm, this time for 20 minutes at 4°C. The supernatant containing the nuclear extract was removed and quantitated for protein. The extract could now be subjected to either EMSA or western blot.

2.16 Virus culture

2.16.1 Growth and purification of virus

A 5L culture (Joklik's media/10% NBCS) of 293 spinner cells ({~3-4x10^5 cells/ml}) was spun down at 4,000rpm for 30 minutes at 4°C. Upon removal of supernatant, the pellets were pooled together and infected with an appropriate amount of relevant virus inoculum in 500ml of Joklik's media. After 1 hour incubation spinning at 37°C, the infection was made upto 5L of Joklik's media with a final concentration of 2% NBCS. The spinner cells were then left to become infected over a period of 2-4 days at 37°C. Just before the cells look likely to burst, they were centrifuged at 4,000rpm for 30 minutes at 4°C. The pellets were pooled together again, washed in PBS and resuspended in 25ml of Tris-HCl pH 7.5. The suspension was then subjected to 3 freeze-thaw cycles, before being transferred to new tubes containing an equal volume of arcton. Following continuous shaking for 10 minutes, an emulsion formed that was centrifuged at 4,000rpm for 15 minutes at 4°C. The tube consisted of 3 layers of which the top layer containing the virus was removed and gently laid onto a caesium chloride gradient. The gradient had been set-up in special 14ml centrifuge tubes ({precleaned with 70% ethanol}, Beckman) containing a bottom layer of 3ml 3M CsCl (made up in STE buffer and filter-sterilised) and a second layer of 2ml 2M CsCl (also made up in STE buffer and filter-sterilised). The tubes were spun at 30,000rpm for 90 minutes at 20°C in a Beckman ultracentrifuge. After centrifugation the virus appeared as a thin blue band between the 3M CsCl and 2M CsCl. This was carefully extracted using a 1ml syringe with a fine needle. After overnight dialysis against PBS at 4°C, glycerol was added to a final concentration of 10% before the virus was stored in 100µl aliquots at -70°C.

2.16.2 Determination of virus titre by plaque assay

293 spinner cells were seeded onto 60mm petri-dishes and allowed to form an even monolayer of approximately 70% confluency. A range of serial dilutions in Joklik's media from 10^{-3} to 10^{-9} of the relevant virus stock were prepared in duplicate. Upon removal of media from the dishes, 100µl of the appropriate dilution was added dropwise to infect the cells at 37°C. Also, 100µl of Joklik's media was used to set-up a control mock infection. After 1 hour, 4ml of a molten overlay mix was added gently to the petri-dishes and left to set at room temperature. The molten mix was made up as a 50ml solution at 40°C composed of 35ml Joklik's media/2% NBCS, 400µl $MgCl_2$ and 15ml molten 3% SeaPlaque Low Melting Point Agarose (NuSieve) made up in dH_2O. After setting, the petri-dishes were put in the incubator at 37°C. After 3 days, another 2ml of the molten mix was added to the petri-dishes, allowed to set at room temperature and left for a further 4 days in the 37°C incubator. The cells were then fixed in PBS/10% formaldehyde and left overnight in a sealed container at room temperature. The agarose overlay was then removed and the cells were stained for 1 hour with 0.1% crystal violet (made up in 10% methanol). The petri-dishes were then washed with water, inverted and left to dry. Plaques were then counted to calculate the virus titre in p.f.u.(plaque forming units) using the formula; number of plaques/well*10* dilution.

2.16.3 Virus infection of cells

Cells were set-up in $75cm^3$ flasks until approximately 80% confluent. Upon removal of media and a PBS wash, 5ml of virus in DMEM/2%NBCS was added to the cells at a titre of 10 p.f.u./cell. The virus was left to infect for the

appropriate time points at 37°C before the cells were subjected to RNA extraction. If the timepoint was longer than 1 hour, a further 5ml of DMEM/2%NBCS was added to the cells. For reporter assays, cells were set-up in 60mm petri-dishes and transfected 24 hours before virus infection with 2μg of reporter plasmid. The infection was then performed in proportion to the $75cm^3$ flasks.

2.17 RNA extraction

After virus infection, cells were harvested by cell scraping into 400μl PBS, before a 3 minute centrifugation at 1,200rpm. Total cell RNA was then isolated from the cells using the Promega SV Total RNA Isolation System. This was performed in accordance with manufacturer's instructions.

2.18 Northern blot analysis

20μl samples composed of 10μg total RNA, 1x MOPS (made from a 10x buffer; 0.4M Morpholinopropanesulphonic acid pH 7.0, 0.1M Na-acetate and 10mM EDTA pH 8.0), 18.5% formaldehyde, 50% formamide, 4% Ficoll and 0.5% bromophenol blue were heated to 90°C for 5 minutes. The samples were cooled on ice, before being loaded on a 1% agarose gel containing 1x MOPS and 2.2M formaldehyde. The gel was run for 3 hours in 1x MOPS buffer at 50mA. The gel was then soaked 4 times in DEPC-treated water (0.1%DEPC {diethyl pyrocarbonate} was added to deionised water overnight and then autoclaved for 20 minutes). Following this, the separated RNA was transferred onto BA85 nitrocellulose membrane (Schleicher & Scheull) via a capillary blot in 10x SSC

buffer (Saline-sodium citrate buffer; 1.5M NaCl, 0.3M Na-citrate) over a period of 16-24 hours. After transfer, the membrane was soaked in 2x SSC, before being baked in a vacuum oven at 80°C for 2 hours. The membrane was then pre-hybridised in 7ml PerfectHyb Plus solution for 5 minutes at 68°C. On addition of approximately $1x10^6$ cpm of the appropriate radiolabelled cDNA probe, the membrane was hybridised at 68°C for 2 hours. The membrane was then subjected to two 20 minute high stringency buffer washes (0.5x SSC, 0.1% SDS) at 68°C, before a final 20 minute wash at room temperature in ultra-high stringency buffer (0.1x SSC, 0.1% SDS). The blots were then wrapped in plastic and exposed to X-ray film using an intensifying screen at -70°C.

2.19 Preparation of probe for northern blot analysis

To release the IκBα cDNA, 1μg of pcDNA3/IκBα/SV-5 was digested with 10u BamHI and 12u EcoRI restriction enzymes for 2 hours (containing 1x Buffer 2 {10mM Tris-HCl pH 7.9, 10mM $MgCl_2$, 50mM NaCl and 1mM DTT} and 0.1μg/μl BSA) at 37°C. On addition of 1x DNA loading buffer, the digest was run on a 1% agarose gel at 70V in 1x TBE running buffer. After 30 minutes, the gel was visualised using ultraviolet light (Kozutsumi *et al.,* 1989) and the appropriate band was excised. The band containing IκBα was then purified and eluted into TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA) using the QIAGEN gel extraction kit. 25-50ng of the IκBα DNA was then radiolabelled with (α^{32}P)-dCTP using the ^{T7}QuickPrime Kit (Amersham Pharmacia) in accordance with manufacturer's instructions. To rid the probe of any incorporated nucleotides, the probe was then purified on a Sephadex G50 DNA

Grade column (Amersham Pharmacia). The probe was then eluted into 40µl 1x STE buffer and ready to use in the northern blot.

2.20 Indirect immunofluorescence analysis of cells

Cells were cultured on 13mm glass coverslips (Scientific Laboratory Supplies Ltd.) in 12 well plates and subjected to transient transfections as necessary. On completion of transfection, cells were washed twice in PBS containing 1mM $MgCl_2$ and 1mM $CaCl_2$. Cells were then fixed with PBS/3% Paraformaldehyde for 10 minutes, washed in PBS three times, and fixation quenched by two 10 minute PBS/0.1M glycine incubations. After a 5 minute PBS wash, cells were then permeabilised for 10 minutes in PBS/0.1% Triton-X-100. Following three more 5 minute PBS washes, the cells were blocked in PBS/0.2% BSA for 10 minutes. Immunostaining with the relevant concentration of primary antibodies was performed for 1 hour in PBS/0.2% BSA before being subjected to three 5 minute PBS/0.2% BSA washes. The cells were then incubated for a further 30 minutes with the corresponding fluorescently labelled secondary antibodies diluted in PBS/0.2% BSA at 1:200 dilution. The cells were given a final three 5 minute PBS washes before the coverslips were gently mounted onto slides (Scientific Laboratory Supplies Ltd.) using Moviol (Calbiochem) and stored overnight at 4°C. Fluorescence microscopy was carried out with a NIKON MICROPHOT-FXA. Images were captured with a Spot CCD camera and processed using the Adobe Photoshop 5.0 program.

3. RESULTS

3.1 Investigation into effects of amino terminal residues of IκBα on signal-induced activation of NF-κB.

3.1.1 Selection of IκBα residues for mutagenesis.

In most cell types the transcription factor, NF-κB is sequestered in the cytoplasm by the protein inhibitor, IκB. Activation of NF-κB occurs in response to external signals that result in the phosphorylation and ubiquitination of IκB, prior to its subsequent degradation by the proteasome. This allows NF-κB to translocate to the nucleus where it can regulate transcription of the appropriate genes. The heterodimer p50/RelA, existing in its inactive state bound to IκBα, is one of the most frequently occurring and extensively studied forms of NF-κB. Targeting of human IκBα by extracellular stimuli leads to phosphorylation at serines 32 (S32) and 36 (S36), followed by ubiquitination at lysines 21 (K21) and 22 (K22) (DiDonato, Mercurio et al. 1996; Rodriguez, Wright et al. 1996). These sites of modification in IκBα also appear in other species. Hence, it seems apparent that the amino terminus of IκBα performs a significant role in NF-κB regulation. A comparison of the human IκBα amino terminus with that of IκBα protein sequences encoded by murine, rat, avian, bovine, porcine and trout displayed a high level of homology (Figure 8). This suggests that IκBα is an evolutionarily conserved protein. Therefore, to identify further residues of importance, amino acids 14 to 42 of human IκBα (Figure 9) were selected to generate a range of alanine mutants by a polymerase chain reaction strategy (described in Materials & Methods, Section 2.6).

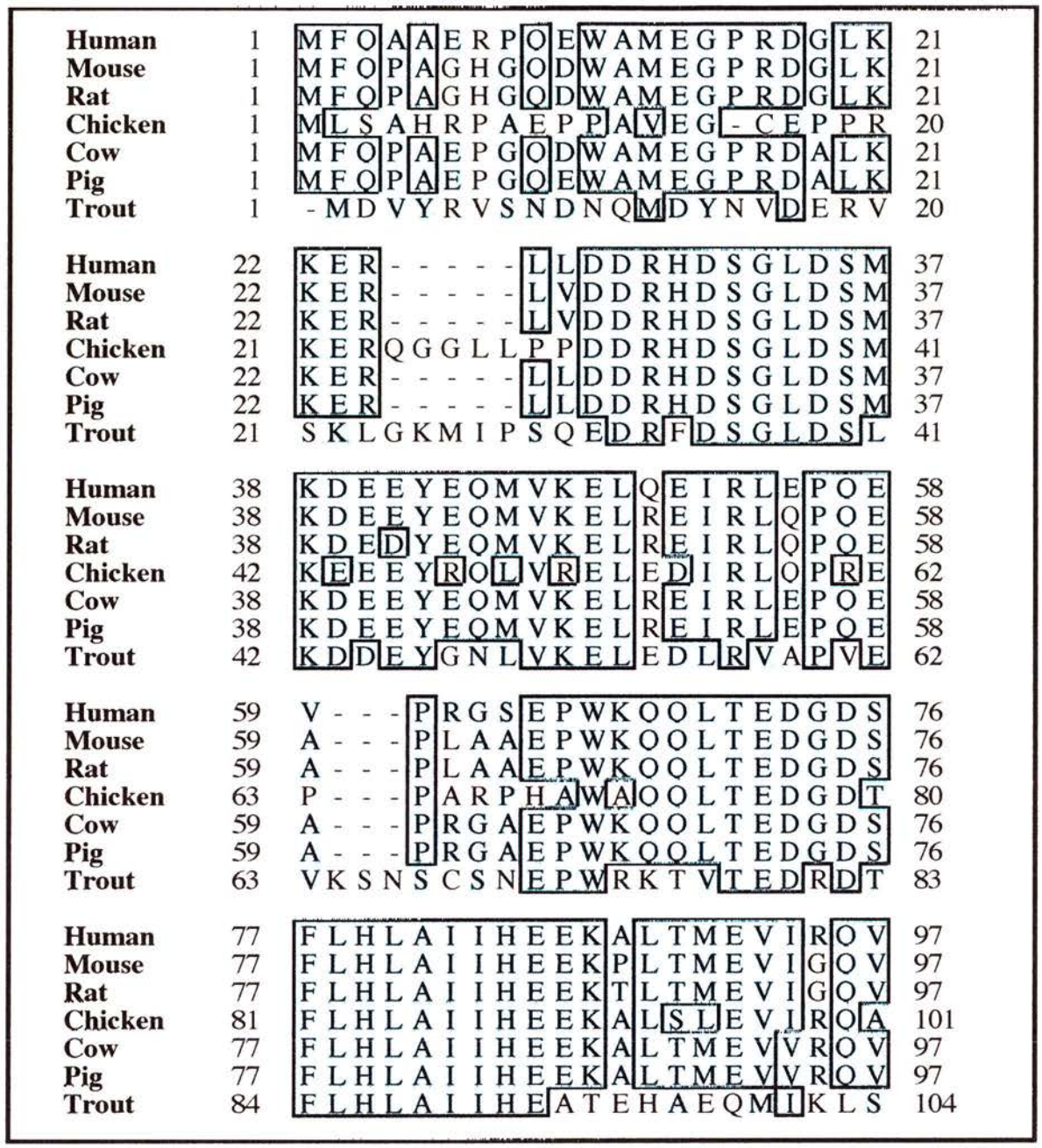


Figure 8. Amino terminal alignment of human IκBα with different species.

The protein sequence of IκBα from each species was obtained from the NCBI website (www.ncbi.nlm.nih.gov). The amino terminal regions of each protein were aligned using the ClustalX program and displayed in the SeqVu program. Regions of homology are shown in blue whilst regions of identity are shown in boxed blue.

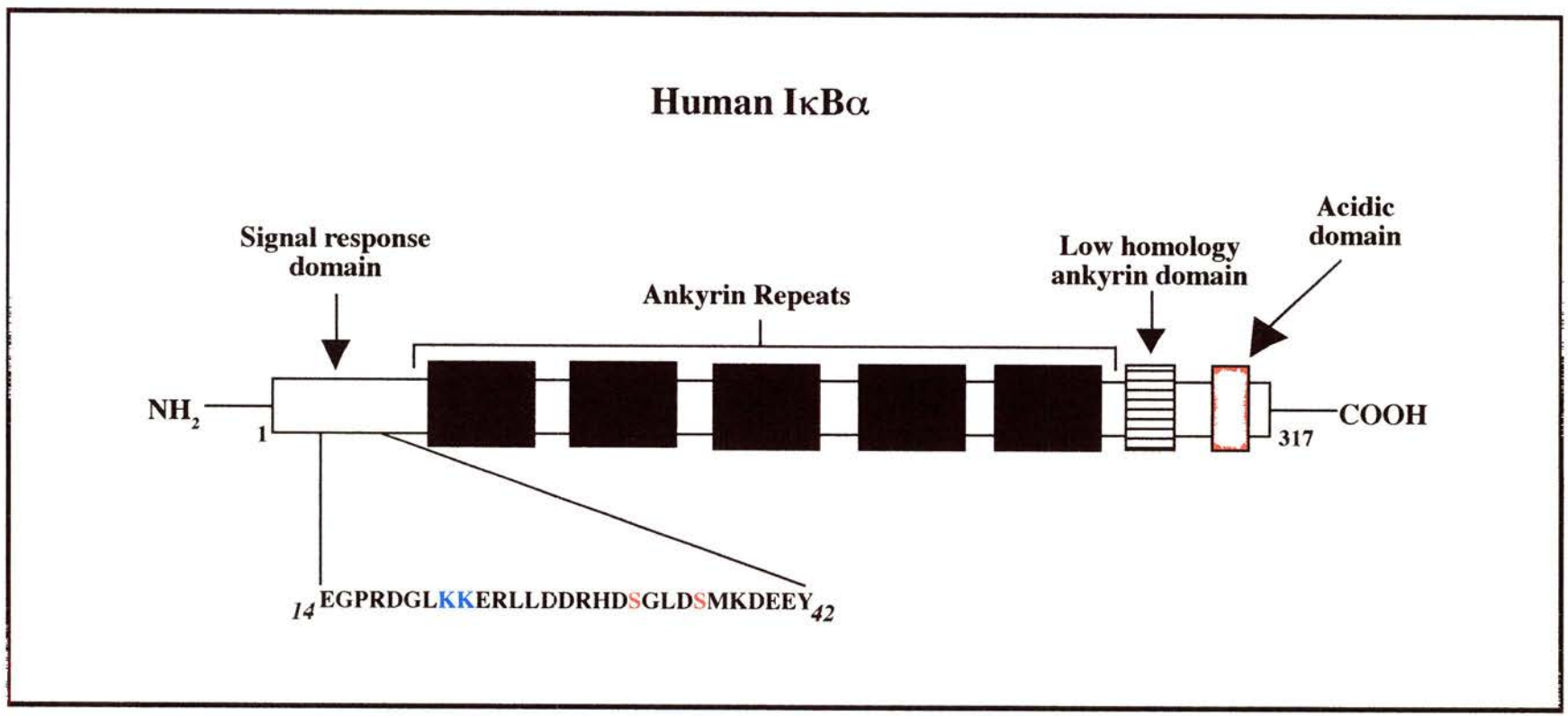


Figure 9. Schematic representation of human IκBα.

This displays the region of the N-terminus encompassing residues changed to alanine by site-directed mutagenesis. Lysine residues 21 and 22 which are ubiquitinated and serine 32 and 36 which are phosphorylated during signal-induced degradation of IκBα are highlighted in blue and red respectively.

3.1.2 The effect of wild-type and mutant IκBα proteins on NF-κB dependant transcription in response to LMP-1.

Analysis of the IκBα mutants was performed by investigating their effects on signal-induced activation of NF-κB transcription. To induce NF-κB activity, an NF-κB dependant luciferase reporter plasmid, 3EnhConALuc (3ECL) was co-transfected into cos7 cells along with IκBα expression plasmids and an expression plasmid encoding an NF-κB activator, LMP-1 (latent membrane protein-1 from the Epstein-Barr Virus). Reporter activity was measured without LMP-1 (Figure 10A) and with co-expression of LMP-1 (Figure 10B), 36 hours post-transfection. To confirm that LMP-1 resulted in activation of NF-κB, 3EnhConALuc was co-transfected with empty plasmid pcDNA3 or an expression plasmid encoding LMP-1. LMP-1 expression resulted in a 35-fold activation of reporter activity when compared to the same transfection lacking LMP-1. To provide evidence that the activation was NF-κB dependant, LMP-1 was transfected with ConALuc [CL] (a luciferase reporter lacking the NF-κB enhancer). Luciferase expression was negligible compared to that observed with 3EnhConALuc and was not altered by LMP-1 expression. To ensure that expression of IκBα was consistent, IκBα was checked by Western Blotting (Figure 11 displays residues 30-38).

To examine the effects of the mutants on NF-κB transactivation, their inhibition was compared to that of wild-type IκBα (Figure 12). Transfection of the expression plasmid, wild-type IκBα as expected resulted in a decrease in LMP-1 induced reporter activity when compared to the control lacking the wild-type IκBα plasmid (Figure 10B). As this form of IκBα was not mutated, its

Figure 10. LMP-1 induced NF-κB transactivation in cos7 cells co-transfected with IκBα mutants

A. Cos7 cells were co-transfected with the reporter plasmid, 3EnhConALuc (not indicated on graph) and either control plasmid, pcDNA3 or one of the IκBα expression plasmids. A control transfection involving reporter plasmid, ConALuc [CL] and pcDNA3 was also performed (first bar). Luciferase activity was assayed 36 hours post-transfection and expressed as RLU/μg of cell lysate protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation.

A
cos7(-LMP)
RLU/μg protein
20000
15000
10000
5000
0
Transfected Plasmid
CL
-
WT
S32A/S36A
E14A
G15A
P16A
R17A
D18A
G19A
L20A
K21A
K22A
E23A
R24A
L25A
L26A
D27A
D28A
R29A
H30A
D31A
S32A
G33A
L34A
D35A
S36A
M37A
K38A
D39A
E40A
E41A
Y42A

B. This experiment was similar to that performed in Figure 10A, but LMP-1 was also co-transfected. Again these experiments were representative of at least 3 experiments repeated in triplicate with the error bars calculated using 1 standard deviation.

