Structure-function analysis of NF-κB1 p105

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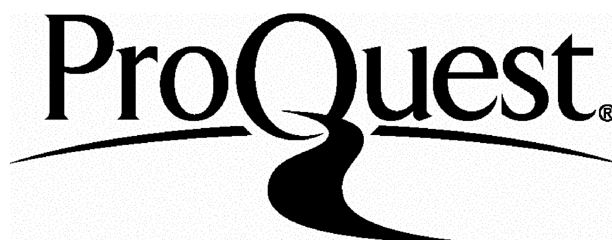

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Abstract

NF-κB transcription factors regulate the expression of genes that promote immunity, inflammation and cell survival. The p105 precursor protein of NF-κB1 p50 acts as an NF-κB inhibitory protein (IκB), retaining associated NF-κB/Rel subunits inactive in the cytoplasm of cells. The pro-inflammatory cytokine tumor necrosis factor-α (TNFα) and bacterial lipopolysacharide (LPS) stimulate p105 proteolysis via IκB kinase (IKK) mediated phoshorylation of p105. Subsequent ubiquitination of p105 precedes its degradation by the proteasome, releasing associated NF-κB/Rel subunits to translocate to the nucleus.

In this study, it is demonstrated that p105 binds to IKK via the death domain (DD) motif in its C-terminus. Furthermore, the p105 DD is required for the efficient phosphorylation and proteolysis of p105 induced by IKK or in response to TNFα stimulation. These data suggest that the p105 DD is a docking domain that couples p105 to signal-induced proteolysis.

The MAP 3-kinase tumor progression locus-2 (TPL-2), which is required for the activation of MEK1/2 MAP 2-kinases in response to LPS in macrophages, associates stoichiometrically with p105. Here, it is demonstrated that TPL-2 stability relies on its high affinity association with p105 through two interaction sites. While the TPL-2 C-terminus binds to residues 497-538 of p105, the p105 DD interacts with the TPL-2 kinase domain. Binding to the p105 DD inhibits TPL-2 MEK kinase activity, but concomitant interaction of the TPL-2 C-terminus with residues 497-538 of p105 is required for efficient TPL-2 inhibition by p105. Consequently, the C-terminally truncated form of TPL-2 is insensitive to p105 regulation *in vivo*, which may explain why such a mutation is oncogenic. These data indicate that in addition to its role as a precursor for p50 and a cytoplasmic inhibitor of NF-κB, p105 is a negative regulator of TPL-2.

Publications arising from this thesis

Beinke, S., Belich, M. P., and Ley, S. C. (2002). The death domain of NF-kappa B1 p105 is essential for signal-induced p105 proteolysis. J Biol Chem *277*, 24162-24168.

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Abbreviations

AP-1	activator protein 1
APC	antigen presenting cell
ATP	adenosine tri-phosphate
β-TrCP	β-transducin repeat containing protein
BAFF	B cell activating factor
BCR	B cell antigen receptor
BMDM	bone marrow-derived macrophages
BSA	bovine serum albumin
bZIP	basic region leucine zipper protein
cAMP	cyclic adenosine mono-phosphate
CARD	caspase recruitment domain
CD	cluster of differentiation
CKII	casein kinase II
Cot	cancer osaka tyroid
CRD	cysteine-rich domain
DD	death domain
DED	death effector domain
DNA	desoxyribonucleic acid
E	gestation day
ERK	extracellular signal-related kinase
EV	empty vector
FADD	Fas-associated death domain
FL	FLAG
GRR	glycine-rich region
GSK3	glycogen synthetase kinase

GST	glutathione-*S*-transferase
h	hours
HA	haemaglutinine
HAT	histone acetyl transferase
HDAC	histone deacetylase
HRP	horse raddish peroxidase
HTLV-1	human T-cell leukemia virus-1
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobin
IKK	IκB kinase
IL	interleukin
IL-1R	IL-1 receptor
iNOS	inducible nitric oxide synthetase
IP	incontinentia pigmenti
IRAK	IL-1 receptor associated kinase
IVP	*in vitro* precipitation
JNK	Jun amino-terminal kinase
KD	kinase-inactive
LFA-1	leukocyte function-associated antigen-1
LPS	lipopolysacharide
LT	lymphotoxin
MAP	mitogen activated protein
MAP 2-kinases	MAP kinase kinase
MAP 3-kinases	MAP kinase kinase kinases
MAPK	MAP kinase
MAPKAPK	MAPK-activated protein kinase
MBIP	MUK binding inhibitory protein

MCP-1	macrophage chemoattractant protein-1
M-CSF	macrophage-colony stimulating factor
MEK	MAPK ERK kinase
MEKK	MEK kinase
MHC	major histocompatibility complex
min	minutes
MIP-1α	macrophage inflammatory protein-1α
MKK	MAP kinase kinases
MKKK	MAP kinase kinase kinases
mRNA	messenger RNA
MUK	MAPK upstream kinase
MyD88	myeloid differentiation factor 88
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
NIK	NF-κB-inducing kinase
NIMR	National Institute for Medical Research
NLS	nuclear localisation signal
NO	nitric oxide
$p90^{rsk}$	p90 ribosome S6 kinase
PAGE	polyacrylamid gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEST	proline/glutamate/serine/threonine-rich
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C

PP2A	protein phosphatase 2A
PRR	pattern recognition receptor
PVDF	polyvinylidene difluoride
RHD	Rel-homology domain
RIP	receptor-interacting protein
RKIP	RAF kinase inhibitory protein
RNA	ribonucleic acid
SCF	SKp1-Cullin-F-box
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SODD	silencer of death domains
STAT	signal transducer and activator of transcription
TAB	TAK1 binding protein
TAK1	transforming growth factor-β activated kinase 1
TCR	T cell antigen receptor
T_H1	T helper type 1
T_H2	T helper type 2
TIR	Toll-like/IL-1 receptor homology
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TPL-2	tumor progression locus-2
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
VCAM-1	vascular cell adhesion molecule-1
V_{max}	maximal velocity
WT	wild type

1. Introduction

1. Introduction

1.1. Receptors in immunity and inflammation

The immune system protects vertebrates from invading pathogens by both innate and adaptive immune responses. Both achieve the recognition of "non self" molecules by specialised receptors, but use different effector mechanisms to finally eliminate pathogens that carry these molecules.

Innate immune responses can be activated very rapidly in response to infection and provide the first line of defence (Janeway and Medzhitov, 2002). The innate immune system relies on germ line encoded pattern recognition receptors (PRRs), which recognise general pathogen-associated molecular patterns (PAMPs). PAMPs are essential components of pathogens with little variability and are absent in mammals (e.g. lipopolysacharide (LPS), peptidylglycans, lipoproteins, unmethylated bacterial DNA or double-stranded RNA).

Ten members of the Toll-like receptor (TLR) family have been identified, which specifically mediate responses to particular PAMPs in mammals (Medzhitov, 2001). TLRs are expressed by a broad range of cells (e.g. monocytes/macrophages, dendritic cells, neutrophils or endothelial cells), which come in close contact with the pathogen at the site of antigen encounter. They are transmembrane receptors and mediate responses to extracellular PAMPs. Ligation of TLRs activates nuclear factor (NF) -κB and mitogen activated protein (MAP) kinase signalling pathways. The nucleotide-binding domain/leucine-rich repeat proteins, NOD1 and NOD2, have been suggested to be involved in the recognition of intracellular PAMPs and also activate NF-κB and MAP kinase signalling pathways (Girardin et al., 2002).

Pathogen recognition induces the up-regulation of the pro-inflammatory cytokines tumor necrosis factor-α (TNFα) and interleukin (IL) -1. TNFα and IL-1 affect most cell types and their biological functions are remarkably similar (Dinarello, 1996; Vassalli, 1992). However, the two cytokines and their receptors are not related in sequence.

TNFα can bind to both the p55 TNF receptor (TNFR1) and the p75 TNF receptor (TNFR2). However, the majority of biological functions of TNFα is mediated through the activation of NF-κB and MAP kinase signalling pathways by TNFR1 (Wallach et al., 1999). Upon ligation TNFR1 also activates a caspase-cascade leading to apoptosis, but this is usually suppressed by the simultaneous induction of anti-apoptotic proteins via the induction of NF-κB. IL-1 binds to the IL-1 type 1 receptor (IL-1R1), which also triggers NF-κB and MAP kinase signalling pathways (O'Neill, 2000).

The major source of TNFα is cells of the monocyte/macrophage lineage, with T lymphocytes, neutrophiles, mast cells and endothelial cells also contributing under different circumstances (Vassalli, 1992). The production and release of TNFα can be induced by all potentially noxious stimuli. *In vivo*, TNFα is the most rapidly produced pro-inflammatory cytokine and acts as a key inductor and central coordinator of the innate immune response (see Diagram 1). TNFα up-regulates the production of pro-inflammatory cytokines, including TNFα itself. This initiates an auto-regulatory positive feedback loop, which plays an important role for the amplification of inflammatory immune responses. Furthermore, TNFα induces the expression of adhesion molecules (e.g. vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), leukocyte function-associated antigen-1 (LFA-1), selectins) and the secretion of chemokines (e.g. IL-8, macrophage inflammatory protein-lα (MIP-1α), macrophage chemoattractant protein-1 (MCP-1), RANTES, eotaxin), which attract mononuclear phagocytes and granulocytes to the site of infection. In macrophages, TNFα induces the expression of inducible nitric oxide synthetase (iNOS), which produces nitric oxide (NO)

and enhances the killing ability of macrophages. Activation of macrophages by TNFα also induces the up-regulation of the major histocompatibility complex (MHC) and co-stimulatory molecules (e.g. CD80, CD86), as well as the secretion of effector cytokines (e.g. IL-6, IL-12, Lymphotoxin (LT) α and β), thereby facilitating the activation of adaptive immune responses.

Adaptive immune responses involve a highly diverse repertoire of somatically rearranged antigen receptors expressed on B and T cells (B cell antigen receptors or BCRs and T cell antigen receptors or TCRs), which recognise virtually every possible structure of foreign protein antigens. Each B or T cell produces receptors of a single specificity. Recognition by TCRs requires phagocytosis and proteolysis of the antigen by antigen presenting cells (APCs; e.g. macrophages, dendritic cells or B cells), which present the antigenic polypeptide bound to the MHC on their surface. BCR and TCR ligation leads to the Ca^{2+}-dependent activation of nuclear factor of activated T cells (NF-AT), as well as the induction of NF-κB and MAP kinase signalling pathways. In concert, activation of these signalling pathways induces the expression of proteins that support lymphocyte proliferation and differentiation. The expansion and differentiation of antigen specific T or B cell clones in response to primary antigen encounter requires 1-2 days.

The main effector function of mature B cells in adaptive immunity is the secretion of antibodies (also referred to as immunoglobin (Ig)), which have a similar structure and the same specificity as the respective BCR. Antibodies bind to pathogens or their toxic products, which elicited the immune response, in the extra cellular spaces of the body, thereby neutralising the pathogen or recruiting phagocytic cells and molecules of the complement system to eliminate them. T cells differentiate into either $CD8^+$ effector T cells, which specifically eliminate cells that host pathogens, or $CD4^+$ helper T cells, which facilitate B cell responses.

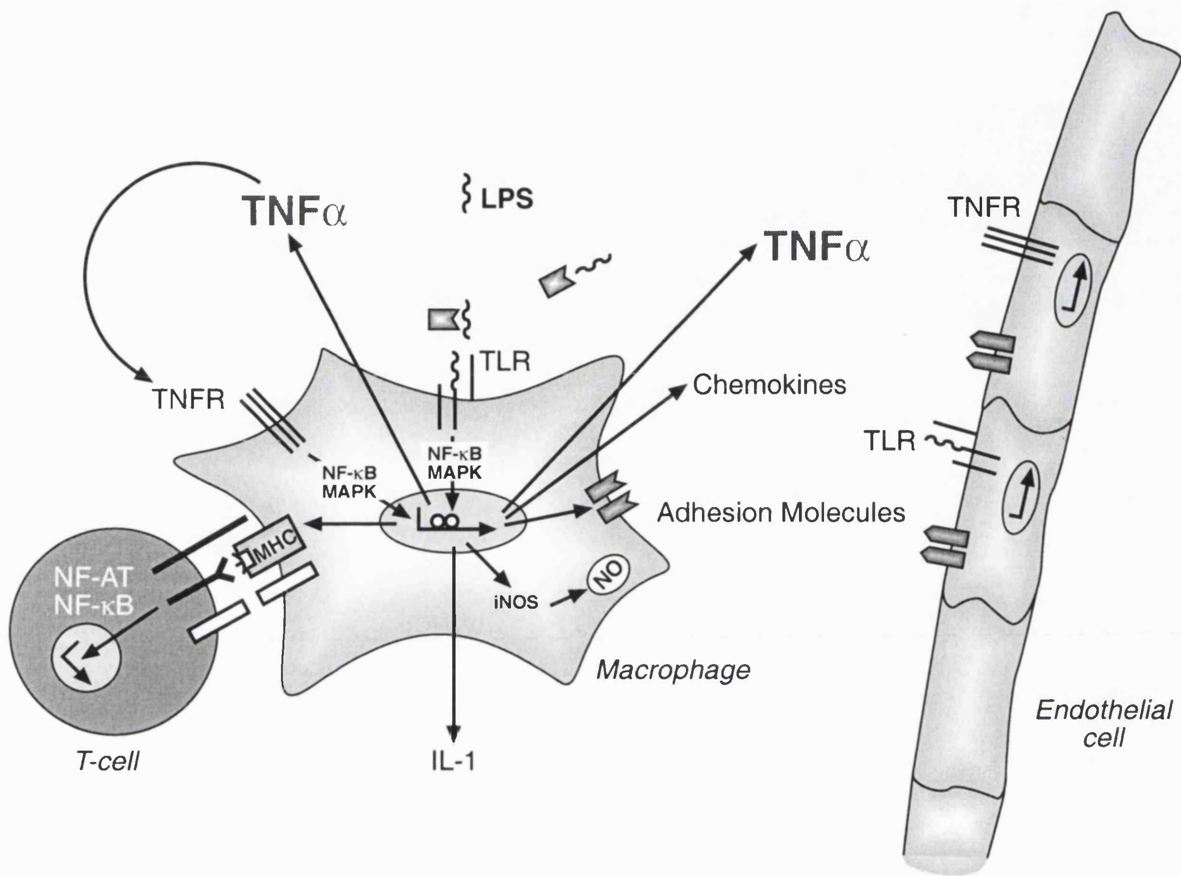


Diagram 1. NF-κB and MAP kinase signalling pathways in immune cell activation
This diagram illustrates signals that induce NF-κB and MAP kinase signalling pathways and downstream effector proteins.

1.2. NF-κB signalling pathways

1.2.1 Structure and function of NF-κB

Nuclear factor (NF) -κB is an eukaryotic transcription factor that is ubiquitously expressed. It was first described in 1986 as a nuclear protein required for the B cell specific transcription of the immunoglobin kappa light chain (Sen and Baltimore, 1986). However, subsequent research revealed that NF-κB is an inducible factor, which is involved in the regulation of an exceptionally large number of genes, most importantly during immune responses, inflammation or lymphoid organ development (Baeuerle and Henkel, 1994; Baldwin, 1996; Ghosh et al., 1998; Siebenlist et al., 1994). NF-κB is also important for the prevention of apoptosis, which is vital for its function in immune responses (Karin and Lin, 2002).

The term NF-κB describes a family of transcriptional regulators composed of hetero- and homodimeric complexes of NF-κB/Rel proteins (Baldwin, 1996; Siebenlist et al., 1994). All NF-κB/Rel proteins contain a conserved Rel-homology domain (RHD) that contributes to dimerisation and DNA binding. In mammalian cells, this family comprises RelA (p65), RelB, c-Rel, NF-κB1 p50 and NF-κB2 p52 (see Diagram 3). NF-κB1 p50 and NF-κB2 p52 are produced as larger inactive precursor molecules of 105kDa (p105) and 100kDa (p100), respectively (Mercurio et al., 1993; Rice et al., 1992). p50 or p52 are derived from the N-terminal domain of their precursors via processing of the C-terminal half by the 26S proteasome (Betts and Nabel, 1996; Heusch et al., 1999; Lin et al., 1998a; Palombella et al., 1994).

In unstimulated cells, NF-κB is retained inactive in the cytoplasm via the interaction with inhibitory proteins termed IκBs (see Diagram 2) (Baldwin, 1996; Siebenlist et al., 1994). IκBs contain multiple ankyrin repeats that bind to RHDs of NF-κB/Rel proteins and can mask their nuclear translocation signal (NLS) thereby preventing

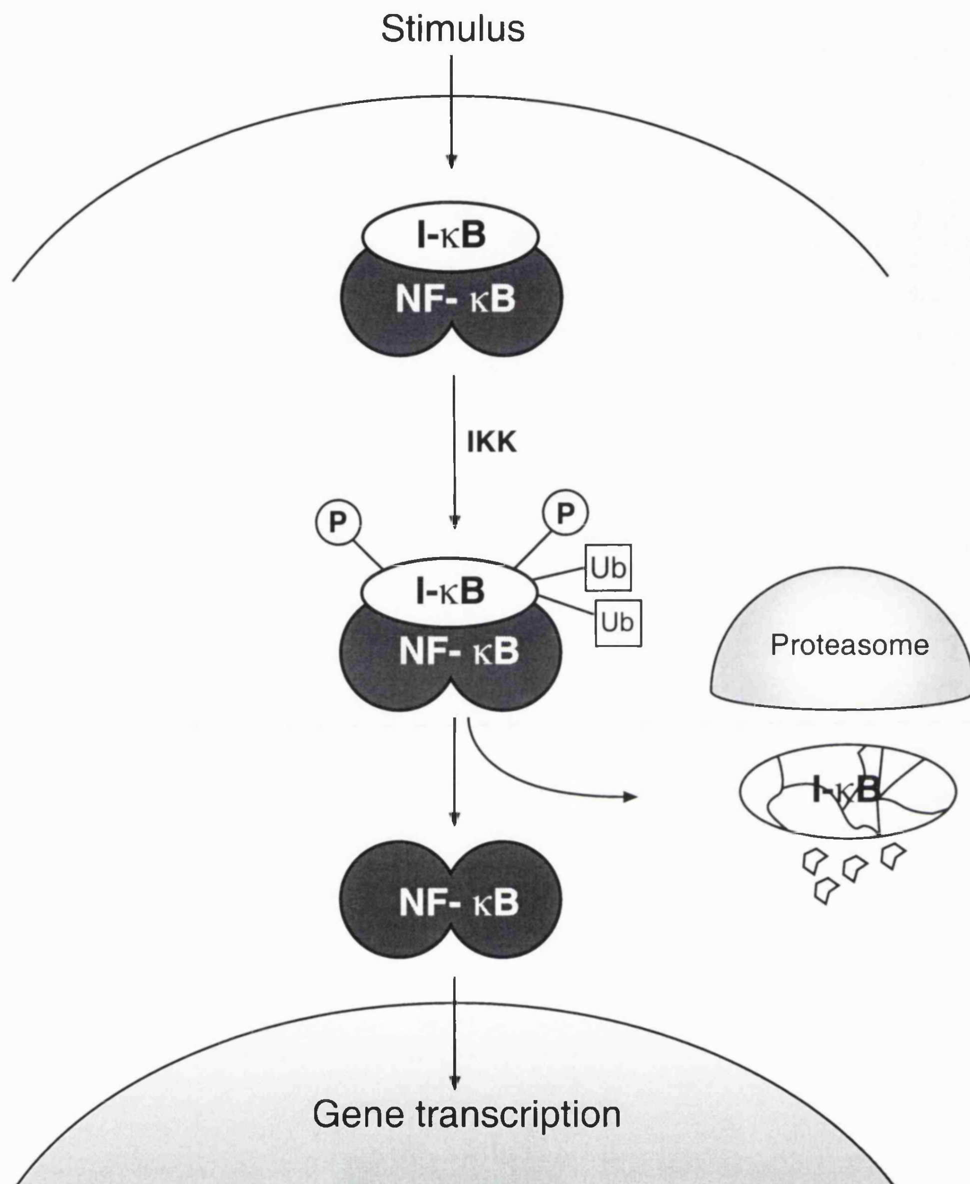


Diagram 2. NF-κB activation.
This diagram gives a general overview about the activation of NF-κB: NF-κB is retained in the cytoplasm of unstimulation cells by IκB. Stimuli induce IκB phosphorylation by IKKs, which results in IκB ubiquitination and its degradation by the proteasome. As a consequence, free NF-κB translocates to the nucleus.

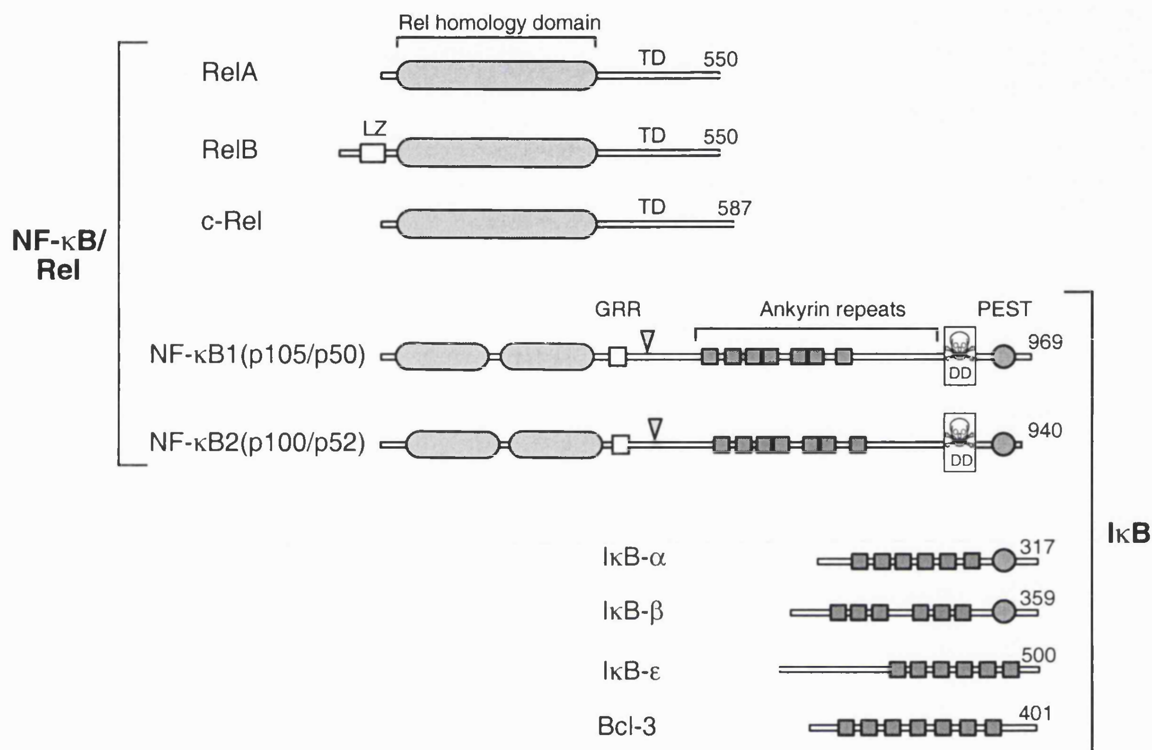


Diagram 3. NF-κB and IκB family members.
NF-κB proteins include RelA, RelB, c-Rel, NF-κB1 p50 and NF-κB2 p52. NF-κB1 p50 and NF-κB2 p52 are produced as larger precursor proteins (NF-κB1 p105 and NF-κB2 p100). IκB proteins include NF-κB1 p105 and NF-κB2 p100, IκBα, IκBβ, IκBε and Bcl-3. The positions of the Rel homology domain, glycine-rich region (GRR), ankyrin repeats, death domain (DD) and proline/glutamate/serine/threonine-rich (PEST) region are indicated.

nuclear uptake. This family of structurally related proteins includes IκBα, IκBβ, IκBε, together with the precursors NF-κB1 p105 and NF-κB2 p100 and the IκB-like protein BCL-3 (see Diagram 3). In response to appropriate stimulation with agonists, IκBs are phosphorylated by the high molecular weight IκB kinase (IKK) complex (Ghosh et al., 1998; Karin and Ben-Neriah, 2000). Subsequent ubiquitination leads to their degradation by the proteasome, which allows NF-κB dimers to translocates into the nucleus and regulate gene expression. In addition, the modification of transcriptional activity of nuclear NF-κB dimers by association with other regulatory proteins is regulated by NF-κB/Rel subunit phosphorylation.

1.2.2 NF-κB/Rel proteins

1.2.2.1 NF-κB/Rel dimerisation and DNA binding

With the exception of RelB, all members of the NF-κB/Rel family can homo- or heterodimerise with each other (Baeuerle and Henkel, 1994; Siebenlist et al., 1994). RelB forms hetero-dimers with p50 and p52, but can not form homodimers. Crystal structures of the p50 homo-dimer and RelA/p50 hetero-dimer reveal that the RHDs of these proteins are comprised of two immunoglobin-like domains connected by a flexible linker (Baltimore and Beg, 1995; Chen et al., 1998; Ghosh et al., 1995; Muller et al., 1995). Dimerisation occurs via inter-digitating hydrophobic site chains exclusively of the C-terminal domain, while loops between β-strands of both N- and C-terminal domains contribute to DNA binding. The overall structure of NF-κB bound to DNA resembles the shape of a butterfly that grips DNA by enveloping it. Different NF-κB dimers exhibit different affinities for the 10 base pair κB-sites with the consensus sequence 5'-GGGRNNYYCC-3' in promotors of NF-κB responsive genes (R is purine, Y is pyrimidin and N is any base) (Kunsch et al., 1992). Variation in the base pair composition of the

κB-sites contributes to specificity to one or the other NF-κB dimer, which is presumably the basis for different NF-κB dimers regulating different genes.