B
cos7(+LMP)
RLU/μg protein
20000
15000
10000
5000
0
Transfected Plasmid
CL
3ECL
WT
S32A/S36A
E14A
G15A
P16A
R17A
D18A
G19A
L20A
K21A
K22A
E23A
R24A
L25A
L26A
D27A
D28A
R29A
H30A
D31A
S32A
G33A
L34A
D35A
S36A
M37A
K38A
D39A
E40A
E41A
Y42A

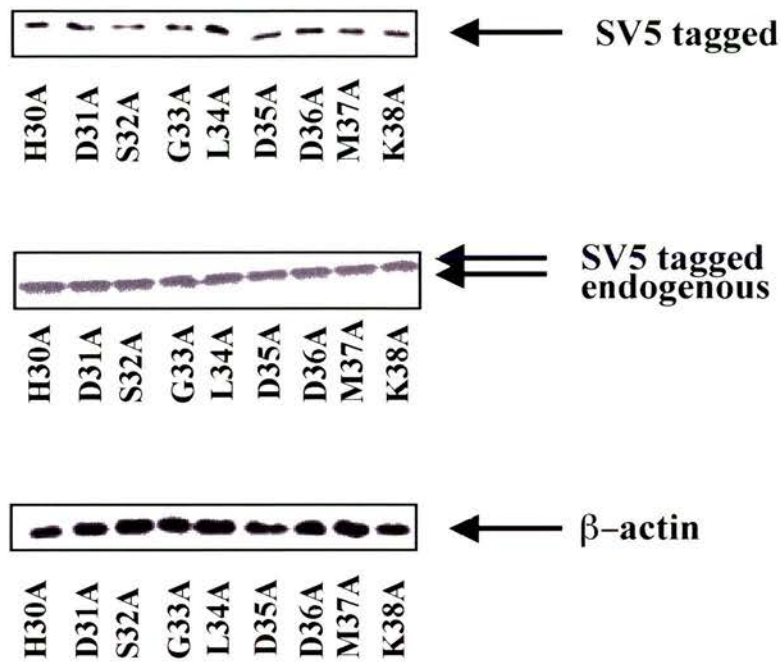


Figure 11. Western Blot depicting expression levels of IκBα

Western blot demonstrates that equal amounts of protein are produced by expression vectors encoding mutant I κBαforms (containing SV5 tag). Equal amounts of cell lysate were fractionated by SDS-PAGE and transferred to P.V.D.F membrane. The top blot shows the lysate probed with the SV5 antibody. The second blot shows lysate probed with IκB antibody. This shows that exogenous IκB was overexpressed in relation to endogenous IκB. Lysate was also probed with β–actin antibody (lower blot).

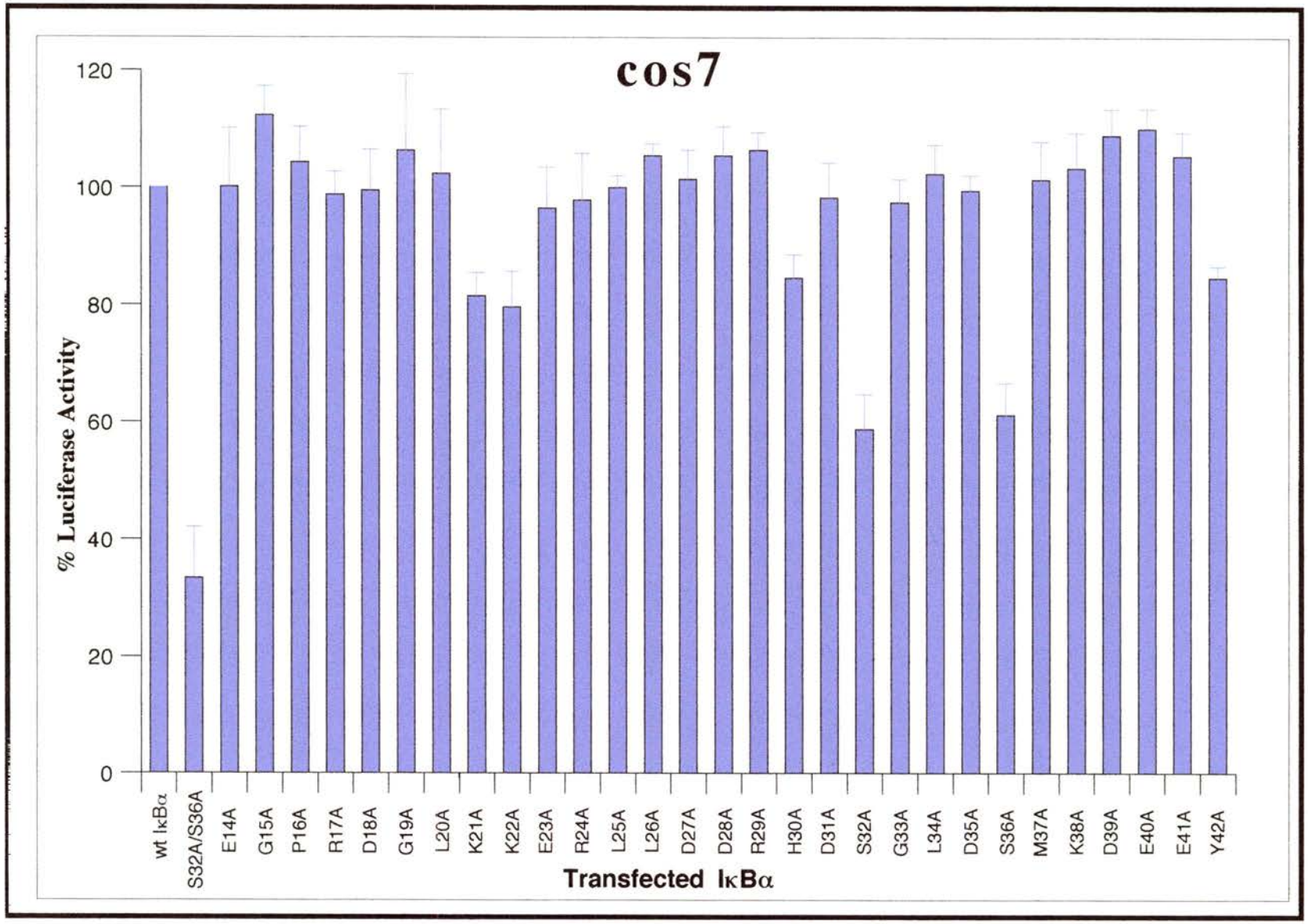


Figure 12. Effect of N-terminal IκBα mutants on NFκB activation by LMP-1 in cos7 cells.
The inhibitory capacities of wild-type and mutated IκBα proteins corresponding to LMP-1 induced cultures from figure 10B are expressed as a percentage of the luciferase activity of cos7 cells co-transfected with wild-type IκBα , 3EnhConALuc and LMP-1, which was arbitarily set to 100%.

reporter activity was taken as the arbitrary value of 100% (Figure 12). Therefore, against this standard, we could compare the inhibition of reporter activity generated by the mutant forms of IκBα. All the mutant plasmids inhibited LMP-1 induced NF-κB activity (when compared to LMP-1 reporter activity of 3EnhConALuc lacking IκBα (Figure 10B)). Of the plasmids examined, the mutants expressing K21A, K22A, H30A, S32A, S36A, S32A/S36A and Y42A forms of IκBα resulted in reduced activity in comparison to that exhibited by wild-type IκBα. From these results the amino acid change at position 30 from histidine to alanine was the only novel mutation to exert a considerable reduction on NF-κB transactivation. As expected, mutant IκBα plasmids that had knocked out the phosphorylation sites, S32A, S36A, S32A/S36A, and the ubiquitination sites K21A, K22A, inhibited NF-κB dependant activation substantially. Also, tyrosine 42 which had previously been suggested as an alternative phosphorylation site to the serine residues involved in IκBα degradation, showed a significant reduction in NF-κB transactivation. In contrast, glycine15, aspartic acid 39 and glutamic acid 40 showed notable increases in NF-κB activation (Figure 12). The remainder of the IκBα mutants displayed similar inhibitory capacities to wild-type IκBα.

3.1.3 Mutant IκBα proteins respond similarly to different activators of NF-κB.

To ensure the effects of the alanine mutations observed were not specific to cos7 cells (derived from monkey kidney cells), HeLa cells, (derived from human cervical carcinoma) were also transfected with the IκBα mutants which were shown previously (Figure 12) to cause a significant reduction in NF-κB activity. Overall, the luciferase activity observed in HeLa cells (Figure 13A) was lower than that seen with cos7 cells, however the effects of the IκBα mutations (Figure 13B) show a similar trend in both cell lines when stimulated with LMP-1.

The effect of these IκBα mutants were then further examined using alternative NF-κB stimuli in both cos7 and HeLa cell types. Luciferase activity and inhibitory capacities using receptor mediated stimuli, TNF-α (Figure 14 and 15) and IL1β (Figure 16 and 17) are shown respectively. Although both NF-κB activators attained higher levels of luciferase activity, results displaying their inhibitory capacities were consistent with findings achieved using LMP-1. Although these three types of stimuli, induce NF-κB activation by differing pathways, they all converge on the IKK complex, which leads to the phosphorylation of IκBα.

3.1.4 Discussion

Many transcription factors appear to be controlled by phosphorylation of their DNA binding subunits. This can either enhance or reduce their DNA binding, dimerisation or even their transcriptional activity. However, control of

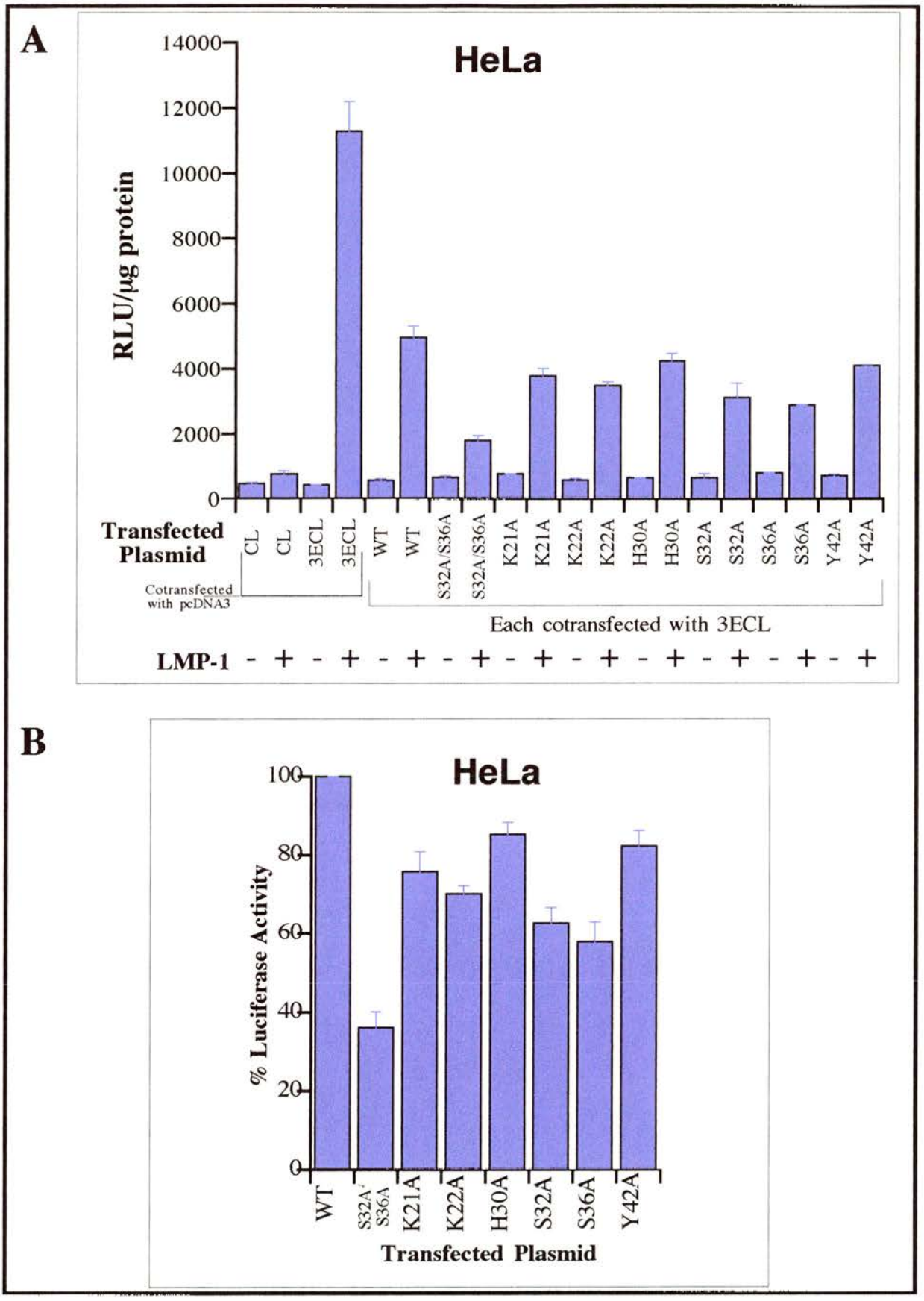


Figure 13. Effects of IκBα mutants during LMP-1 induced transfections in HeLa cells. A. HeLa cells were transfected with or without LMP-1 (indicated by + or -), reporter plasmid 3EnhConALuc, and the relevant IκBα expression plasmid. After 36 hours, luciferase activity was measured as RLU/µg of cell lysate protein. A set of controls were also performed using pcDNA3 and ConALuc [CL]. These results are representative of 3 experiments done in triplicate. Again error bars were measured at 1 standard deviation. B. The inhibitory capacities of LMP-1 induced transfections are expressed as a percentage of the transfection involving wild-type IκBα in figure 13A which was set at 100%.

Figure 14. Effect of IκBα mutants on NF-κB activation by TNF-α in cos 7 cells.

A. Cos7 cells were co-transfected with the reporter plasmid, 3EnhConALuc and either control plasmid, pcDNA3 or one of the IκBα expression plasmids. A control transfection involving reporter plasmid, ConALuc [CL] and pcDNA3 was also performed. Transfected cells were induced with 10ng/ml TNF-α (indicated by +) or left unactivated (indicated by -) for the last 8 hours in culture before measuring the luciferase activity in cell lysates. Luciferase activity was measured in relation to μg of protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation. B. The amounts of luciferase activity by TNF-α induced cos7 cells transfected with the IκBα expression plasmids (See Figure 14A) are expressed as a percentage of the 100% value designated by the transfection involving wild-type IκBα.

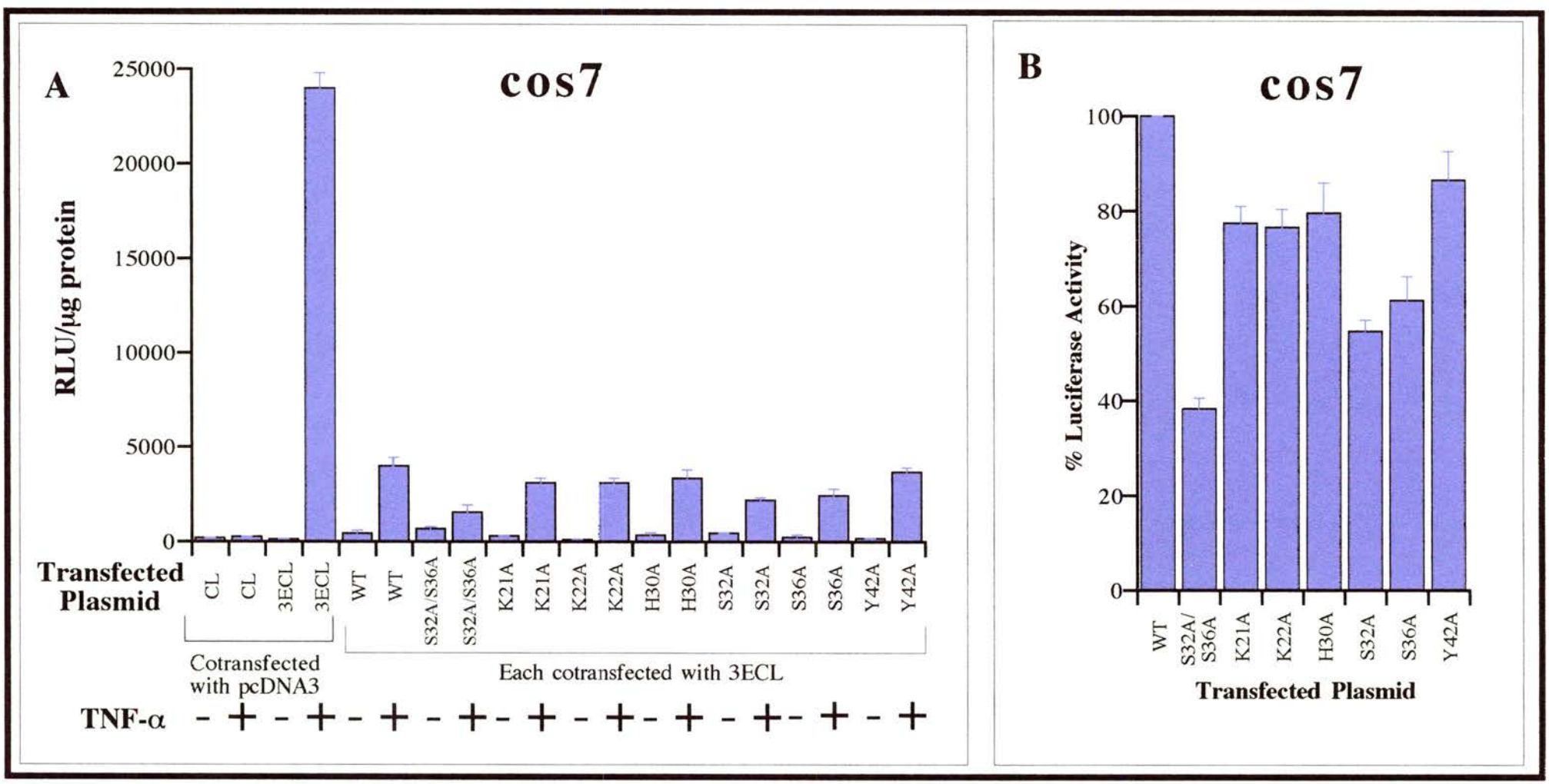

A
cos7
RLU/μg protein
25000
20000
15000
10000
5000
0
Transfected Plasmid
CL
CL
3ECL
3ECL
WT
WT
S32A/S36A
S32A/S36A
K21A
K21A
K22A
K22A
H30A
H30A
S32A
S32A
S36A
S36A
Y42A
Y42A
Cotransfected with pcDNA3
Each cotransfected with 3ECL
TNF-α - + - + - + - + - + - + - + - + - + - +
B
cos7
% Luciferase Activity
100
80
60
40
20
0
WT
S32A/ S36A
K21A
K22A
H30A
S32A
S36A
Y42A
Transfected Plasmid

Figure 15. Effect of IκBα mutants on NF-κB activation by TNF-α in HeLa cells.

A. Cos7 cells were co-transfected with the reporter plasmid, 3EnhConALuc and either control plasmid, pcDNA3 or one of the IκBα expression plasmids. A control transfection involving reporter plasmid, ConALuc [CL] and pcDNA3 was also performed. Transfected cells were induced with 10ng/ml TNF-α (indicated by +) or left unactivated (indicated by -) for the last 8 hours in culture before measuring the luciferase activity in cell lysates. Luciferase activity was measured in relation to μg of protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation. B. The amounts of luciferase activity by TNF-α induced cos7 cells transfected with the IκBα expression plasmids (See Figure 15A) are expressed as a percentage of the 100% value designated by the transfection involving wild-type IκBα.

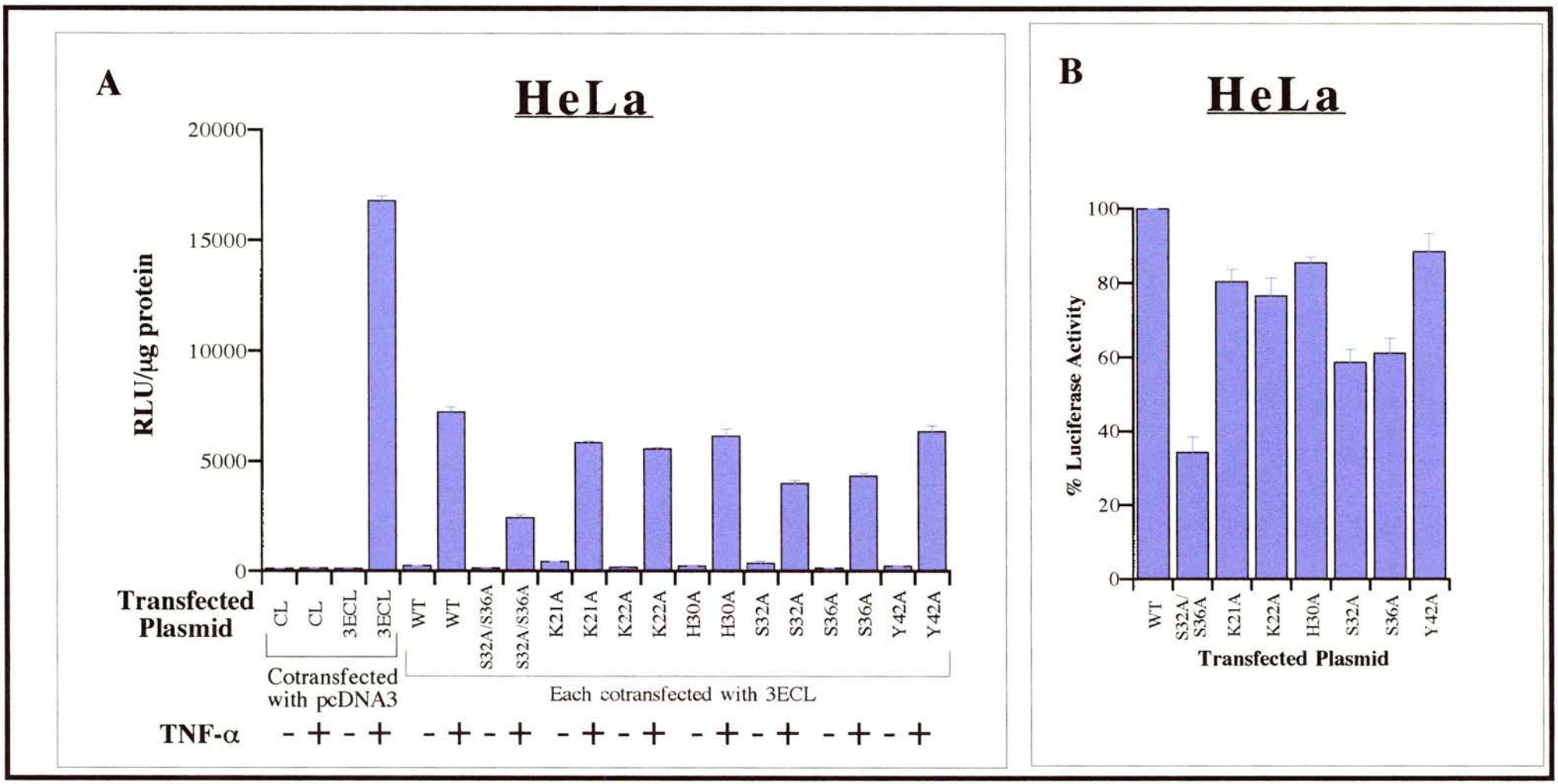

A
HeLa
RLU/μg protein
20000
15000
10000
5000
0
Transfected Plasmid
CL
CL
3ECL
3ECL
WT
WT
S32A/S36A
S32A/S36A
K21A
K21A
K22A
K22A
H30A
H30A
S32A
S32A
S36A
S36A
Y42A
Y42A
Cotransfected with pcDNA3
Each cotransfected with 3ECL
TNF-α - + - + - + - + - + - + - + - + - + - +
B
HeLa
% Luciferase Activity
100
80
60
40
20
0
WT
S32A/S36A
K21A
K22A
H30A
S32A
S36A
Y42A
Transfected Plasmid

Figure 16. Effect of IκBα mutants on NF-κB activation by IL1-β in cos7 cells.