1.2.2.2 Generation of p50 and p52

p50 and p52 are generated as the transcriptionally inactive longer precursor proteins p105 and p100, respectively (Bours et al., 1990; Ghosh et al., 1990; Kieran et al., 1990; Meyer et al., 1991; Neri et al., 1991; Schmid et al., 1991). The N-terminal half of the precursors is identical to the active NF-κB/Rel subunits. Processing of the precursor proteins results in the complete degradation of the C-termini leaving the N-terminal transcription factor domain intact. p50 and p52 production has been proposed to occur co-translationally on polysomes (Heusch et al., 1999; Lin et al., 1998a; Lin et al., 2000; Mordmuller et al., 2003). However, the physiological relevance of this mechanism is not clear since most other studies find that the majority of cellular p50 and p52 is produced post-translational by processing of p105 or p100, respectively (Belich et al., 1999; Claudio et al., 2002; Coope et al., 2002; Dejardin et al., 2002; Donald et al., 1995; Mellits et al., 1993; Orian et al., 2000; Xiao et al., 2001b).

A number of evidence indicate that proteolysis of p105 is mediated by the proteasome. It was demonstrated that p105 proteolysis is adenosine triphosphate (ATP) dependent, requires ubiquitination and can be blocked by proteasome inhibitors *in vitro* and *in vivo* as well as by mutations of the proteasome in yeast (Fan and Maniatis, 1991; Palombella et al., 1994; Sears et al., 1998). Furthermore, proteasome enriched cytosolic fractions can facilitate p105 processing *in vitro* (Orian et al., 1995). Similarly, the proteasome was found to mediate processing of p100 to p52 (Betts and Nabel, 1996; Heusch et al., 1999).

p105 processing to p50 occurs constitutively, but p105 proteolysis is also induced in response to cellular stimulation. However, it is not entirely clear whether signal-induced p105 proteolysis results in increased processing to p50 or in the complete degradation of p105. Some studies report increased processing of p105 in response to TNFα (Mellits et al., 1993; Naumann and Scheidereit, 1994), LPS (Donald et al., 1995) or phorbol 12-myristate 13-acetate (PMA)/ionomycin (MacKichan et al., 1996), while others fail to detect an increase in p50 levels equivalent to the decrease in p105 levels (Belich et al., 1999; Harhaj et al., 1996; Zheng et al., 1993). Most stimuli tested induce p105 proteolysis with slower kinetics than IκBα degradation (0.5-4 hours) (Donald et al., 1995; Lang et al., 2003). p100 is not constitutively processed to p52 and p100 processing is completely unaffected by most stimuli that induce degradation of IκBα. However, p100 processing occurs in response to LTβ, CD40 and B cell activating factor (BAFF) within 3-6 hours (Claudio et al., 2002; Coope et al., 2002; Dejardin et al., 2002; Kayagaki et al., 2002).

Partial proteolysis of proteins by the proteasome is a rare mechanism that only applies to p105, p100 and a few other proteins, including the distant yeast homologues of the NF-κB precursor proteins SPT23p and Mga2p (Hoppe et al., 2000; Rape et al., 2001). Flexible unstructured regions composed of amino acids with short side chains seem to be necessary for partial degradation of proteins by the proteasome (Rape and Jentsch, 2002). A glycine-rich region (GRR) is required for 105 and 100 processing (Betts and Nabel, 1996; Heusch et al., 1999; Lin and Ghosh, 1996; Orian et al., 1999). The GRR could act as a stop signal that halts proteasomal degradation proceeding from the C-terminus.

Two lysines 441 and 442 and a potential ubiquitin ligase recognition motif C-terminal to the p105 GRR are also required for constitutive ubiquitination and processing of p105 (Orian et al., 1999). Matouschek and collegues proposed a model for proteasomal degradation, whereby the proteasome unravels proteins sequentially from the degradation

signal (Lee et al., 2001). Accordingly, an alternative model for p105 processing bases on a proteolytic cleavage of the precursor protein before the C-terminus is degraded. Therefore, the GRR could function as a cleavage site. However, a potential endoproteolytic activity needs to be associated with the 26S proteasome, because the 26S proteasome clearly can provide all proteolytic activities necessary for p50 processing *in vitro* (Coux and Goldberg, 1998). Indeed, the proteasome was recently demonstrated to catalyse endoproteolytic cleavage of polypeptide bonds (Liu et al., 2003). Processing substrates may enter the proteasome by hairpin loop formation to be proteolytically cleaved by the active site of the proteasome (Lee et al., 2001; Liu et al., 2003). Unfolding of the GRRs of p105 and p100, as well as regions of low sequence complexity in related domains of SPT23p and Mga2p, probably does not require much energy and the lack of bulky side chains may facilitate the passage into the 20S proteasome barrel. Interestingly, the yeast proteins SPT23p and Mga2p are cleaved before their C-termini are degraded (Rape et al., 2001).

This model implies uni-directional proteolysis from the cleavage site into the C-terminus. Accordingly, tightly folded domains or protein interaction can prevent proteasomal degradation (Lee et al., 2001). Indeed, processing of p105 and SPT23p/Mga2p is dependent on homo-dimerisation of the N-terminal Ig-like domains of the transcription factors (Lee et al., 2001; Lin et al., 2000; Rape et al., 2001). Therefore, the globular structure of the dimerised transcription factors probably rescues them from proteasomal degradation. The generation of the p50 and p52 NF-κB/Rel subunits from inactive precursor proteins provides an additional regulatory step in the activation of NF-κB.

1.2.2.3 Transactivation potential of NF-κB/Rel dimers

RelA and c-Rel function as potent transcriptional activators, while RelB can activate transcription only in certain cell types. p50 and p52 lack intrinsic transactivation capacity. This implies that homodimers of p50/p50 or p52/p52 without any co-factors are transcriptionally repressive, while dimers including RelA, RelB or c-Rel are transcriptionally activating. Once liberated from the inhibitory IκB proteins the capacity of NF-κB/Rel dimers to induce transcription is also a regulated process. Binding affinity to both DNA and nuclear cofactors is often regulated by direct phosphorylation of NF-κB/Rel dimers.

Protein kinase A (PKA) (Zhong et al., 1997; Zhong et al., 1998), Akt/protein kinase B (PKB) (Madrid et al., 2001; Madrid et al., 2000), casein kinase II (CKII) (Bird et al., 1997; Wang et al., 2000) and phosphatidylinositol 3-kinase (Sizemore et al., 1999) have been implicated in NF-κB/Rel subunit phosphoryalation. Knockout studies revealed that glycogen synthetase kinase 3β (GSK3β) (Hoeflich et al., 2000), protein kinase C (PKC) ζ (Leitges et al., 2001), IKK (Sizemore et al., 2002) and Tbk/t2k/NAK (Bonnard et al., 2000) are required for in the induction of transactivation capacity in response to a wide variety of stimuli, while NF-κB-inducing kinase (NIK) regulates NF-κB transcriptional competence specifically in response to LTβ (Yin et al., 2001).

RelA It was first shown that RelA is phosphorylated after cellular stimulation (Naumann and Scheidereit, 1994). RelA phosphorylation regulates the recruitment of histone acetyl transferases (HATs), such as CBP/300 and p/CAF, to nuclear RelA containing NF-κB dimers, which modify the secondary structure of target DNA sites to facilitate NF-κB binding (Gerritsen et al., 1997; Merika et al., 1998; Perkins et al., 1997; Sheppard et al., 1999; Zhong et al., 1998). Nuclear RelA can also interact with distinct histone deacetylase (HDAC) isoforms, which negatively regulates gene

expression (Ashburner et al., 2001; Chen et al., 2001; Ito et al., 2000; Lee et al., 2000; Zhong et al., 2002).

p50/p52 p50 and p52 lack a transactivation domain and can not directly recruit HATs (Sheppard et al., 1999). Consistent with this, over-expression of p50 causes a decrease in the expression of NF-κB dependent genes, such as TNFα (Baer et al., 1998). Analysis of naturally occurring promotor mutants has confirmed that binding of p50 homodimers to the TNFα promotor negatively regulates TNFα expression (Udalova et al., 2000). Most nuclear NF-κB complexes in unstimulated cells were shown to consist of p50 homodimers associated with HDAC-1. These $(p50)_2$/HDAC complexes repress the transcription of a subset of NF-κB-dependent genes, but can be replaced within certain target gene promotors by complexes containing p50 and RelA, which is phosphorylated by PKA (Zhong et al., 2002). However, p50 and p52 homodimers can promote transcription *in vivo* via the interaction with the nuclear, IκB-like protein Bcl-3, which recruits associated HATs, such as Tip60 (Dechend et al., 1999). The transcriptional activation potential of p50 and p52 homodimers, therefore, seems to be dependent on the availability of Bcl-3 and is likely to be cell type and stimulus specific.

1.2.2.4 Genetic analysis of NF-κB/Rel protein function

RelA The p50/RelA heterodimer was the first form of NF-κB to be identified and is the most abundant NF-κB dimer in most cell types. The deletion of the *relA* gene in mice causes embryonic death at gestation day (E) 15.5-16.5 due to massive apoptosis of liver parenchymal cells, which especially affects hepatocytes (Beg et al., 1995a). TNFα readily kills macrophages and fibroblast from RelA-/-, but not wild type mice, and RelA-/-TNFα-/- or RelA-/-TNFR1-/- double mutants are born with grossly normal morphology (Alcamo et al., 2001; Doi et al., 1999). Thus, RelA is important for

the protection from TNFα-induced apoptosis, a function that is not compensated by other NF-κB dimers.

Adoptive transfer of RelA-/- hematopoietic stem cells into irradiated SCID mice results in a reduced number of B cells, which exhibit impaired proliferative responses and reduced immunoglobin secretion (Doi et al., 1997). More severe lymphopoietic defects with complete absence of B cells is found in lethally irradiated mice reconstituted with double deficient RelA-/-NF-κB1-/- fetal liver stem cells (Horwitz et al., 1999). This illustrates that RelA also has specfic functions in B cell development and function.

NF-κB1 p50 The involvement of p50/RelA in the transcription of a wide variety of genes and the importance of the RelA subunit in the prevention of apotptosis during embryonic development would suggest severe histopathological defects in mice deficient for NF-κB1. However, these mice, which do not produce either the p105 precursor or p50, develop normally, suggesting a great degree of functional compensation by other NF-κB family members for p50 in the p50/RelA dimer (Sha et al., 1995).

Nevertheless, NF-κB1-deficient mice are unable to clear the intracellular bacterium *Listeria monocytogenes* and are more susceptible to infection with the extra-cellular gram-positive bacterium *Streptococcus pneumoniae*. However, they respond normally to challenges with extra-cellular gram-negative bacteria *Haemophilus influenza* and *Escherichia coli* K1. NF-κB1-/- B cells display impaired proliferation in response to LPS and soluble CD40 ligand, but not in response to membrane-bound CD40 ligand, anti-IgM or anti-IgD-dextran. They also display defective IgG3, IgE and IgA class switching (Snapper et al., 1996b). These observations suggest an important role for p50 in B cell proliferation and maturation as well as in cellular immune responses. Interestingly, NF-κB1-/- mice are more resistant to murine encephalomycarditis virus, correlating with increased β-interferon production by NF-κB1-/- fibroblasts implicating a

negative regulatory function of p50 homodimers in regulation of the expression of the β-interferon gene. Furthermore, NF-κB1-/- mice are completely devoid of allergic airway inflammation in a murine model for asthma (Yang et al., 1998). This results from a lack of $CD4^+$ T helper type 2 (T_H2) differentiation of NF-κB1-/- T cells, which is due to impaired IL-4, IL-5 and IL-13 production, correlating with the failure to induce the expression of the transcription factor GATA-3 (Das et al., 2001). NF-κB1-/- mice are also refractory to the induction of both a chronic (collagen-induced) and acute (methylated BSA/IL-1 induced) arthritis model, suggesting p50 is important for the pathology of this inflammatory disease (Campbell et al., 2000).

NF-κB2 p52 Analyses of NF-κB2 knockout mice indicates a particular role for p52 in the maintenance of the peripheral B cell pool and organisation of B cell compartments in secondary lymphoid organs. Deletion of *NF-κB2* in mice results in disrupted splenic architecture characterised by diffuse B cell areas, indiscreet follicles and the absence of the perifollicular marginal zone (Caamano et al., 1998). The number of B cells in the spleen and other lymphoid organs is reduced and NF-κB2-/- B cells show mild proliferative defects in responses to CD40 ligand, LPS and anti-IgD-dextran. NF-kB2-/- mice are also deficient in the formation of germinal centres and Peyer's patches, and their T cell-dependent and independent responses are reduced (Yilmaz et al., 2003). These mice also display increased susceptibility to infection by *L. monocytogenes* (Caamano et al., 1998).

NF-κB1/2 p50/p52 The phenotype of NF-κB1 and NF-κB2 double knockout mice suggest these proteins are partially redundant (Iotsova et al., 1997). Impaired bone remodelling leading to osteopetrosis and incomplete odontosis in these mice correlates with a decrease in the total number of osteoclasts. This indicates an additional role of p50/p52 in marcrophage/osteoclast differentiation These mice also display myoloid

hyperplasia, early thymus regression, lack of $CD4^+$ and $CD8^+$ T cells in the periphery and progressive loss of B cells.

RelB RelB-/- mice are impaired in the formation of germinal centers, the marginal zone of the spleen and Peyer's patches (Weih et al., 2001; Yilmaz et al., 2003). Consistent with this, RelB is required for normal production of antigen specific IgG in response to T-dependent and independent stimuli (Snapper et al., 1996a). This phenotype is very similar to that of NF-κB2 knockout mice, suggesting that these Rel subunits functionally cooperate.

RelB knockout mice also display multifocal inflammatory cell infiltration in several organs accompanied with myeloid hyperplasia and splenomegaly (Weih et al., 1995). Crossing RelB-deficient mice with mice that lack either T and B cells or only T cells revealed that this inflammatory phenotype is dependent on T cells (Weih et al., 1996). Moreover, RelB-/- mice are susceptible to *L. monocytogenes* and are unable to mount protective immune responses to *choriomeningitis virus*, indicating defects in T cell/macrophage interaction and $CD8^+$ cytotoxic T cell responses (Weih et al., 1997). RelB is also required for a normal delayed-type hypersensitivity response, an immune function that requires the intact function of epidermal antigen presenting (Langerhans) and antigen specific $CD4^+$ T helper type 1 (T_H1) cells (Weih et al., 1995). Thus, RelB is not only essential in the correct development of the hematopoietic system, but is also involved in humoral and cellular immune responses.

c-Rel Knockout mice for c-Rel develop normally but are specifically impaired in humoral and cellular immune responses (Gerondakis et al., 1996; Kontgen et al., 1995). Proliferation of c-Rel-/- B and T cells in response to most mitogenic stimuli is impaired, and serum levels of IgG1, IgG2a, IgG2b and IgG3 are decreased in c-Rel-/- mice. Resident peritoneal macrophages killed bacteria poorly due to reduced expression

of inducible nitric oxide synthetase (iNOS), which correlates with diminished nitric oxide (NO) production. This makes c-Rel-/- mice susceptible to infection with the intracellular parasite *Leichmania major*. Mice with an homozygous deletion of the trans-activating C-terminus of c-Rel (c-Rel$^{\Delta CT/\Delta CT}$) display similar defects in immune functions but with a more severe phenotype than mice lacking the entire c-Rel protein (Carrasco et al., 1998). Additionally, c-Rel$^{\Delta CT/\Delta CT}$ animals present histopathological alterations of hemopoietic tissues, such as an enlarged spleen due to lymphoid hyperplasia, extramedullary hematopoiesis and bone marrow hypoplasia. Thus, in c-Rel$^{\Delta CT/\Delta CT}$ mice, the lack of c-Rel activity is less efficiently compensated by other NF-κB proteins, which may result from c-Rel$^{\Delta CT/\Delta CT}$ acting as a dominant negative protein.

1.2.3 NF-κB regulation by IκB proteins

1.2.3.1 Function and specificity of IκB proteins

IκB proteins contain six to seven ankyrin repeats of approximately 33 amino acids, with which they bind to the RHD of NF-κB/Rel proteins (see Diagram 3) (May and Ghosh, 1998). The small IκB proteins, IκBα, IκBβ and IκBε contain six centrally located ankyrin repeats and an N-terminal domain, which is required for signal-induced degradation. IκBα and IκBβ also contain a C-terminal PEST region, which is involved in their constitutive protein turnover. NF-κB1 p105 and NF-κB2 p100 contain seven ankyrin repeats in their C-terminal half and also function as IκB proteins in addition to their function as precursor proteins for p50 or p52, respectively (Hatada et al., 1993; Mercurio et al., 1993; Naumann et al., 1993; Rice et al., 1992). IκBγ is produced by transcriptional initiation from an alternative promotor in the p105 gene and is identical in sequence to the C-terminal IκB-like region of p105 (Gerondakis et al., 1993; Inoue et al., 1992).

The association with IκB proteins retains NF-κB dimers inactive in the cytoplasm of unstimulated cells. The prevailing view is that this is due to masking of the NLS in the C-terminus of the RHD of NF-κB/Rel proteins (Beg et al., 1992; Ganchi et al., 1992; Henkel et al., 1992; Zabel et al., 1993). Crystal structures for IκBα bound to p50/RelA RHD dimers reveal that each repeat consists of two closely packed helices followed by a loop and a tight hairpin turn (Huxford et al., 1998; Jacobs and Harrison, 1998). Multiple ankyrin repeats form a curved cylindrical stack facing the NF-κB RHDs with the loops forming finger like extensions. In the complex, IκBα directly sequesters the NLS of RelA, which is not ordered in NF-κB bound to DNA, as an α-helical segment (Huxford et al., 1998; Jacobs and Harrison, 1998).

IκBα IκBα was the first member of this family to be cloned and is the best characterised IκB protein (Davis et al., 1991; Haskill et al., 1991). IκBα interacts with heterodimers of p50 and p52 complexed with RelA and c-Rel, as well as homo- and heterodimers of RelA and c-Rel. Most stimuli that have been implicated in NF-κB activation induce the rapid degradation of IκBα within minutes, thereby quickly releasing associated NF-κB dimers to translocate to the nucleus and induce gene transcription (Henkel et al., 1993). However, IκBα is efficiently re-synthesised through the auto-regulatory induction of IκBα mRNA transcription by NF-κB (Brown et al., 1993; Chiao et al., 1994; Le Bail et al., 1993; Sun et al., 1993). Newly synthesised IκBα enters the nucleus and removes NF-κB from DNA (Arenzana-Seisdedos et al., 1995). A non classical nuclear localisation signal in the second ankyrin repeat of IκBα as well as a transport mechanism involving the IκBα ankyrin repeats and associated proteins have been suggested to be responsible for nuclear import of free IκBα (Sachdev et al., 2000; Turpin et al., 1999).

Recent studies show that IκBα:NF-κB complexes constantly shuttle between cytoplasm and nucleus (Huang et al., 2000; Rodriguez et al., 1999; Tam et al., 2000). It is

most likely that the NLS of p50, which is not covered by IκBα in the complex contributes to its nuclear uptake (Jacobs and Harrison, 1998; Malek et al., 2001). However, the strong nuclear export signal of RelA together with nuclear export signals in the N- and C-terminus of IκBα removes IκBα:NF-κB complexes immediately from the nucleus by a CRM1-dependent mechanism, so that the default position of the complexes is in the cytoplasm (Harhaj and Sun, 1999b; Huang et al., 2000; Huang and Miyamoto, 2001; Johnson et al., 1999; Rodriguez et al., 1999; Tam et al., 2000). Therefore, IκBα is involved in both the steady state inhibition as well as the post-induction attenuation of NF-κB activation.

IκBα knockout mice develop normally, but after birth exhibit psoriasis-like skin defects and extensive granulopoiesis, which results in death typically by 8-10 days (Beg et al., 1995b; Klement et al., 1996). Haematopoietic cells from these mice display elevated basal and sustained induced levels of NF-κB in the nucleus. mRNAs of some but not all genes thought to be regulated by NF-κB are induced in these cells, consistent with IκBα regulating only a subset of NF-κB dimers and the equivalent responsive genes. Signal-induced NF-κB activation in IκBα-/- embyonic fibroblast is minimally effected and correlates with the degradation of IκBβ, suggesting that IκBβ can substitute for IκBα in this cell type.

IκBβ IκBβ has a similar binding affinity for RelA and c-Rel containing NF-κB complexes as IκBα (Chu et al., 1996; Thompson et al., 1995). Genetic complementation studies revealed that IκBβ can largely substitute for IκBα (Cheng et al., 1998). Thus, mice that have the *IκBα* gene replaced by the *IκBβ* gene and express *IκBβ* under the *IκBα* promotor do not show the obvious abnormal phenotype of the IκBα knockout mice. However, most defects in IκBα-/- mice are not complemented by endogenous IκBβ. Therefore, IκBα and IκBβ acquire their specific functions as a result of their divergent expression patterns.

LPS, IL-1 and the human T-cell leukemia virus-1 (HTLV-1) Tax protein induce IκBβ degradation with slower kinetics than IκBα degradation (Good and Sun, 1996; McKinsey et al., 1996; Thompson et al., 1995). Therefore, IκBβ was proposed to regulate the persistent activation of NF-κB in a cell type and stimulus dependent manner. κB-Ras proteins, which display homology to the small GTPase Ras, have been identified to specifically associate with IκBβ and appear to cause the slow kinetics of IκBβ degradation (Fenwick et al., 2000). In unstimulated cells, IκBβ is hyper-phosphorylated and IκBβ:NF-κB complexes reside exclusively in the cytoplasm because the hyper-phosphorylated IκBβ masks both NLS of associated NF-κB/Rel proteins (Malek et al., 2001). Basal phosphorylation of IκBβ involves CKII-mediated phosphorylation of serine 313 and serine 315 in the PEST region (Chu et al., 1996; McKinsey et al., 1997). IκBβ, which is newly synthesised during the post-induction phase, appears unphosphorylated (Suyang et al., 1996). Unphosphorylated IκBβ binds NF-κB in the cytoplasm, but fails to mask its NLS and DNA binding domains. Therefore, it translocates together with NF-κB to the nucleus and forms a ternary complex with DNA, which may shield associated NF-κB from feedback inhibition by IκBα (Tran et al., 1997).

IκBε Degradation of IκBε is also induced with slow kinetics (Whiteside et al., 1997). IκBε was found to interact specifically with RelA/RelA and RelA/c-Rel complexes and is most likely involved in the expression of specific genes, whose promotors bind preferentially RelA and c-Rel complexes, e.g. IL-8 (Kunsch and Rosen, 1993; Whiteside et al., 1997). IκBε-null mice develop normally and display only minor abnormalities including up-regulation of IgM and IgG1 Ig-isotypes and constitutively elevated *IL-1*, *IL-1R antagonist* and *IL-6* mRNA levels. The failure of observable augmentation of constitutive nuclear NF-κB-binding activity is probably due to compensatory mechanisms involving IκBα and IκBβ, which are up-regulated in several organs.

NF-κB1 p105 p105 has been shown to have a higher affinity for p50 than other members of the NF-κB/Rel family (Hatada et al., 1993; Liou et al., 1992). Deletion of the IκB-like C-terminal half of p105 (*nf-κb1*$^{\Delta C/\Delta C}$) in mice results in an increase of nuclear NF-κB activity in several organs, which consists mainly of p50 homodimers. This indicates that p105 is required to specifically control p50 homodimers and other IκBs are not able to compensate for the loss of the p105 (Ishikawa et al., 1998). Proteolysis of p105 in response to TNFα coincides with the nuclear appearance of p50 homodimers complexed with Bcl-3, which is independent of IκBα degradation (Heissmeyer et al., 1999). Therefore, signal-induced proteolysis of p105 is an important mechanism to specifically activate p50 homodimers, presumably inducing the expression of a specific subset of genes.

nf-κb1$^{\Delta C/\Delta C}$ mice display a severe hyper-inflammatory phenotype with enlarged spleens and lymph nodes, perivascular infiltration in lung and liver as well as increased susceptibility to opportunistic infections. Correspondingly, B cell proliferation in response to α-IgM and LPS is enhanced. However, T cell functions are moderately reduced, and cytokine production in macrophages is severely impaired. Therefore, analysis of these mice clearly demonstrates the dual function of nuclear p50 homodimers functioning either as transcriptional activators or repressors depending on the cell type.

NF-κB2 p100 Deletion of the C-terminal half of p100 (*nf-κb2*$^{\Delta C/\Delta C}$) in mice results in the enhanced nuclear activity of p52 homodimers, suggesting that p100 is particularly important for the control of p52 (Ishikawa et al., 1997). The pathophysiological consequences of increased nuclear p52 in these mice are gastric hyperplasia, atrophy of thymus and spleen, enlarged lymph nodes and increased granulopoiesis. Splenic T cells of *nf-κb2*$^{\Delta C/\Delta C}$ mice have increased proliferation responses and increased cytokine production.

p100 was also shown to be the bone fide inhibitor for RelB (Solan et al., 2002). Indeed, anti-CD40 and LTβ stimulation, which induces p100 processing, results the nuclear translocation of p52/RelB dimers (Coope et al., 2002; Yilmaz et al., 2003).

Bcl-3 Bcl-3 is not involved in the retention of NF-κB in the cytoplasm of cells. It is a nuclear protein, which binds specifically to nuclear p50 and p52 homodimers (Bours et al., 1993; Hatada et al., 1992). There are two proposed mechanisms by which Bcl-3 modulates their transcriptional effector function. First, Bcl-3 has been shown to be able to activate transcription by removing inhibitory homodimers from DNA (Franzoso et al., 1993; Nolan et al., 1993). Second, it recruits proteins, e.g. the HAT Tip60, which provide transactivation potential to homodimers (Bours et al., 1993; Dechend et al., 1999; Fujita et al., 1993). Bcl-3 knockout mice fail to clear infection with the intracellular bacterium *L. monocytogenes* and succumb upon infection with the gram-positive bacterium *S. pneumoniae* (Schwarz et al., 1997). They display defects in humoral immune responses characterised by a reduction in B cells, disorganised B and T cell areas in the spleen and impaired formation of germinal centres. This phenotype is most similar to that of $nf\text{-}\kappa b2^{-/-}$ mice and suggests that Bcl-3 mainly functions together with p52 terms of gene activation.

1.2.3.2 Targeting IκBα for the proteasome

The rapid degradation of IκBα in response to potent NF-κB stimuli, e.g. TNFα, IL-1 and LPS, requires phosphorylation of serines 32 and 36 in its N-terminus (Brockman et al., 1995; Brown et al., 1995; DiDonato et al., 1996; Traenckner et al., 1995; Whiteside et al., 1995). The two phosphorylated serines lie in a sequence motif DS*GψXS* (* marks phospho-serines, ψ is hydrophobic), which represents one of the best defined recognition motives for RING E3 ubiquitin ligases and is conserved between IκBα and

DSGΨxS

32 36

IκBα 24 RLLDDRHDSGLDSMKDE
IκBβ 11 ADADEWCDSGLGSLGPD
IκBε 10 EAEESQYDSGIESLRSL
NF-κB2/p100 858 PSTEVKEDSAYGSQSVE

NF-κB1/p105 919 RDSDSVCDSGVETSFRK

927 932

GRR Ankyrin repeats PEST

p105 DD

Diagram 4. IκB destruction boxes.
Target phosphorylation sites of IKK in IκBα, IκBβ, IκBε, p100 and p105. Position of phosphorylated serines are indicated for IκBα and p105. The p105 destruction box resides in the PEST region of p105.

IκBβ of all species so far examined (see Diagram 4) (Yaron et al., 1997). The equivalent serines in IκBβ have similarily been shown to be required for its signal-induced degradation (Weil et al., 1997).

Phospho-IκBα is recognised by β-transducin repeat containing protein (β-TrCP), a F-box WD repeat protein that is the receptor subunit of the SKp1-Cullin-F-box $(SCF)^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex (Kroll et al., 1999; Spencer et al., 1999; Winston et al., 199; Yaron et al., 1998). The concerted action of the $SCF^{\beta\text{-}TrCP}$ complex and a specific E2, UbcH5, results in the attachment of ubiquitin chains to two specific lysines (lysine 21 and 22) N-terminal to the recognition site (Baldi et al., 1996; Chen et al., 1995; Scherer et al., 1995). This modification then targets IκBα for rapid degradation by the 26S proteasome releasing associated NF-κB/Rel dimers to translocate to the nucleus. Inhibition of the proteasome efficiently blocks NF-κB activation indicating that neither phosphorylation nor ubiquitination results in the release of NF-κB from IκBs (Alkalay et al., 1995; DiDonato et al., 1995; Lin et al., 1995).

1.2.3.3 The IKK complex – structure and function

A cytokine responsive kinase activity specific for the regulatory serines 32 and 36 in IκBα was purified as a 700-900kDa protein complex (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). Some activity was also found at a size of 300kDa (Zandi et al., 1997). Three components of the high molecular weight IκB kinase (IKK) complex were subsequently identified by means of protein purification, micro sequencing and molecular cloning: IKK1, IKK2 and NEMO.

IKK1 and IKK2 (also referred to as IKKα and IKKβ), which are 85 and 87kDa proteins, respectively, are 50% identical (DiDonato et al., 1997; Mercurio et al., 1997;

Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). They contain an N-terminal kinase domain as well as leucine zipper and helix-loop-helix motifs. IKK1 and IKK2 serve as the catalytic subunits of the IKK complex.

The third component of the 700-900kDa IKK complex, NEMO (also designated as IKKγ), is a 48kDa regulatory subunit (Mercurio et al., 1999; Rothwarf et al., 1998). NEMO was also identified by genetic complementation of a cell line unresponsive to NF-κB activating stimuli (Yamaoka et al., 1998). Furthermore, NEMO is identical to FIP-3, which interacts with an adenovirus protein (Ad E3-14.7K), which had been shown to prevent TNFα-induced cytolysis (Li et al., 1999d). NEMO does not contain a catalytic domain but is composed mostly of three α-helical regions containing a leucine zipper and a zinc finger motif in its C-terminus. NEMO exist in either a dimeric, trimeric or tetrameric form (Agou et al., 2002; Rothwarf et al., 1998; Tegethoff et al., 2003). It is possible that the ability of NEMO to bind to itself and to interact with IKK1 and IKK2 is sufficient to account for the formation of the 700-900kDa IKK complex (Miller and Zandi, 2001). However, NEMO may also recruit other components into the complex.

The prevalent form of active IKK in mammalian cells contains both catalytic subunits (DiDonato et al., 1997; Mercurio et al., 1999; Mercurio et al., 1997; Rothwarf et al., 1998). However, studies with recombinant IKK1 and IKK2 expressed in insect cells indicate that IKK1 and IKK2 can form both hetero- and homodimers (Mercurio et al., 1999; Zandi et al., 1998). Indeed, in IKK1 or IKK2-deficient cell lines, homodimeric forms of the remaining catalytic subunit are complexed with NEMO (Hu et al., 1999; Li et al., 1999a; Li et al., 1999c; Li et al., 1999e; Tanaka et al., 1999). In NEMO-deficient cells, IKK1 and IKK2 elute on a gel filtration column with a molecular size of 300kDa, suggesting that the low molecular weight IKK complex may consist solely of IKK1 and IKK2 dimers (Yamaoka et al., 1998). A high molecular weight complex containing IKK2

but not IKK1 was detected in HeLa cells (Mercurio et al., 1999). Therefore, IKK complexes presumably display heterogeneity in terms of their subunit composition.

The kinase domains of IKK1 and IKK2 are similar to those of serine threonine kinases. Mutation of a highly conserved lysine (Lys44) in the ATP binding site generates catalytically inactive forms of IKK1 and IKK2 (Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1998; Zandi et al., 1997). Considerable evidence indicates that IKK activation is dependent on phosphorylation. Purified IKK is inactivated upon incubation with protein phosphatase 2A (PP2A), whereas treatment of HeLa cells with the PP2A inhibitor, okadaic acid, results in the activation of IKK (DiDonato et al., 1997). Like other kinases, IKK1 and IKK2 contain an activation loop in their kinase domains. This region contains specific sites, whose phosphorylation causes a conformational change that results in kinase activation (Johnson et al., 1996). Replacement of serines 177 and 181 in the activation loop of IKK2 with alanines prevents activation of IKK2 (Delhase et al., 1999; Mercurio et al., 1997). Replacement of these serines with glutamic acid (which mimics phospho-serine) results in constitutive IKK2 activity (Mercurio et al., 1997). Similarily, mutation of serine 176 to alanine inactivates IKK1, whereas substitution with glutamic acids results in its full activation (Ling et al., 1998).

In vitro both catalytic subunits of the IKK complex, IKK1 and IKK2, can phosphorylate the regulatory serines in IκBα (Mercurio et al., 1997; Zandi et al., 1998; Zandi et al., 1997). However, functional analyses of IKK1 and IKK2 by gene targeting in mice demonstrate that only IKK2 mediates IκBα degradation in response to pro-inflammatory stimuli (Li et al., 1999a; Li et al., 1999e; Tanaka et al., 1999). IKK2-/- cells exhibit very little NF-κB activation in response to TNFα or IL-1 and are more sensitive to TNFα-induced apoptosis than wild type cells. As a result, IKK2 knockout mice die at E13.5 to 14.5 due to massive apoptosis in the liver. This phenotype is essentially identical to that of mice lacking RelA (Beg et al., 1995a), which die at E15.5 to 16.5, or mice

deficient in both RelA and p50 (Horwitz et al., 1997), which die at E13.5 to14.5. IKK1 knockout mice are born alive, but die within 4 hours after birth (Hu et al., 1999; Li et al., 1999b; Takeda et al., 1999). IKK1-deficient mice display a defect in keratinocyte differentiation, which results in a thickened epidermis that causes fusion of extremities to the bodies of these animals. However, the function of IKK1 in keratinocyte differentiation is not dependent on IKK kinase activity or NF-κB activation (Hu et al., 2001). TNFα, IL-1 or LPS-induced IκBα degradation and IKK activation are normal in IKK1-deficient cells.

The C-terminus of NEMO is required for IKK activation induced by TNFα and IL-1, but not for basal IKK activity (Mercurio et al., 1999; Rothwarf et al., 1998), suggesting it may be required for the recognition by activators of IKK. Consistent with this hypothesis is the observation that over-expression of full-length NEMO or deletion mutants that contain its C-terminus inhibit activation of IKK or NF-κB in response to pro-inflammatory stimuli (Li et al., 1999d; Mercurio et al., 1999). NF-κB activation in response to TNFα, IL-1 or LPS is completely ablated in NEMO-deficient cells (Makris et al., 2000; Rudolph et al., 2000; Schmidt-Supprian et al., 2000). NEMO is an X-chromosome linked gene and NEMO-deficient male knockout mice die at E11.5–E12.5 from enhanced liver apoptosis due to the lack of TNFα-induced NF-κB activation. However, heterozygous NEMO -/+ female mice develop a unique dermatopathy similar to the human X-linked disorder incontinentia pigmenti (IP) characterised by keratinocyte hyper-proliferation, skin inflammation and increased apoptosis (Makris et al., 2000; Schmidt-Supprian et al., 2000). Human IP is also caused by NEMO mutation (Smahi et al., 2000).

1.2.3.4 Upstream of IKK

Over-expressed in cells, IKK1 or IKK2 can auto-phosphorylate the regulatory serines in their activation loops and exhibit strong activity in the absence of additional stimulation (Li et al., 1998; Zandi et al., 1998; Zandi et al., 1997). Therefore, it is possible that IKK auto-phosphorylation is sufficient for IKK activation. Physiologically, auto-phosphorylation may play a central role in response to the Tax protein of HTLV-1. Tax, which can stabilise dimer formation of proteins (Bex et al., 1998), has been shown to directly interact with NEMO (Chu et al., 1999; Harhaj and Sun, 1999a; Jin et al., 1999) and may bring two IKK catalytic domains in close contact, thereby inducing their auto-activation.

Alternatively, IKK activation may be mediated by upstream kinases. PKC isoforms (Lallena et al., 1999) and the MAP 3-kinase family members NIK (Lin et al., 1998b; Woronicz et al., 1997), MEK kinase (MEKK) 1 (Lee et al., 1998; Nemoto et al., 1998), MEKK2 (Zhao and Lee, 1999), MEKK3 (Zhao and Lee, 1999), TPL-2/Cot (Lin et al., 1999), transforming growth factor-β activated kinase 1 (TAK1) (Ninomiya-Tsuji et al., 1999; Sakurai et al., 1999) and Akt/PKB (Ozes et al., 1999; Romashkova and Makarov, 1999) have been implicated in the activation of the IKK complex. The activation loop motif in IKK1 and IKK2 is similar to those found in many MAP 2-kinases (S-X-X-X-S, see section 1.3). It is, therefore, not surprising that MAP 3-kinases can activate IKKs when over-expressed in cells. Catalytically inactive forms of MAP 3-kinases can bind to IKKs upon over-expression and function as potent inhibitors of their activation by pro-inflammatory stimuli. However, it is unclear whether these activities reflect their physiological function. Nevertheless, given the multiple and diverse upstream stimuli that activate NF-κB through IKK, it seems reasonable to predict that several physiological relevant IKK kinases exist.

Genetic studies are more definitive in delineating the physiological function of MAP 3-kinases in specific pathways that lead to NF-κB activation. Deletion of MEKK3 in mice disrupts TNFα-induced IκBα degradation and NF-κB activation, suggesting that MEKK3 may be upstream of IKK and function as the IKK kinase in the TNFR signalling pathway (Yang et al., 2001). Interestingly, PKCζ and PKCβ were demonstrated to be specifically required for TCR or BCR-induced NF-κB activation, respectively (Saijo et al., 2002; Su et al., 2002; Sun et al., 2000). However, knockout mice for MEKK1, NIK and TPL-2/Cot do not display an obvious defect in NF-κB activation in response to pro-inflammatory cytokines or LPS (Dumitru et al., 2000; Xia et al., 2000; Yin et al., 2001; Yujiri et al., 2000).

1.2.3.5 Signal-induced p100 processing

Slow processing of p100 is specifically induced by LTβ, BAFF and CD40 (Claudio et al., 2002; Coope et al., 2002; Dejardin et al., 2002; Kayagaki et al., 2002; Yilmaz et al., 2003). Interestingly, p100 processing is impaired in mice that carry the alymphoplasia (aly) mutation, which causes an amino acid substitution in the carboxy-terminal interaction domain of NIK and effects its interaction with IKK1 (Claudio et al., 2002; Coope et al., 2002; Dejardin et al., 2002; Matsushima et al., 2001; Shinkura et al., 1999; Yilmaz et al., 2003). Furthermore, NIK interacts with p100 and over-expressed NIK induces p100 processing, which requires two serines that reside in a motif homologous to the IκBα degradation box in the p100 C-terminus (Xiao et al., 2001b). NIK-induced p100 processing is dependent on IKK1 but not IKK2, suggesting that NIK functions upstream of IKK1 (Senftleben et al., 2001). LTβ requires IKK1 but not IKK2 or NEMO to induce p100 processing (Dejardin et al., 2002; Yilmaz et al., 2003) and BAFF-induced p100 processing is also NEMO-independent (Claudio et al., 2002).

Therefore, NIK may specifically activate IKK1 in a noncanonical NF-κB pathway, which is induced by LTβ, BAFF and CD40 to induce processing of p100. The retroviral oncoprotein Tax induces p100 processing via the recruitment of IKK1 to p100, but this is dependent on NEMO (Xiao et al., 2001a).

1.2.3.6 Signal-induced p105 proteolysis

Potent NF-κB stimuli such as TNFα, IL-1 and LPS induce slow proteolysis of p105. The p105 PEST region contains a motif $DS^{927}GVETS^{932}$, which is very similar to the sequence of N-terminal destruction boxes of IκBα, IκBβ, IκBε and p100 (see Diagram 4). Work from our laboratory has recently demonstrated that serines 927 and 932 of p105 are phosphorylated in response to TNFα stimulation and mutation of either residue to alanine blocks TNFα-induced p105 proteolysis (Lang et al., 2003; Salmeron et al., 2001). Over-expressed in cells, IKK2 can induce proteolysis of p105, which requires serines 927 and 932 in the PEST region, but not the N-terminal ubiquitination domain that is involved in constitutive p105 processing to p50 (Heissmeyer et al., 1999; Lang et al., 2003; Orian et al., 2000; Salmeron et al., 2001). Both IKK1 and 2 can directly phosphorylate p105 on serines 927 and 932 *in vitro* (Lang et al., 2003). Deficiency for either catalytic IKK subunit only marginally impairs TNFα and IL-1-induced p105 proteolysis in mouse embryonic fibroblasts, suggesting that IKK1 and 2 can substitute for each other in p105 phosphorylation *in vivo* (Salmeron et al., 2001). However, NEMO-deficient mouse embryonic fibroblast and a mutant pre-B cell line lacking NEMO are completely impaired in TNFα, IL-1 or LPS-induced p105 proteolysis, respectively (Heissmeyer et al., 2001; Lang et al., 2003; Salmeron et al., 2001). Together, these data indicate that phosphorylation of p105 on serines 927 and 932 by a NEMO-containing IKK complex is a crucial step in signal-induced p105 proteolysis. However, it is not clear

whether physiologically one or both catalytical subunits, IKK1 and IKK2, mediate p105 phosphorylation. The motif containing phosphorylated p105 serines 927 and 932 function as a recognition site for $SCF^{\beta\text{-}TrCP}$ ligase, which mediates TNFα-induced p105 ubiquitination and triggers its subsequent proteolysis by the proteasome (Heissmeyer et al., 2001; Lang et al., 2003; Orian et al., 2000). Lower affinity of β-TrCP to its recognition sequence in p105 than to the IκBα destruction box may affect the efficiency of p105 ubiquitination and slow down p105 proteolysis (Lang et al., 2003).

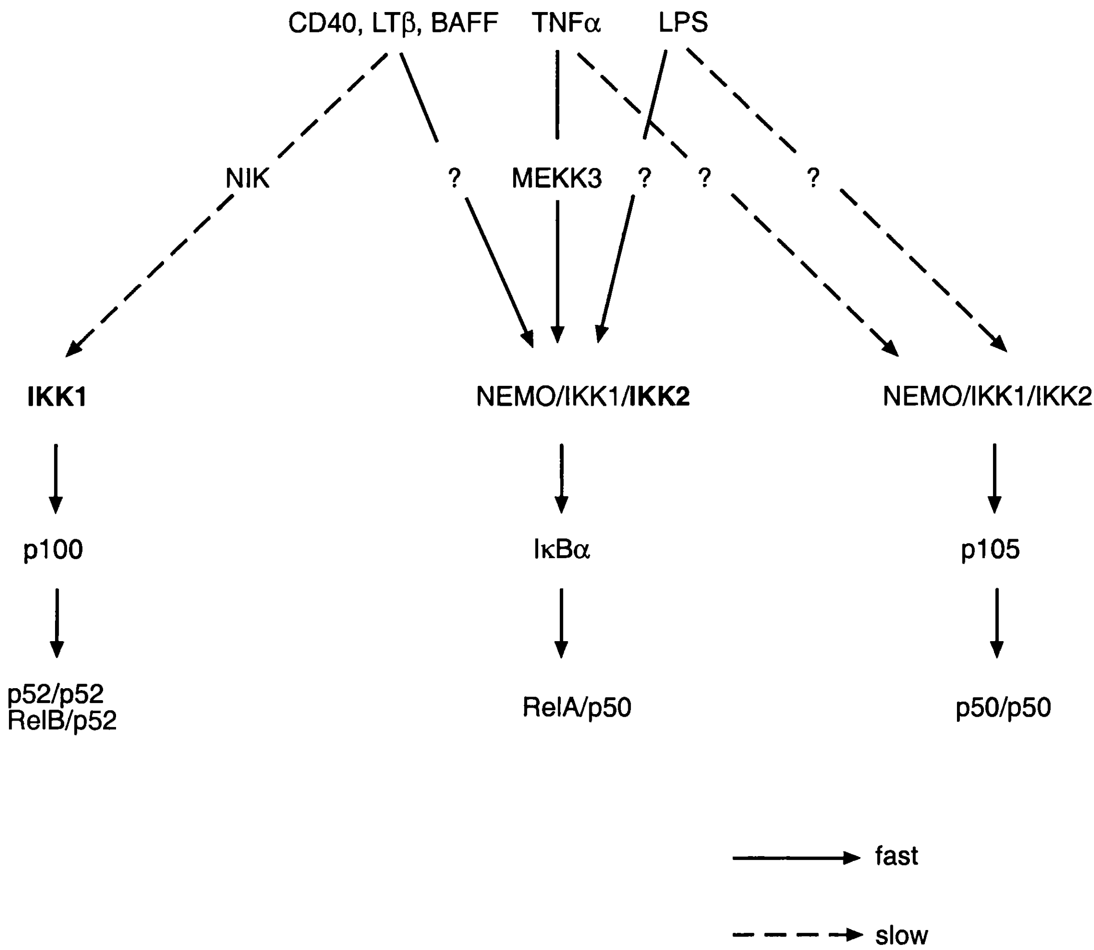


Diagram 5. Heterogenicity of NF-κB signalling pathways
LPS and TNFα induce the classical NF-κB pathway that involves NEMO, the catalytic activity of IKK2, rapid degradation of IκBα and nuclear translocation of RelA/p50. CD40/LTβ/BAFF also activate this pathway, but additionally induce a noncanonical pathway via IKK1 that results in slow processing of p100 and liberation of p52 and RelB. MEKK3 and NIK are required for TNFα-induced IκBα and CD40/LTβ/BAFF-induced p100 processing, respectively, and may act as IKK kinases in these pathways. It is not known whether other kinases upstream of IKK exist downstream of other stimuli. LPS/TNFα-induced p105 proteolysis is slow, involves NEMO and presumably controls p50, but it is unclear whether there is a prevalent role for either IKK1 or IKK2 in signal-induced p105 proteolysis.

1.3. MAP kinase signalling pathways

In addition to NF-κB, TNFα, IL-1 and LPS activate mitogen activated protein (MAP) kinases of the extracellular signal-related kinase (ERK), Jun amino-terminal kinase (JNK) and p38 families. MAP kinases respond to a wide variety of stimuli and have diverse physiological functions in the regulation of gene expression, cell proliferation and apoptosis (Chang and Karin, 2001; Dong et al., 2002; Hazzalin and Mahadevan, 2002). Upon activation, MAP kinases can translocate into the nucleus and often modulate the activity of transcription factors by phosphorylation of serine and threonine residues that are followed by a proline residue, designating MAP kinases as proline-directed kinases. Activator protein 1 (AP-1) is a common transcriptional regulator that is affected by all MAP kinases (Shaulian and Karin, 2002). AP-1 comprises a number of dimeric basic region leucine zipper proteins (bZIP) that belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra2), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) subfamilies. In promotors, AP-1 recognises either 12-O-tetradecanoylphorbol-13-acetate response elements (5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGAC/GTCA).

ERK The mammalian ERK family comprises ERK1 and ERK2. Upon activation, ERK1 and ERK2 phosphorylate and activate several nuclear transcription factors including Elk1, Ets1, Sap1a, c-myc, Tal and signal transducer and activator of transcription (STAT) proteins (Widmann et al., 1999). Ets transcription factors are involved in the induction of *fos* genes, whose products heterodimerise with Jun proteins to form AP-1. ERK also phosphorylates substrates in the cytoplasm such as the MAP kinase-activated protein kinase (MAPKAPK) ribosome S6 kinase ($p90^{rsk}$). Phosphorylation of $p90^{rsk}$ by ERK leads to its activation and translocation to the nucleus, where it activates c-fos by phosphorylation in the C-terminal trans-repression domain (Chen et al., 1996). $p90^{rsk}$ also phosphorylates GSK3, which results in its inactivation

(Sutherland et al., 1993). Active GSK3 has been shown to negatively regulate c-Jun and the transient inactivation of GSK3 has been proposed to enable the rapid activation of c-Jun and AP-1 (Boyle et al., 1991; Eldar-Finkelman et al., 1995).

JNK Members of the JNK family (JNK1, JNK2 and JNK3) phosphorylate transcription factors including c-Jun, ATF2, NFAT4, Elk-1, DPC-4 and p53 (Davis, 2000). c-Jun phosphorylation by JNK results in an increase in the formation of Jun/Jun homodimers and Jun/ATF2 heterodimers and makes c-Jun more resistant to ubiquitin-dependent degradation (Musti et al., 1997). Phosphorylation of c-Jun by JNK requires a specific docking site in c-Jun (Kallunki et al., 1994). However, heterodimerisation of c-Jun with other AP-1 components that lack a docking site enables phosphorylation of these proteins by JNK (Kallunki et al., 1996). JNK phosphorylation of ATF2 results in increased transcriptional activity (Gupta et al., 1995).

p38 There are four mammalian p38 kinase isoforms: p38α, p38β, p38γ and p38δ. p38 can phosphorylate the activation domain of Elk-1, which is also phosphorylated by ERK and JNK (Whitmarsh et al., 1997). Elk-1, therefore, integrates signals from all three MAP kinase pathways. Furthermore, p38 phosphorylates ATF2 in the activation domain, which results in increased transcriptional activity (Fuchs et al., 2000). p38 is also involved in post-transcriptional regulation of gene expression by phosphorylating and activating MAPKAPK-2 (Rouse et al., 1994). Activation of MAPKAPK-2 is required for AU-rich region mediated mRNA stabilisation and LPS-mediated TNFα production (Kotlyarov et al., 1999; Winzen et al., 1999).

MAP kinase regulation. MAP kinases are part of a three-component kinase module, which is conserved from yeast to human. Activation of MAP kinases is induced through sequential phosphorylation of the upstream kinases in this module. MAP kinase kinases (MAP 2-kinases, MKKs) are dual-specific kinases that phosphorylate a Thr-X-

Tyr motif in the activation loop of MAP kinases, which leads to their activation (Gartner et al., 1992). MAP kinase kinase kinases (MAP 3-kinases, MKKKs) activate MAP 2-kinases through phosphorylation of serines in a conserved motif (S-X-X-X-S) in their activation loop.

MAP 2-kinases are highly specific for their downstream MAP kinase targets. Thus, the MAP 2-kinases MAPK/ERK kinase (MEK) 1 and 2 specifically activate ERK1 and 2, while MKK4/7 activate JNKs, and MKK3/6 activate p38 kinases (see Diagram 6). Direct interactions between MAP 2-kinases and MAP kinases play a critical role in maintaining specificity of signal transmission. Additionally, scaffolding proteins, which organise MAP kinases in specific protein complexes with their upstream kinases contribute to specificity (van Drogen and Peter, 2002).

Each MAP 2-kinase can be activated by number of MAP 3-kinases when over-expressed in cells (Widmann et al., 1999). This may partially occur as an artefact of over-expression. However, genetic studies indicate that physiologically different MAP 3-kinases confer responsiveness of MAP 2-kinases to distinct stimuli. Thus, the MEK/ERK pathway is activated by RAF-1 in response to multiple hormones, growth and differentiation factors (Widmann et al., 1999), while TPL-2 activates MEK/ERK in response to LPS (see section 1.6). The large number of MAP 3-kinases, therefore, may allow a diversity of inputs from numerous stimuli to feed into specific MAP kinase pathways. Scaffolding proteins also recruit specific MAP 3-kinases to MAP kinase modules. Kinase supressor of Ras (KSR), for example, associates constitutively with MEK but binds to RAF-1 and ERK upon stimulation (Morrison, 2001).

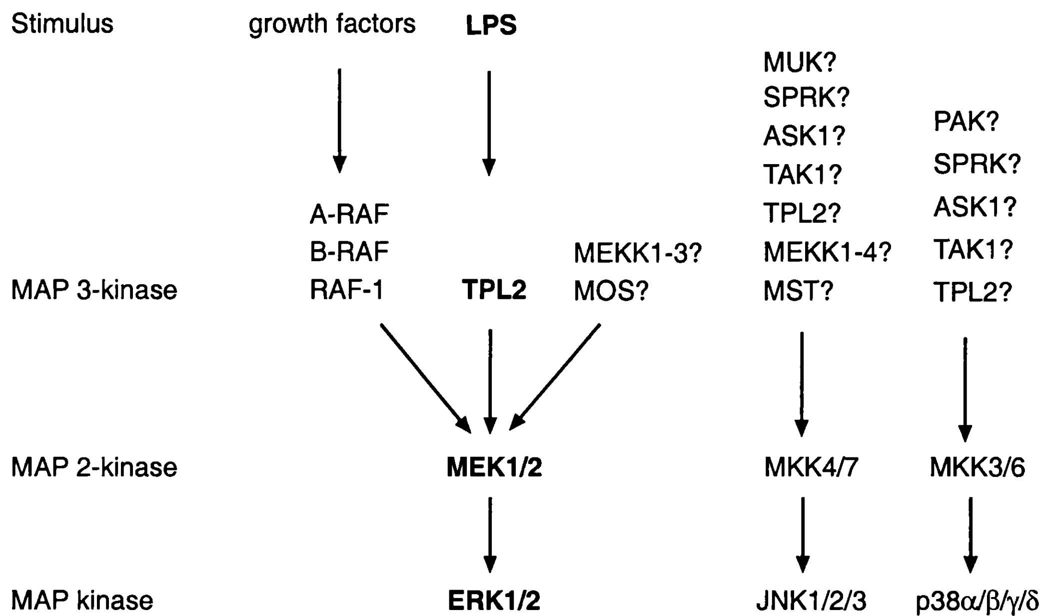


Diagram 6. MAP kinase signalling pathways.
MAP kinase signalling pathways consist of a three-component kinase module, which is activated by consecutive phosphorylation and comprises a MAP 3-kinase (MAP3K), a MAP 2-kinase (MAP2K) and a MAP kinase (MAPK). Different members of MAP kinases families and their specific upstream MAP 2-kinases, which can be activated by multiple MAP 3-kinases are indicated. This diagram is based on (Widmann et al, 1999).