A. Cos7 cells were co-transfected with the reporter plasmid, 3EnhConALuc and either control plasmid, pcDNA3 or one of the IκBα expression plasmids. A control transfection involving reporter plasmid, ConALuc [CL] and pcDNA3 was also performed. Transfected cells were induced with 2ng/ml IL1-β (indicated by +) or left unactivated (indicated by -) for the last 8 hours in culture before measuring the luciferase activity in cell lysates. Luciferase activity was measured in relation to μg of protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation. B The amounts of luciferase activity by IL1-β induced cos7 cells transfected with the IκBα expression plasmids(See Figure 16A) are expressed as a percentage of the 100% value designated by the transfection involving wild-type IκBα.

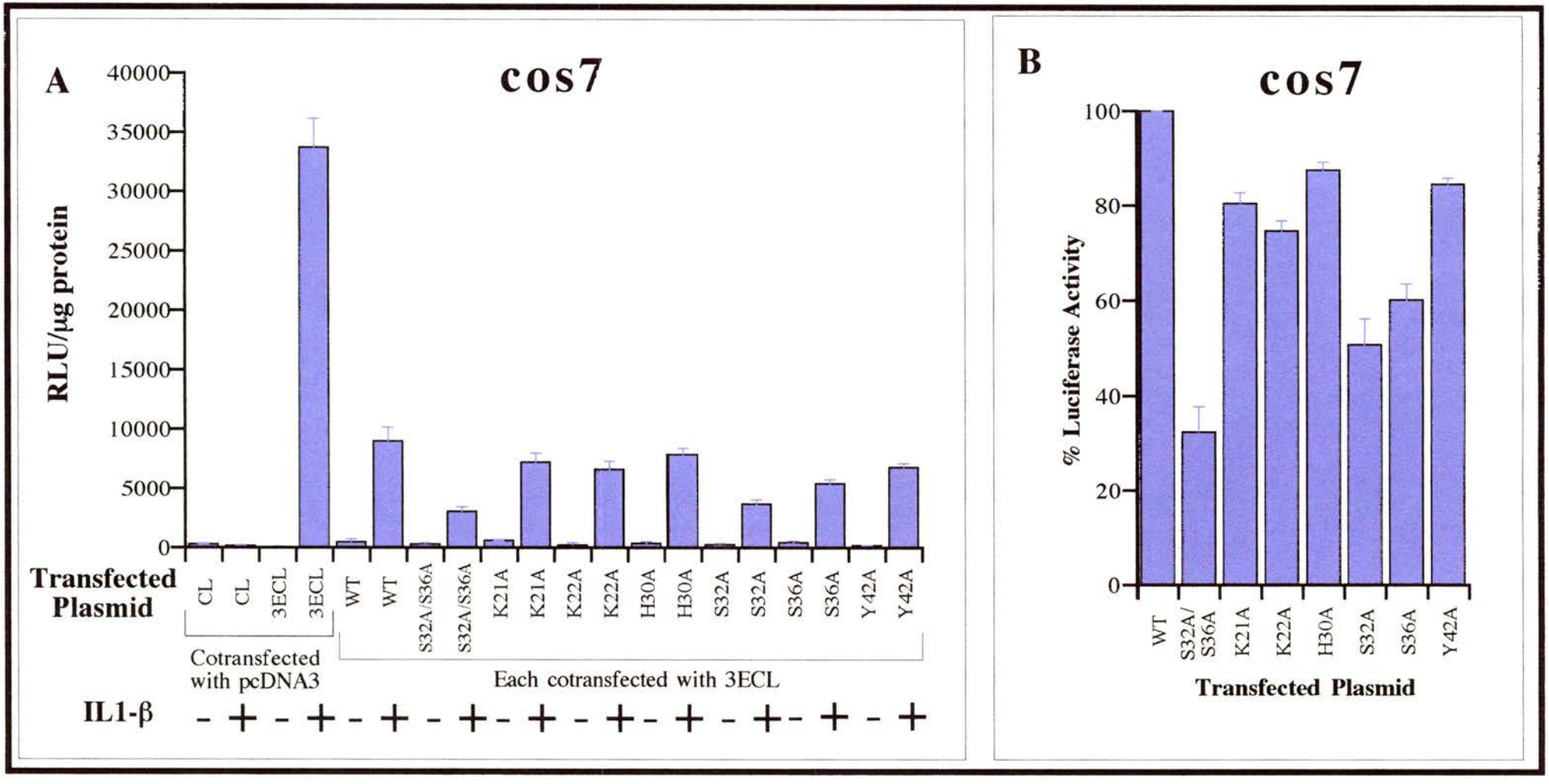
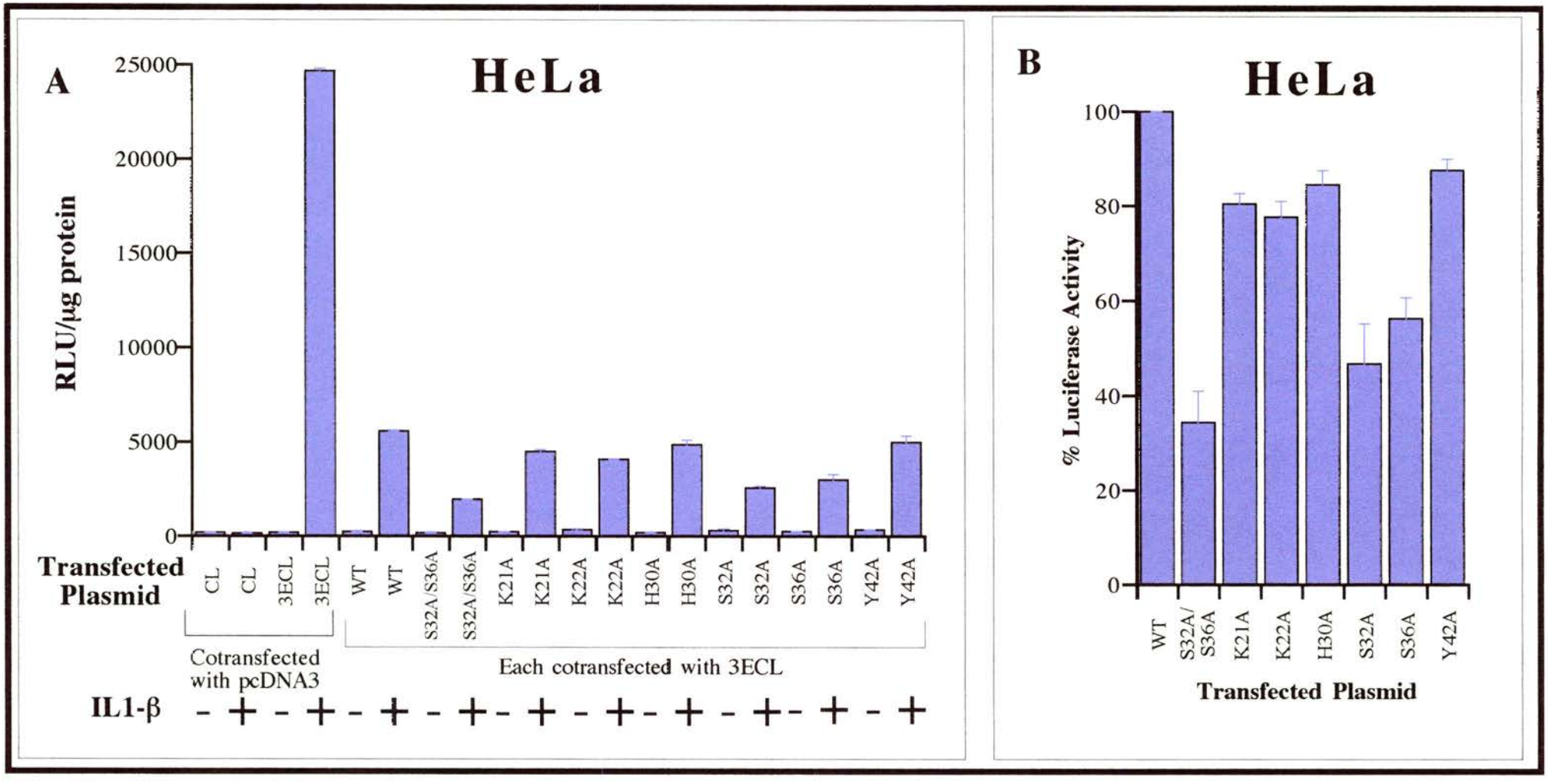

A
cos7
RLU/μg protein
40000
35000
30000
25000
20000
15000
10000
5000
0
Transfected Plasmid
CL
CL
3ECL
3ECL
WT
WT
S32A/S36A
S32A/S36A
K21A
K21A
K22A
K22A
H30A
H30A
S32A
S32A
S36A
S36A
Y42A
Y42A
Cotransfected with pcDNA3
Each cotransfected with 3ECL
IL1-β
- + - + - + - + - + - + - + - + - + - +
B
cos7
% Luciferase Activity
100
80
60
40
20
0
WT
S32A/S36A
K21A
K22A
H30A
S32A
S36A
Y42A
Transfected Plasmid

Figure 17. Effect of IκBα mutants on NF-κB activation by IL1-β in HeLa cells.

A. Cos7 cells were co-transfected with the reporter plasmid, 3EnhConALuc and either control plasmid, pcDNA3 or one of the IκBα expression plasmids. A control transfection involving reporter plasmid, ConALuc [CL] and pcDNA3 was also performed. Transfected cells were induced with 2ng/ml IL1-β (indicated by +) or left unactivated (indicated by -) for the last 8 hours in culture before measuring the luciferase activity in cell lysates. Luciferase activity was measured in relation to μg of protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation. B The amounts of luciferase activity by IL1-β induced cos7 cells transfected with the IκBα expression plasmids(See Figure 17A) are expressed as a percentage of the 100% value designated by the transfection involving wild-type IκBα.


A
HeLa
RLU/μg protein
25000
20000
15000
10000
5000
0
Transfected Plasmid
CL
CL
3ECL
3ECL
WT
WT
S32A/S36A
S32A/S36A
K21A
K21A
K22A
K22A
H30A
H30A
S32A
S32A
S36A
S36A
Y42A
Y42A
Cotransfected with pcDNA3
Each cotransfected with 3ECL
IL1-β
- + - + - + - + - + - + - + - + - + - +
B
HeLa
% Luciferase Activity
100
80
60
40
20
0
WT
S32A/ S36A
K21A
K22A
H30A
S32A
S36A
Y42A
Transfected Plasmid

the transcription factor NF-κB's activation relies initially on the phosphorylation of its inhibitory subunit, IκB. Phosphorylation by IκB kinase leads to ubiquitination before the proteasome-mediated degradation of IκB and the subsequent release of NF-κB to the nucleus. Consequently IκB is the focal point of many external stimuli requiring an NF-κB transcriptional response. The phosphorylation at two serine residues and ubiquitination at two lysine residues have been located in the amino terminus of IκB. Comparison of IκB amongst multiple species revealed that the amino acids involved in phosphorylation and ubiquitination were highly conserved and in addition so was the majority of the amino terminus. Previous deletion analysis studies of the amino terminus of IκB have shown it is not involved in binding to NF-κB, inhibiting DNA binding of NF-κB or even cytoplasmic retention (Jaffray, Wood et al. 1995). In fact the amino terminus is in an exposed proteolytically sensitive region when bound to NF-κB, which is susceptible to the protease (Jaffray, Wood et al. 1995). Hence, this has raised the possibility that the amino terminus functions as a signal response domain. Furthermore the homology suggests the possibility of a recognition motif required for phosphorylation by the IκB kinase complex. This prompted the investigation of the residues within and surrounding these modification sites and their role in NF-κB activation. Amino acids 14-42 of IκBα were converted into a series of alanine mutants and the effect of such mutations on NF-κB transcription were examined. To examine their effects, a reporter assay tool was utilised rather than a Western Blot. The reporter assay is much more sensitive and easier to observe effects in than in comparison to a Western Blot.

Generally, the inhibitory capacities were identical irrespective of whether TNF-α, IL1-β or LMP-1 was used as the stimuli. As expected the co-transfection of plasmid encoding wild-type IκBα resulted in a substantial decrease in NF-κB activation. Amino acids that were involved in IκB modifications further inhibited activation in comparison to the wild-type. The supersuppressor S32A/S36A IκBα mutant resulted in the greatest inhibition, approximately 70% reduction compared to that of wild-type IκBα. Transfection of this mutant would be expected to abolish 100% of induced NF-κB activity, as it has knocked out the phosphorylation sites required to degrade IκBα. However, it is probable that some of the NF-κB pool is associated with endogenous wild-type IκBα in addition to the mutant. Therefore, upon stimulation the former could release NF-κB and escape binding to any of the excess mutant and activate the reporter. Single serine changes resulted in inhibition to a lesser degree, which agrees with data that both serines 32 and 36 are required to evoke the accumulative inhibition. Although not hugely significant, serine 32 alone seemed to inhibit NF-κB activity more when compared to serine 36, which suggests it may be more important in triggering IκBα degradation. Mutating the lysines at positions 21 and 22 to knock out the ubiquitination sites also inhibited NF-κB activation but not as significantly as when the phosphorylation sites were mutated. This difference in the level of inhibition of NF-κB activity could be explained by phosphorylation occurring prior to ubiquitination. Therefore a single lysine mutant would be able to undergo phosphorylation at serine 32 and 36, before ubiquitination on the adjacent lysine allowing at least some IκB degradation, whereas in the serine mutants, IκB would encounter difficulty passing the phosphorylation stage.

Recent work has implicated the presence of a DSG***S motif present in IκBα which is necessary for the ubiquitin-mediated proteolysis of IκBα (Yaron, Hatzubai et al. 1998). However, in these studies aspartic acid 31 and glycine 33 showed a similar level of inhibition to that seen with wild-type IκBα. It is not clear why this is the case, however Hattori *et al.* showed that these residues did not effect phosphorylation of IκBα but rather the ubiquitination process (Hattori, Hatakeyama et al. 1999). So although the results do not agree totally, it seems apparent that these residues are not required by an IκB kinase.

Instead, histidine 30 and tyrosine 42 were the only other residues that showed an inhibition stronger than that of wild-type IκBα. Histidine 30 lies between the lysine and serine residues and loss of the imidazole ring could cause a slight structural change that could effect either the phosphorylation or ubiquitination process resulting in this inhibition. The stronger inhibition shown with the tyrosine 42 mutant is interesting as this residue has been implicated in a phosphorylation involving an alternative pathway. Although not fully understood, activation via pervanadate involves tyrosine phosphorylation of IκBα which does not cause IκBα degradation but rather its dissociation from NF-κB. This pathway does not involve the IκB kinase which is targeted by TNF-α, IL1-β and LMP-1. It is possible that the IκB kinase pathway does require the tyrosine for its phosphorylation on serine 32 and 36, but TNF-α, IL1-β and LMP-1 may in the short timeframe trigger off the pervanadate pathway upstream of their own effects on the IKK complex.

As these studies were to identify residues that inhibit NF-κB transcription, glycine 15, aspartic acid 39 and glutamic acid 40, all of which did not inhibit as strongly as the wild-type IκBα were not tested with TNF-α or

IL1-β. The change to alanine probably altered the structure hindering phosphorylation by the IκB kinase.

As a result of these studies into the amino terminus of IκBα it has been confirmed that serine 32 and 36, lysine 21 and 22 are necessary for signal-induced activation of NF-κB via the IκB kinase. Histidine 30 is potentially involved in recognition by the IκB kinase but the role of tyrosine 42 is unclear due to its role in an alternative pathway. Results here also contradict those that suggest the need for an aspartate and glycine at positions 31 and 33. Thus further work has to be carried out to identify whether these residues can in conjunction with S32A and S36A mutations generate a stronger suppressor than the supersuppressor S32A/S36A IκBα mutant.

3.2 Investigation into effects of tumour suppressor protein p53 on NF-κB dependent transcription.

3.2.1 Effect of p53 on NF-κB transcriptional activation

The transcription factor NF-κB is activated by a variety of extracellular signals and performs an important role in regulating immune, inflammatory and anti-apoptotic responses. In 2000, it was reported that p53, both a tumour suppressor and transcription factor, was capable of activating NF-κB (Ryan, Ernst et al. 2000). Ryan *et al.* used p53-inducible Saos2 cell lines to examine the effects of p53 on NF-κB DNA binding activity. These osteogenic sarcoma cell lines did not express any endogenous p53, but upon induction by doxycycline (a tetracycline analogue), expression of p53 could be induced. This resulted in an elevation in NF-κB DNA binding activity. Furthermore, p53-dependent apoptosis was not observed in p65 $^{-/-}$ mice Also, this paper suggested that p53 stimulated NF-κB activation by an alternative pathway to that mediated by tumour necrosis factor (TNF-α). This involved phosphorylation of IκB by mitogen activated 90 kDa ribosomal S6 kinase ($p90^{rsk}$) via the MAP kinase cascade, rather than the conventional IKK complex. Presently, this kinase has been identified upon stimulation by 12-*O*-tetradecanoyl phorbol 13-acetate, only to target phosphorylation of serine 32 but not the additional phosphorylation of serine 36 utilised by TNF-α (Ghoda, Lin et al. 1997; Schouten, Vertegaal et al. 1997).

To identify whether any of the N-terminal IκBα mutants generated previously (Chapter 3.1.1) had an effect on p53 mediated NF-κB activation, reporter assays were carried out to examine the transcriptional response of NF-κB to p53 (Figure 18, 19 & 20). Three cell lines with different p53 phenotypes were obtained to evaluate the effect of p53 on NF-κB dependent transcriptional activity. The first cell line, human foreskin fibroblasts was a primary cell line

Figure 18. Effect of wild-type p53 on NF-κB activity in HFF cell line.
Human foreskin fibroblasts (HFF) were co-transfected with one of the following three reporter plasmids by the lipofectamine method.; 3EnhConALuc, PG13L or ConALuc and a plasmid encoding either wild-type p53, mutant p53 R175H, mutant p53 R273H, LMP-1 or pcDNA3. Luciferase activity was assayed 14 hours post-transfection and expressed as RLU/μg of cell lysate protein. (- This indicates background luciferase activity in untransfected cells.) These experiments were performed 3 times in triplicate and the standard error measured to one standard deviation.