As described earlier MAP 3-kinases may also play an important role in the activation of the IKK complex and NF-κB signalling. Therefore, in some cases MAP 3-kinases may represent branch points in the regulation of MAP kinase and NF-κB signalling pathways.

1.4. TNF receptor signalling

TNFR1 contains a death domain (DD) in its cytoplasmic tail, which is crucial for TNFR signal transduction (Tartaglia et al., 1993). DDs belong to the death domain superfamily of protein interaction domains, which also includes the death effector domains (DED) and the caspase recruitment domains (CARD) (Aravind et al., 1999). In an uninduced state the TNFR1 DD is complexed with silencer of death domains (SODD), a protein, which prevents TNFR1 signalling (Jiang et al., 1999). SODD dissociates upon stimulation and the adapter protein TNF receptor-associated death domain (TRADD) then binds via its DD to the TNFR DD (Hsu et al., 1995). TRADD is required for the recruitment of at least the three further signalling proteins: Fas-associated death domain (FADD), receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2) (Hsu et al., 1996a; Hsu et al., 1996b).

The DD of FADD forms a homo-typic protein interaction with the DD of TRADD. FADD was first identified as a protein that interacts with the cytoplasmatic domain of Fas/Apo, a member of the TNFR superfamily that is primarily involved in the induction of apoptosis. TNFR1 also induces apoptosis through the recruitment of FADD (Hsu et al., 1996b). FADD mediates the activation of caspase-8, which in turn activates a caspase-cascade leading to cytochrome-c release and apoptosis (Rath and Aggarwal, 1999).

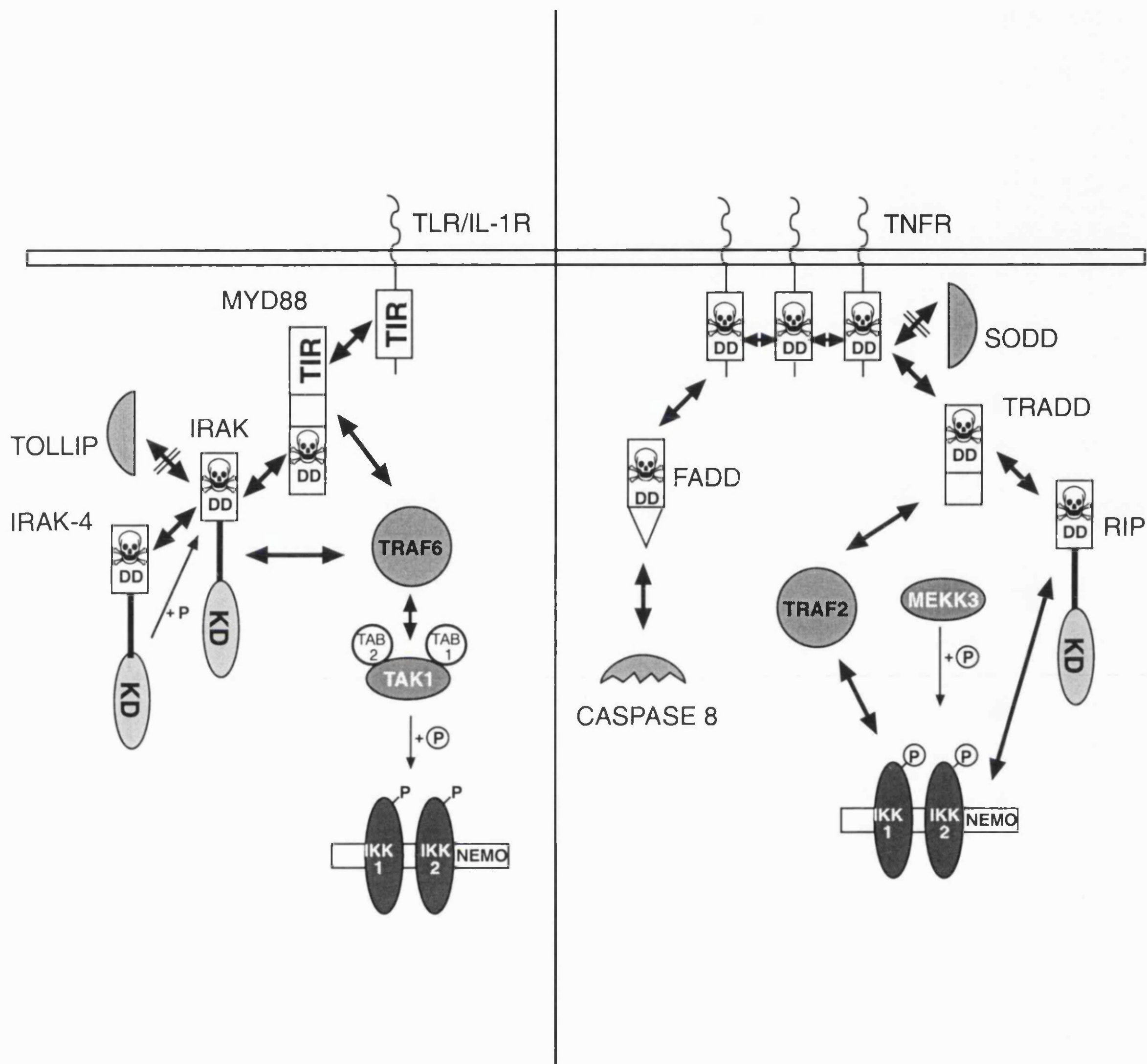


Diagram 7. TLR/IL-1R and TNFR signalling

Upon stimulation multiple signalling proteins are recruited to TLRs/IL-1Rs and TNFRs. Protein interactions are indicated by arrows and protein dissociations by crossed arrows. Death domains (DD) and TLR/IL-1 R homology (TIR) domains are indicated. Phosphorylation is indicated by arrows + P.

RIP is a serine threonine kinase that contains a C-terminal DD, which binds to the TRADD DD, an intermediate domain and a N-terminal kinase domain. Knockout experiments have shown that RIP is required for TNFα-induced NF-κB, but not MAPK activation (Kelliher et al., 1998). Interestingly, while the DD and the intermediate domain of RIP are required, the kinase domain of RIP is dispensable for NF-κB activation. RIP may, therefore, function solely as an adapter protein.

TRAF2 contains a C-terminal TRAF domain and at the N-terminus a ring finger and several zinc finger motives, which are required for TNFα-induced NF-κB activation (Baud et al., 1999; Liu et al., 1996; Rothe et al., 1995; Song et al., 1997). TRAF2 knockout mice, which are completely deficient in TNFα-induced JNK activation, display only a partial defect in NF-κB activation (Lee et al., 1997). Another TRAF protein, TRAF5, compensates for TRAF2 deficiency in NF-κB signalling, as TRAF2-TRAF5 double knockout mice are completely defective in TNFα-induced NF-κB activation (Tada et al., 2001).

The mechanism by which RIP and TRAF2 activate IKK is not clear. TRAF2 associates with MEKK1, but this seems to be important for the activation of the JNK (Chadee et al., 2002; Xia et al., 2000; Yujiri et al., 2000). It has been demonstrated that TRAF2 mediates the recruitment of the IKK complex to the activated TNFR1 (Devin et al., 2000; Devin et al., 2001; Zhang et al., 2000). TRAF2 directly interacts with IKK1 and IKK2, which requires the ring finger containing N-terminal domain of TRAF2 and the leucine zipper of the IKKs. RIP is not required for the recruitment of IKK to the receptor complex, but interacts with NEMO upon stimulation, which is necessary for IKK activation. The chaperone Cdc37/Hsp90, which was identified as an additional component of the IKK complex, may also be required for assembly, translocation and activation of the IKK complex (Chen et al., 2002). Induced oligomerisation of the IKK complex via RIP, NEMO or IKK1/2 is sufficient for IKK activation (Poyet et al., 2000).

Therefore, the induced proximity of IKK1 and IKK2 by the recruitment into the TNFR complex may be sufficient for trans-phosphorylation and auto-activation of IKK. Alternatively, recruitment of IKK into the TNFR complexes may facilitate its activation by MEKK3.

1.5. IL-1/Toll-like receptor signalling

IL-1R1 and the TLRs contain a homologous region in their intracellular tail, designated as the Toll-like/IL-1 receptor homology (TIR) domain, which is required for signal transduction. A central mediator of IL-1R/TLR signalling is the adaptor protein myeloid differentiation factor 88 (MyD88), which binds to the receptors via its TIR domain in a homotypic interaction upon stimulation (Adachi et al., 1998; Kawai et al., 1999; Muzio et al., 1997; Wesche et al., 1997). MyD88 also contains a DD, which binds to the DD of the serine threonine kinase IL-1 receptor associated kinase (IRAK) and mediates its recruitment to the activated receptor complexes (Cao et al., 1996a; Huang et al., 1997; Wesche et al., 1997). Similar to RIP, the kinase domain of IRAK is not required for NF-κB activation (Knop and Martin, 1999; Maschera et al., 1999). IRAK in turn forms a complex with the recently identified serine threonine kinase IRAK-4 and TRAF6 resulting in its hyper-phosphorylation, translocation to the cytoplasm and eventual degradation by the proteasome (Burns et al., 2003; Jiang et al., 2002; Li et al., 2002; Suzuki et al., 2002; Yamin and Miller, 1997).

TRAF6 plays an important role for IL-1 and LPS-induced activation of NF-κB and JNK (Lomaga et al., 1999). The C-terminal TRAF domain of TRAF6 is required for the complex formation with IRAK and its zinc finger and ring finger mediate IL-1/LPS-induced NF-κB activation (Baud et al., 1999; Cao et al., 1996b; Deng et al., 2000; Kobayashi et al., 2001). TRAF6 has been suggested to facilitate IKK activation by

functioning as an ubiquitin ligase, which mediates the assembly of unique poly-ubiquitin chains linked through lysine 63 (K63), in conjunction with an ubiquitin-conjugating enzyme (E2) complex composed of Ubc13 and Uev1A (Mms2) (Deng et al., 2000). K63-ubiquitinated TRAF6 was shown to be able to activate a complex of the MAP 3-kinase TAK1 and TAK1 binding protein (TAB) 2, which in turn can phosphorylate the activation loops of IKK2 and MKK6 leading to the activation of both NF-κB and p38 (Wang et al., 2001). Consistent with this TRAF6 forms a complex with pre-assembled TAK1-TAB1-TAB2 at the membrane, which then translocates to the cytoplasm (Jiang et al., 2002; Takaesu et al., 2000). However, a role of TAK1 in IL-1/LPS-induced IKK activation remains to be confirmed genetically.

1.6. Tumor progression locus-2 (TPL-2)

The rat serine / threonine kinase tumor progression locus-2 (TPL-2) was initially identified as a target for provirus integration in Moloney murine leukemia virus-induced T cell lymphomas (Patriotis et al., 1993). The provirus integrates into the last intron of the TPL-2 gene, which results in enhanced expression of a truncated TPL-2 mRNA transcript encoding a protein that is altered at its C-terminus (Makris et al., 1993). A C-terminally truncated form of the human homologue of TPL-2, known as cancer osaka tyroid (Cot), was independently identified as a transforming gene for a human thyroid carcinoma cell line (Chan et al., 1993). Transgenic mice expressing the truncated form of TPL-2 under the control of a T cell-specific promoter develop T cell lymphoblastic lymphomas, confirming its oncogenic potential (Ceci et al., 1997).

TPL-2/Cot encodes a serine/threonine kinase expressed as two isoforms of 46kDa and 52kDa as a result of alternative initiation of translation (Aoki et al., 1993). TPL-

2/Cot mRNA is mainly expressed in lymphoid tissues and gut endothelium in the adult (Aoki et al., 1993; Makris et al., 1993; Ohara et al., 1993).

Studies in cell lines have demonstrated that over-expressed TPL-2 activates the ERK, JNK and p38 MAP kinase pathways (Chiariello et al., 2000; Patriotis et al., 1994; Salmeron et al., 1996). *In vitro* experiments indicate that this activity results from TPL-2 acting as a MAP 3-kinase, which directly phosphorylates and activates the respective MAP 2-kinases for each of the MAP kinase pathways (Chiariello et al., 2000; Salmeron et al., 1996). However, the physiological relevance of the MAP 3-kinase activity of TPL-2 has only recently been established by analysis of TPL-2 knockout mice (Dumitru et al., 2000). Thus, LPS activation of MEK1 and 2, which phosphorylate and activate ERK1 and 2, is blocked in bone marrow-derived macrophages (BMDMs) from TPL2-deficient mice (Dumitru et al., 2000). In contrast, LPS activation of JNK and p38 in the knockout macrophages is normal, demonstrating that in these cells TPL-2 is physiologically a MAP 3-kinase only for the ERK MAP kinase cascade. Because of the MEK/ERK signalling defect, LPS induction of TNFα production is dramatically reduced in TPL2-deficient macrophages due to defective transport of TNFα mRNA from the nucleus to cytoplasm (Dumitru et al., 2000). Induction of COX-2 by LPS in macrophages is also abrogated in the absence of TPL2 (Eliopoulos et al., 2002). As a result of these defects, TPL-2-/- mice are resistant to LPS/D-galactosamine-induced endotoxin shock (Dumitru et al., 2000). A TPL-2-/- background also strongly attenuates the development of TNFα-mediated pathology in a mouse model for Crohn's inflammatory bowl disease (Kontoyiannis et al., 2002).

TPL-2 also activates NFAT and NF-κB upon over-expression and was implicated in IL-2 production during T lymphocyte activation. (Ballester et al., 1997; Tsatsanis et al., 1998a; Tsatsanis et al., 1998b). NF-κB activation by over-expressed TPL-2 results from its activation of the IKK complex (Lin et al., 1999). However, many MAP 3-kinases

activate the IKK complex when over-expressed (Karin and Ben-Neriah, 2000), presumably due to the similarity of the activating phospho-acceptor sites on its two component kinase subunits, IKK 1 and IKK2, to those in MAP 2-kinases (Delhase et al., 1999). Crucially, TPL-2 knockout mice have no obvious defects in T cell activation or NF-κB activation (Dumitru et al., 2000). Therefore, activation of IKK by TPL-2 may be an over-expression artefact and TPL-2 is unlikely to have a function in T cells activation.

Our laboratory has previously demonstrated that TPL-2 binds stoichiometrically to the C-terminal half of NF-κB1 p105, and that over-expressed TPL-2 induces proteolysis of p105 (Belich et al., 1999). TNFα stimulation of p105 proteolysis is triggered by IKK complex phosphorylation of p105 (Lang et al., 2003; Salmeron et al., 2001). It is, therefore, possible that the stimulatory effect of over-expressed TPL-2 on p105 proteolysis is mediated via the activation of the endogenous IKK complex. However, it was also demonstrated that kinase-inactive TPL-2 blocks only TNFα-induced p105 proteolysis, but not TNFα-induced IκBα degradation in Jurkat cells (Belich et al., 1999). Therefore, it remains to be elucidated whether TPL-2 has a physiological function specifically in signal-induced p105 proteolysis.

1.7. Specific aims of this study

Most NF-κB activating stimuli induce the rapid activation of the IKK complex and degradation of IκBα. However, TNFα, IL-1 or LPS-induced p105 proteolysis is inefficient and occurs with relatively slow kinetics. Therefore, distinct mechanisms must exist to regulate signal-induced p105 proteolysis.

Sequence homology analysis reveals that the p105 C-terminus contains a DD (Diagram 8A) (Feinstein et al., 1995; Schultz et al., 1998). DDs commonly mediate

protein interactions between receptors and adapter proteins in signalling pathways leading to the induction of NF-κB, AP-1 and apoptosis. Therefore, we aimed to investigate whether the p105 DD mediates the interaction of p105 with upstream signalling proteins and whether this is important for signal-induced p105 proteolysis.

TPL-2 has been implicated in p105 proteolysis, but also activates the MEK/ERK MAP kinase signalling pathway in response to LPS in macrophages (Belich et al., 1999; Dumitru et al., 2000). TPL-2 stoichiometrically associates with p105, but the consequence of this interaction for TPL-2 function is not known. *In vitro* TPL-2 binding to p105 requires its C-terminus, which when deleted activates its oncogenic potential. Therefore, we aimed to further characterise the nature of the physical interaction of TPL-2 and p105 as well as its consequence for TPL-2-induced p105 proteolysis and MEK activation.

A

			α1		α2		α3			α4		α5		α6	
hNF-κB1 p105	814	DV	KLQLYKLLEI	PDP	DKNWATLAQKL	G[illegible]G	IL.NNAFRLS	PA		PSKTLMDNYEVSG		GTVRELVEALR	QMG	YTEAIEVIQ	AASS
hNF-κB2 p100	871	QN	LEQLLDGPEA		QGSWAELAERL	GLR	SL VDTYRQT	TS		PSGSLLRSYELAG		GDLAGLLEALS	DMG	LEEGVRLLR	GPET
hMYD88	32	RL	SLFLNVRTQV		AADWTALAEEM	DFE	YLEIRQLETQ	AD		PTGRLLDAWQGRP	G	ASVGRLLELLT	KLG	RDDVLLELG	PSI
hIRAK	26	VM	CRFYKVMDAL	E	PADWCQFAALI	VRD	QTELRLCERS	GQ		RTASVLWPWINRN		ARVADLVHILT	HLQ	LLRARDII	
dTube	36	VE	DNDIYRLAKI	LDEN	SC.WRKLMS.I	IPK	GMDVQACSGA	GCLNFPAEIKKGFKY	*	KSQMMIDEWKTSG	KLNER	PTVGVLLQLLV	QAE	LFSAADFVA	LDFL
dPelle	40	PV	RAQLCAHLDA		LDVWQQLATAV	KLY	PDQVEQISSQ	KQRGRS		ASNEFLNIWGGQY	N	HTVQTLFALFK	KLK	LHNAMRLIK	DYV
hp75NGFR	341	AK	REEVEKLLNG	SA	GDTWRHLAGEL	GYQ	PEHIDSFTHE	AC		PVRALLASWATQD	S	ATLDALLAALR	RIQ	RADLVESLC	SES
hFas/Apol	226	DV	DLSKYITTIA	GVMT	LSQVKGFVRKN	GVN	EAKIDEIKND	NVQD.TAE		QKVQLLRNWHQLH	GKKE	A.YDTLIKDLK	KAN	LCTLAEKIQ	TIIL
hTNFR1	352	DT	DDPATLYAVV	ENVP	PLRWKEFVRRL	GLS	DHEIDRLELQ	NGRC.LRE		AQYSMLATWRRRT	PRRE	ATLELLGRVLR	DMD	LLGCLEDIE	EALC
hRIP	290	TT	SLTDKHLDPI	RENL	GKHWKNCARKL	GFT	QSQIDEIDHD	YERDGLKE		KVYQMLQKWVMRE	GIKG	ATVGKLAQALH	QCS	RIDLLSSLI	YVS
hTRADD	211	NR	PLSLKDQQTF	ARSV	GLKWRKVGRSL	QRG	CRALRDPALD	SLAYEYEREGLYE		QAFQLLRRFVQAE	GRR	ATLQRLVEALE	ENE	LTSLAEDLL	GLT

*TAQDVFQIDEAANRLPPDQS

B

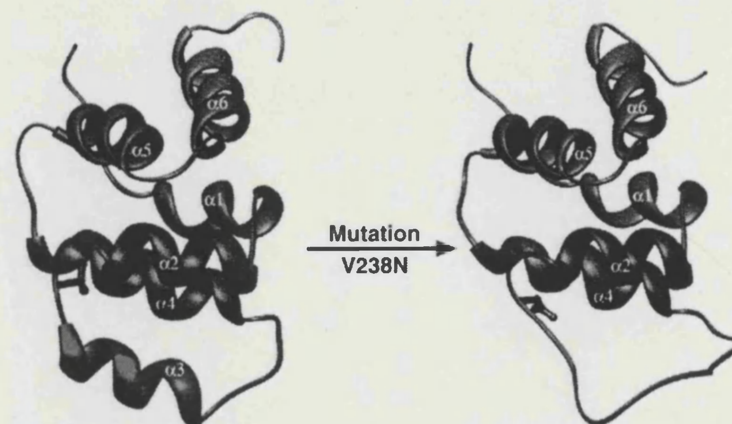


Diagram 8. Structure of death domains.
(A) Alignment of death domain protein sequences (reproduced from Feinstein et al., 1995). The first residue within the individual proteins is indicated. Homologous residues to the lpr mutation in Fas are highlighted. **(B)** Ribbon illustration of the NMR structure of the wilde type or lpr Fas death domain, respectively (reproduced from Eberstadt et al., 1997). α-helices 1-6 are indicated.

2. Results

2. Results

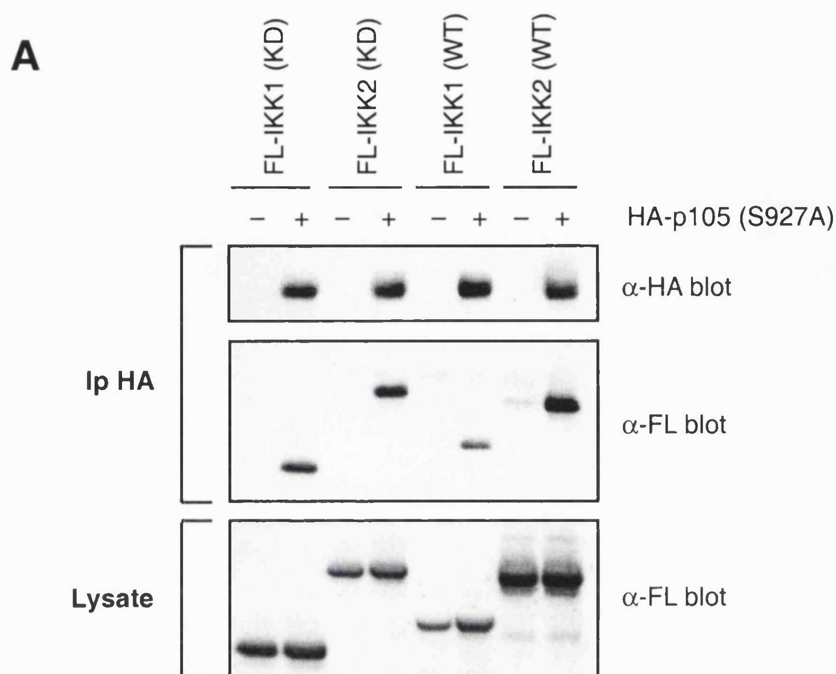
2.1. The p105 DD is a docking domain required for signal-induced p105 proteolysis

2.1.1 IKK1 and IKK2 interact with NF-κB1 p105 in transiently transfected 293 cells

The IKK complex is essential for TNFα induced p105 proteolysis and directly phosphorylates p105 on serines 927 and 932 (Lang et al., 2003; Salmeron et al., 2001). To analyse whether p105 can interact with the IKK complex, Flag-tagged IKK1 or IKK2 (FL-IKK1 or FL-IKK2) were co-expressed with HA-tagged $p105_{S927A}$ (HA-$p105_{S927A}$) or control empty vector (EV) in 293 cells. HA-$p105_{S927A}$ lacks one of the critical regulatory phosphorylation sites involved in signal-induced p105 proteolysis, and therefore, its expression levels are not affected when co-expressed with active IKK subunits (Salmeron et al., 2001). Interestingly, both FL-IKK1 and FL-IKK2 specifically co-immunoprecipitated with HA-$p105_{S927A}$ at similar levels (Figure 1A). Kinase-inactive (KD) point mutants of FL-IKK1 and FL-IKK2 interacted with HA-$p105_{S927A}$ to a similar extent as the wild type proteins (Figure 1A). However, it should be noted that the kinase inactive form of IKK1 lacks 17 C-terminal residues (H. Nakano, personnel, communication). No association was detected between Myc-p105 and HA-NEMO (Figure 1B). Therefore, both FL-IKK1 and FL-IKK2 bind to HA-p105 and this interaction is unlikely to be bridged by endogenous NEMO.

2.1.2 p105 interacts with the endogenous IKK complex

To determine whether p105 binds to the IKK complex at physiological expression levels, the endogenous IKK complex was immunoprecipitated with anti-IKK1/2 antibody



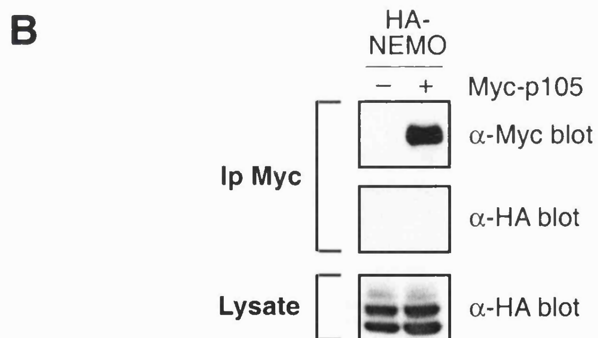


Figure 1. p105 interacts with co-expressed IKK1 and IKK2 in 293 cells.
(A) 293 cells were co-transfected with the indicated expression vectors and cultured for 36h. HA-p105S927A was immunoprecipitated from cell lysates with anti-HA mAb and Western blotted. Blots were sequentially probed with the indicated antibodies. **(B)** HA-NEMO was immunoprecipitated from lysates of 293 cells as in A and Western blots probed sequentially with anti-Myc and anti-HA mAbs.

from extracts of $HeLa_{Ohio}$ cells, pre-treated with MG132 inhibitor to block proteasome mediated p105 proteolysis. Low levels of p105 specifically co-immunoprecipitated with IKK1/2 from control cells and slightly increased levels from cells stimulated for 15min with TNFα (Figure 2). Similar results were obtained in 293 cells and THP-1 monocytes and with cells cultured in the absence of MG132 (data not shown). Thus, the endogenous IKK complex constitutively associates at low stoichiometry with endogenous p105 and this interaction is marginally increased by TNFα stimulation over a period of 15min during which maximal p105 serine 927 and 932 phosphorylation occurs (Lang et al., 2003; Salmeron et al., 2001).

2.1.3 The p105 DD is required for the interaction of p105 with the IKK complex

To define the sites of interaction for FL-IKK1 and FL-IKK2 on p105, two HA-p105 mutants were generated in which the C-terminus was deleted to remove the PEST region or PEST plus DD (see Figure 3A). These mutants were then transiently expressed in 293 cells with kinase-inactive FL-IKK1 or FL-IKK2, which do not affect HA-p105 expression levels and interact with HA-$p105_{S927A}$ similar to their wild type counterparts (Figure 1A). Removal of the PEST region (HA-$p105_{1\text{-}892}$), which contains the IKK target site (Lang et al., 2003; Salmeron et al., 2001), had little effect on the amount of FL-IKK1 immunoprecipitating with HA-p105 (Figure 3B). There was a small reduction in the interaction of FL-IKK2 with HA-$p105_{1\text{-}892}$ compared to wild type HA-p105. However, further deletion to remove the DD (HA-$p105_{1\text{-}801}$) reduced the amount of co-immunoprecipitating FL-IKK1 and FL-IKK2 to control levels (Figure 3B). A p105 mutant containing an internal deletion (residues 802-892), which specifically removed the DD, HA-$p105_{\Delta DD}$, was also severely impaired in its ability to bind to both FL-IKK1 and FL-IKK2 compared to wild type HA-p105. HA-$p105_{\Delta DD}$ constantly ran as a doublet on SDS-PAGE. *In vitro* phosphatase treatment of immunoprecipitates demonstrated the

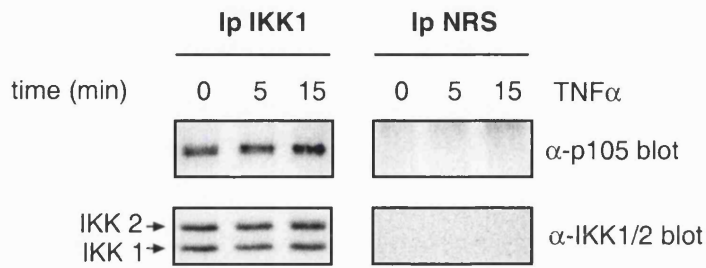


Figure 2. Endogenous p105 interacts with IKK1 and IKK2.
HeLa cells were pre-treated for 30min with MG132 to block proteasome function. Cells were then cultured for 5 and 15min with TNFα or control medium before lysis. The IKK complex was isolated from cell lysates by immunoprecipitation with anti-IKK1/2 antiserum. Non-immune rabbit serum (NRS) was used for control immunoprecipitations. A Western blot of immunoprecipitated protein was sequentially probed for p105 and IKK1/2.

slower migrating form corresponds to a phosphorylated form of the lower HA-p105$_{\Delta DD}$ band (data not shown). It was not possible to test whether the p105 DD was sufficient for IKK1/2 binding, as the p105 DD (FL-p105$_{802-892}$) was not expressed at significant levels in 293 cells (data not shown). Nevertheless, the data in this section demonstrate that the p105 DD is required for IKK binding to p105. The PEST region contributes to binding to a limited extent and probably accounts for the low level of FL-IKK1/2 binding to HA-p105$_{\Delta DD}$. This may be due to the interaction of the IKK2 kinase domain with its target motif on p105 in the 293 cell over-expression system.

It was important to rule out the possibility that DD deletion mutants failed to bind IKK1 and IKK2 due to gross structural alterations in p105. The Fas DD and other DDs comprises six anti parallel, amphipathic α-helices (see Diagram 8) (Huang et al., 1996; Weber and Vincenz, 2001). A naturally occurring mutation in the DD of Fas (V238N) in *lpr* mice leads to the accumulation of auto-reactive T lymphocytes and the development of a systemic lupus erythematosus-like autoimmune disease (Watanabe-Fukunaga et al., 1992). This mutation locally unfolds the Fas DD in the region corresponding to α-helix three in the wild type protein with no detectable conformational changes in other regions of the DD (see Diagram 8B) (Eberstadt et al., 1997). V238N Fas cannot bind to its downstream signalling intermediate FADD and therefore cannot trigger apoptosis (Huang et al., 1996). To determine whether α-helix three in the p105 DD is involved in binding to IKK, leucine 841 of p105 was mutated to alanine to generate the HA-p105$_{L841A}$ mutant (see Figure 3A). HA-p105$_{L841A}$ was also impaired in its ability to bind to both kinase-inactive FL-IKK1 and FL-IKK2 compared to wild type HA-p105 when over-expressed in 293 cells, although not to such a great extent as HA-p105$_{\Delta DD}$ (Figure 3C). These data indicate that α-helix three of p105 DD is involved in binding to the IKK complex. The reduced binding of HA-p105$_{L841A}$ to IKK1 and IKK2 also suggests that gross conformational changes do not account for the inability of DD deletion mutants of

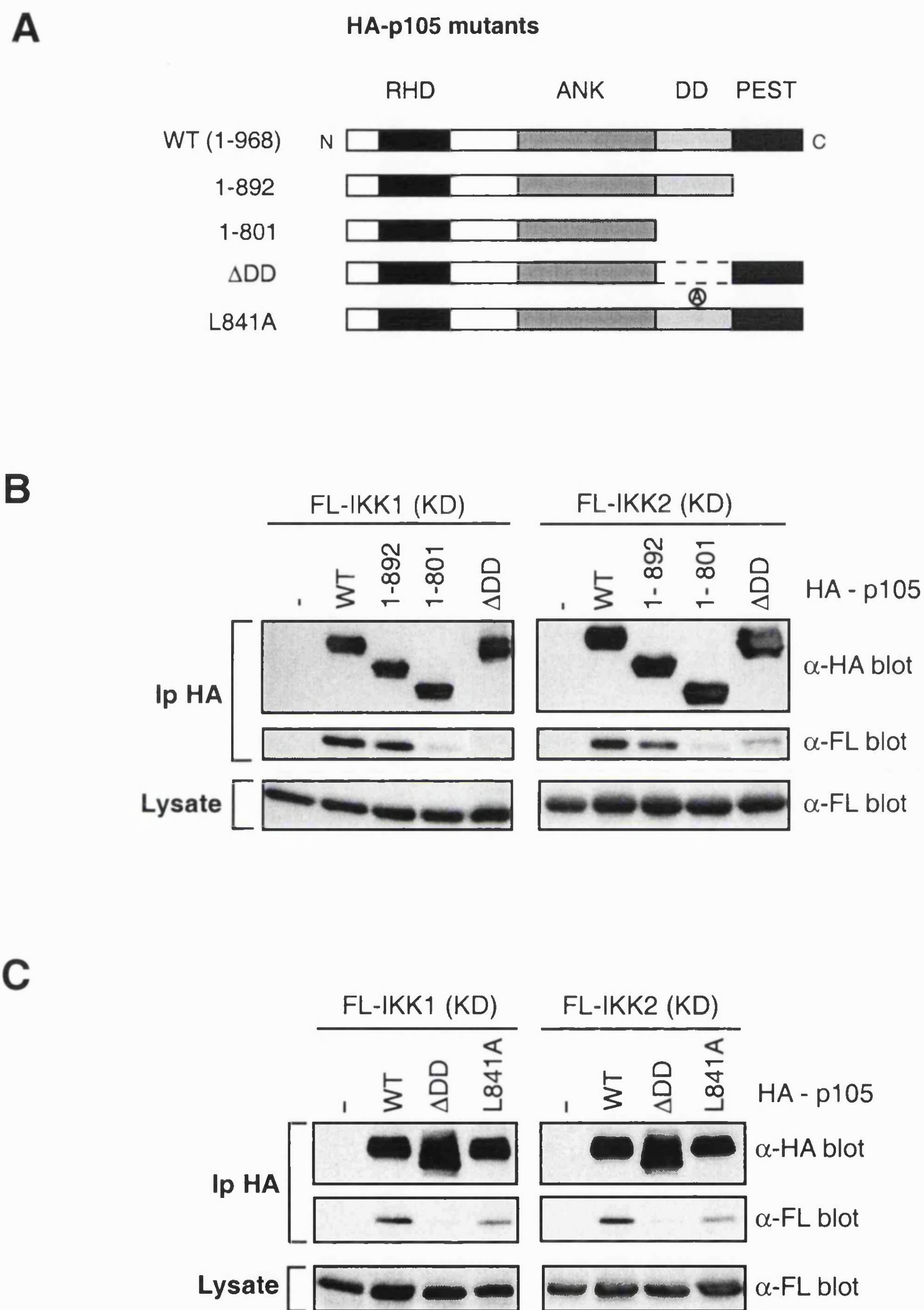
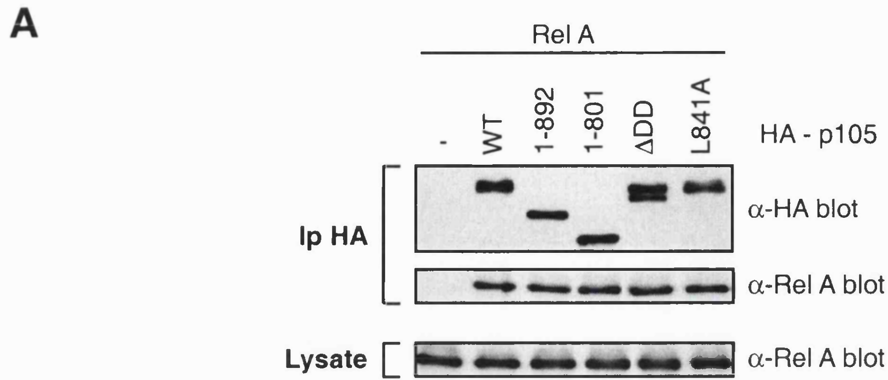


Figure 3. The p105 death domain is required for p105 binding to IKK1 and IKK2. **(A)** Schematic diagram of p105 point and deletion mutants. The relative positions of the Rel homology domain (RHD), ankyrin repeats (ANK), death domain (DD) and PEST region are shown. **(B and C)** 293 cells were co-transfected with vectors encoding FL-IKK1 (KD) or FL-IKK2 (KD), as indicated, and wild type, deletion or point mutants of HA-p105. Lysates were prepared from cells after 36h culture and immunoprecipitated with anti-HA mAb. Western blots of immunoprecipitated protein (Ip HA) and total cell lysates (Lysate) were probed with the antibodies shown.



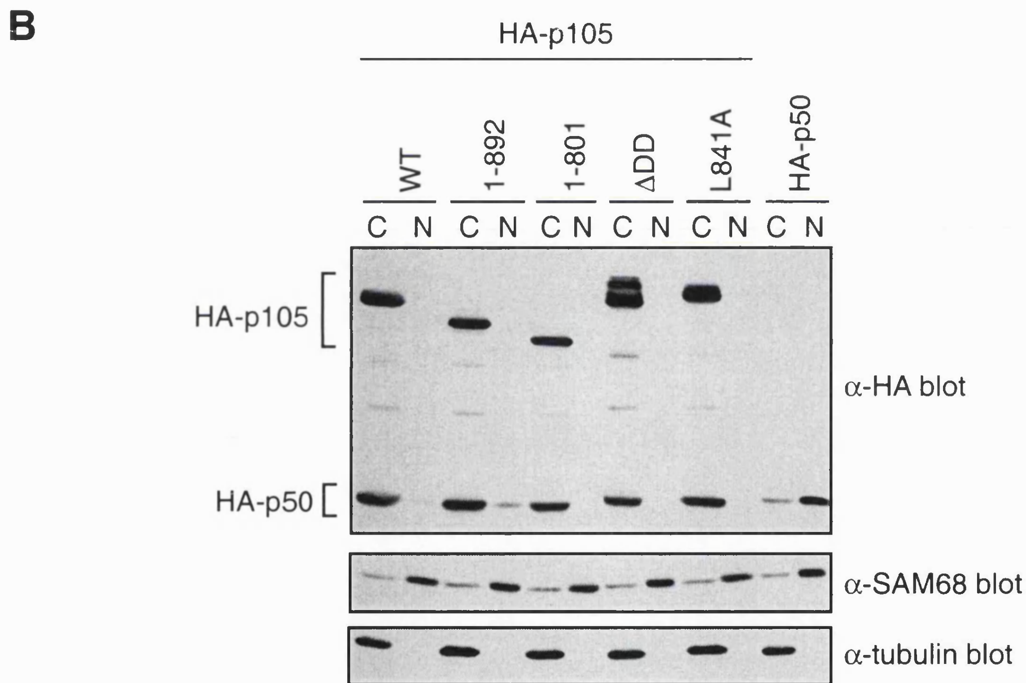
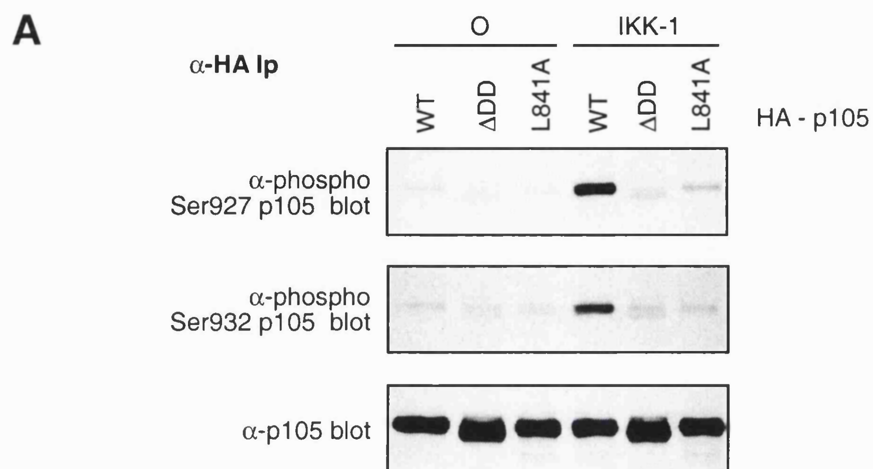


Figure 4. p105 death domain mutants bind to RelA and are retained in the cytoplasm. **(A)** 293 cells were co-transfected with vectors encoding RelA and wild type, deletion or point mutants of HA-p105. Lysates were prepared from cells after 36h culture and immunoprecipitated with anti-HA mAb. Western blots of immunoprecipitated protein (Ip HA) and total cell lysates (Lysate) were probed with the antibodies shown. **(B)** 293 cells were transfected with vectors encoding the indicated HA-p105 constructs. Nuclear (N) and cytoplasmic (C) fractions were prepared, resolved by 8% SDS-PAGE, Western blotted and sequentially probed with the antibodies shown. SAM68 and tubulin were used as controls for nuclear or cytoplasmatic fractionations, respectively.

HA-p105 to bind FL-IKK1/2. Consistent with this hypothesis, all of the p105 mutants could bind to RelA with equal efficiency (Figure 4A). In addition, the p105 mutants exclusively localised to the cytoplasm similar to the WT, while HA-p50 localised to the nucleus (Figure 4B). Thus, in agreement with previous data deletion of the PEST region and/or DD did not affect function of ankyrin repeats of p105 (Blank et al., 1991).

2.1.4 A functional p105 DD is required for efficient serine 927/932 phosphorylation *in vitro* by IKK1 or IKK2

The p105 DD facilitates the interaction between p105 and the IKK complex. In many cases, the specificity of protein kinases is determined by docking sites within the substrate, distinct from the phospho-acceptor site (Holland and Cooper, 1999). Such docking sites recruit kinases to their correct substrates and enhance their fidelity and efficiency of action. Serines 927 and 932 are essential for signal-induced proteolysis of p105 and are directly phosphorylated by purified IKK1 and IKK2 *in vitro* (Salmeron et al., 2001; Lang et al.,2003). In initial functional experiments, therefore, the role of the DD in phosphorylation of p105 serines 927 and 932 by IKK *in vitro* was investigated. To do this, HA-p105, HA-$p105_{\Delta DD}$ or HA-$p105_{L841A}$ were transiently expressed in 293 cells and isolated by immunoprecipitation with anti-HA mAb. The immunoprecipitated proteins were then phosphorylated by recombinant IKK1 or IKK2 protein and Western blotted with anti-phospho Ser927 p105 and anti-phospho Ser932 p105 antibody (Figure 5A and B). Both IKK1 and IKK2 clearly phosphorylated serine 927 and 932 of wild type HA-p105. In contrast, IKK1 and IKK2 induced little detectable serine 927 and 932 phosphorylation of either HA-$p105_{\Delta DD}$ or HA-$p105_{L841A}$. Immunoblotting with anti-p105 antibody confirmed that similar levels of HA-p105 were immunoprecipitated in each



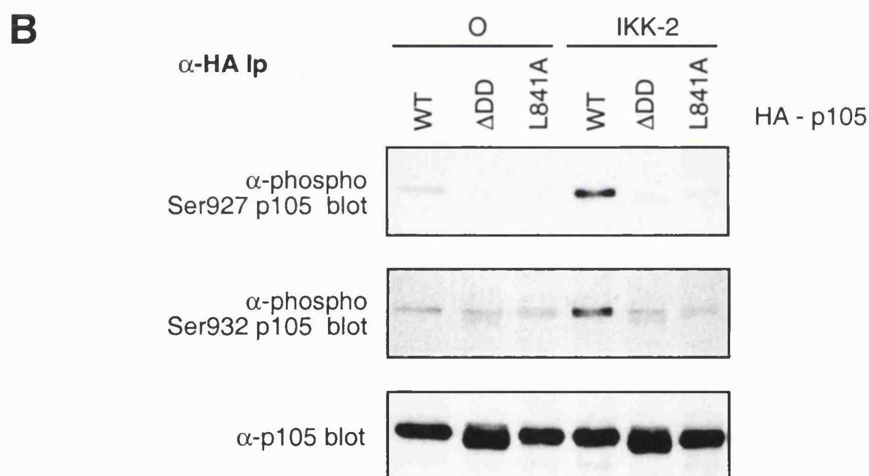


Figure 5. Phosphorylation of p105 serine 927/932 by IKK1 and IKK2 *in vitro* requires a functional p105 death domain.
(A and B) 293 cells were transfected with HA-p105, HA-p105ΔDD or HA-p105L841A. Lysates were prepared after 24h culture and immunoprecipitated with anti-HA mAb. Immunoprecipitates were phosphorylated *in vitro* with IKK1 or IKK2 and then resolved by 8% SDS-PAGE and Western blotted. Blots were sequentially probed with anti-phospho Ser927 p105, anti-phospho Ser932 p105 and anti-p105 antibodies.

case. These experiments indicate that the DD is essential for both IKK1 and IKK2 to efficiently phosphorylate HA-p105 *in vitro*.

2.1.5 Efficient p105 proteolysis induced by over-expressed IKK2 requires a functional p105 DD

FL-IKK2 triggers degradation of HA-p105 when co-expressed in 3T3 cells (Salmeron et al., 2001). Therefore, to determine whether the p105 DD is important for the IKK complex to regulate p105 proteolysis *in vivo*, an expression vector encoding FL-IKK2 or an empty vector with no insert (EV) was co-transfected in 3T3 cells with vectors encoding wild type, ΔDD or L841A HA-p105. Whole cell lysates of transfected cells were Western blotted with anti-HA mAb, which recognises the N-terminal HA-tag of HA-p105 as well as its processed product HA-p50 (Figure 6A). In FL-IKK co-transfected cells the amount of wild type HA-p105 remaining was reduced compared to EV co-transfected cells, while the amount of HA-p50 remained the same (Figure 6A). This is illustrated in a shift in the ratio of the amounts of HA-p50/HA-p105 (Figure 6B). In contrast, FL-IKK2 co-expresssion hardly induced a decrease in HA-p105$_{\Delta DD}$ or HA-p105$_{L841A}$ levels, and therefore, caused no obvious shift in their ratios to HA-p50. Similar amounts of IKK2 were expressed in each case. These data suggest that a functional p105 DD is required for IKK2 to induce p105 proteolysis when co-expressed in cells.

To confirm this, the turnover of HA-p105, HA-p105$_{\Delta DD}$ or HA-p105$_{L841A}$ co-expressed with FL-IKK2 in 3T3 cells was then determined in pulse-chase metabolic labelling experiments (Figure 7A). The half-life of wild type HA-p105 (mean $t_{1/2}$ = 9.7h) was dramatically reduced by FL-IKK2 co-expression (mean $t_{1/2}$ = 2.5h), as previously shown (Salmeron et al., 2001). Analysis of the 4h point confirmed that the reduction in remaining HA-p105 induced by FL-IKK2 co-expression was significant ($p = 0.001$; n =

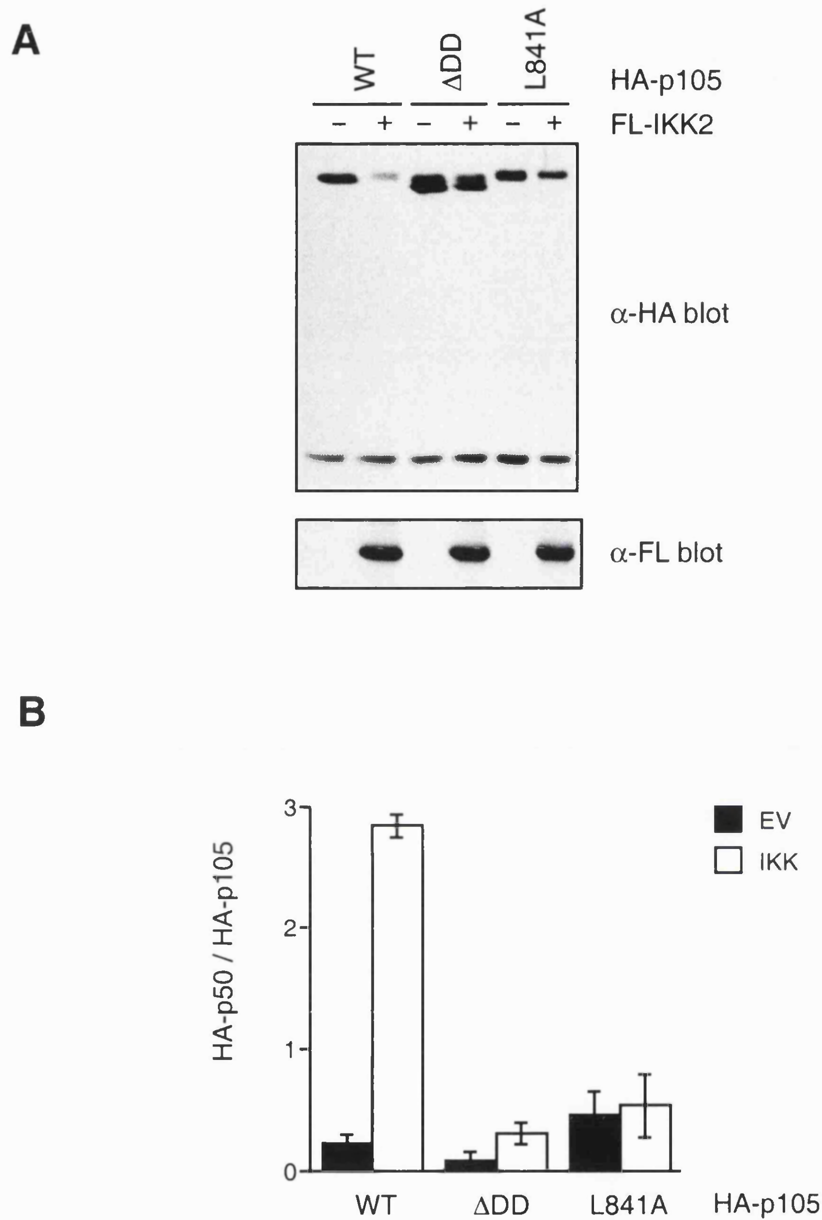
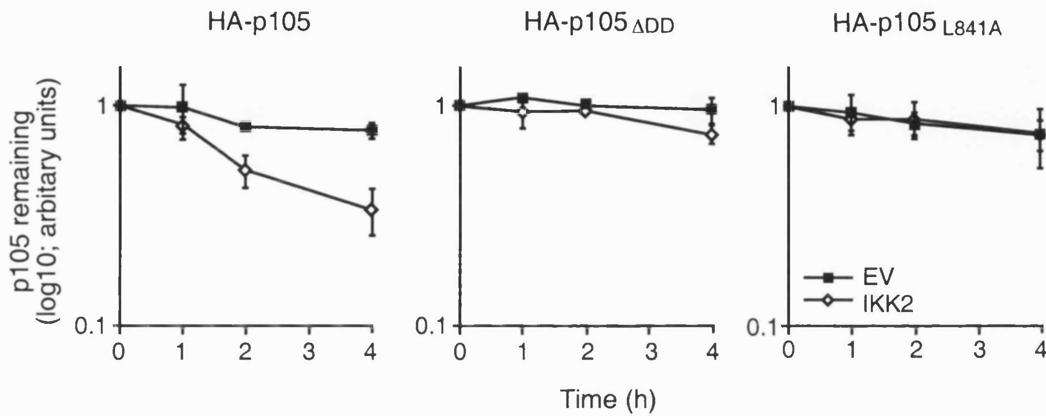


Figure 6. Induction of a p50/p105 ratio shift by IKK2 requires the p105 death domain. (A) 3T3 cells were transiently co-transfected with expression vectors encoding wild type (WT) HA-p105 or the indicated HA-p105 DD mutants and FL-IKK2 or an empty vector with no insert (EV). After 24h, total cell lysates were prepared, resolved by 8% SDS-PAGE, Western blotted and probed with the indicated antibodies. **(B)** Replicates of experiments as in A were quantified by densiometric imaging and the HA-p50/HA-p105 ratio calculated (n=3).