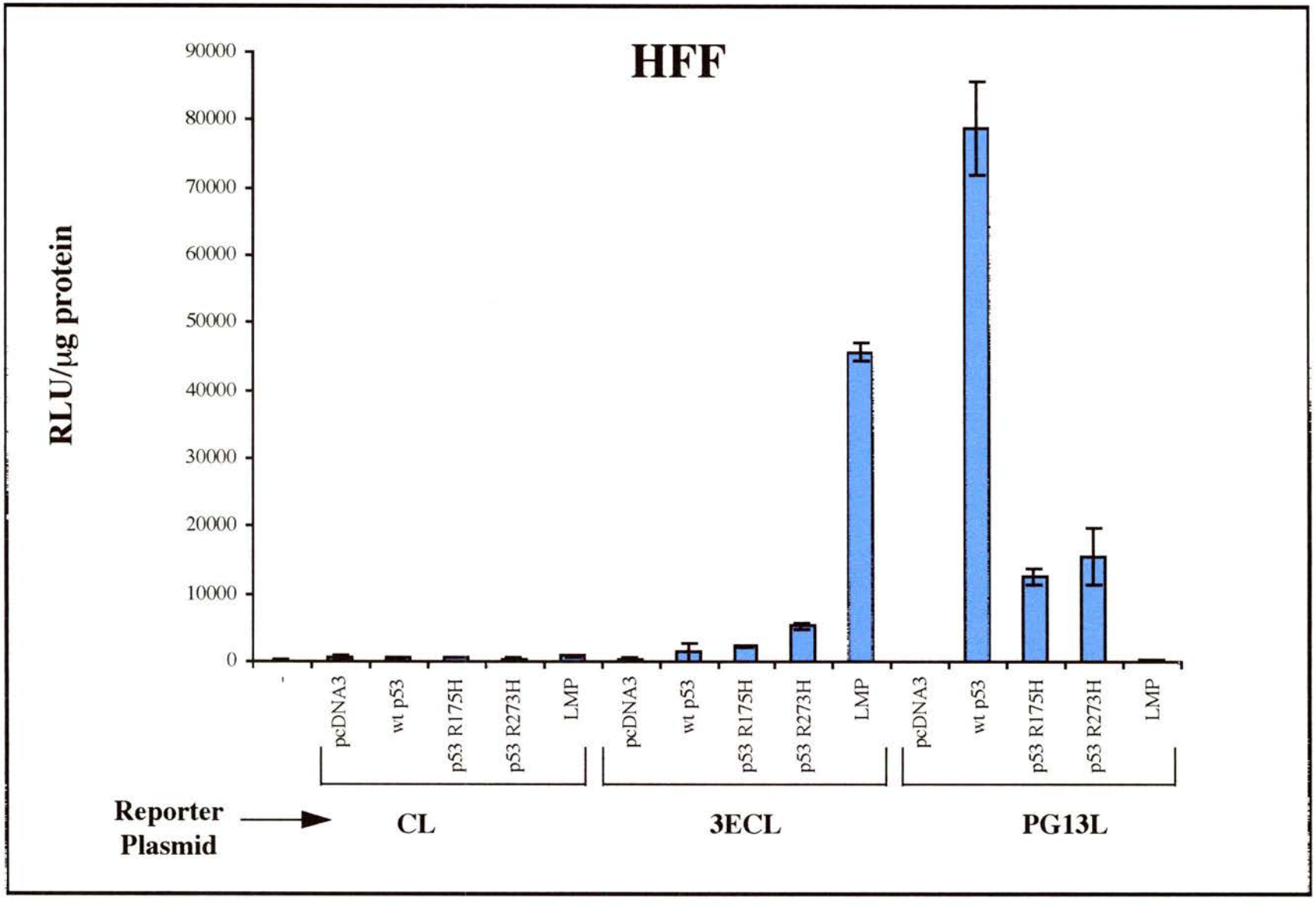

HFF
RLU/µg protein
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
-
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
Reporter Plasmid
CL
3ECL
PG13L

Figure 19. Effect of wild-type p53 on NF-κB activity in Saos2 cell line.
Saos2 cells were co-transfected with one of the following three reporter plasmids by the lipofectamine method.; 3EnhConALuc, PG13L or ConALuc and a plasmid encoding either wild-type p53, mutant p53 R175H, mutant p53 R273H, LMP-1 or pcDNA3. Luciferase activity was assayed 14 hours post-transfection and expressed as RLU/μg of cell lysate protein. (- This indicates background luciferase activity in untransfected cells.) These experiments were performed 3 times in triplicate and the standard error measured to one standard deviation.

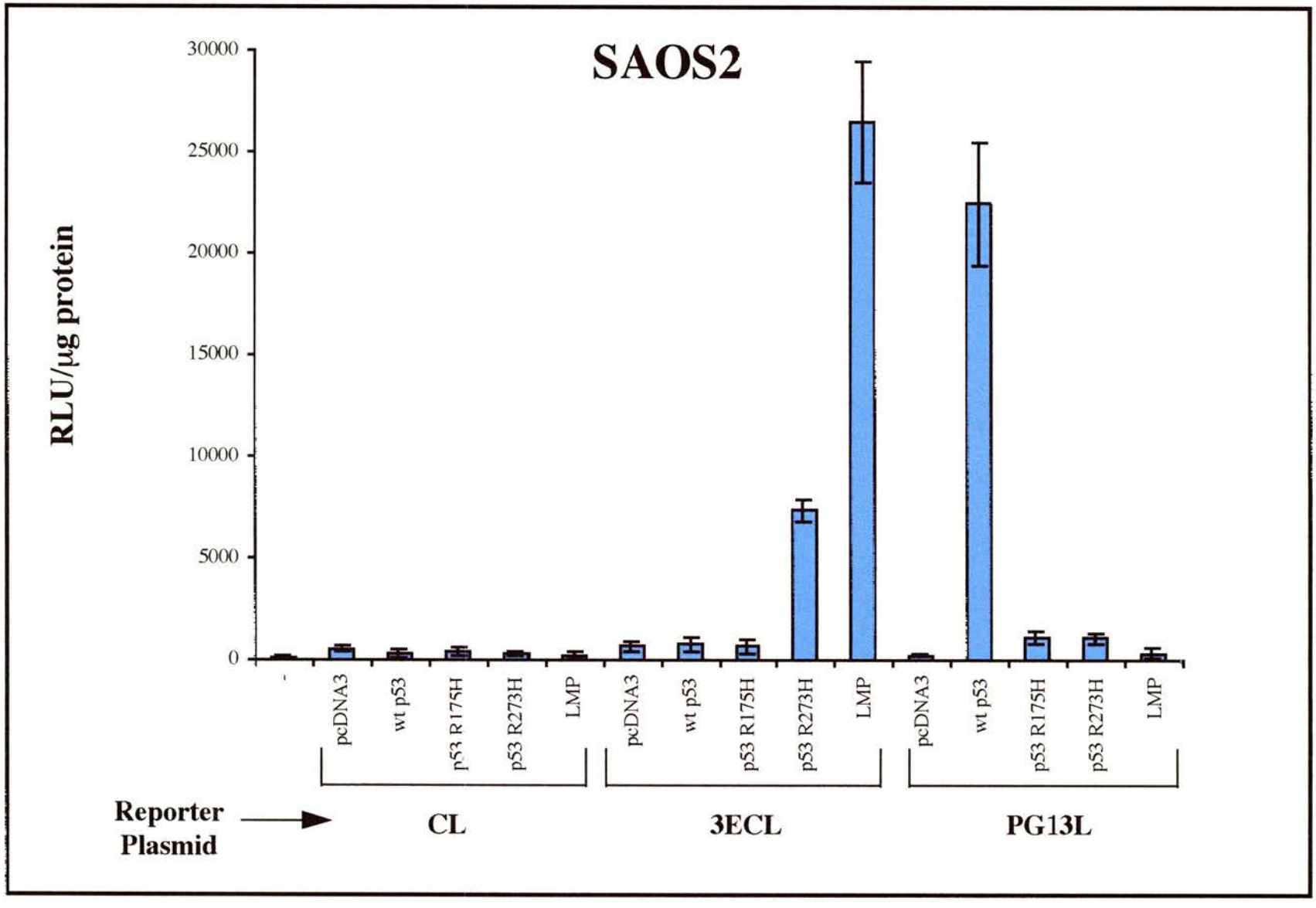
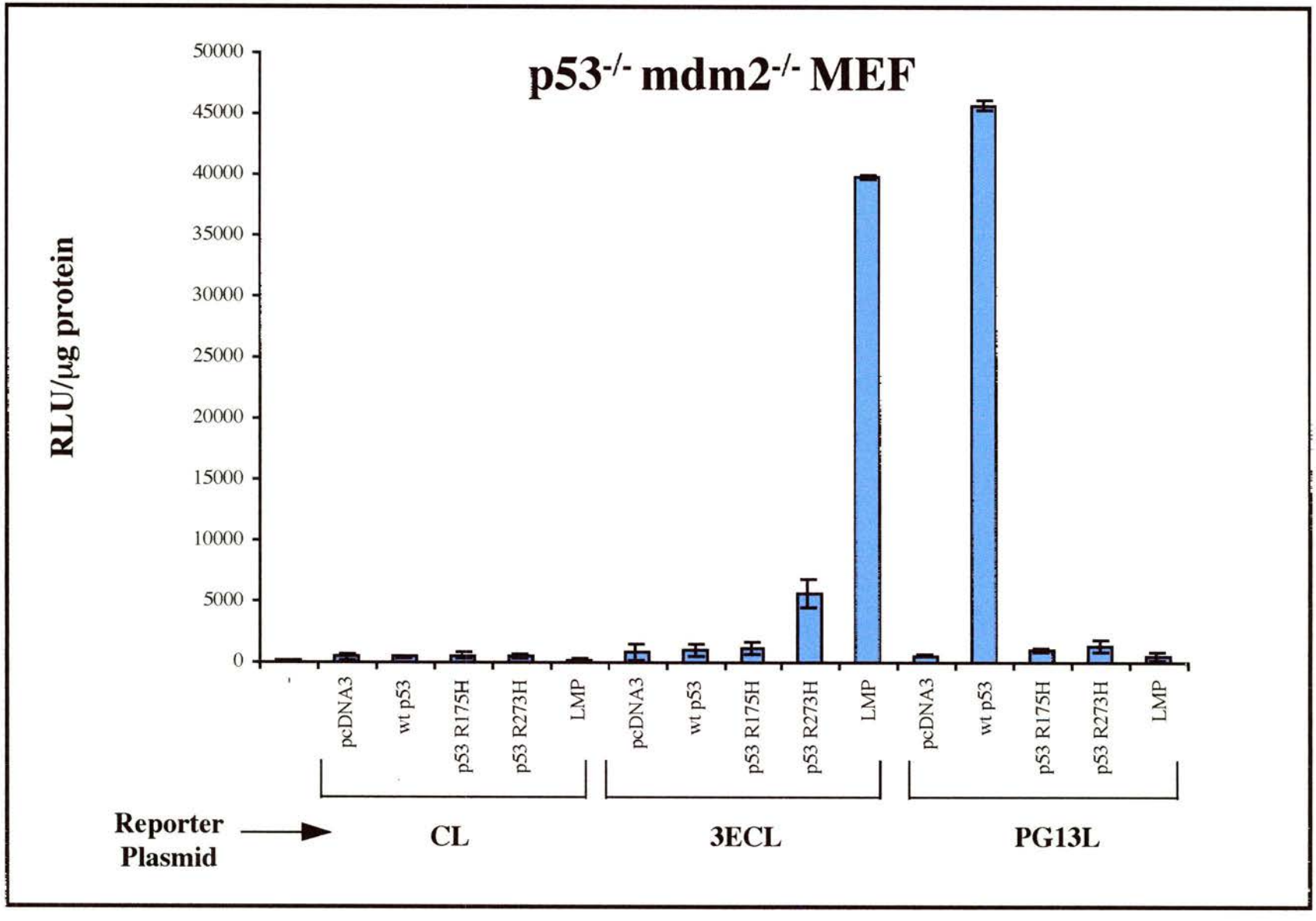

SAOS2
RLU/µg protein
30000
25000
20000
15000
10000
5000
0
-
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
Reporter Plasmid
CL
3ECL
PG13L

Figure 20. Effect of wild-type p53 on NF-κB activity in $p53^{-/-}$ $mdm2^{-/-}$ MEF.
$p53^{-/-}$ $mdm2^{-/-}$ mouse embryo fibroblasts were co-transfected by the lipofectamine method with one of the following three reporter plasmids; 3EnhConALuc, PG13L or ConALuc and a plasmid encoding either wild-type p53, mutant p53 R175H, mutant p53 R273H, LMP-1 or pcDNA3. Luciferase activity was assayed 14 hours post-transfection and expressed as RLU/μg of cell lysate protein. (- This indicates background luciferase activity in untransfected cells.) These experiments were performed 3 times in triplicate and the standard error measured to one standard deviation.


p53-/- mdm2-/- MEF
RLU/μg protein
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
-
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
pcDNA3
wt p53
p53 R175H
p53 R273H
LMP
Reporter Plasmid
CL
3ECL
PG13L

that had wild-type p53. The other two cell lines, Saos 2 (osteogenic sarcoma) and $p53^{-/-}$ $mdm2^{-/-}$ MEFs (mouse embryo fibroblasts) both did not contain any endogenous p53. Furthermore, the $p53^{-/-}$ $mdm2^{-/-}$ cell line also lacked Mdm2, the negative regulator of p53. Three expression plasmids encoding wild-type p53 (wt p53), p53 with an arginine to histidine change at residue 175 (p53 R175H) and p53 with an arginine to histidine change at residue 273 (p53 R273H) were introduced into the cell lines by the lipofectamine method to test their effects on NF-κB activation. The mutation at p53 R175H disrupts the tetrameric structure of p53 as it is involved with stabilising two loops (L2 and L3) via a zinc atom which is critical for organising the core domain of p53. However, the mutation at p53 R273H does not disrupt the structure of p53, but rather its binding to DNA. Each plasmid was co-transfected into each cell line with one of the following luciferase reporter plasmids, NF-κB dependent 3EnhConALuc (3ECL), p53 dependent PG13L and the control ConALuc (CL). Also an expression plasmid encoding the NF-κB activator, LMP-1 (latent membrane protein-1) was transfected with each of the reporter plasmids in the three cell lines to act as a positive control for NF-κB activation while pcDNA3 was correspondingly utilised as a negative control for both p53 and NF-κB dependent activation. 14 hours post-transfection, the cells were assayed for reporter activity.

The first striking result from these reporter assays was presented in the HFF cell line (Figure 18) Although, it was apparent that wild-type p53 activated p53 dependent transcription, it did not activate any NF-κB dependent transcription. Furthermore, these results were confirmed in both Saos2 (Figure 19) and $p53^{-/-}$ $mdm2^{-/-}$ cell lines (Figure 20). This was clearly unexpected as

Ryan *et al.*, had shown p53 to induce NF-κB DNA binding activity and therefore it was assumed wild-type p53 would activate NF-κB transcription. Moreover, the mutant p53 R273H was shown to activate NF-κB, a five-fold activation in HFFs, eight-fold in Saos2 and five-fold in $p53^{-/-}$ $mdm2^{-/-}$ cell lines, respectively. However, the mutant p53 R175H did not display any NF-κB activation in any of the cell lines. As expected these two mutants did not show any p53 dependent transcription in either Saos2 or $p53^{-/-}$ $mdm2^{-/-}$ cell lines. However, in HFF, these mutants stimulated p53 transcriptional activity. This activity was not observed in those transfections involving LMP-1 or pcDNA3.

As expected none of the expression plasmids had any influence on the ConALuc luciferase reporter in any of the cell lines. Furthermore, control pcDNA3 did not induce either NF-κB or p53 dependent activity in HFF, Saos2 or $p53^{-/-}$ $mdm2^{-/-}$ cell lines. But LMP-1 induced NF-κB activity but not p53-dependent activity in all three cell lines. Levels of p53 expression were checked by western blot to be at similar levels (data not shown).

In summary, it has been shown that mutant p53 R273H activates NF-κB whereas wild-type p53 does not.

3.2.2 The effect of wild-type p53 and mutant p53 R273H on NF-κB DNA binding.

To confirm the NF-κB transcriptional activities observed upon transfection by wild-type p53 and p53 R273H, an electrophoretic mobility shift assay (EMSA) was performed to analyse their effects on NF-κB binding activity.

Nuclear extracts were obtained from Saos2 cells transfected with expression plasmids encoding wild-type p53 and mutant p53 R273H, respectively. This was because NF-κB transcriptional activation depends on NF-κB translocating to the nucleus and binding to DNA. Thus if NF-κB is activated it should be present in the nucleus. Saos2 nuclear extracts transfected with the expression plasmids encoding positive control, LMP-1 and negative control pcDNA3 were also collected. 5μg of each extract was then incubated in a binding mix in the presence of a ^{32}P labelled double-stranded oligonucleotide which contained a recognition site for NF-κB. These binding assays were also performed in the presence of either p50 or p65 antibody. Following incubation, the complexes were separated on a non-denaturing PAGE, the gel subsequently dried down and exposed to a phosphorimager plate.

The results demonstrated by the EMSA (Figure 21) correspond with the results displayed in Section 3.2.1. Wild-type p53 did not induce NF-κB DNA binding activity (lane 2). However, mutant p53 R273H generated some NF-κB DNA binding activity (lane 3). Furthermore lane 7 and lane 11 show that these complexes can bind the p65 and p50 antibodies, respectively. As expected LMP-1 induced significant NF-κB activity (lane 4) which was also capable of

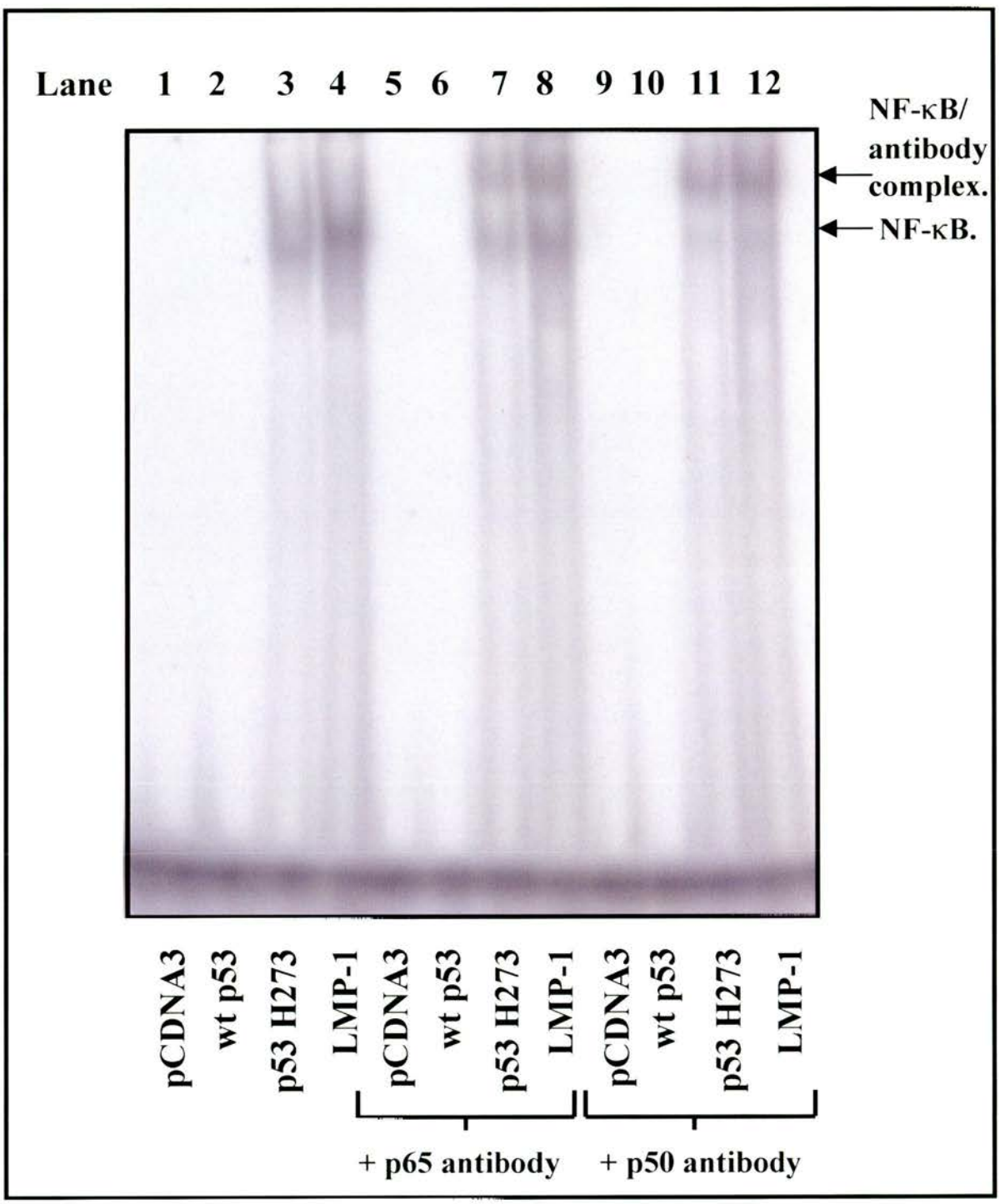


Figure 21. Mutant p53 R273 induces NF-κB DNA binding activity.

EMSA reactions were performed on 5 μg of appropriately transfected nuclear extracts of Saos2 cells. Each assay reaction was also repeated in the presence of p65 and p50 antibody.

reacting with the p65 (lane 8) and p50 antibody (lane 12). By contrast, pcDNA3 did not induce any NF-κB binding (lane 1). Thus these results confirm that transfection of mutant p53 R273H induces NF-κB transcription whereas wild-type p53 does not.

To confirm these results, indirect immunofluorescence of the cells was performed to identify the cellular localisation of NF-κB. Saos2 cells were transfected by lipofectamine with plasmids encoding wild-type p53 and mutant p53 R273H. These cells were then grown on coverslips for 4 hours in Optimem before being changed to D-MEM containing 10% FCS for the next 18 hours to allow a 24 hour transfection period. Following cell fixation, permeabilisation and blocking, endogenous NF-κB and exogenous p53 proteins were monitored by indirect immunofluorescence. In this process, the primary antibody recognises the appropriate protein, whilst the species specific secondary antibody, conjugated to either fluorescein isothiocyanate (FITC) or Texas-Red (TR), binds the primary antibody and upon microscopy with a NIKON MICROPHOT-FXA generates a fluorescent signal. The images were captured with a Spot CCD camera and processed using the Adobe Photoshop 5.0 program (Figure 22).

In these pictures (Fig. 22A-D), the cells were labelled with two different fluorescent antibodies to enable recognition of both endogenous NF-κB and exogenous p53 proteins in the same cell. The p53 proteins were recognised by the anti-mouse FITC secondary antibody binding the primary monoclonal DO-1 antibody whilst NF-κB was identified by a secondary anti-rabbit polyclonal TR antibody binding the primary polyclonal NF-κB antibody.

Figure 22A shows wild-type 53 localised in the nucleus of the cells, which would agree with a role in being transcriptionally active. Figure 22B shows that in these same cells NF-κB is predominantly cytoplasmic. This would confirm that wild-type p53 does not induce NF-κB activity. Mutant p53 H273 (figure 22C), although not as strongly labelled as wild-type 53 is also localised to the nucleus. In these circumstances, NF-κB is also nuclear (Figure 22D).

Therefore these results enhance our observations in the DNA binding assay (Figure 21), in that it is mutant p53 R273H which stimulates NF-κB DNA binding whilst wild-type p53 does not.

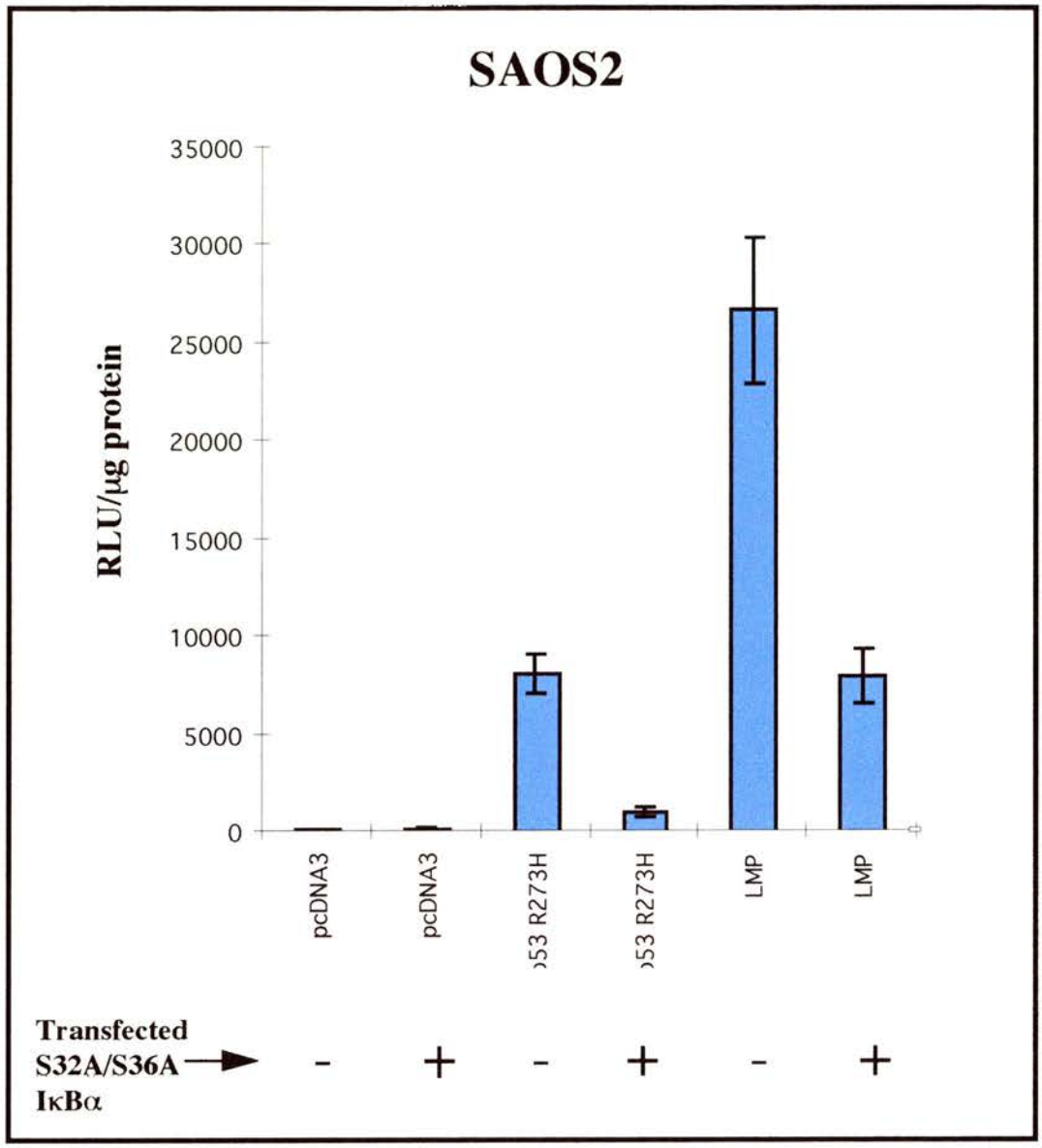
3.2.3 IκBα S32/S36A mutant inhibits NF-κB dependent transcription by p53 R273H.

Reporter assays were performed to detect whether mutant p53 R273H induced NF-κB transcription could be inhibited by the supersupressor of TNF-α induced NF-κB transcription, IκB-α S32A/S36A. Plasmids expressing mutant p53 R273H and IκB-α S32A/S36A were co-transfected by lipofectamine into Saos2 cells with the NF-κB dependent luciferase reporter plasmid 3ECL. This same transfection was also performed without the supersuppressor IκB-α S32A/S36A.

14 hours post-transfection, luciferase activity was measured (Figure 23). The supersuppressor was capable of inhibiting this transcriptional activation by approximately 85%.

Figure 23. Effect of supersuppressor IκBα S32A/S36A on mutant p53 R273H induced NF-κB activity in Saos2 cell line.

Saos2 cell lines were co-transfected with NF-κB dependant luciferase reporter plasmid, 3EnhConALuc and a plasmid encoding for mutant p53 R273H, LMP-1 or pcDNA3 by the lipofectamine method. Furthermore, these transfections were also carried out with or without the presence(indicated by - or + respectively) of supersuppressor IκBα S32A/S36A. Luciferase activity was assayed 14 hours post-transfection and expressed as RLU/μg of cell lysate protein. This is representative of three independent experiments each performed in triplicate with the standard error measured to one standard deviation.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RLU/µg protein
35000
30000
25000
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15000
10000
5000
0
pcDNA3
pcDNA3
53 R273H
53 R273H
LMP
LMP
Transfected
S32A/S36A
IκBα
-
+
-
+
-
+

Negative and positive controls involving pcDNA3 and LMP-1 for this transfection was also assayed for reporter activity. As expected, pcDNA3 did not activate any luciferase activity by 3ECL, with or without IκB-α S32A/S36A whereas LMP-1 did manage to activate NF-κB dependent luciferase activity which was significantly inhibited in the transfection involving IκB-α S32A/S36A.

These transfection experiments were also performed with control luciferase reporter ConALuc (instead of 3ECL), which displayed no significant transcriptional activity (data not shown).

3.2.4 IκBα mRNA levels are not effected by wild-type p53

Our observations showing wild-type p53 not able to induce any NF-κB transcriptional activity did not concur with results published by Ryan *et al.* However, one possibility was that wild-type p53 did induce NF-κB activity, but this activity was inhibited by an unknown mechanism. For example, a huge increase in levels of IκBα expression could inhibit any subsequent NF-κB activity. This could occur as IκBα gene expression is under the control of NF-κB (de Martin, Vanhove et al. 1993; Le Bail, Schmidt-Ullrich et al. 1993). To address this situtation, IκBα mRNA levels were analysed by Northern Blot, following viral infection of Saos2 cells with an adenovirus expressing wild-type p53. This was done over various time points in the first hour and a further time point at 8 hours. A control experiment was also performed using an adenovirus expressing β-galactosidase.

Figure 24A displays that there is no significant change over time in the steady-state level of IκBα transcript following infection by an adenovirus

expressing wild-type p53. This result was backed up by that involving the adenovirus expressing β-galactosidase (Figure 24B). Reporter assays also showed that the adenovirus expressing wild-type p53 was transcriptionally active (Figure 24C). This further supported our results that wild-type p53 did not induce NF-κB activity.

3.2.5 Effect of p53-inducible cell lines on NF-κB activity

As our results disagreed with those observed in Ryan *et al.*, we managed to obtain the p53-inducible Saos2 cell lines used in their paper to perform our reporter assays upon (Ryan, Ernst et al. 2000). Also, we acquired a similar cell line which expressed the mutant p53 R169H. 12 hours prior to inducing the cell line transfections with each of the reporter plasmids, ConALuc, 3ECL and PG13 were set-up in the two cell lines. The cells were then induced by doxycycline for 24 hours. Duplicate transfections were also executed but not induced by doxycycline.

Figure 25A shows that wild-type p53 induced by doxycycline was not capable of activating NF-κB dependent transcription but was capable of activating p53 dependent transcription. The Saos2 p53 R169H inducible cell line (Figure 25B) did not activate any of the luciferase reporters upon induction by doxycycline. These results corresponded with our observation that wild-type p53 did not activate NF-κB dependent transcription.

To establish whether any NF-κB DNA binding activity was induced under these conditions, nuclear extracts were obtained from both cell lines that had been induced by doxycycline for 24 hours. 5μg of each extract was then incubated in a binding mix in the presence of a ^{32}P labelled double-stranded

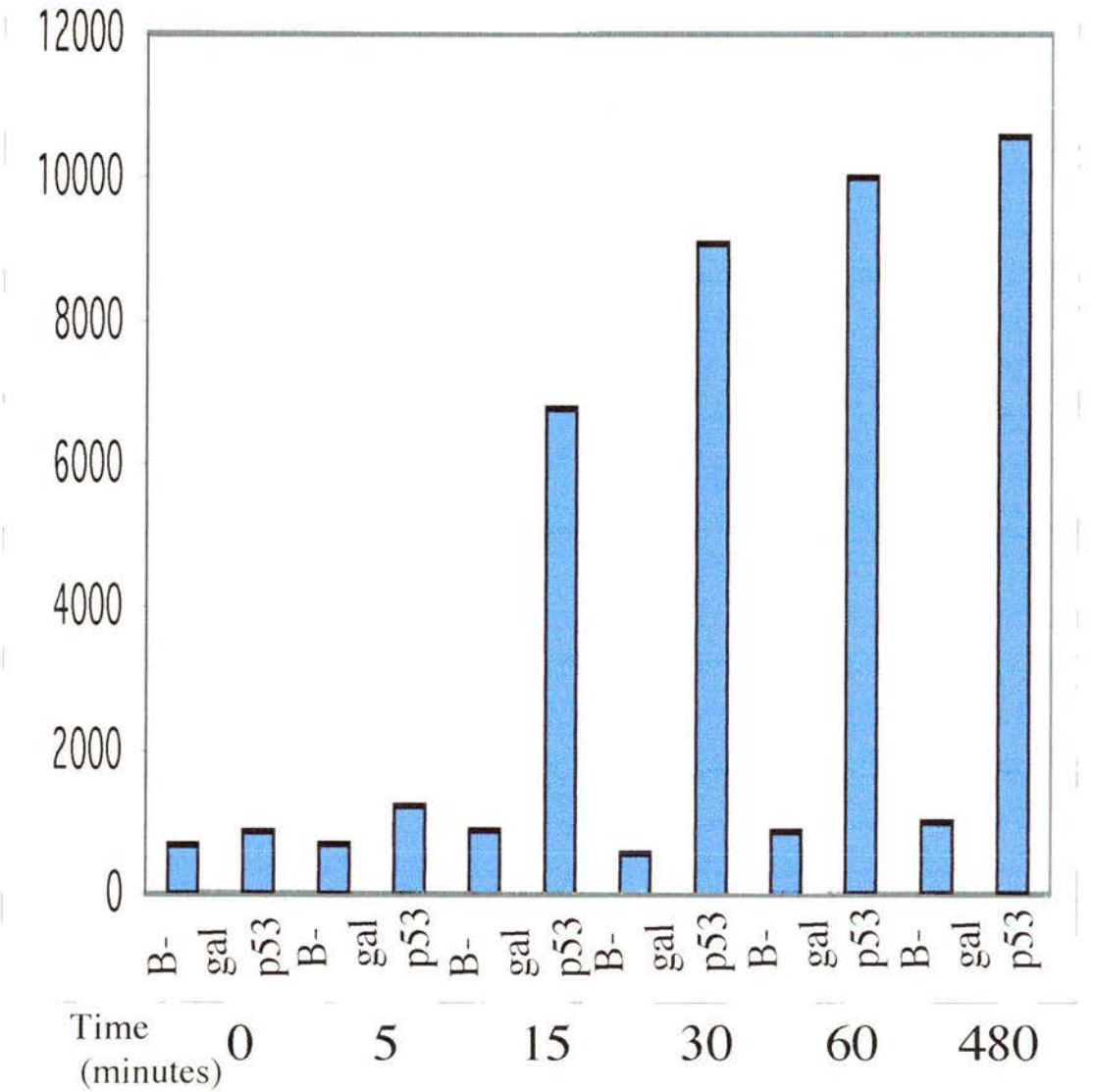
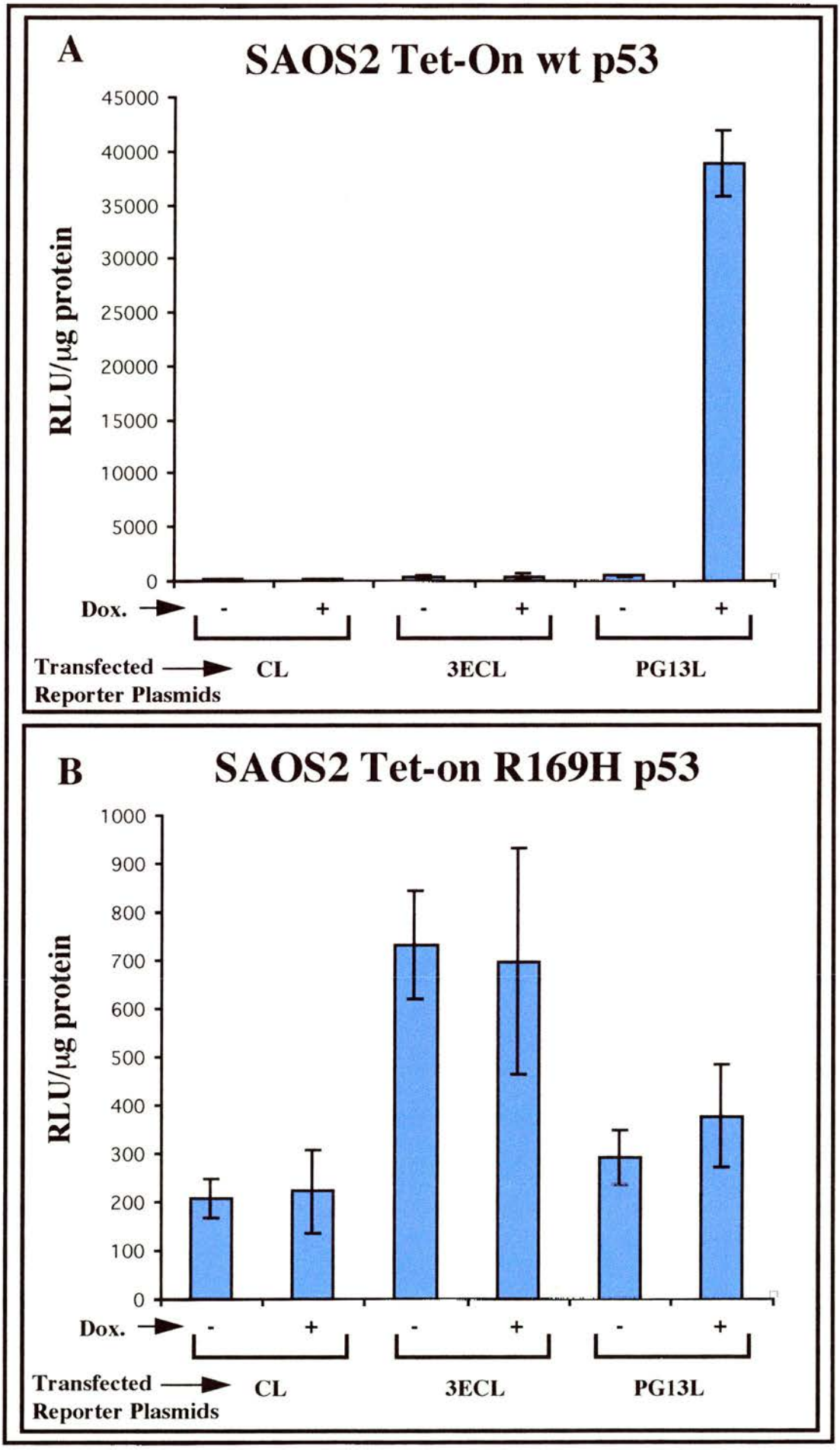


Figure 24C This graph shows that p53 virus is activating transcriptionally a p53-luciferase reporter in the timeframe that samples for the Northern blot were taken.

Figure 25. NF-κB activity in Saos2 cells expressing doxycycline induced wild-type p53

A. Saos2 Tet-on wild-type p53 cell line was transfected with one of three reporter plasmids; 3EnhConALuc (3ECL), PG13L or ConALuc (CL) by the lipofectamine method. After 12 hours, the cells were induced with doxycycline or left unactivated. Luciferase activity was assayed 24 hours post-induction and expressed as RLU/μg of cell lysate protein. This is representative of three independent experiments each performed in triplicate with the standard error measured to one standard deviation. B. The same experiment illustrated in Figure 25A was repeated in the Saos2 Tet-on R169H p53 cell line.


A
SAOS2 Tet-On wt p53
RLU/µg protein
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Dox.
-
+
-
+
-
+
Transfected
Reporter Plasmids
CL
3ECL
PG13L
B
SAOS2 Tet-on R169H p53
RLU/µg protein
1000
900
800
700
600
500
400
300
200
100
0
Dox.
-
+
-
+
-
+
Transfected
Reporter Plasmids
CL
3ECL
PG13L

oligonucleotide which contained a recognition site for NF-κB. These binding assays were also performed in the presence of either p50 or p65 antibody. Following incubation, the complexes were separated on a non-denaturing PAGE, the gel subsequently dried down and exposed to a phosphorimager plate.

Figure 26 shows that doxycycline induced wild-type p53 did stimulate NF-κB DNA binding activity (Lane 6) and that this complex was capable of binding the p65 and p50 antibodies (Lane 7 and 8). This corresponded to the results indicated by Ryan *et al.* Whereas upon doxycycline inducement mutant p53 R169H did not initiate any NF-κB binding activity (Lane 2-4). Control nuclear extracts from uninduced Saos2 wild-type p53 (5) and Saos2 p53 R169H (Lane 1) cell lines also did not exhibit NF-κB DNA binding activity.

This result suggests there may be a link between the two transcription factors but it is one of a repressive nature as no NF-κB transcriptional activation was observed.

3.2.6 Adriamycin stimulation of NF-κB activity is not p53 dependent

Adriamycin has been identified as a potent inducer of both p53 and NF-κB transcription factors. These transcription factors are induced in response to DNA damage mediated by adriamycin. To establish whether a relationship existed between both NF-κB and p53 activation in response to adriamycin (e.g. was NF-κB activation, p53 dependent), reporter assays were carried out in various cell lines with different NF-κB and p53 characteristics (results illustrated in Figure 27 & 28, respectively).

The following transfections were performed in each cell line. Prior to an 8 hour stimulation with adriamycin or TNF-α, cells were transfected for 14