A



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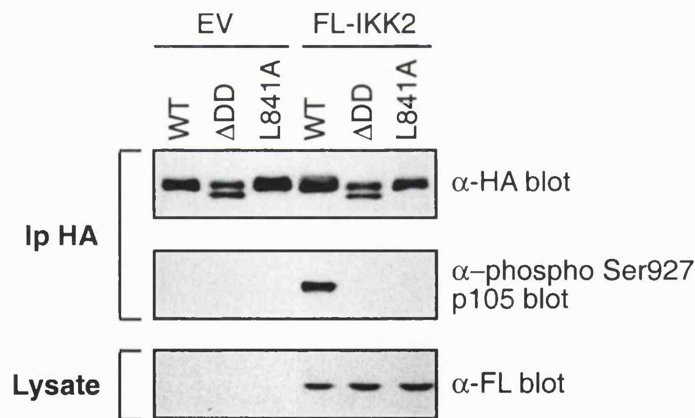


Figure 7. The p105 death domain is required for IKK2-induced p105 proteolysis and serine 927 phosphorylation of p105.

(A) 3T3 cells were transiently co-transfected with expression vectors encoding wild type (WT) HA-p105 or the indicated HA-p105 DD mutants and FL-IKK2 or an empty vector with no insert (EV). After 24h, cells were metabolically pulse-labelled with [35S]methionine/[35S]cysteine (30min) and then chased for the times indicated. Anti-HA immunoprecipitates were resolved by 8% SDS-PAGE and revealed by fluorography. Amounts of labelled HA-p105 were quantified by densitometric imaging and normalised against total amounts of HA-p105 determined by Western blotting and probing with anti-HA mAb (n = 3). **(B)** 3T3 cells were co-transfected as in A. Proteasome activity was blocked by addition of MG132 for the last 4h of culture. Western blots of anti-HA immunoprecipitates were probed sequentially with anti-phospho Ser927 p105 and anti-HA antibodies.

3). However, FL-IKK2 co-expression had little effect on the turnover of HA-p105$_{\Delta DD}$ (mean $t_{1/2}$ + FL-IKK2 = 9.9h) or HA-p105$_{L841A}$ (mean $t_{1/2}$ + FL-IKK2 = 9.5h). Consistent with this, there was no significant difference in the amount of HA-p105 remaining -/+ FL-IKK2 at 4h for either HA-p105$_{\Delta DD}$ ($p = 0.054$; $n = 3$) or HA-p105$_{L841A}$ ($p = 1.0$; $n = 3$). Thus, the DD is required for over-expressed FL-IKK2 to efficiently induce HA-p105 proteolysis.

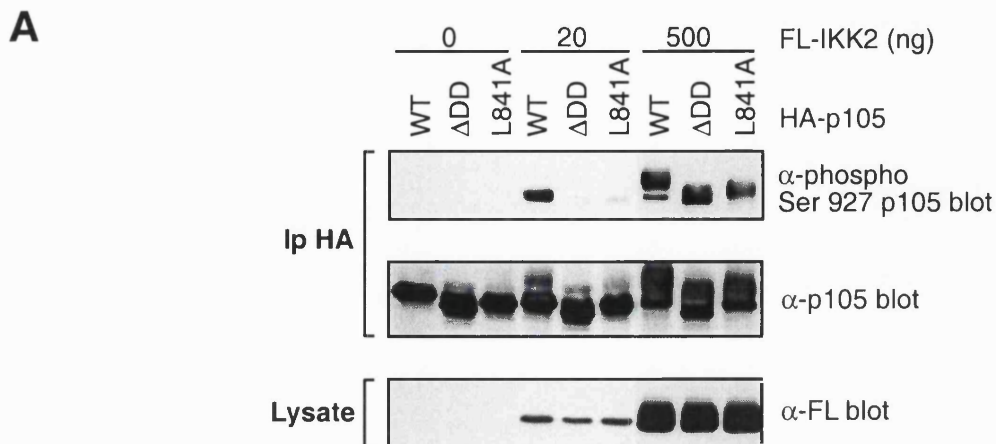
FL-IKK2 triggers degradation of HA-p105 by phosphorylating serine 927 and 932 in the p105 PEST region (Lang et al., 2003; Salmeron et al., 2001). The role of the p105 DD in FL-IKK2 mediated phosphorylation of p105 when co-transfected in 3T3 cells was determined by and Western blotting of anti-HA immunoprecipitates with anti-phospho Ser927 p105 antibody (Figure 7B). FL-IKK2 co-expression clearly induced phosphorylation of wild type HA-p105. However, FL-IKK2 co-expression did not induce detectable phosphorylation of either HA-p105$_{\Delta DD}$ or HA-p105$_{L841A}$, although similar amounts of HA-p105 were immunoprecipitated in each case and FL-IKK2 was expressed at the same level for each transfection. The DD of p105 is, therefore, essential for its efficient phosphorylation on serine 927 by FL-IKK2 *in vivo*, explaining why the half-life of HA-p105 DD mutants is unaffected when co-expressed with FL-IKK2.

2.1.6 The DD is not required for HA-p105 proteolysis induced by high concentrations of FL-IKK2

The previous data were consistent with the hypothesis that the DD of p105 functions as a docking site for IKK1 and IKK2. The DD, therefore, may increase the local concentration of IKK on p105, facilitating serine 927 and 932 phosphorylation. This model predicts that high concentrations of transfected FL-IKK2 might overcome the requirement for the DD to trigger phosphorylation of HA-p105. Accordingly, FL-IKK2

was expressed at high (500ng plasmid) and low (20ng plasmid) concentrations together with wild type and DD mutants of HA-p105. 293 cells were used for this experiment as much higher levels of transiently expressed protein can be obtained than is possible in 3T3 fibroblasts. Four hours before lysis, proteasome function was blocked by addition of MG132. HA-p105 was immunoprecipitated and the level of phosphorylation of serine 927 determined by Western blotting (Figure 8A). When expressed at a low concentration, FL-IKK2 clearly induced serine 927 phosphorylation of wild type HA-p105, but not either HA-p105$_{\Delta DD}$ or HA-p105$_{L841A}$. However, at the high FL-IKK2 concentration, all three HA-p105 proteins were phosphorylated on serine 927. Similar levels of HA-p105 were immunoprecipitated in each case. Thus, high concentrations of FL-IKK2 induce p105 serine 927 phosphorylation independently of the p105 DD. Binding to the p105 DD, therefore, appears to increase the effective concentration of IKK available to phosphorylate p105, thereby increasing its efficiency of action.

To rule out the possibility that the p105 DD has other functions in p105 proteolysis additional to its role to facilitate efficient IKK-mediated p105 phosphorylation (e.g. ubiquitination or proteasome attachment), we also tested whether high concentration of IKK2 can induce proteolysis of p105 independently of the p105 DD. In this respect, high (500ng) and low (20ng) concentrations of FL-IKK2 were expressed together with wild type HA-p105, HA-p105$_{\Delta DD}$ or HA-p105$_{L841A}$ in 293 cells, and p105 and p50 levels in whole cell lysates were determined by Western blotting with antibody against the N-terminal HA-tag. Bands were quantified by densiometric analysis, and the p50/p105 ratio was calculated (Figure 8B). Low levels of FL-IKK2 induced a p50/p105 ratio change for wild type HA-p105, but not for HA-p105$_{\Delta DD}$ or HA-p105$_{L841A}$ as expected. However, high levels of FL-IKK2 induced a p50/p105 ratio change for all



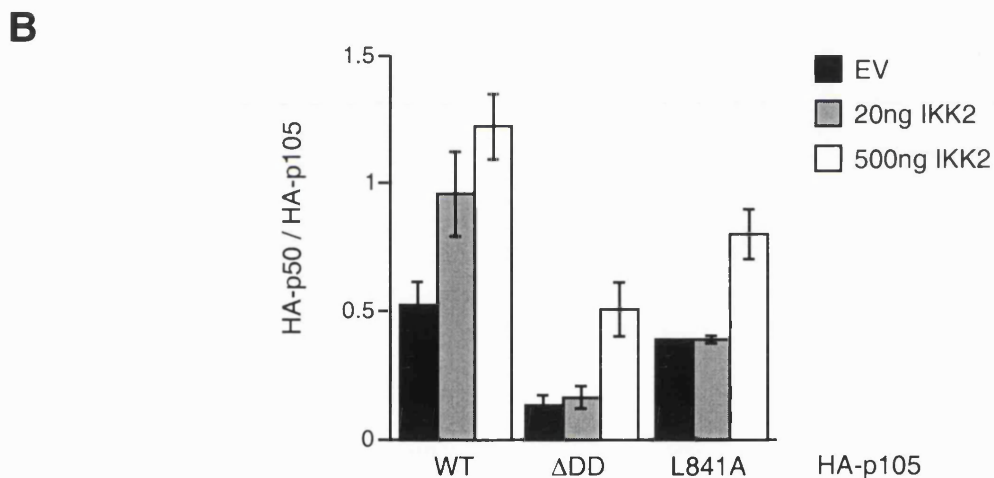


Figure 8. High levels of over-expressed IKK2 induce p105 proteolysis independent of the p105 death domain.
(A) 293 cells were co-transfected with expression vectors encoding wild type or DD mutant HA-p105 and the indicated amount of FL-IKK2 vector or EV. The total plasmid added for each transfection was kept constant by addition of EV. Cell lysates were prepared after 24h culture. Anti-HA immunoprecipitates were resolved by 8% SDS-PAGE, Western blotted and sequentially probed for phospho Ser927 p105 and HA-p105. **(B)** 293 cells were transfected as in A. Total cells lysates were prepared, resolved by 8% SDS-PAGE and Western blotted for HA-p50 and HA-p105 using anti-HA mAb. Replicates of three experiments were quantified by densiometric imaging and the ratio of HA-p50/HA-p105 calculated.

three p105 proteins. Preliminary results using pulse chase metabolic labelling confirmed that high levels of co-expressed IKK2 (500ng) indeed induce the proteolysis of HA-$p105_{\Delta DD}$ or HA-$p105_{L841A}$ to a similar extend as wild type HA-p105 (data not shown). These data suggest that although the 105 DD facilitates the interaction of p105 with the IKK complex and subsequent phosphorylation of p105, it is not required for downstream events during p105 proteolysis.

2.1.7 p105 DD is essential for TNFα-induced degradation of p105

It was important to demonstrate that the DD is required for proteolysis of p105 induced by physiological stimulation with a cytokine, as this process involves p105 phosphorylation by the endogenous IKK complex. To this end, HeLa cells were transfected with plasmids encoding HA-p105, HA-$p105_{\Delta DD}$ or HA-$p105_{L841A}$ and stable clones isolated after G418 selection. Pulse-chase experiments were then carried out using clones with expression levels similar to endogenous p105 (Figure 9A). TNFα increased the rate of turnover of wild type HA-p105 (mean $t_{1/2}$ – TNFα = 5.5h; mean $t_{1/2}$ + TNFα = 0.9h) as expected (Salmeron et al., 2001). At 90min, the amount of HA-p105 remaining was significantly reduced with TNFα ($p = 0.0004$; $n = 3$). In contrast, TNFα stimulation did not alter the turnover of HA-$p105_{\Delta DD}$ (mean $t_{1/2}$ + TNFα = 4.6h) and at 90min there was no significant difference in the amount of HA-$p105_{\Delta DD}$ remaining -/+ TNFα ($p = 0.225$; $n = 3$). Similarly, TNFα stimulation did not induce proteolysis of HA-$p105_{L841A}$ or a significant reduction in the amount of HA-$p105_{L841A}$ remaining after 90min chase ($p = 0.604$; $n = 3$). However, the basal turnover of HA-$p105_{L841A}$ was consistently increased relative to wild type HA-p105 (mean $t_{1/2}$ = 1.1h). The reason for this increase is unclear.

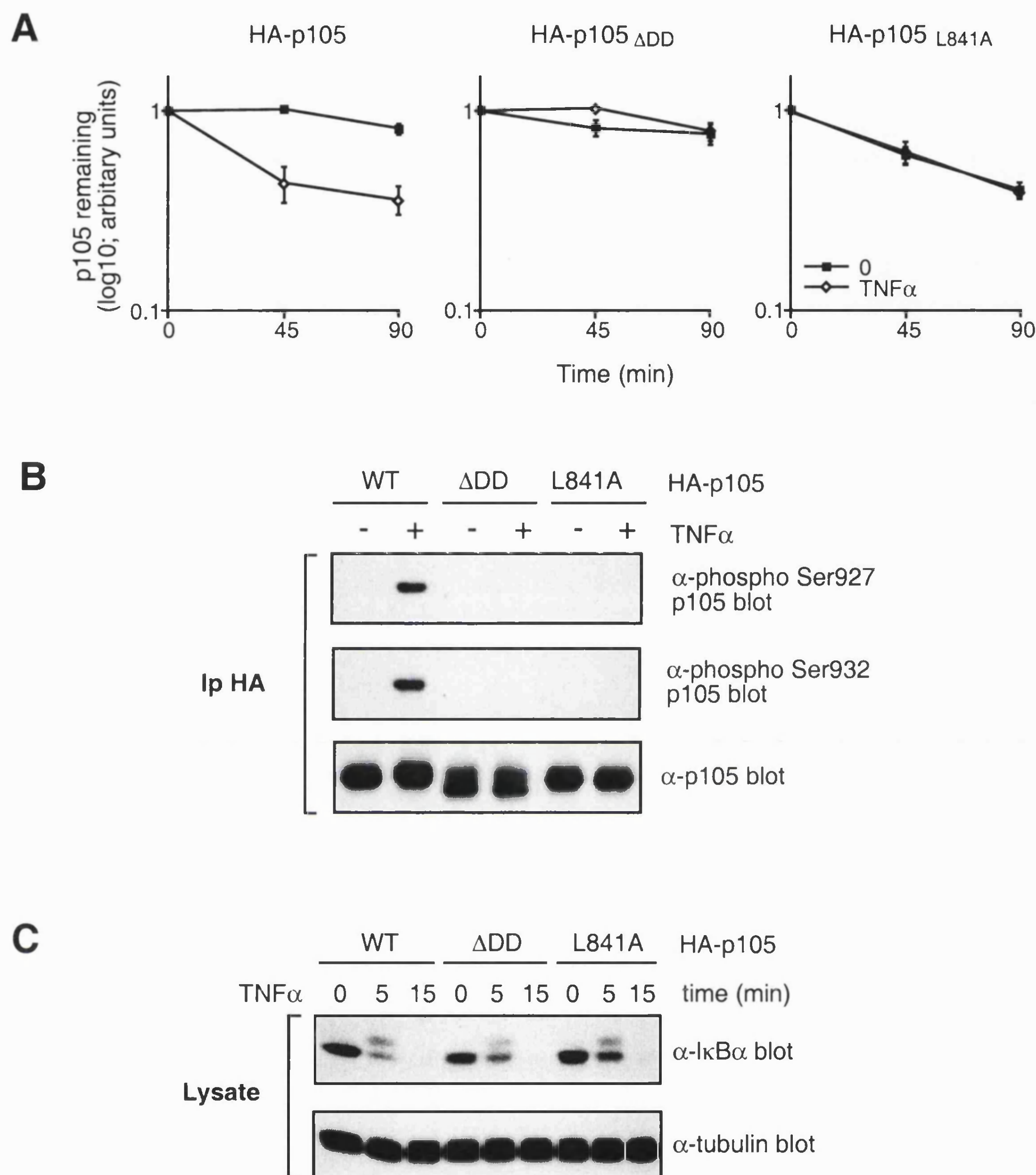


Figure 9. The p105 death domain is essential for TNFα-induced serine 927/932 phosphorylation and proteolysis of p105.

(A) Clones of HeLa cells stably transfected with HA-p105, HA-p105ΔDD or HA-p105L841A were metabolically pulse-labelled with [35S]methionine/[35S]cysteine (30min) and then chased for the times indicated in complete medium (0) or complete medium supplemented with TNFα. Anti-HA immunoprecipitates were resolved by 8% SDS-PAGE and revealed by fluorography and quantified by densitometric imaging (n = 3). **(B)** The HeLa clones in A were pre-treated with MG132 for 30min prior to stimulation with TNFα or control medium (15min). Anti-HA immunopecipitates of cell lysates were Western blotted and probed sequentially with anti-phospho Ser927 p105, anti-phospho Ser932 p105 and anti-p105 antibodies. **(C)** The HeLa clones in A were stimulated for the times indicated in complete medium (0) or complete medium supplemented with TNFα. Whole cell lysates were prepared, resolved by 12% SDS-PAGE, Western blotted and probed with the indicated antibodies.

The ability of TNFα to induce p105 serine 927 and 932 phosphorylation in each of the clones was also determined by Western blotting (Figure 9). To do this p105 mutants were isolated by a two-step anti-HA mAb immunoprecipitation as described in the Materials and Methods section, which disrupted the interaction of transfected and endogenous p105. Serine 927/932 phosphorylation of HA-p105 was clearly induced after 15min TNFα stimulation. However, TNFα-induced serine 927/932 phosphorylation was completely abrogated for both HA-$p105_{\Delta DD}$ and HA-$p105_{L841A}$.

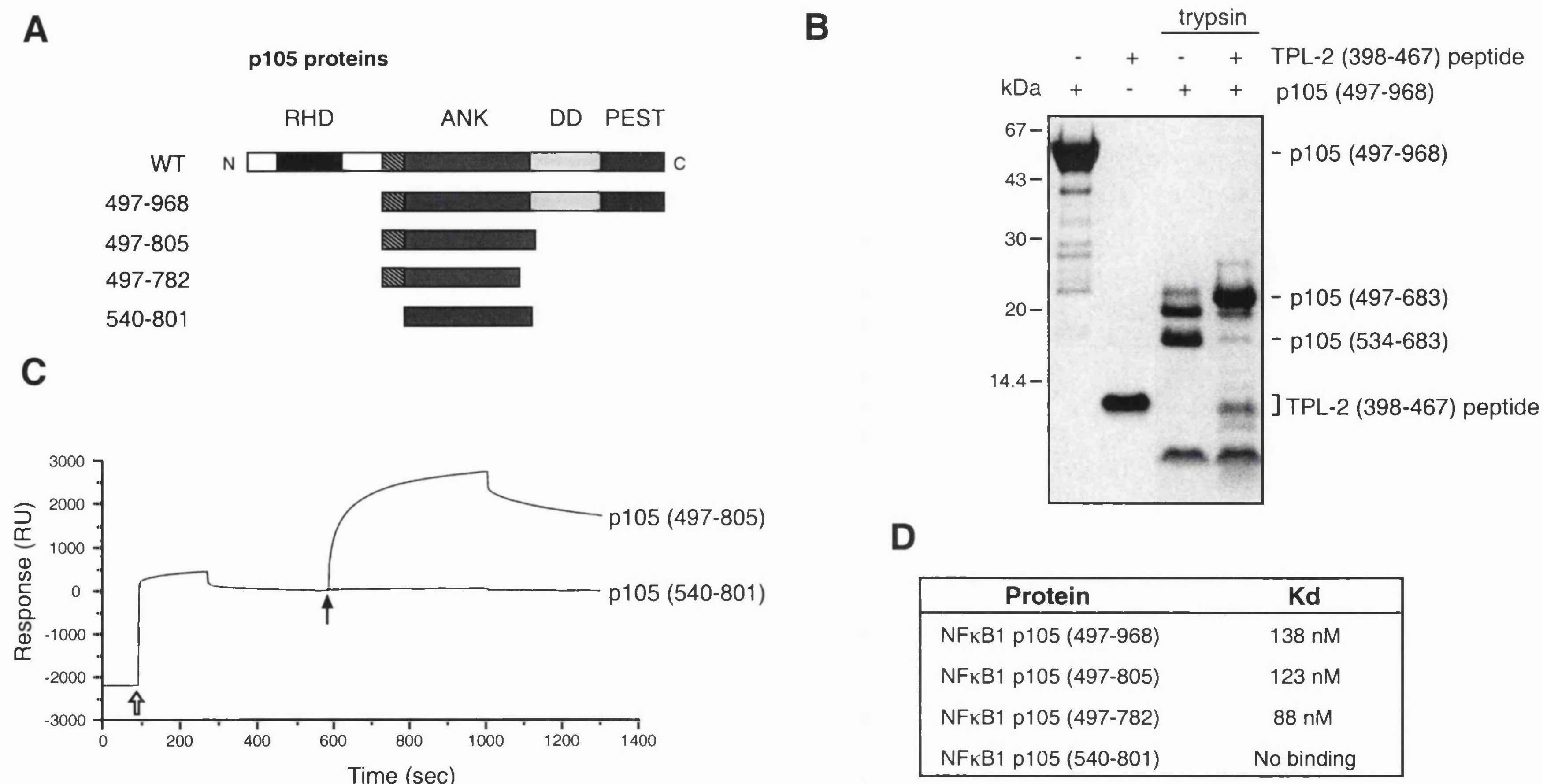
Control experiments demonstrated that IκBα was similarly degraded following TNFα stimulation in all three clones (Figure 9C). The TNFα signalling pathway controlling the IKK complex was, therefore, intact in both the HA-$p105_{\Delta DD}$ and HA-$p105_{L841A}$ clones.

Together, these data demonstrate that the p105 DD is absolutely required for TNFα-induced phosphorylation of the p105 destruction box and subsequent p105 degradation, confirming the importance of the DD in regulating signal-induced p105 proteolysis. Since IKK-mediated phosphorylation of p105 is essential for cytokine-induced p105 degradation (Salmeron et al., 2001), this is likely to reflect the role of the DD in facilitating IKK-mediated phosphorylation of p105.

2.2. Characterisation of TPL-2 binding to p105

2.1.8 The TPL-2 C-terminus binds to residues 497-539 of p105

Deletion of its C-terminal 70 amino acids activates the oncogenic potential of TPL-2 (Ceci et al., 1997). Interestingly, it was previously demonstrated that this region of TPL-2 is also required for its high affinity binding to the C-terminal half of p105 *in vitro* (Belich et al., 1999). Experiments carried out in collaboration by Jürgen Deka (Division



Protein	Kd
NFκB1 p105 (497-968)	138 nM
NFκB1 p105 (497-805)	123 nM
NFκB1 p105 (497-782)	88 nM
NFκB1 p105 (540-801)	No binding

Figure 10. The TPL-2 C-terminus binds to residues 497-539.
(A) Schematic diagram of recombinant p105 proteins. **(B)** Recombinant p105(497-968) protein was digested with trypsin +/- TPL-2(398-497) peptide for 60min, resolved by 10% Bis-Tris gel electrophoresis and Western blotted. Protein fragments were visualized by Coomassie Brilliant blue staining. The mobilities of p105497-968 and the fragments corresponding to p105(497-683) and p105(534-683) are indicated. **(C)** Surface plasmon resonance analysis of the interaction of p105(497-805) and p105(540-801) protein with biotinylated TPL-2(398-497) peptide, immobilized on a Streptavidin-coated sensor surface. Open arrow denotes the injection of biotinylated TPL-2 C-terminal peptide, closed arrow denotes injection of indicated p105 protein. **(D)** Binding affinities of TPL-2(398-497) peptide for the indicated recombinant p105 proteins, as determined by surface plasmon resonance.

of Molecular Structure, NIMR) aimed to identify the actual binding site of the TPL-2 C-terminus on p105. To do this, recombinant protein encoding the C-terminus of p105 (residues 497-968) (see Figure 10A) was digested with trypsin in the presence and absence of a synthetic peptide corresponding to the C-terminus of TPL-2 (residues 398-467). In the absence of TPL-2 peptide, a 16kDa fragment accumulated in the digest (Figure 10B), which was identified by N-terminal sequencing and mass spectrometry to correspond to amino acids 534-683 of p105. A larger fragment, with an apparent MW of 20kDa, was generated in the presence of TPL-2 peptide, which was identified as p105 amino acids 497-683 (Figure 10B). Thus, the binding of TPL-2 peptide prevents access of the protease to a target site between residue 533 and 534 of p105, suggesting that this region constitutes an important component of the binding site for the C-terminus of TPL-2.

In order to obtain corroborating evidence for the TPL-2 C-terminus binding site on p105, Jürgen Deka carried out surface plasmon resonance experiments using the panel of recombinant p105 proteins shown in Figure 10A. These data revealed that $p105_{540-801}$ did not detectably bind to the TPL-2 peptide (Figure 10C), whereas recombinant p105 proteins that included the segment 497-539 bound to the TPL-2 peptide with nanomolar affinity constants (Figure 10C and D). Taken together, the proteolytic mapping data and binding experiments strongly suggest that residues 497-539 of p105 constitute at least part of a high affinity binding site for the C-terminus of TPL-2.

2.1.9 The TPL-2 kinase domain interacts with the p105 DD

Previous binding experiments with *in vitro* translated protein indicated that the interaction with the TPL-2 C-terminus is required for efficient binding of p105 to TPL-2 (Belich et al., 1999). However, very low levels of C-terminally truncated TPL-2 (TPL-

2ΔC) do co-immunoprecipitate with p105 *in vitro*, which are revealed only on long exposure of autoradiographs (M. Belich and S.C. Ley, personnel communication). To investigate TPL-2 binding to p105 in more detail, it was important to determine whether deletion of the TPL-2 C-terminus also disrupts the interaction of TPL-2 with p105 *in vivo*. To do this, 293 cells were transiently co-transfected with plasmids encoding HA-p105 and TPL-2ΔC or wild type TPL-2. TPL-2ΔC was clearly detected in anti-HA-immunoprecipitates, although in some experiments at lower levels than full length TPL-2 (Figure 11A).

The association of TPL-2ΔC with p105 raised the possibility that TPL-2 contains a second binding site for p105 in addition to its C-terminus. To identify, which additional region of TPL-2 is involved in the interaction with p105, 293 cells were co-transfected with plasmids encoding HA-p105 and a panel of N-terminal deletion mutants of TPL-2ΔC (see Figure 11B). Immunoprecipitation and Western blotting revealed that the N-terminus of TPL-2 is not required for binding to HA-p105 in 293 cells (Figure 11C). A kinase-inactive TPL-2ΔC mutant (TPL-2ΔC_{D270A}), which presumably has an altered structure in the TPL-2 activation loop, co-immunoprecipitated with HA-p105 to a much reduced extent compared to TPL-2ΔC when co-expressed in 293 cells (Figure 11D). These data suggest that the TPL-2 kinase domain is involved in the interaction of TPL-2 and p105, additionally to binding of the TPL-2 C-terminus to residues 497-539 of p105.