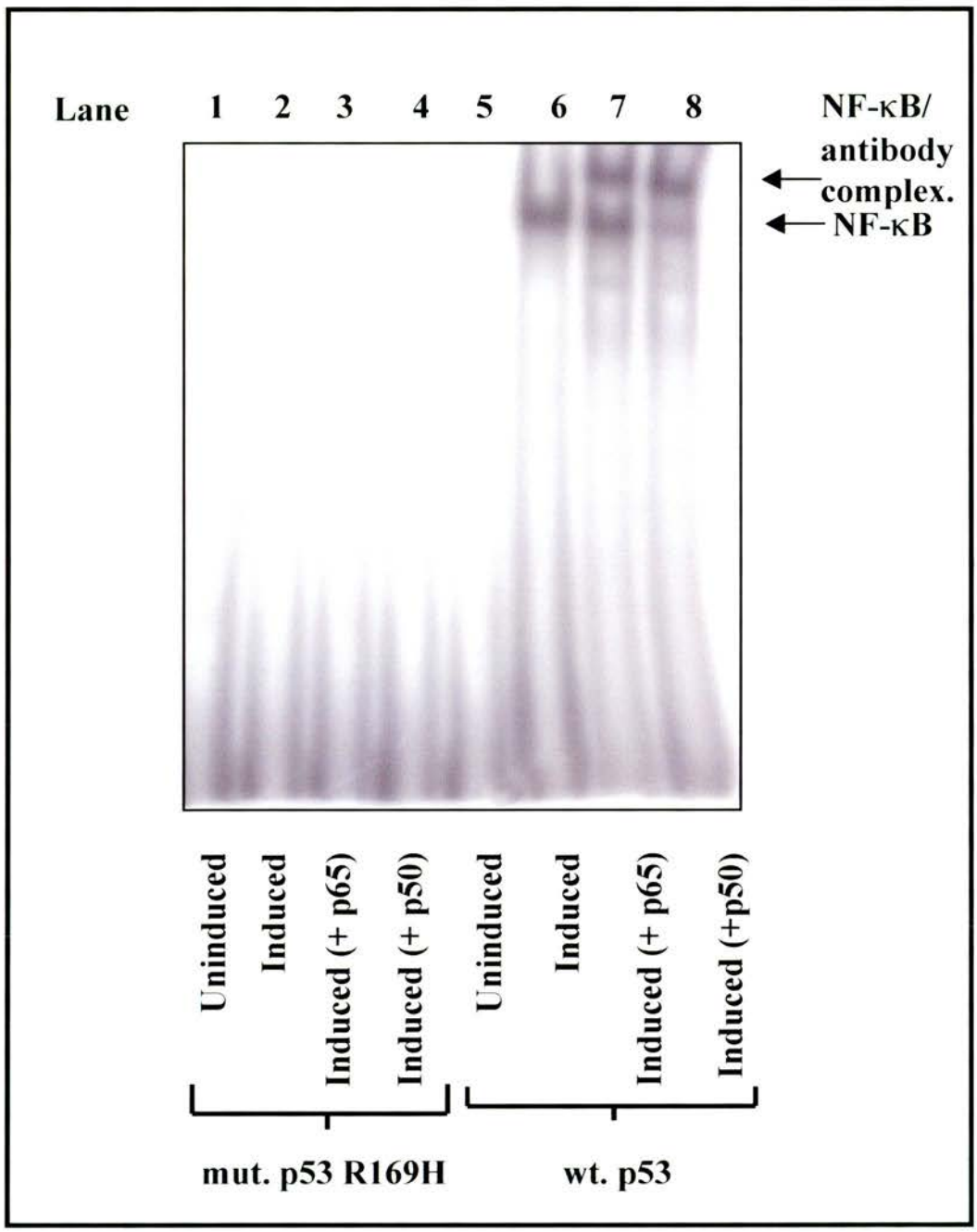
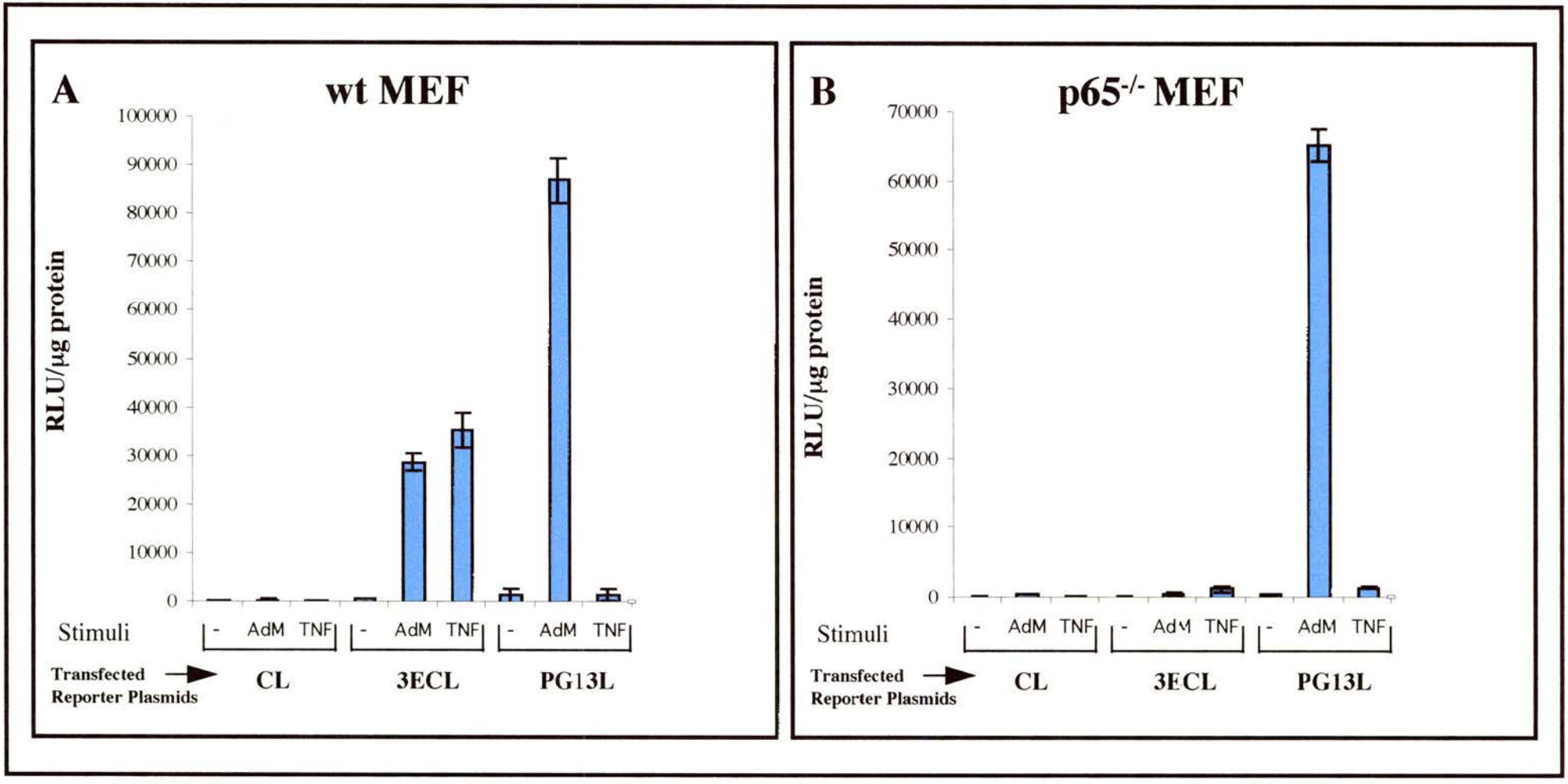


Figure 26. Doxycycline induced cell line expresses wild type p53 to stimulate NF-κB DNA binding activity.

EMSA reactions were performed on 5 μg of nuclear extracts of doxycyline inducible Saos2 cells which expressed wild-type p53 and mutant p53 R169H. These were also repeated in the presence of p65 or p50 antibody. Uninduced extracts from each cell line were also subjected to EMSA (Lane 1 and 5)

Figure 27. Adriamycin activation of NF-κB in cell lines with p65 characteristics.

A. Wild type mouse embryo fibroblasts (wt MEF) were transfected with the reporter plasmid, 3EnhConALuc (3ECL), PG13L or ConALuc (CL) by the lipofectamine method. 14 hours later, transfected cells were induced with 4ng/ml Adriamycin (abbreviated as AdM in figure),10ng/ml TNF-α or left unactivated (indicated by -) for 8 hours in culture before measuring the luciferase activity in cell lysates. Luciferase activity was measured in relation to μg of protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation. B. The same experiment in Figure 27A was repeated in $p65^{-/-}$ mouse embryo fibroblasts.


A
wt MEF
RLU/µg protein
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Stimuli
-
AdM
TNF
-
AdM
TNF
-
AdM
TNF
Transfected
Reporter Plasmids
CL
3ECL
PG13L
B
p65-/- MEF
RLU/µg protein
70000
60000
50000
40000
30000
20000
10000
0
Stimuli
-
AdM
TNF
-
AdM
TNF
-
AdM
TNF
Transfected
Reporter Plasmids
CL
3ECL
PG13L

C. IKK1$^{-/-}$ mouse embryo fibroblasts (IKK1$^{-/-}$ MEF) were transfected with the reporter plasmid, 3EnhConALuc (3ECL), PG13L or ConALuc (CL) by the lipofectamine method. 14 hours later, transfected cells were induced with 4ng/ml Adriamycin, 10ng/ml TNF-α or left unactivated (indicated by -) for 8 hours in culture before measuring the luciferase activity in cell lysates. Luciferase activity was measured in relation to μg of protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation. D. The same experiment in Figure 27C was repeated in IKK2$^{-/-}$ mouse embryo fibroblasts.

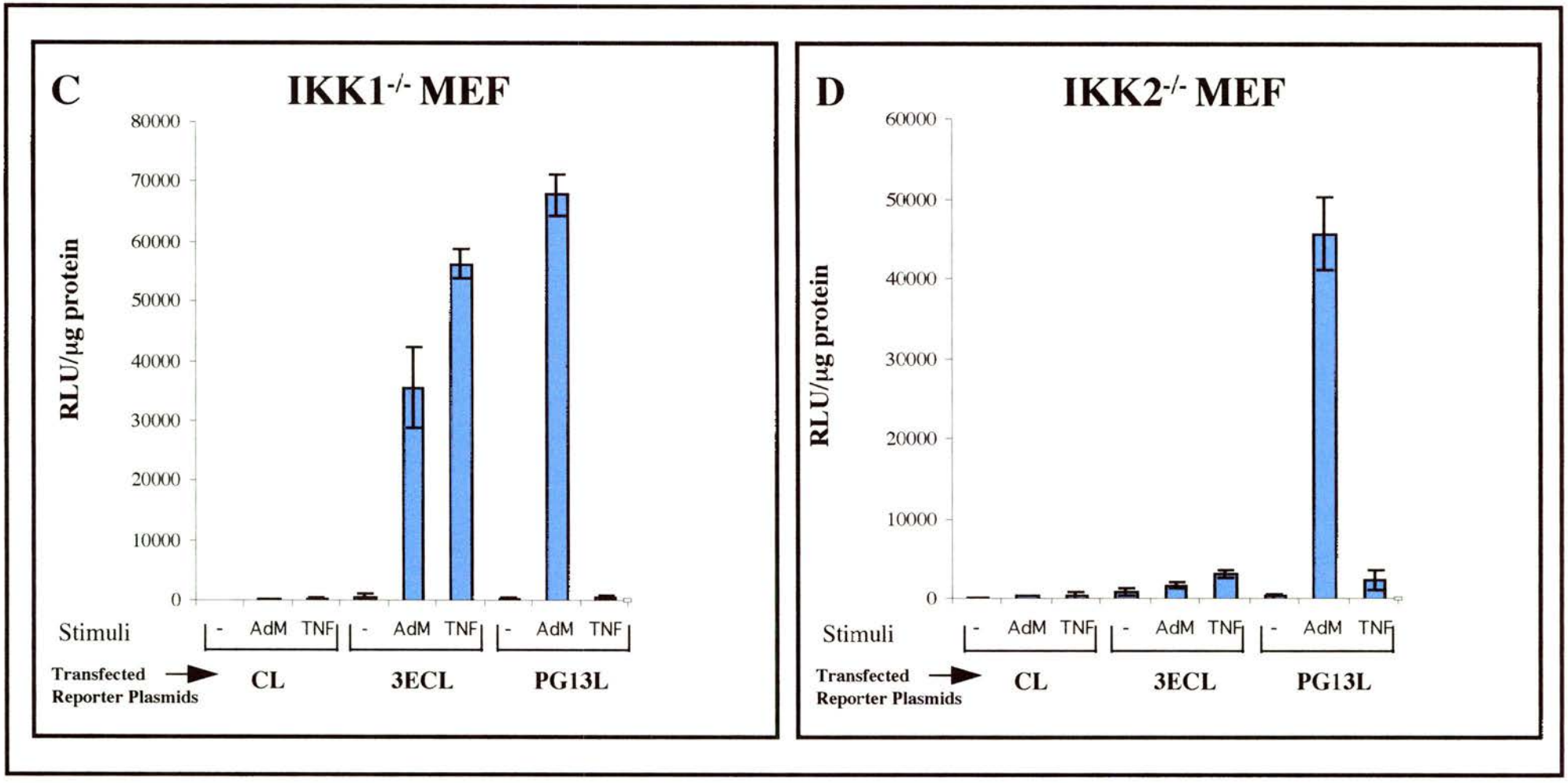
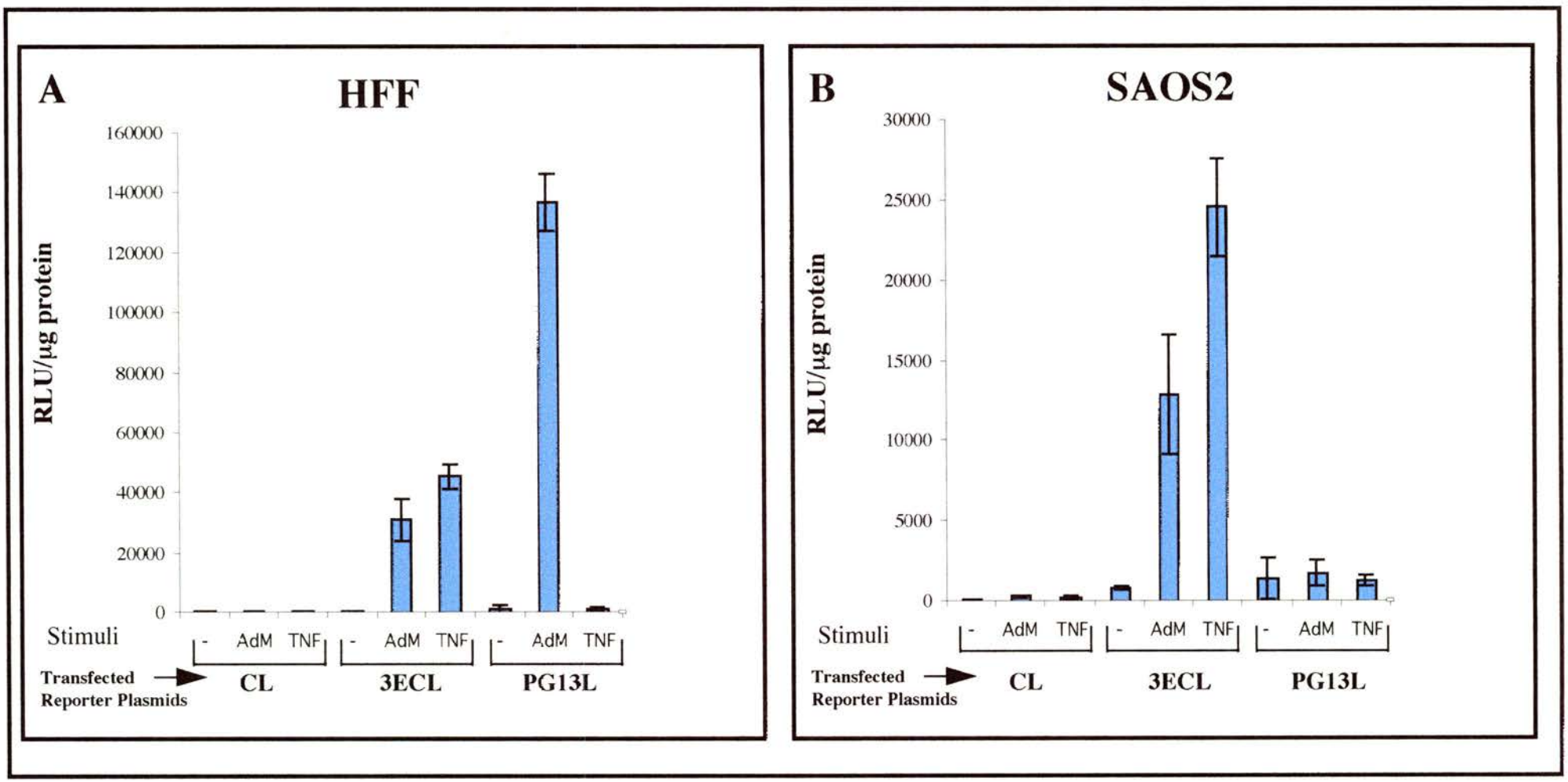

C
IKK1-/- MEF
RLU/μg protein
80000
70000
60000
50000
40000
30000
20000
10000
0
Stimuli
-
AdM
TNF
-
AdM
TNF
-
AdM
TNF
Transfected
Reporter Plasmids
CL
3ECL
PG13L
D
IKK2-/- MEF
RLU/μg protein
60000
50000
40000
30000
20000
10000
0
Stimuli
-
AdM
TNF
-
AdM
TNF
-
AdM
TNF
Transfected
Reporter Plasmids
CL
3ECL
PG13L

Figure 28. Adriamycin activation of NF-κB in cell lines with p53 characteristics.

A. Human foreskin fibroblasts (HFF) were transfected with the reporter plasmid, 3EnhConALuc (3ECL), PG13L or ConALuc (CL) by the lipofectamine method. 14 hours later, transfected cells were induced with 4ng/ml Adriamycin (abbreviated as AdM in figure),10ng/ml TNF-α or left unactivated (indicated by -) for another 8 hours in culture before measuring the luciferase activity in cell lysates. Luciferase activity was measured in relation to μg of protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation. B. The same experiment illustrated in Figure 28A was repeated in Saos2 cells.


A
HFF
RLU/µg protein
160000
140000
120000
100000
80000
60000
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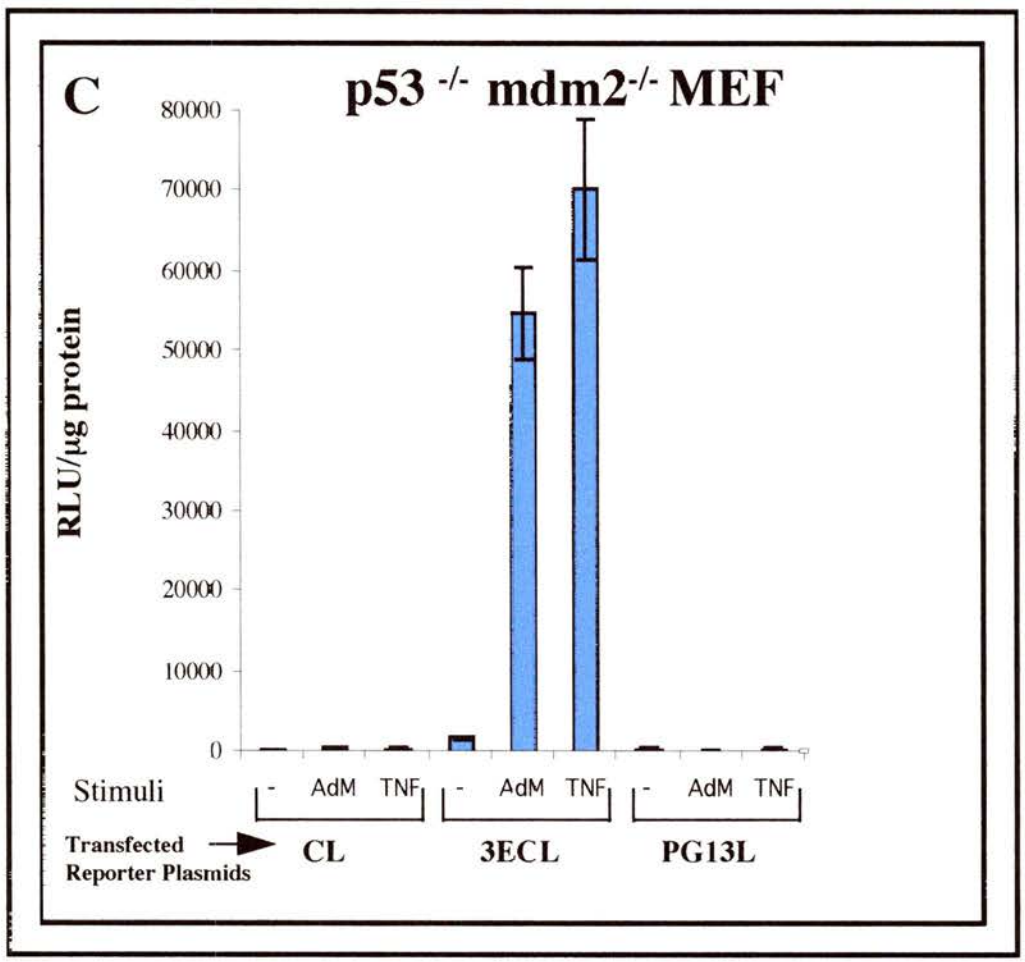
20000
0
Stimuli
-
AdM
TNF
-
AdM
TNF
-
AdM
TNF
Transfected
Reporter Plasmids
CL
3ECL
PG13L
B
SAOS2
RLU/µg protein
30000
25000
20000
15000
10000
5000
0
Stimuli
-
AdM
TNF
-
AdM
TNF
-
AdM
TNF
Transfected
Reporter Plasmids
CL
3ECL
PG13L

C. $p53^{-/-}$ $mdm2^{-/-}$ mouse embryo fibroblasts were transfected with the reporter plasmid, 3EnhConALuc (3ECL), PG13L or ConALuc (CL) by the lipofectamine method. 14 hours later, transfected cells were induced with 4ng/ml Adriamycin,10ng/ml TNF-α or left unactivated (indicated by -) for another 8 hours in culture before measuring the luciferase activity in cell lysates. Luciferase activity was measured in relation to μg of protein. These experiments are representative of at least 3 experiments repeated in triplicate and the error bars are calculated using 1 standard deviation


C
p53 -/- mdm2-/- MEF
RLU/μg protein
80000
70000
60000
50000
40000
30000
20000
10000
0
Stimuli
- AdM TNF
- AdM TNF
- AdM TNF
Transfected Reporter Plasmids
CL
3ECL
PG13L

hours via the lipofectamine method with one of three different reporter plasmids; NF-κB dependent 3ECL, p53 dependent PG13L or ConALuc. A set of equivalent transfections were also assembled which were left unactivated for 8 hours as control.

Figure 27A displays the luciferase activity assayed in wild-type mouse embryo fibroblasts (wt. MEF). Both adriamycin and TNF-α were capable of activating NF-κB activity (29- and 35-fold respectively) which was abolished to basal levels in the $p65^{-/-}$ MEF (Figure 27B). These cells did not contain any p65 subunits. Furthermore, adriamycin could stimulate p53 activity in both cell lines (75 fold in wt MEFs and 140-fold in $p65^{-/-}$ MEFs).

TNF-α is known to induce NF-κB activation via the IKK2 subunit of the IKK complex rather than the IKK1 subunit. Therefore to identify which subunit was transmitting the signal from adriamycin, transfections were repeated in $IKK1^{-/-}$ MEFs (cells lacking the IKK1 subunit) and $IKK2^{-/-}$ MEFs (cells lacking the IKK2 subunit). The results illustrated in Figures 27C and Figure 27D show that adriamycin appears to induce NF-κB activation via the IKK2 subunit. In $IKK1^{-/-}$ MEFs, adriamycin displayed a 35-fold activation of NF-κB which is abolished in the $IKK2^{-/-}$ MEFs. As expected NF-κB activation by TNF-α was normal in $IKK1^{-/-}$ MEFs (55-fold activation) but was reduced to a minimal 2.5 fold activation in the $IKK2^{-/-}$ MEFs. Again, adriamycin was capable of inducing p53 dependent reporter activity in $IKK1^{-/-}$ MEFs (80-fold) and in $IKK2^{-/-}$ MEFs (41.5-fold).

In HFFs (Figure 28A), which contain wild-type p53, adriamycin activated NF-κB 30-fold, while in response to TNF-α a 50-fold activation was generated. Once more, adriamycin activated the p53 dependent luciferase

reporter (180-fold). In the Saos2 and $p53^{-/-}$ $mdm2^{-/-}$ cells, adriamycin activated the NF-κB dependent reporter by 12-fold and 47-fold. However as p53 was lacking in both these cell lines, there was no activation of the p53 dependent luciferase reporter. This established that adriamycin activation of NF-κB was not p53 dependent. TNF-α activated NF-κB 22-fold and 61-fold in the Saos2 and $p53^{-/-}$ $mdm2^{-/-}$ cells, respectively.

The above observations were further validated as adriamycin and TNF-α did not activate the control ConALuc reporter in any cell line. Also, neither NF-κB nor p53 reporters were activated in the absence of inducer. Furthermore, TNF-α did not activate any p53 activity in any of the cell lines.

3.2.7 Effect of $p14^{ARF}$ on NF-κB activity

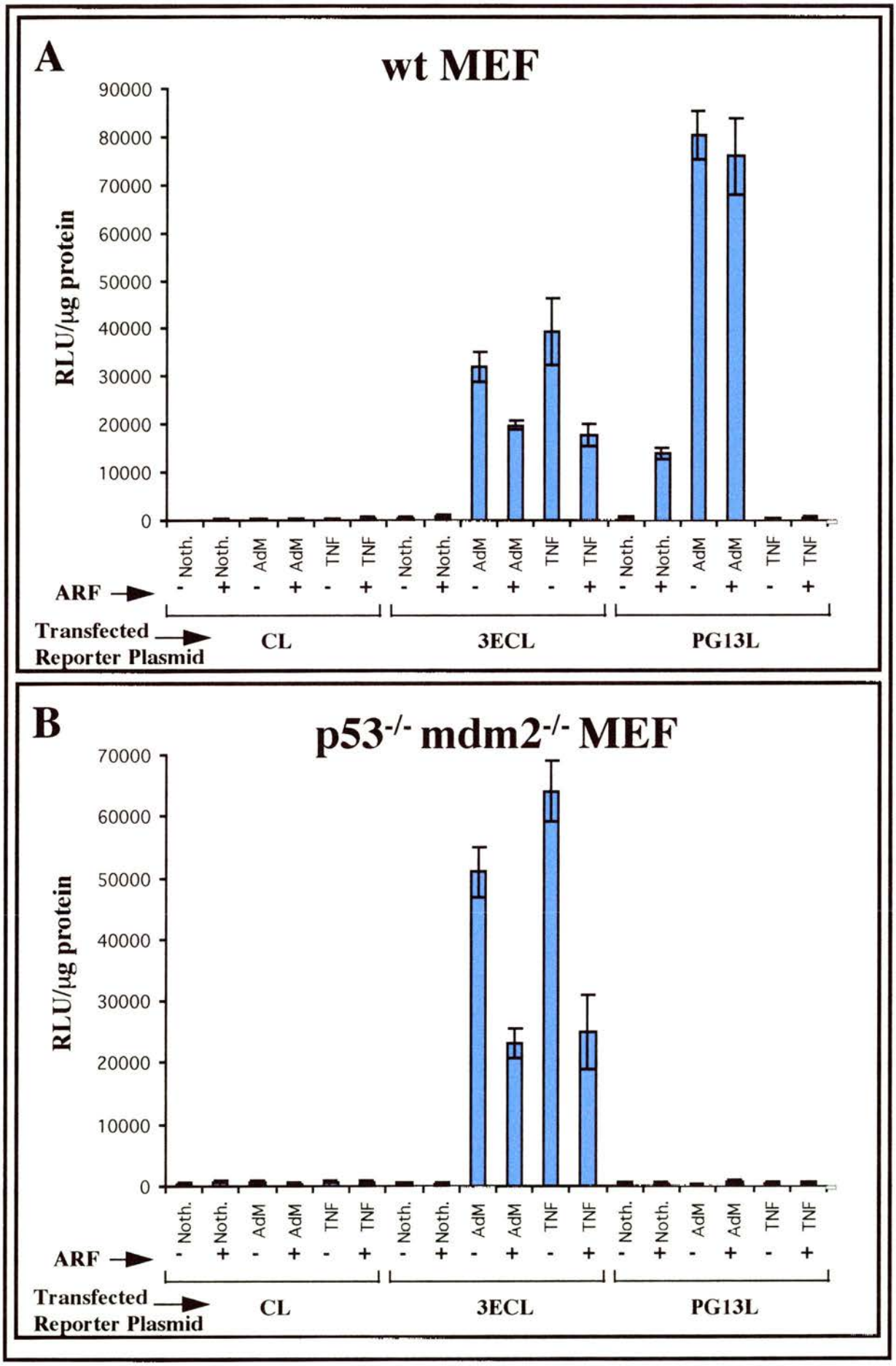
To further enhance our data that wild-type p53 was not required for NF-κB activity, alternative reading frame product, ($p14^{ARF}$) was utilised to determine its effect on both p53 dependent and NF-κB dependent transcription. $p14^{ARF}$ is known to enhance p53-mediated transcriptional activity by interfering with p53's negative regulator Mdm2.

The expression plasmid encoding $p14^{ARF}$ was cotransfected with each of the following luciferase reporters into wild-type mouse embryo fibroblasts (wt MEF); NF-κB dependent 3EnhConALuc (3ECL), p53 dependent PG13L and the control ConALuc (CL). 14hours post-transfection the cells were stimulated with either adriamycin, TNF-α or left unactivated for 8 hours. Duplicate transfections were also performed in the absence of $p14^{ARF}$.

As expected ConALuc was not activated (Figure 29A). However, adriamycin induction of NF-κB activity was reduced by a third in the presence

Figure 29. Effect of $p14^{ARF}$ on NF-κB activity induced by adriamycin or TNF-α

A. Wild-type mouse embryo fibroblasts were transfected with one of three reporter plasmids; 3EnhConALuc, PG13L or ConALuc by the lipofectamine method. Furthermore, in certain experiments, $p14^{ARF}$ (denoted as ARF in figure) was also co-transfected alongside the reporter plasmids(ARF presence indicated by - or + respectively) 14 hours post-transfection, the cells were stimulated for a further 8 hours in the presence of either adriamycin, TNF-α or left unactivated. Luciferase activity was assayed 22 hours post-transfection and expressed as RLU/μg of cell lysate protein. This results are representative of three independant experiments each performed in triplicate with the standard error measured to one standard deviation.


A
wt MEF
RLU/μg protein
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
- Noth.
+ Noth.
- AdM
+ AdM
- TNF
+ TNF
ARF
Transfected Reporter Plasmid
CL
3ECL
PG13L
B
p53-/- mdm2-/- MEF
RLU/μg protein
70000
60000
50000
40000
30000
20000
10000
0
- Noth.
+ Noth.
- AdM
+ AdM
- TNF
+ TNF
ARF
Transfected Reporter Plasmid
CL
3ECL
PG13L

of $p14^{ARF}$. This result was also observed by TNF-α activation of NF-κB where $p14^{ARF}$ decreased NF-κB activity by at least a half. However, the presence of $p14^{ARF}$ did not inhibit adriamycin activation of p53 dependent luciferase reporter PG13L.

To clarify whether this inhibition was dependent on p53, transfections were repeated in the $p53^{-/-}$ $mdm2^{-/-}$ MEFs. Both adriamycin and TNF-α activation of NF-κB was also reduced within the presence of $p14^{ARF}$ in this cell line (Figure 29B). As neither adriamycin or TNF-α could induce p53 activity, it was established that these effects of $p14^{ARF}$ upon NF-κB activity did not involve wild-type p53. ConALuc was also not activated in this cell line.

3.2.8 Discussion

The tumour suppressor p53 plays a pivotal role in the cellular response to DNA damage, by controlling regulation of processes such as DNA repair, cell cycle growth arrest and apoptosis. The transcription factor, NF-κB, also a key regulator of the apoptotic process, was shown to exhibit DNA-binding activity, upon induction by p53 (Ryan, Ernst et al. 2000). This led to an enhancement of p53-mediated apoptosis. Furthermore, this stimulation of NF-κB DNA binding was induced by a pathway involving the $pp90^{RSK}$ kinase.

In our studies, we analysed the effects of p53 upon NF-κB transcriptional activation. Figures 19 and 20 illustrate that when exogenous wild-type p53 is transfected into cell lines lacking p53 (Saos2 and $p53^{-/-}mdm2^{-/-}$ MEFs), there is no activation of NF-κB. However in the same cells, the exogenous p53 was capable of activating p53 dependent transcription from the PG13 luciferase reporter. Thus p53 is transcriptionally active under these

conditions. Even in cells containing endogenous wild-type p53 (HFF), transfection of exogenous wild-type p53 did not result in NF-κB activation (Figure 20).

However the mutant form of p53 (p53 R273H) was capable of activating NF-κB dependent transcription in HFF, Saos2 and $p53^{-/-}mdm2^{-/-}$ MEF cell lines. By comparison mutant p53 R175H was not capable of inducing NF-κB dependent transcription in any of these cell lines.

Surprisingly, both the transcriptionally inactive forms of mutant p53 did display some p53-dependant luciferase activity in HFFs (Figure 18). As neither pcDNA3 (-ve control) nor LMP-1 (+ve control) showed any p53 dependant transcriptional activation, it was possible that the transfection of p53 mutants might somehow induce the wild-type p53 present in the HFFs, especially as this p53-dependant luciferase activity was not observed in equivalent transfections in cell lines lacking p53.

Activation of NF-κB involves signal induced translocation of NF-κB to the cell nucleus. To establish that mutant p53 R273H, induced the nuclear translocation of NF-κB, both a DNA-binding assay (Figure 21) and indirect immunofluorescence analysis (Figure 22) was performed upon Saos2 cells transfected with the plasmid encoding mutant p53 R273H. Correspondingly, the same experiments were performed with wild-type p53, to establish that wild-type p53 did not induce translocation of NF-κB to the nucleus. Figure 21 reveals the presence of NF-κB in the nuclei of Saos2 cells following transfection by mutant p53 R273H (Lane 3). It is probable that this NF-κB form comprises the p50/p65 heterodimer as antibodies against p65 and p50 resulted in a shift-up (Lane 7 and 11). By contrast, transfection of the wild-type form of p53 did not

result in the nuclear presence of NF-κB (Lane 2). To further confirm these results involving wild-type p53, the immunofluorescence pictures in Figure 23A & B demonstrate that cells transfected with wild-type 53 do not bring about nuclear localisation of NF-κB. Figure 23A shows cells containing exogenous p53 in the nucleus. In these same cells, Figure 23B clearly shows endogenous NF-κB to be predominantly cytoplasmic. In the case of mutant p53 R273H, Figures 23C and 23D show both exogenous mutant p53 and endogenous NF-κB to be nuclear, respectively. As mutant p53 R273H is nuclear, this does not suggest it is transcriptionally active, but it is in a stable formation, because the presence of p53 in the cytoplasm usually leads to ubiquitin-mediated degradation.

To establish whether NF-κB activity induced by mutant p53 R273H could be blocked by the super-repressor IκBα S32A/S36A mutant, cotransfections with the two plasmids was performed in Saos2 cells. In comparison to NF-κB activity induced by mutant p53 R273H alone, the presence of IκBα S32A/S36A resulted in an 85% inhibition (Figure 24). This inhibition of activity was even greater than that observed in the inhibition caused by IκBα S32A/S36A upon LMP-1 induced NF-κB activity. This suggests that mutant p53 R273H induces NF-κB activation by targeting the phosphorylation of IκBα. (as accomplished by TNF-α induction of NF-κB).

NF-κB regulates its own autoregulation by transcribing IκBα (inhibitor of IκBα). As reporter assays require at least 6 hours post-transfection before any significant phenomena can be observed, a Northern Blot was performed to analyse the levels of IκBα mRNA within the first hour. This would help to clarify that wild-type p53 did not induce NF-κB activity instantly which could

be repressed by an increase in NF-κB transcription of IκBα at a later timepoint. An adenovirus expressing the wild-type p53 was utilised to infect Saos2 cells. IκB mRNA levels did not differ upon infection over the first hour and a further timepoint at eight hours showed no substantial change in levels. Therefore it was clear that the presence of high levels of wild-type p53 did not trigger any NF-κB activity within a short timeframe. Furthermore IκBα mRNA levels were not upregulated in response to wild-type p53.

As our results involving wild-type p53 did not appear to activate NF-κB, we managed to obtain the special cell lines utilised in the Ryan paper. Once again our reporter assays failed to show any NF-κB activation in response to wild-type p53 induction (Figure 25). However, the DNA binding assay (Figure 26) did display the presence of NF-κB in the nucleus upon p53 induction. In corroboration with the negligible NF-κB reporter activity, it has to be assumed that this form of nuclear NF-κB is in a repressive state. Therefore, it is probable that wild-type p53 induces NF-κB in a repressive form as opposed to the active form stimulation by TNF-α. Why this form of NF-κB was not present in the DNA binding assay in Figure 21, is not apparent. Could it be due to the levels of wild-type p53 that is required to stimulate this phenomena. When we transfect in wild-type p53, we are essentially overexpressing. It is possible that the endogenous levels induced by this specially modified cell line is appropriate to stimulate NF-κB repression.

To further investigate potential p53-induced NF-κB activity, adriamycin a DNA damaging agent and stimulator of both transcription factors was utilised. The reporter assays were performed in cell lines with different NF-κB characteristics (Figure 27) and different p53 characteristics (Figure 28). Figure

27 involved cell lines which were derived from mice that had been crossbred to produce a phenotype that resulted in the homologous deletion of p65, IKK1 and IKK2 (Figures 27B, C and D). Results established that it was essential for the presence of both p65 and IKK2 to enable NF-κB activation in response to adriamycin, as well as TNF-α. IKK1 did not appear to exert any inhibition of NF-κB activity upon stimulation by adriamycin. Although it looked like that adriamycin utilised a similar mechanism to activate NF-κB as TNF-α, it was not established if this was p53 dependent. To evaluate this hypothesis, the equivalent reporter assays were performed in p53 deficient cell lines (Figure 28B & C). Our results displayed no loss of NF-κB activation, thus determining that the pathway leading to NF-κB activation was p53-independent. Furthermore, Figure 27B verified that adriamycin activation of p53 was p65 independent.

The last set of figures (Figure 29) illustrate that $p14^{ARF}$ inhibits NF-κB activity stimulated by either adriamycin or TNF-α. This inhibition of NF-κB is an exciting result given $p14^{ARF}$'s link with p53. $p14^{ARF}$ is known to stimulate p53 dependent transcription by interacting with the inhibitory regulator of p53, mdm2. Although in wild type mouse embryo fibroblasts, it did not enhance adriamycin induction of p53, the activity of p53 was very high such that the influence of $p14^{ARF}$ may not induce much more activity. Thus further experiments are required to explore the role of $p14^{ARF}$ in downregulating NF-κB activity. Cells lacking $p14^{ARF}$ would enable us to confirm this role upon NF-κB dependent transcription.

4. CONCLUSIONS

4.1 The role of the N-terminus of IκBα.

The transcription factor, NF-κB bound to the inhibitory protein IκB exists as a dynamic complex in unstimulated cells.. TNF-α, IL-1β and LMP-1 are well characterised stimulators of NF-κB. In particular, they target the activation of the trimeric complex consisting of the hetrodimeric p50/p65 form of NF-κB bound to IκBα via the IKK complex, resulting in the nuclear translocation of NF-κB and ensuing NF-κB activity. TNF-α induction in the presence of proteasome inhibitors results in the detection of both hyperphosphorylated and ubiquitinated forms of IκBα (Traenckner, Pahl et al. 1995; Roff, Thompson et al. 1996). Subsequently, a mutant containing serine to alanine substitutions at residues 32 and 36 was revealed to inhibit both phosphorylation and ubiquitination of IκBα (Brown, Gerstberger et al. 1995; DiDonato, Mercurio et al. 1996; Roff, Thompson et al. 1996). Further studies revealed that lysine residues 21 and 22 were the primary residues to be involved in ubiquitination (Scherer, Brockman et al. 1995; Rodriguez, Wright et al. 1996). Therefore, although the N-terminus is highly susceptible to proteolytic activity (Jaffray, Wood et al. 1995), and is highly unstructured, its importance as the signal response domain of IκBα is apparent.

The cluster of IκBα modifications occurring over a region spanning 15 residues in the N-terminus, initiated our search for a motif on IκB-α which could be recognised by the IKK complex following stimulation by the pathway shared by LMP, TNF-α and IL-1β. Hence the generation of alanine mutants

encompassing residues 14-42 of the N-terminus of IκBα to identify amino acids of interest.

Although a motif was not characterised, the results obtained confirmed the significance of certain residues within the N-terminus. Foremost, the singular importance of serines 32 and 36, and also lysines 21 and 22 was established. Previous work had determined that when both serines or both lysines are mutated to alanine, there is inhibition of NF-κB activity in response to TNF-α. This work validated these findings by showing when these residues were singly mutated to alanine, their effect on NF-κB activity by the TNF-α pathway was inhibited. However, as expected it was never as great as the inhibition involving the double serine to alanine mutant form of IκBα. Also, our results implicated a requirement for both histidine 30 and tyrosine 42. The importance of histidine 30 was probably more structural than as a result of any type of modification. On the other hand tyrosine 42 was probably due to a phosphorylation modification as it had already been implicated in IκBα phosphorylation but not via the IKK2 pathway.

Yaron *et al.* suggested the importance of aspartic acid 31 and glycine 33 in an IκBα consensus motif DSG**S in interactions with the E3 ubiquitin ligase component β-trcp. (Yaron, Hatzubai et al. 1998). However our results did not show any significant inhibitory effects upon NF-κB activation of the D31A or G33A mutations. Thus although there appears to be a high level of conservation of IκBα between different species (Figure 8), only a handful of these residues in human IκB appear to be significant. Therefore although we have not identified a recognition motif, our results have verified the importance of specific residues in the N-terminus of IκBα in relation to NF-κB transcriptional activation.

4.2 The effect of p53 on NF-κB activity.

The loss of p53-dependent apoptosis in $p65^{-/-}$ mouse embryo fibroblasts implicates a possible link between p53 and p65. However, numerous studies have indicated that NF-κB activation can suppress cell death pathways by inducing the expression of anti-apoptotic genes such as cIAP1 & 2. Furthermore, activation of NF-κB has been shown to be required to protect cells from the apoptotic cascade initiated by TNF-α (Barkett and Gilmore 1999). Therefore what gain could p53 possibly achieve from NF-κB preventing apoptosis, when p53 itself is intent on promoting apoptosis.

In truth, this anti-apoptotic characteristic of NF-κB enables the cell in the advancement of oncogenesis. For example, our findings showed that mutant p53 R273H activated NF-κB transcriptionally. Mutations in p53 are one of the primary requirements for a cell to become tumourigenic. Therefore, in a biological scenario NF-κB activation would benefit cancerous growth of cells by utilising the anti-apoptotic machinery switched on by NF-κB. Hence, this would help to repel the defence mechanisms initiated by the host to remove these cells. Moreover, NF-κB is also known to activate growth factors which would help to increase spread of the cancer.

In equivalent experiments, wild-type p53 did not induce any NF-κB-dependent transcriptional activation. Nevertheless, Figure 26 did show the presence of NF-κB DNA binding activity in Saos2 cells expessing wild-type p53 upon doxycycline induction (as shown previously by Ryan *et al.* 2000). However following reporter assays, it was demonstrated that this NF-κB was

not transcriptionally active (figure 25). To explain this result, it could be possible that wild-type p53 can in certain circumstances induce a repressive form of NF-κB which would prevent transcription of anti-apoptotic genes. This, in turn would enhance p53-dependent apoptosis of cells allowing p53 to function efficiently as a tumour suppressor. Therefore, as p53 has opposing apoptotic roles to NF-κB, it may find it necessary to induce this repressive form of NF-κB, so that it can function efficiently without worrying about any potential drawbacks.

Although, we have suggested ideas as to how wild-type and mutant p53 can activate NF-κB for their own needs, it is not apparent how these effects are being initiated. It is probable that mutant p53 R273H triggers off the IKK2 pathway leading to IκBα phosphorylation and subsequent NF-κB activity (as shown by the inhibitory effects of IκBα S32A/S36A in Figure 23). But as it does not mediate p53-dependent transcription, it is probably via a protein-protein interaction that it is inducing NF-κB activity. In the case for wild-type p53, it is possible that coactivator protein p300/CBP performs a role. It has been previously shown that both p53 and the p65 subunit of NF-κB interact with this protein (Webster and Perkins 1999). Although, how p53 initiates the NF-κB translocation to the nucleus cannot be explained, it is possible that p53 uses up the limited pool of p300/CBP during its p53-dependent transcription. This depletes any coactivator that NF-κB could utilise to function transcriptionally, thus its existence in a repressive state following p53 transactivation. Therefore even although it activates NF-κB activity the fact it is repressive maybe just a consequence of p300/CBP depletion and that it is not actually a targeted function of p53.

To try and establish this antagonistic relationship between NF-κB and p53, the DNA-damaging agent, adriamycin which is known to activate both transcription factors was utilised to try and help interpret what was occurring. Our results (Figures 27 & 28) suggest that via adriamycin, there is no inter-relationship between the two proteins and that both proteins are activated by independent mechanisms. Moreover our results did manage to determine that adriamycin activates NF-κB dependent transcription via the IKK2 subunit of the IKKcomplex.