To determine the p105 binding site for the TPL-2 kinase domain, TPL-2ΔC was co-expressed in 293 cells with the panel of C-terminal deletion mutants of HA-p105 (see Figure 12A) and association was determined by immunoprecipitation and Western blotting. TPL-2ΔC interacted with HA-$p105_{1-892}$, which lacks the PEST region, to a similar degree to full length HA-p105 (Figure 12B). However, TPL-2ΔC did not interact with HA-$p105_{1-801}$, HA-$p105_{\Delta DD}$ or HA-$p105_{L841A}$, which lack a functional p105 DD.

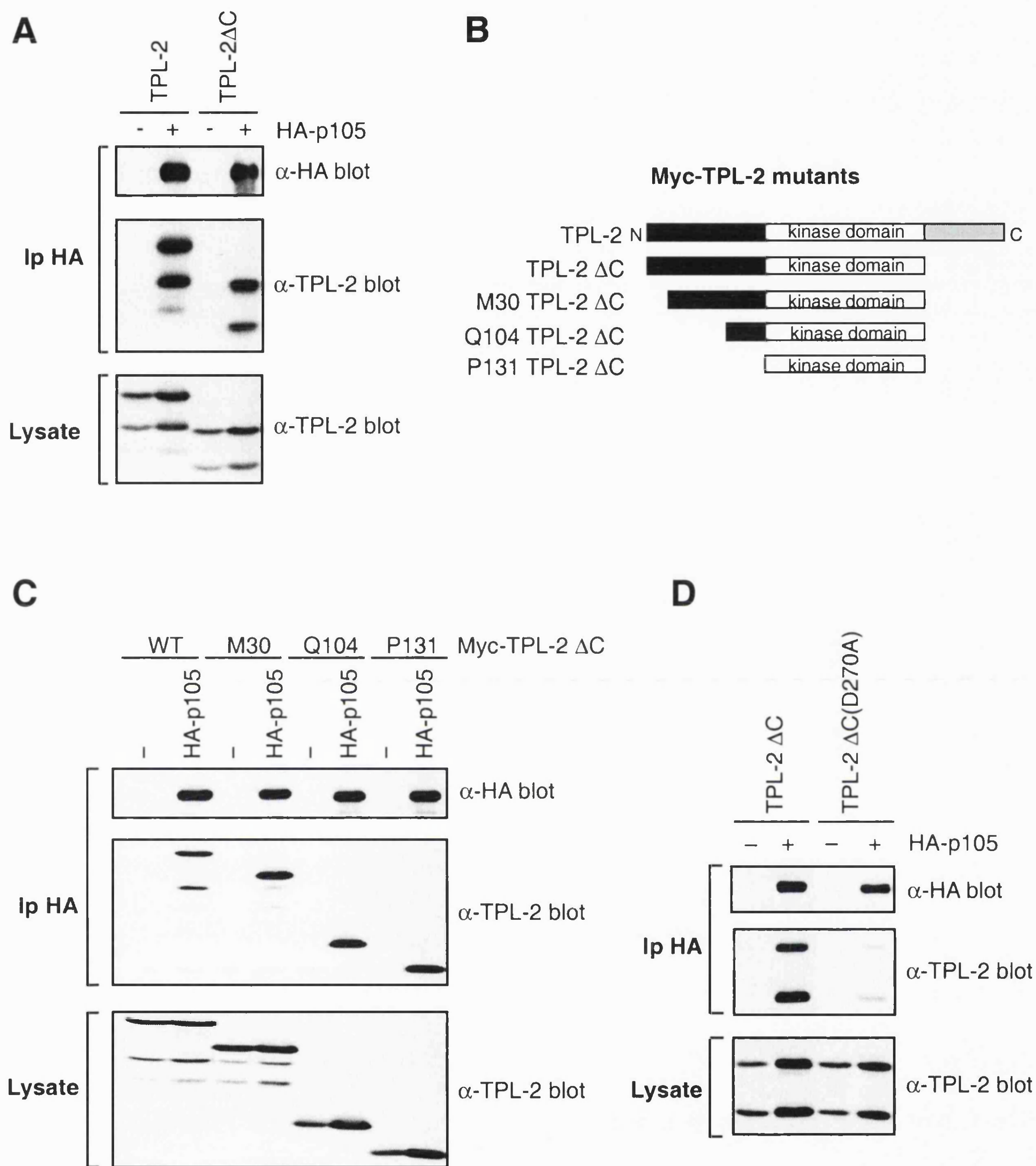
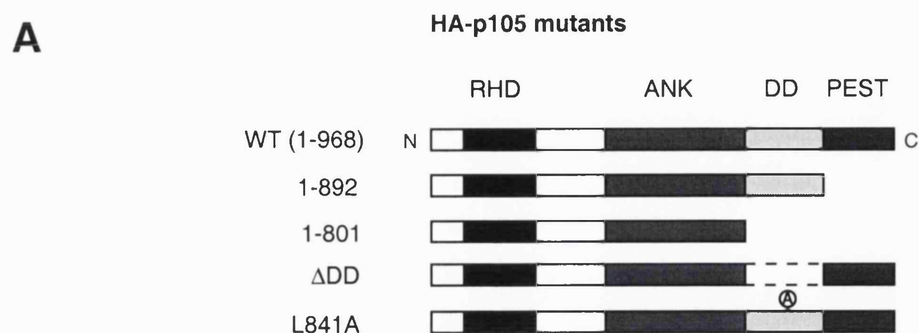
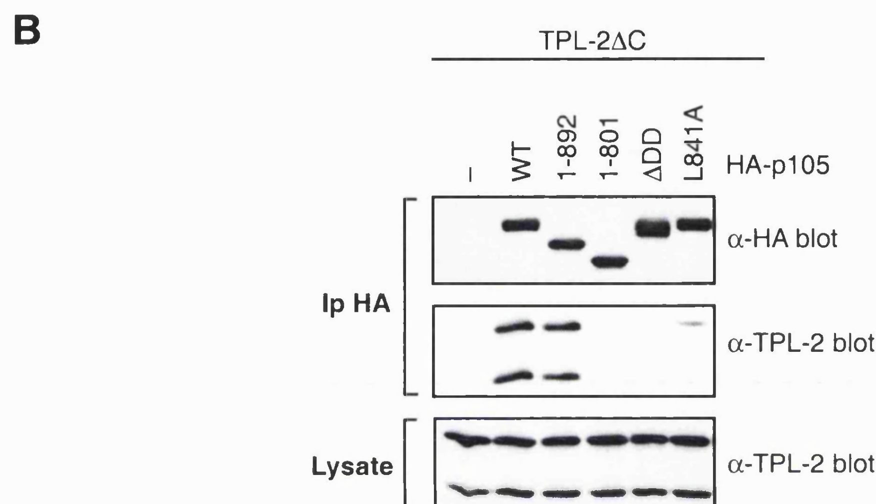


Figure 11. The TPL-2 kinase domain is a second binding site for p105.
(A) 293 cells were co-transfected with vectors encoding TPL-2 or TPL-2ΔC and HA-p105. Anti-HA immunoprecipitates and cell lysates were resolved by 8% SDS-PAGE, Western blotted and probed for HA-p105 and TPL-2. **(B)** Schematic diagram of Myc-TPL-2 deletion mutants. M30 corresponds to the natural internal initiation site (Aoki et al., 1993). **(C and D)** 292 cells were co-transfected with vectors encoding the indicated Myc-TPL-2 mutants and HA-p105 or EV (-). Anti-HA immunoprecipitates and cell lysates were resolved by 8% SDS-PAGE, Western blotted and probed with the indicated antibodies. In A, C and D, the amounts of vector encoding TPL-2 or TPL-2ΔC were adjusted to achieve equal protein expression in lysates +/- HA-p105.





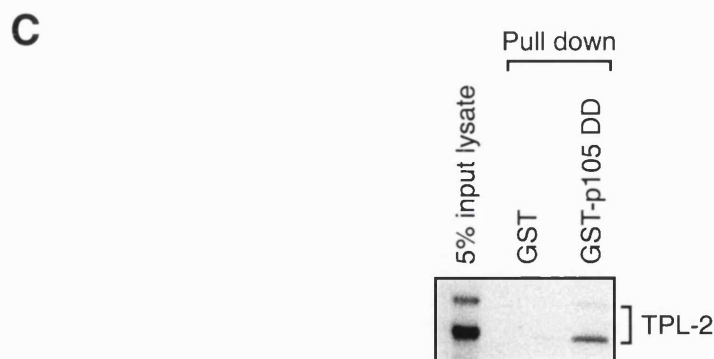


Figure 12. The kinase domain of TPL-2 directly interacts with the p105 death domain. **(A)** Schematic diagram of HA-p105 mutants. The relative positions of the Rel homology domain (RHD), ankyrin repeats (ANK), death domain (DD) and PEST region are shown. **(B)** 293 cells were co-transfected with TPL-2ΔC and the indicated HA-p105 mutants. Anti-HA mAb immunoprecipitates and cell lysates were resolved by 8% SDS-PAGE, Western blotted and probed with the indicated antibodies. The amounts of vector encoding TPL-2ΔC were adjusted to achieve equal protein expression in lysates +/- HA-p105. **(C)** TPL-2 was synthesised and labelled with [35S]methionine/[35S]cysteine by *in vitro* cell-free translation. Pull-downs were then performed with GST-p105 DD (p105 residues 808-892) or GST control. Isolated protein was resolved by 8% SDS-PAGE and revealed by fluorography.

Thus, the p105 DD is essential for the interaction of p105 with TPL-2ΔC. A GST-p105DD fusion protein also associated with *in vitro* translated TPL-2 (Figure 12C), further suggesting a direct interaction between TPL-2 and the p105 DD. Together, these data demonstrate that the p105 DD is the binding site for the TPL-2 kinase domain.

2.1.10 The p105 DD and residues 497-538 of p105 contribute equally to the interaction with TPL-2

To determine the relative contribution of the two TPL-2 binding sites on p105, additional HA-p105 constructs (see Figure 13A) were generated with residues 497-538 deleted alone (HA-$p105_{\Delta 497\text{-}538}$) or in combination with deletion (HA-$p105_{\Delta 497\text{-}538;\ \Delta DD}$) or point mutation (HA-$p105_{\Delta 497\text{-}538;\ L841A}$) of the p105 DD. These p105 constructs and HA-p105 constructs, in which the p105 DD alone was mutated (HA-$p105_{\Delta DD}$ and HA-$p105_{L841A}$), were individually co-expressed with wild type TPL-2 in 293 cells and immunoprecipitated from cell lysates with anti-HA antibody. The extent of co-immunoprecipitation of TPL-2 was reduced by deletion or point mutation of the DD compared with wild type HA-p105 (Figure 13B). Deletion of residues 497-538 of p105 reduced association with TPL-2 to a similar extent. However, no association of TPL-2 was detected with either of the double binding site mutants. Control experiments demonstrated that all of the HA-p105 mutants were cytoplasmic similar to wild type, indicating that altered subcellular localisation did not contribute to their differential association with TPL-2 and the overall structure of the proteins was not affected (Figure 13C, see also Figure 4B). Therefore, both binding sites contribute equally to the interaction of TPL-2 and p105.

The TPL-2 C-terminus is required for TPL-2 binding to p105 *in vitro* (Belich et al., 1999). Therefore, TPL-2 binding to wild type HA-p105, HA-$p105_{\Delta DD}$,

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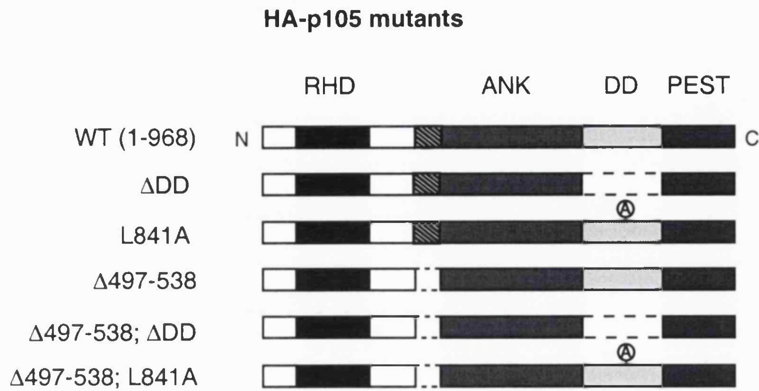

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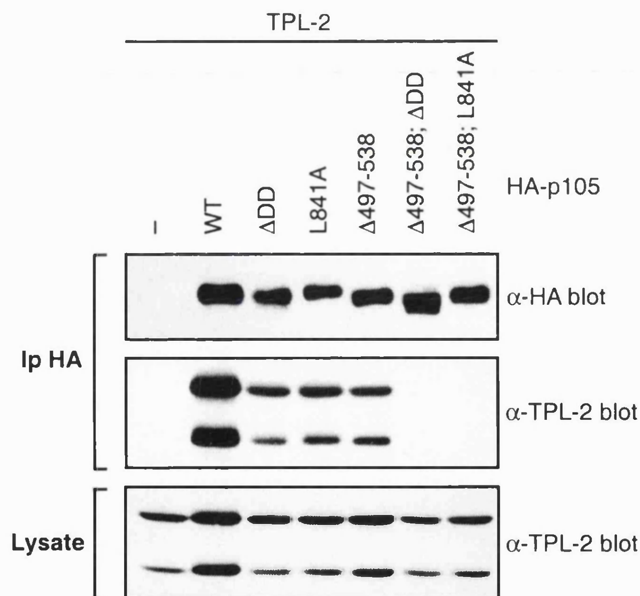

Figure 13. The p105 death domain and residues 497-538 of p105 equally contribute to TPL-2 binding to p105.

(A) Schematic diagram of HA-p105 mutants. The relative positions of the Rel homology domain (RHD), residues 497-538, ankyrin repeats (ANK), death domain (DD) and PEST region are shown. **(B)** 293 cells were co-transfected with vectors encoding TPL-2 and the indicated HA-p105 mutants or EV. In this experiment, to assay the interaction between TPL-2 and HA-p105 under more stringent conditions, cells were lysed and immunoprecipitated in RIPA buffer. Anti-HA mAb immunoprecipitates and cell lysates were resolved by 8% SDS-PAGE, Western blotted and probed sequentially with the indicated antibodies. The amount of TPL-2 expression vector was adjusted so that similar steady-state levels of protein expression in cell lysates were obtained +/- HA-p105.

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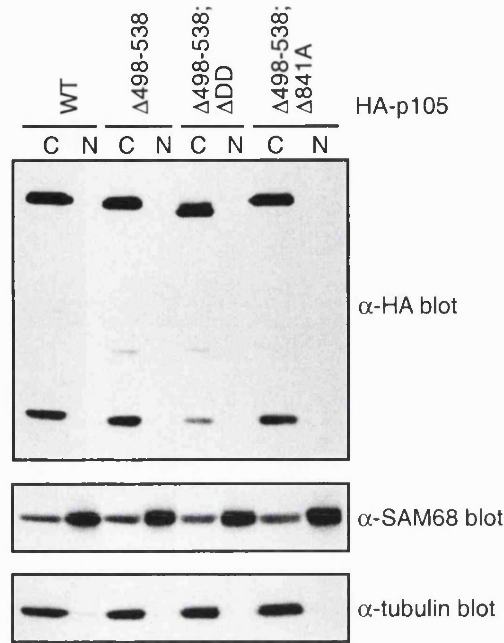

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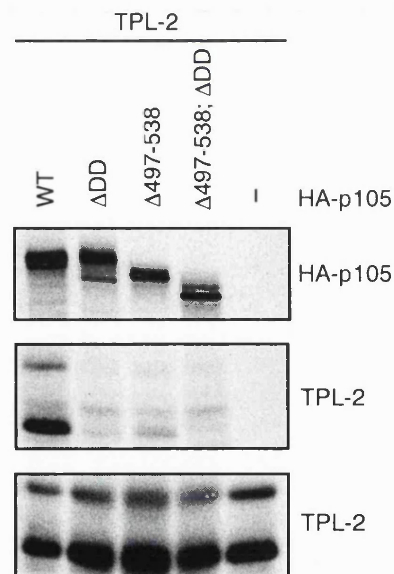

Figure 13. The p105 death domain and residues 497-538 of p105 equally contribute to TPL-2 binding to p105.

(C) 293 cells were transfected with vectors encoding the indicated HA-p105 constructs. Nuclear (N) and cytoplasmic (C) fractions were prepared resolved by 8% SDS-PAGE, Western blotted and probed sequentially with the antibodies shown. SAM68 and tubulin were used as controls for nuclear or cytoplasmatic fractionations, respectively. **(D)** TPL-2 and the indicted HA-p105 mutants or alone was synthesised and labelled with [35S]methionine/[35S]cysteine by *in vitro* cell-free translation. Anti-HA mAb immunoprecipitates were resolved by 8% SDS-PAGE and revealed by fluorography.

HA-p105$_{\Delta 497-538}$ or p105$_{\Delta 497-538;\ \Delta DD}$ was tested with *in vitro* translated protein produced using a reticulocyte lysate system. In this experiment, deletion of either residues 497-538 or the p105 DD severely impaired co-immunoprecipitation of TPL-2 with p105 similar to deletion of both binding sites (Figure 13D). Only on long exposures of the autoradiograph little amounts of TPL-2 were detected in HA-p105$_{\Delta DD}$, HA-p105$_{\Delta 497-538}$ immunoprecipitates, while no TPL-2 was found to associate with HA-p105$_{\Delta 497-538;\ \Delta DD}$ (data not shown). Therefore, both binding sites on p105 make an essential contribution to TPL-2 binding *in vitro*. This indicates that the second binding site strongly increases the affinity between TPL-2 and p105, while the affinity of either binding site on their own is not sufficient for the interaction to be readily detected in the *in vitro* reticulocyte lysate system.

Together these data indicate that each of the identified binding sites makes a substantial contribution to TPL-2 association with p105 and both are required for optimal complex formation. This implies that the binding affinity of TPL-2 kinase domain for the p105 DD is of a similar order to that observed between the TPL-2 C-terminus and residues 497-539 of p105 (nanomolar range). The overall affinity of TPL-2 for p105, therefore, may be very high (perhaps picomolar), consistent with the finding that all TPL-2 in cells is complexed with p105 (Belich et al., 1999).

2.3. p105 regulates the metabolic stability of TPL-2

Previous experiments have indicated that TPL-2 stoichiometrically associates with the C-terminus of NF-κB1 p105 in HeLa cells (Belich et al., 1999). As TPL-2 is essential for LPS activation of MEK1/2 and ERK1/2 in macrophages (Dumitru et al., 2000), it was important to determine whether a similar complex forms in macrophages. To do this endogenous p105 was immunoprecipitated from lysates of bone marrow-

derived macrophages (BMDM) from Balb/c mice. Western blotting confirmed that TPL-2 specifically co-immunoprecipitated with p105. Furthermore, p105 immunodepletion removed all detectable TPL-2 from lysates (Figure 14A). Thus, similar to the situation in HeLa cells (Belich et al., 1999), all of the cellular pool of TPL-2 is complexed with p105 in resting primary macrophages.

The observation that endogenous TPL-2 does not exist in a p105-free form in cells suggests that p105 might be important for the stability of TPL-2. To investigate this possibility, 293 cells were transiently co-transfected with a fixed quantity of vector encoding TPL-2 and increasing amounts of HA-p105 vector. Expression of HA-p105 resulted in a concentration dependent increase in the amount of TPL-2 detected in cell extracts compared with empty vector (EV) co-transfected cells (Figure 14B). In contrast, co-expression with HA-p100, which does not associate with TPL-2 (data not shown), had little effect on TPL-2 protein levels (Figure 14B). Pulse-chase metabolic labelling demonstrated that HA-p105 co-expression elevated TPL-2 levels by increasing the half-life of the kinase (Figure 14C).

The effect of each of the individual binding sites on p105 for stability of TPL-2 protein in 293 cells was also investigated. Co-expression of HA-p105 containing deletion or point mutation of the DD increased the steady-state level of TPL-2 to a lesser extent than co-expression with wild type HA-p105 (Figure 14D). Similarly, HA-p105 lacking residues 497-538 resulted in lower levels of TPL-2. Moreover, HA-p105 mutants lacking both binding sites had no effect on the levels of co-transfected TPL-2 protein. Pulse-chase metabolic labelling experiments confirmed that HA-p105$_{\Delta 497\text{-}538;\ \Delta DD}$ did not alter the half-life of co-expressed TPL-2 (Figure 14C). Therefore, the optimal effect of p105 on TPL-2 protein stabilisation requires association via both binding sites.

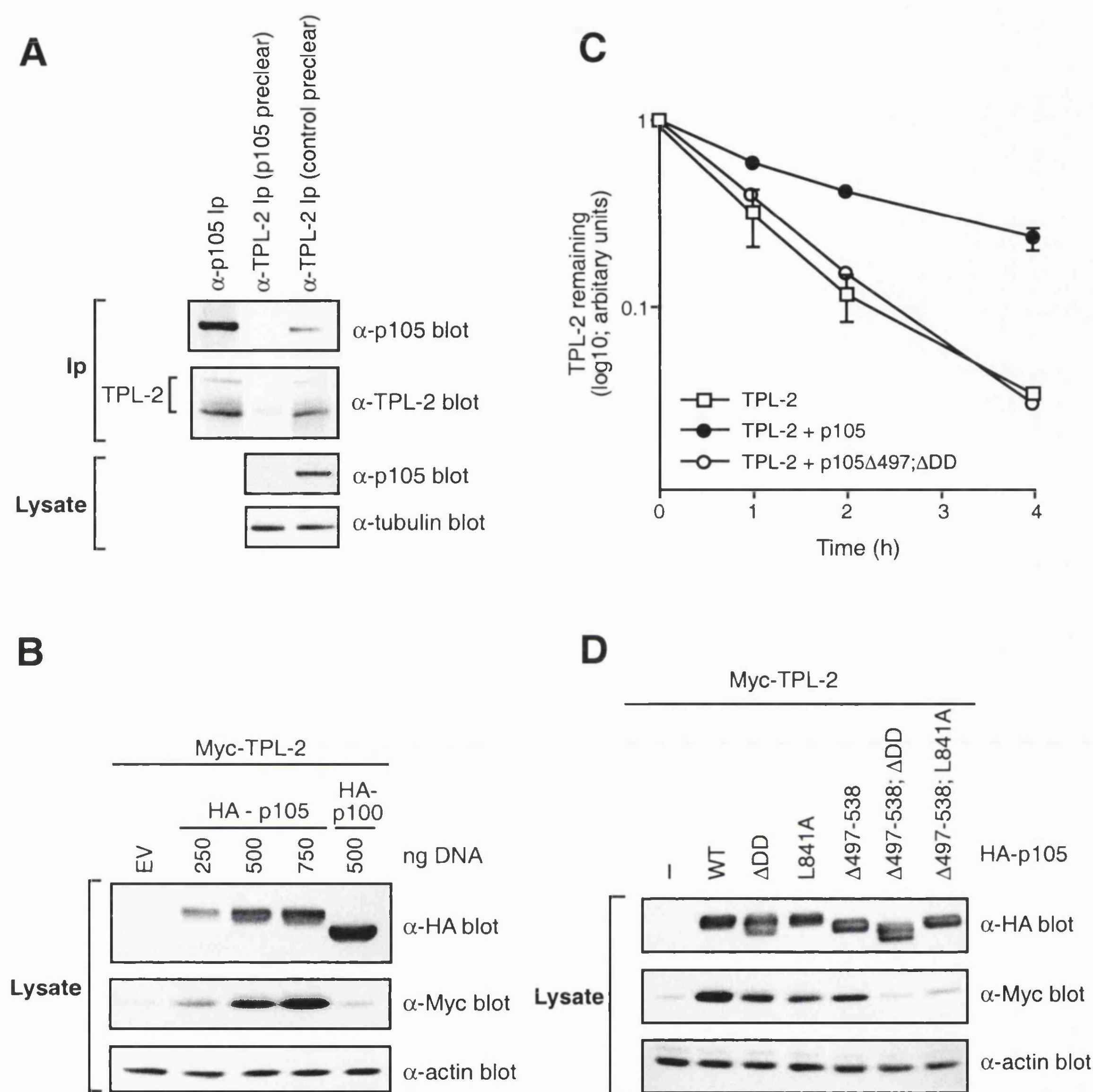

Figure 14. TPL-2 metabolic stability is regulated by p105.