The experiments involving $p14^{ARF}$ did however manage to formulate a link between the two opposing transcription factors. What gain $p14^{ARF}$ obtains from inhibiting NF-κB activity is not apparent. But from the perspective of p53, inhibition of NF-κB activity appears to be of beneficial consequence. $p14^{ARF}$ is known to induce p53 dependent transactivation. One pathway involves sequestering mdm2 so that it cannot bind p53, thus liberating p53 to function transcriptionally. (Weber, Taylor et al. 1999). As explained before, NF-κB activates an anti-apoptotic response. Therefore as well as activating p53, $p14^{ARF}$ inhibiting NF-κB activity (Figure 29B), is further enhancing p53 activation of the pro-apoptotic pathway. Interestingly though in these circumstances, there appears to be no direct link between NF-κB and p53. In $p53^{-/-}mdm2^{-/-}$ MEFs, activation of NF-κB by both TNF-α and adriamycin was inhibited. Furthermore, even although $p14^{ARF}$ interacts with mdm2, Saos2 cells which contain endogenous mdm2, exhibited the same inhibitory response to NF-κB upon stimulation by TNF-α or adriamycin (data not shown). Therefore the presence of neither p53 or mdm2 is required for $p14^{ARF}$ inhibition of NF-κB. But these preliminary results suggest that $p14^{ARF}$ could be the critical link between the

opposing effects of p53 upon NF-κB. On one side it can activate p53-dependent transcription whilst on the other side it can result in the inhibition of NF-κB activity.

Therefore it seems there are many factors to consider before linking p53 to NF-κB. It is clear that a better understanding between p53 and NF-κB is required, to enable us to comprehend fully the implications their intricate relationship takes on the life or death of a cell (Figure 30).

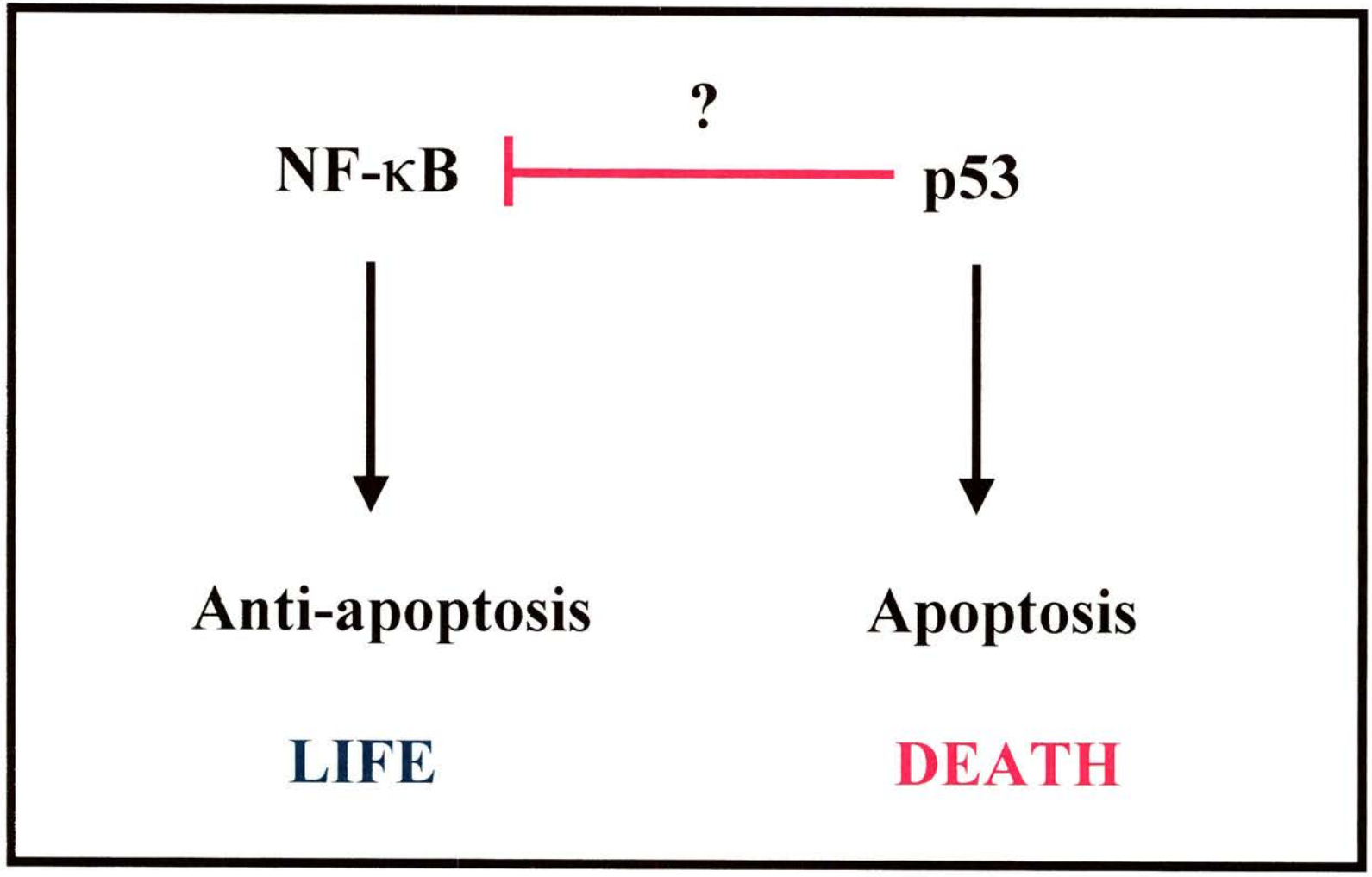


Figure 30 Is there a link between p53 and NF-κB in determining the fate of a cell?

5. APPENDIX

5.1 Internal Mutagenic Primers

E14A-IκBα

Upstream 5' -TGGGCCATGGCGGGCCCCCGC- 3'

Downstream 5' -GCGGGGGCCCGCCATGGCCCA- 3'

G15A-IκBα

Upstream 5' -GCCATGGAGGCGCCCCGCGAC- 3'

Downstream 5' -GTCGCGGGGCGCCTCCATGGC- 3'

P16A-IκBα

Upstream 5' -ATGGAGGGCGCCCGCGACGGG- 3'

Downstream 5' -CCCGTCGCGGGCGCCCTCCAT- 3'

R17A-IκBα

Upstream 5' -GAGGGCCCCGCCGACGGGCTG- 3'

Downstream 5' -CAGCCCGTCGGCGGGGCCCTC- 3'

D18A-IκBα

Upstream 5' -GGCCCCCGCGCCGGGCTGAAG- 3'

Downstream 5' -CTTCAGCCCGGCGCGGGGGCC- 3'

G19A-IκBα

Upstream 5' -CCCCGCGACGCGCTGAAGAAG- 3'

Downstream 5' -CTTCTTCAGCGCGTCGCGGGG- 3'

L20A-IκBα

Upstream 5' -CGCGACGGGGCGAAGAAGGAG- 3'

Downstream 5' -CTCCTTCTTCGCCCCGTCGCG- 3'

K21A -IκBα

Upstream 5' -GACGGGCTGGCGAAGGAGCGG- 3'

Downstream 5' -CCGCTCCTTCGCCAGCCCGC- 3'

K22A -IκBα

Upstream 5' -GGGCTGAAGGCGGAGCGGCTA- 3'

Downstream 5' -TAGCCGCTCCGCCTTCAGCCC- 3'

E23A -IκBα

Upstream 5' -CTGAAGAAGGCGCGGCTACTG- 3'

Downstream 5' -CAGTAGCCGCGCCTTCTTCAG- 3'

R24A -IκBα

Upstream 5' -AAGAAGGAGGCGCTACTGGAC- 3'

Downstream 5' -GTCCAGTAGCGCCTCCTTCTT- 3'

L25A -IκBα

Upstream 5' -AAGGAGCGGGCACTGGACGAC- 3'

Downstream 5' -GTCGTCCAGTGCCCGCTCCTT- 3'

L26A -IκBα

Upstream 5' -GAGCGGCTAGCGGACGACCGC- 3'

Downstream 5' -GCGGTCGTCCGCTAGCCGCTC- 3'

D27A -IκBα

Upstream 5' -CGGCTACTGGCCGACCGCCAC- 3'

Downstream 5' -GTGGCGGTCGGCCAGTAGCCG- 3'

D28A -IκBα

Upstream 5' -CTACTGGACGCCCGCCACGAC- 3'

Downstream 5' -GTCGTGGCGGGCGTCCAGTAG- 3'

R29A-IκBα

Upstream 5' -CTGGACGACGCCCACGACAGC- 3'

Downstream 5' -GCTGTCGTGGGCGTCGTCCAG- 3'

H30A-IκBα

Upstream 5' -GACGACCGCGCCGACAGCGGC- 3'

Downstream 5' -GCCGCTGTCGGCGCGGTCGTC- 3'

D31A-IκBα

Upstream 5' -GACCGCCACGCCAGCGGCCTG- 3'

Downstream 5' -CAGGCCGCTGGCGTGGCGGTC- 3'

S32A-IκBα

Upstream 5' -CGCCACGACGCCGGCCTGGAC- 3'

Downstream 5' -GTCCAGGCCGGCGTCGTGGCG- 3'

G33A-IκBα

Upstream 5' -CACGACAGCGCCCTGGACTCC- 3'

Downstream 5' -GGAGTCCAGGGCGCTGTCGTG- 3'

L34A-IκBα

Upstream 5' -GACAGCGGCGCGGACTCCATG- 3'

Downstream 5' -CATGGAGTCCGCGCCGCTGTC- 3'

D35A-IκBα

Upstream 5' -AGCGGCCTGGCCTCCATGAAA- 3'

Downstream 5' -TTTCATGGAGGCCAGGCCGCT- 3'

S36A-IκBα

Upstream 5' -GGCCTGGACGCCATGAAAGAC- 3'

Downstream 5' -GTCTTTCATGGCGTCCAGGCC- 3'

M37A-IκBα

Upstream 5' -CTGGACTCCGCGAAAGACGAG- 3'

Downstream 5' -CTCGTCTTTCGCGGAGTCCAG- 3'

K38A-IκBα

Upstream 5' -GACTCCATGGCAGACGAGGAG- 3'

Downstream 5‘ -CTCCTCGTCTGCCATGGAGTC- 3’

D39A-IκBα

Upstream 5‘ -TCCATGAAAGCCGAGGAGTAC- 3’

Downstream 5‘ -GTACTCCTCGGCTTTCATGGA- 3’

E40A-IκBα

Upstream 5‘ -ATGAAAGACGCGGAGTACGAG- 3’

Downstream 5‘ -CTCGTACTCCGCGTCTTTCAT- 3’

E41A-IκBα

Upstream 5‘ -AAAGACGAGGCGTACGAGCAG- 3’

Downstream 5‘ -CTGCTCGTACGCCTCGTCTTT- 3’

Y42A-IκBα

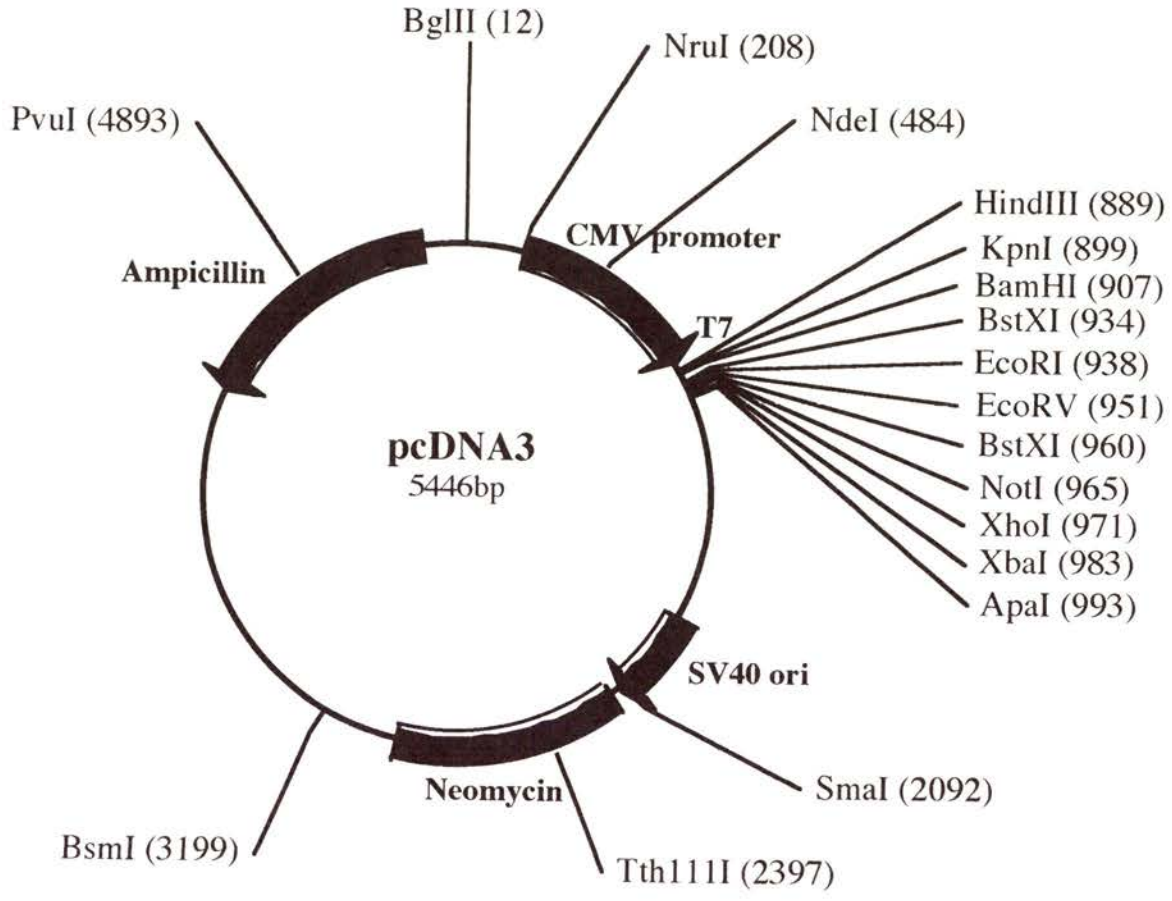
Upstream 5‘ -GACGAGGAGGCCGAGCAGATG- 3’

Downstream 5‘ -CATCTGCTCGGCCTCCTCGTC- 3’

S32A/S36A-IκBα

Upstream 5‘ -GCCACGACGCCGGCCTGGACGGCGTCCAGGC- 3’

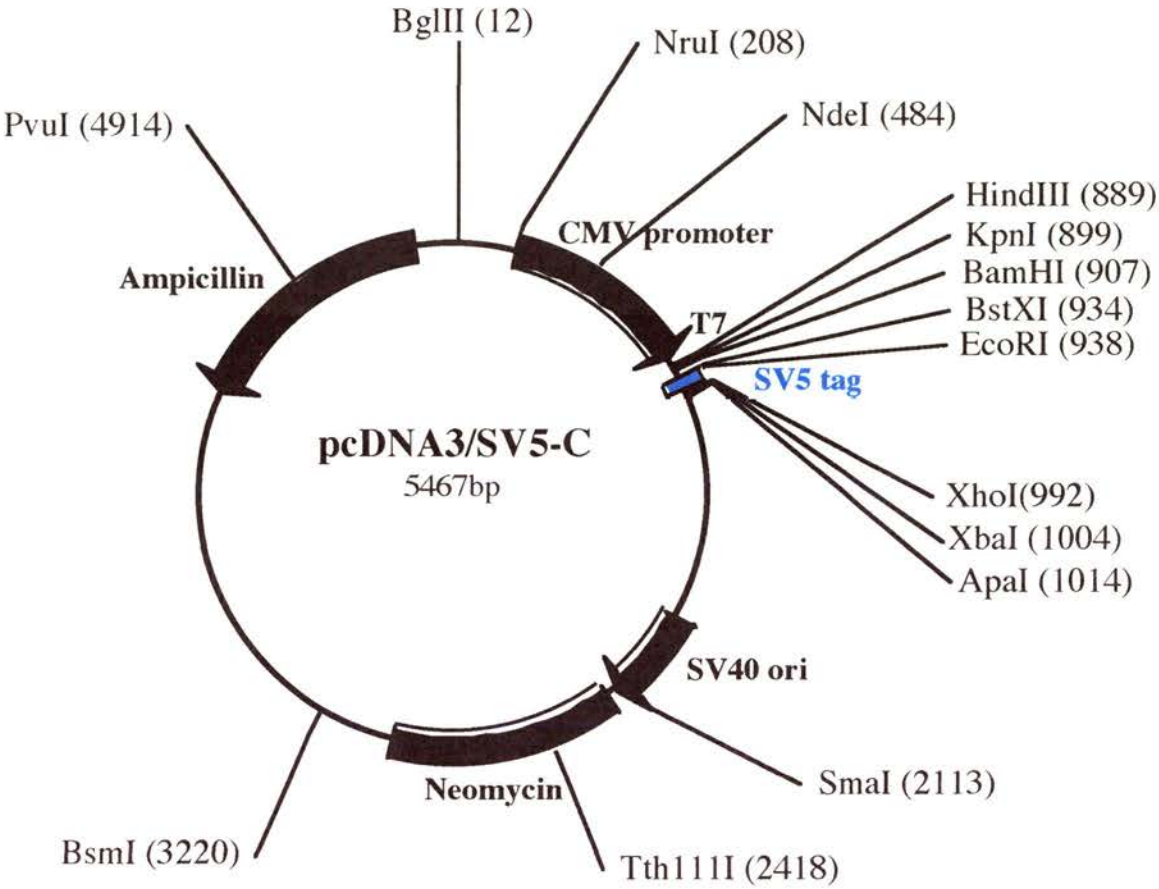
Downstream 5‘ -GCCTGGACGCCGTCCAGGCCGGCGTCGTGGC- 3’



Comments for pcDNA3 (5446 nucleotides)

Supplier: Invitrogen

CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH ploy A: bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: 1984-2069
Neo^R ORF: bases 2151-2932
SV40 poly A: bases 3120-3250
pUC19 backbone: bases 3272-5446
Amp^R ORF: bases 4450-5310



Cloning Primers:

Upstream (EcoRI): 5' - AATTCGGAAAGCCGATCCCAAACCCTTTGCTGGGATTGG ACTCCACCTAGTGAC - 3'

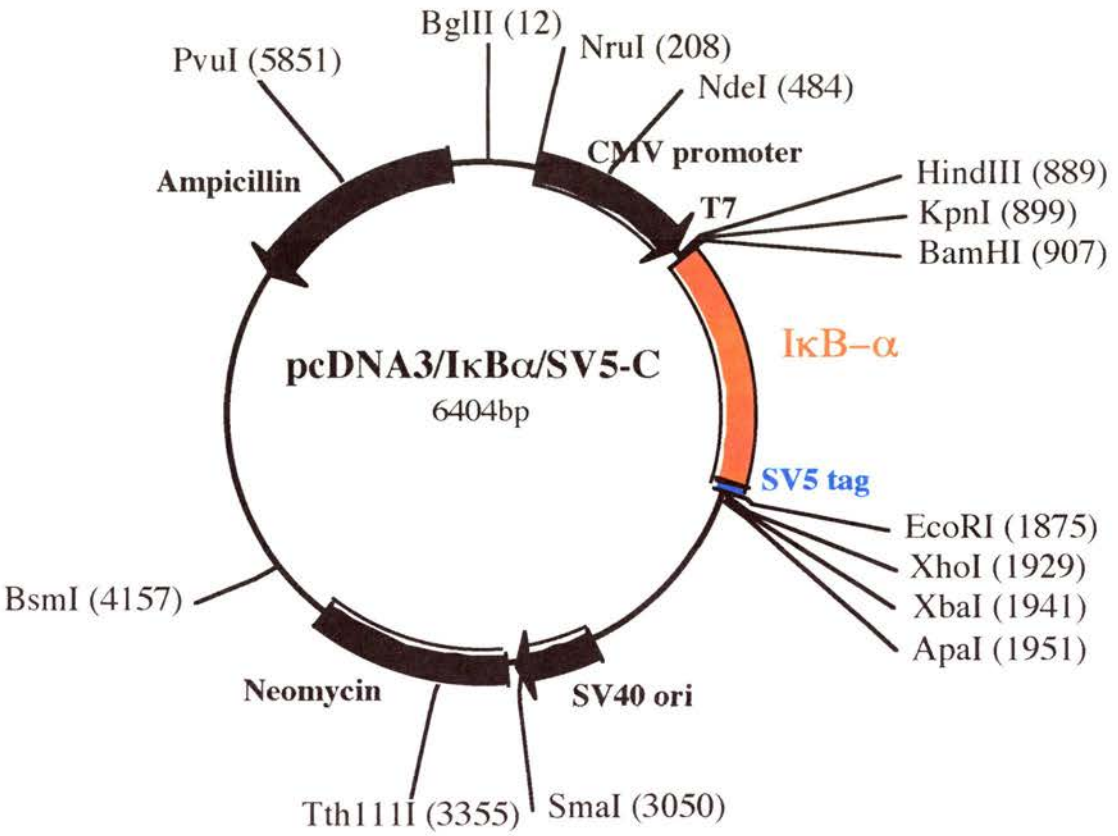
Downstream (XhoI): 5' - TCGAGTCACTAGGTGGAGTCCAATCCCAGCAAAGGGT TTGGGATCGGCTTTCCG - 3'

SV5-P-k tag:

Peptide: IPNPLLGLD

Antibody:

Mouse 336 monoclonal antibody against SV5-P-k peptide tag obtained from R.E. Randall, University of St. Andrews (Hanke *et al.*, 1992).



Cloning Primers:
Upstream (BamHI): 5' -GTACTAGGATCCATGTTCCAGGCGGCCGAG- 3'
Downstream (EcoRI): 5' -GCCGCGGAATTCTAACGTCAGACGCTGGCCTCC- 3'

IκBα:
Organism: *Homo sapiens*
Coding region: 951bp
Amino acids: 1-317
GB accession number: M69043

Antibodies:
Mouse 336 monoclonal antibody against SV5-P-k peptide obtained from R.E. Randall, University of St. Andrews (Hanke *et al.*, 1992).

Mouse MAD 10B monoclonal antibody which recognises an epitope located between amino acids 21 and 48 of IκB-α (Jaffray *et al.*, 1995).

See overleaf for internal primers used in creating the various IκB-α mutants.

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