(A) Endogenous p105 was removed from lysates of BMDMs by immunoprecipitation with anti-p105 antibody. TPL-2 expression in pre-cleared lysates (lower panels) was determined by re-immunoprecipitation with anti-TPL-2 antibody and Western blotting. TPL-2 resolves as two bands due to alternative initiation (Aoki et al., 1993). **(B)** 293 cells were transiently transfected with 0.25mg of Myc-TPL-2 plasmid and the indicated amounts of HA-p105 plasmid or HA-p100 plasmid. Total plasmid DNA was adjusted to 1mg with empty vector (EV). Expression of TPL-2, HA-p105 and HA-p100 was determined by Western blotting of cell lysates. **(C)** 293 cells were co-transfected with expression vectors encoding Myc-TPL-2 and indicated HA-p105 constructs or EV. After 24h, cells were metabolically pulse-labelled with [35S]methionine/[35S]cysteine (30min) and then chased for the times indicated. Anti-HA mAb immunoprecipitates were resolved by 8% SDS-PAGE and revealed by fluorography. Amounts of immunoprecipitated Myc-TPL-2 were quantified by densitometric imaging (p105 and EV n = 3, p105Δ497;ΔDD n=1). **(D)** Cell lysates of 293 cells co-transfected with fixed ratios of the expression vectors encoding TPL-2 (0.25mg DNA) and the indicated HA-p105 mutants (0.75mg DNA). Cell lysates were resolved by 8% SDS-PAGE, Western blotted and probed sequentially with the

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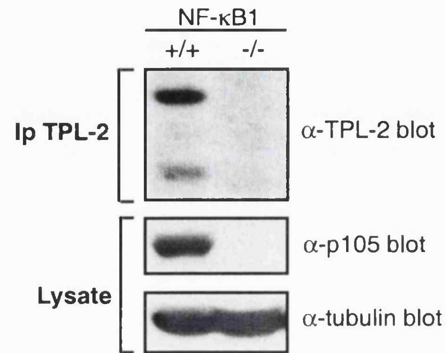

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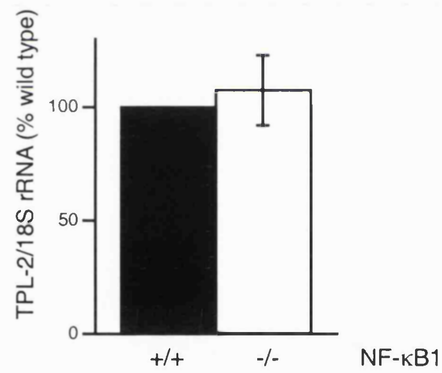


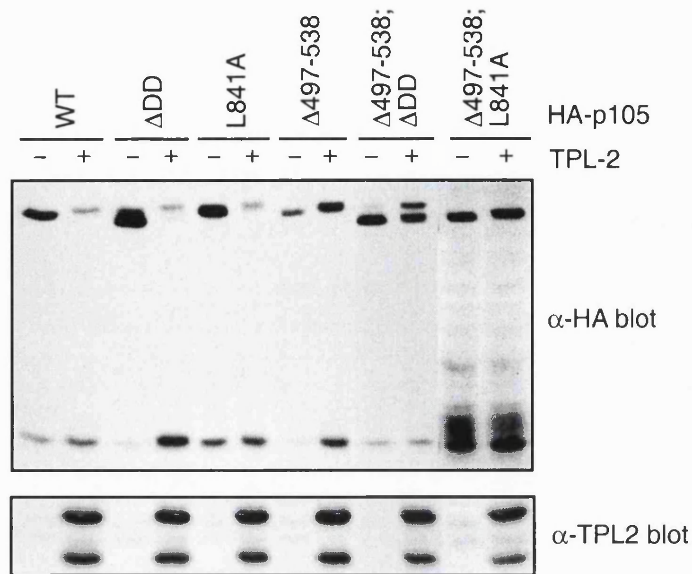
Figure 14. TPL-2 metabolic stability is regulated by p105.
(E) Lysates from NF-κB1-deficient and wild type 3T3 fibroblasts were subjected to immunoprecipitation with anti-TPL-2 antibody. Isolated TPL-2 was resolved by 8% SDS-PAGE and revealed by Western blotting.
(F) TPL-2 mRNA levels in total RNA isolated from NF-κB1-deficient and wild type 3T3 fibroblasts were assayed by semi-quantitative PCR. 18S ribosomal RNA was used as an internal control. Scanned data from three experiments are presented.

To determine whether p105 regulates the steady-state levels of endogenous TPL-2 protein, cell lysates were prepared from 3T3 fibroblasts derived from wild type and NF - κB1-deficient mice. TPL-2 was readily detected by Western blotting of anti-TPL-2 immunoprecipitates from wild type fibroblasts (Figure 14E) and was associated with endogenous p105 (data not shown). In contrast, no TPL-2 was detected in the NF-κB1 knockout fibroblasts, although semi-quantitative RT-PCR demonstrated that these cells expressed similar levels of *tpl-2* mRNA to wild type cells (Figure 14F). Thus, NF-κB1 expression appears important for the maintenance of steady-state levels of endogenous TPL-2 protein, consistent with the results of the 293 co-expression experiments.

2.4. TPL-2-induced p105 proteolysis depends on its binding to p105

Over-expressed TPL-2 induces p105 proteolysis in 3T3 cells (Belich et al., 1999). Since all TPL-2 is complexed with p105 in cells, we aimed to test the relevance of TPL-2 binding to p105 for TPL-2-induced p105 proteolysis. To this end, wild type HA-p105, HA-$p105_{\Delta 497\text{-}538;\ \Delta DD}$ or HA-$p105_{\Delta 497\text{-}538;\ L841A}$ were co-expressed with TPL-2 or empty vector in 3T3 cells, and first, the HA-p50/HA-p105 ratio was determined by Western blotting of whole cell lysates with anti-HA antibody (Figure 15A and B). Co-expression of TPL-2 induced a shift in the ratio between HA-p50 and wild type HA-p105, but not in the ratio between HA-p50 and HA-$p105_{\Delta 497\text{-}538;\ \Delta DD}$ or HA-$p105_{\Delta 497\text{-}538;\ L841A}$, which do not bind to TPL-2. Similar amounts of TPL-2 were co-expressed in each case. Pulse chase metabolic labelling experiments confirmed that co-expression of TPL-2 in 3T3 cells reduced the half-life of wild type HA-p105, but not HA-$p105_{\Delta 497\text{-}538;\ \Delta DD}$ (Figure 15C). In fact, co-expression of TPL-2 resulted in an increase in the levels of labelled HA-$p105_{\Delta 497\text{-}538;\ \Delta DD}$ within the first two hours of the chase period, which is probably due to

A



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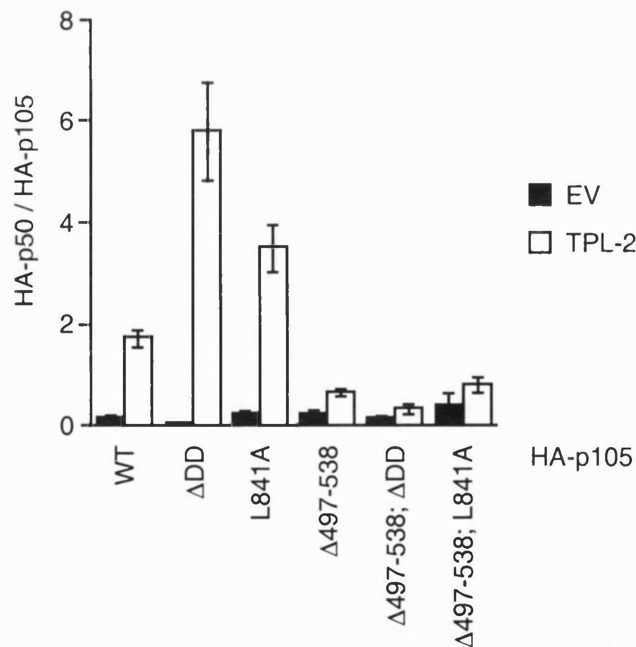


Figure 15. Binding to p105 is required but the p105 death domain is inhibitory for TPL-2-induced p105 proteolysis.

(A) 3T3 cells were transiently co-transfected with expression vectors encoding wild type (WT) HA-p105 or the indicated HA-p105 mutants and TPL-2 or an empty vector with no insert (EV). After 24h, total cell lysates were resolved by 8% SDS-PAGE, Western blotted and probed with anti-HA antibody. **(B)** Bands were quantified by densiometric imaging and ratios of HA-p50/HA-p105 calculated (n=3)..

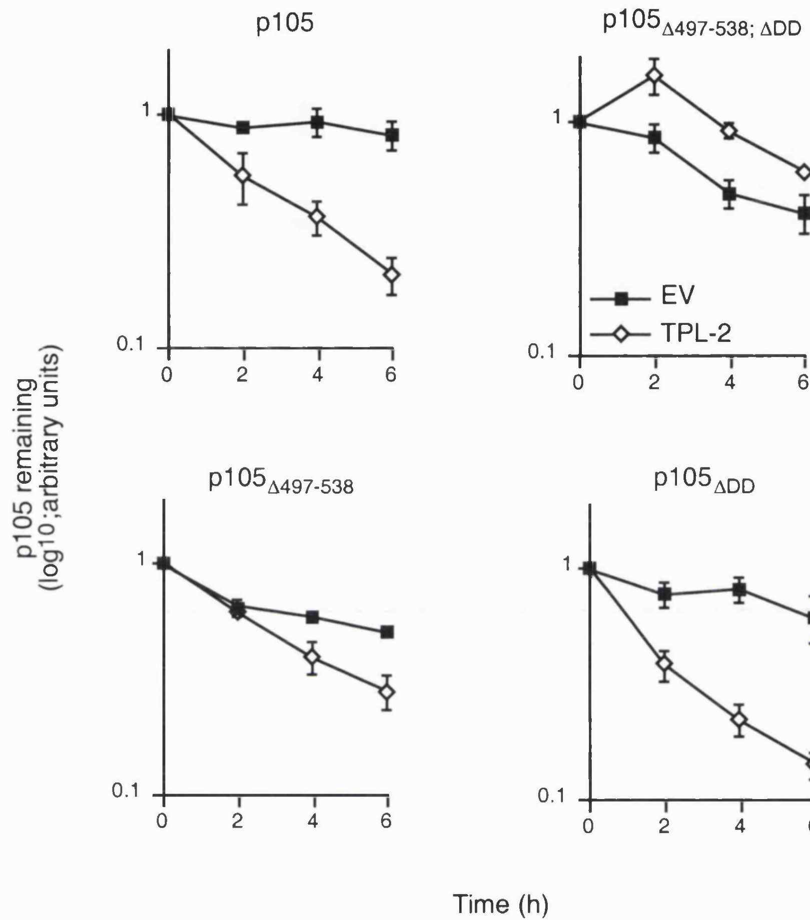


Figure 15. Binding to p105 is required but the p105 death domain is inhibitory for TPL-2-induced p105 proteolysis.
(C) 3T3 cells were transiently transfected with expression vectors encoding wild type (WT) HA-p105 or the indicated HA-p105 mutants and TPL-2 or an empty vector with no insert (EV). After 24h, cells were metabolically pulse-labelled with [35S] methionine/[35S]cysteine (30min) and then chased for the times indicated. Anti-HA mAb immunoprecipitates were resolved by 8% SDS-PAGE and revealed by fluorography. Amounts of labelled HA-p105 were quantified by densitometric imaging and normalised against total amounts of HA-p105 determined by Western blotting and probing with anti-HA mAb (n = 3).

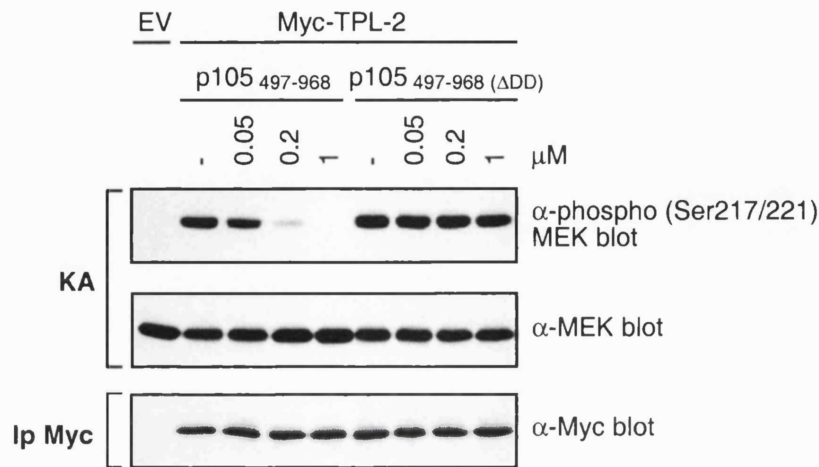
the induction of the promotor of the p105 expression vector by TPL-2. This is presumably not obvious for wild type HA-p105 because TPL-2 simultaneously induces its proteolysis. These data indicate that TPL-2 binding to p105 is required for TPL-2-induced p105 proteolysis.

Furthermore, deletion of the binding site for the TPL-2 C-terminus on p105 strongly impaired the induction of a HA-p50/HA-p105$_{\Delta497-538}$ ratio shift by TPL-2 co-expression, although it was not completely abrogated (Figure 15A and B). The half-life of HA-p105$_{\Delta497-538}$ determined by pulse chase metabolic labelling was marginally reduced by TPL-2 compared to empty vector co-expression (Figure 15C), suggesting that binding of the TPL-2 C-terminus to the residues 497-538 is important for TPL-2 induced p105 proteolysis.

Surprisingly, TPL-2 co-expression induced an even greater ratio shift between HA-p50 and HA-p105$_{\Delta DD}$ or HA-p105$_{L841A}$ than between HA-p50 and wild type HA-p105 (Figure 15A and B). In pulse chase metabolic labelling experiments, the half-life of HA-p105$_{\Delta DD}$ was confirmed to be more potent reduced by TPL-2 than the half-life of wild type HA-p105 with a significant difference at 4 hours ($p=0.02$, $n=3$, Figure 15C). Thus, binding of the TPL-2 kinase domain to the p105 DD is inhibitory for TPL-2 induced p105 proteolysis.

2.5. p105 inhibits TPL-2 MEK kinase activity

Data presented above demonstrated that p105 is an obligate binding partner for TPL-2 in cells. The p105 DD binds to the TPL-2 kinase domain and inhibits TPL-2-induced p105 proteolysis in 3T3 cells. This raised the interesting possibility that TPL-2 kinase activity might be regulated by the interaction of TPL-2 with p105.



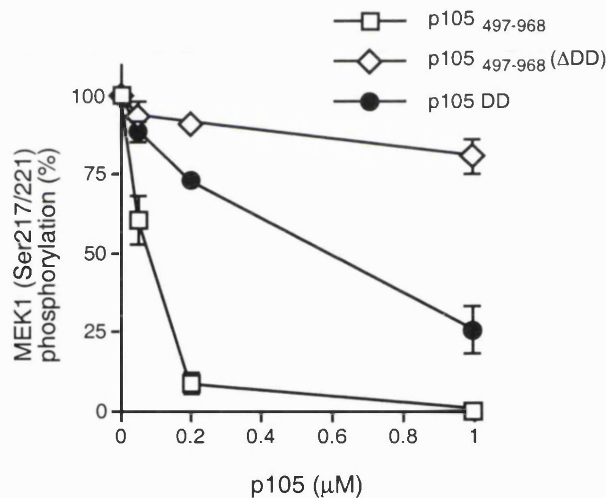


Figure 16. The p105 death domain inhibits TPL-2 MEK kinase activity.
(A) Myc-TPL-2 was immunoprecipitated from lysates of transfected 293 cells and then pre-incubated with the different amounts of the indicated recombinant p105 proteins or control buffer (-). *In vitro* kinase assays (KA) were then performed using GST-MEK1(K207A) as a substrate and phosphorylation determined by probing Western blots of reaction mixtures (KA) with an anti-phospho MEK1/2 Ser217/Ser221 antibody. Equal loading of GST-MEK1(K207A) protein was confirmed by re-probing blots with anti-MEK1/2 antibody. Western blotting of anti-Myc immunoprecipitates demonstrated that similar amounts of TPL-2 were assayed in each reaction (lower panel). **(B)** MEK1 phosphorylation in replicates of the experiments shown in A and C was quantified by densitometric imaging (n = 3). Data are presented as percentages of control MEK kinase activity.

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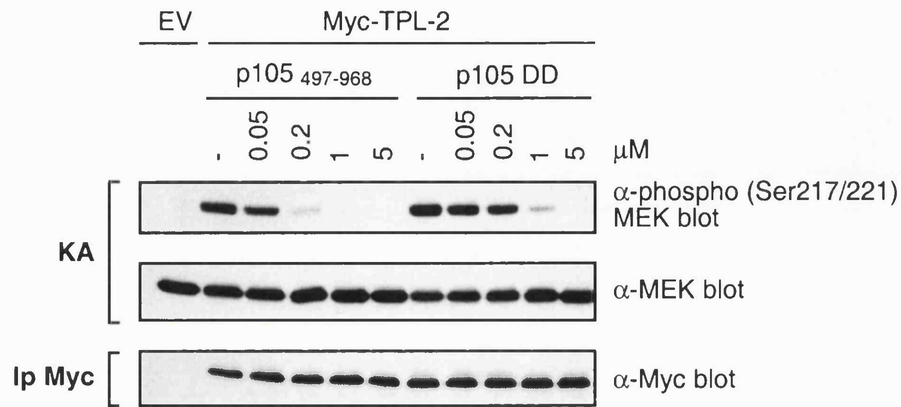

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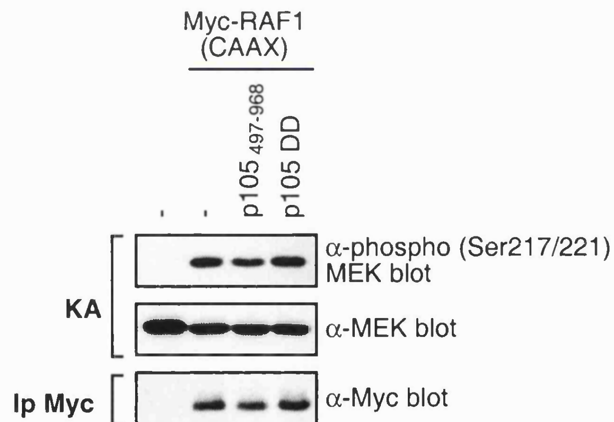
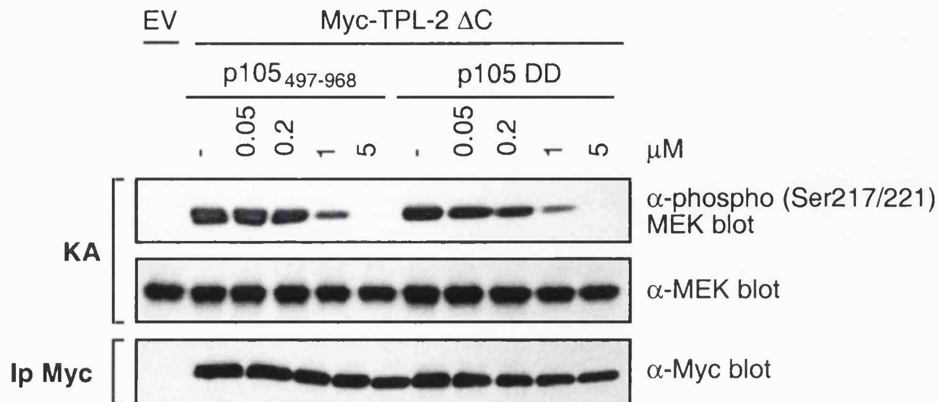


Figure 16. The p105 death domain inhibits TPL-2 MEK kinase activity.
(C) Myc-TPL-2 was immunoprecipitated from lysates of transfected 293 cells. MEK kinase assays were carried out as described in Figure 16A, in the presence of the indicated amounts of recombinant p105 proteins. **(D)** Myc-Raf1(CAAX) was immunoprecipitated from lysates of transfected 293 cells and MEK kinase activity assayed as in A. Recombinant p105 protein was added to a final concentration of 5mM.

To investigate this, Myc-TPL-2 was purified by immunoprecipitation from lysates of transiently transfected 293 cells and its kinase activity assayed *in vitro,* using GST-MEK1(K207A) protein as a substrate. Only a small fraction of over-expressed Myc-TPL-2 is associated with p105 (data not shown), presumably due to titration of endogenous p105 protein. The effect of p105 binding on Myc-TPL-2 MEK kinase activity was investigated by incubation of TPL-2 immunoprecipitates with recombinant $p105_{497-968}$ protein prior to *in vitro* kinase assay. Control experiments confirmed that $p105_{497-968}$ protein bound to TPL-2 *in vitro* (data not shown). Addition of $p105_{497-968}$ dramatically inhibited TPL-2-induced phosphorylation of serines 217 and 221 in the activation loop of MEK in a dose-dependent fashion (Figure 16A and B). 50% inhibition (IC50) was achieved at 0.09μM $p105_{497-968}$, which represents a molar $p105_{497-968}$/GST-MEK ratio of 0.6:1. The inhibitory effect of $p105_{497-968}$ was specific since the MEK kinase activity of activated Raf1 (Myc-Raf1(CAAX); (Leevers et al., 1994)) was unaffected by addition of $p105_{497-968}$ protein (Figure 16D). Thus, the C-terminal half of p105 can inhibit TPL-2 MEK kinase activity *in vitro.*

Addition of $p105_{497-968(\Delta DD)}$ protein, which lacks the p105 DD, did not affect Myc-TPL-2 MEK kinase activity at the equivalent molar concentrations to $p105_{497-968}$ (Figure 16A and B), suggesting that interaction of the p105 DD with the kinase domain of Myc-TPL-2 inhibited its kinase activity. This hypothesis was tested directly by assaying the effect of addition of recombinant $p105_{808-892}$ protein, which consists solely of the p105 DD. The p105 DD clearly inhibited Myc-TPL-2 MEK kinase activity (Figure 16B and C), although less efficiently than $p105_{497-968}$ protein (TPL-2 IC50 (p105DD) = 0.55μM). As expected, the p105 DD had no effect on Myc-Raf1(CAAX) MEK kinase activity (Figure 16D). These data indicate that the p105 DD mediates the inhibition of TPL-2 MEK kinase activity, but is not sufficient to facilitate full inhibition.

A



B

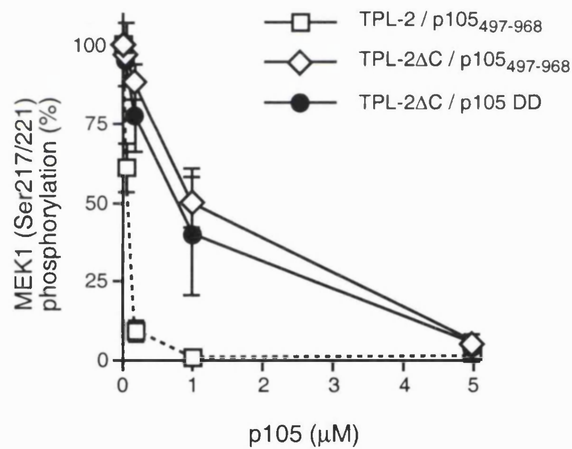
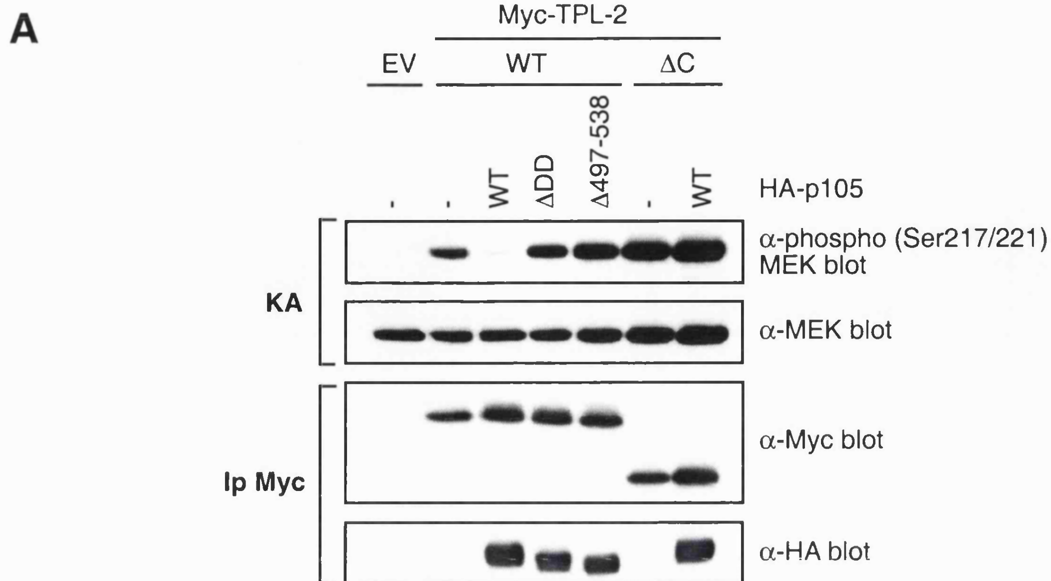


Figure 17. The TPL-2 C-terminus is required for the efficient inhibition of TPL-2 MEK kinase activity by p105.

(A) Myc-TPL-2 and Myc-TPL-2ΔC were isolated by immunoprecipitation from lysates of transfected 293 cells. MEK kinase assays were carried out as described in Figure 16, in the presence of the indicated amounts of recombinant p105 proteins. **(B)** MEK phosphorylation in replicates of the experiment shown in A was quantified by densitometric imaging (n = 3). Data are presented as percentages of control MEK kinase activity. For comparative purposes, the data from Figure 16 showing the effect of p105(497-968) protein on the MEK kinase activity of TPL-2 are included.

The inhibition of TPL-2 MEK kinase activity by $p105_{497-968}$ may be more efficient than the isolated p105 DD as the former protein contains a binding site for the TPL-2 C-terminus. It was, therefore, interesting to determine the sensitivity of TPL-2ΔC catalytic activity to inhibition by p105. The specific activity of Myc-TPL-2ΔC, isolated by immunoprecipitation from transfected 293 cell lysates, was modestly (approximately 2-3 fold) increased relative to wild type Myc-TPL-2 (see Figure 18). However, Myc-TPL-2ΔC MEK kinase activity was significantly less sensitive to inhibition by $p105_{497-968}$ protein (TPL-2ΔC IC50 ($p105_{497-968}$) = 1μM) than full length Myc-TPL-2 (TPL-2 IC50 ($p105_{497-968}$) = 0.09μM) (Figure 17A and B). The isolated p105 DD ($p105_{802-892}$), that lacks the binding site for the TPL-2 C-terminus, inhibited TPL-2ΔC MEK kinase activity with similar efficiency to $p105_{497-968}$ protein (Figure 17A and B), in contrast to its decreased inhibitory effect on wild type Myc-TPL-2 (Figure 16B and C). These data suggest that efficient inhibition of TPL-2 MEK kinase activity by the p105 DD requires binding of the TPL-2 C-terminus to p105.

To determine whether binding to p105 *in vivo* affects TPL-2 kinase activity, Myc-TPL-2 was transiently expressed in 293 cells in the presence and absence of an excess of wild type HA-p105 and isolated by immunoprecipitation with anti-Myc mAb. Myc-TPL-2 MEK kinase activity was dramatically inhibited by co-expression with HA-p105 (Figure 18A). Strikingly, neither HA-$p105_{\Delta DD}$ nor HA-$p105_{\Delta 497-538}$ co-expression affected Myc-TPL-2 activity, and MEK kinase activity of Myc-TPL-2ΔC was also completely unaffected by co-expression with HA-p105. However, Myc-TPL-2ΔC and wild type Myc-TPL-2 clearly associated with HA-p105 or HA-$p105_{\Delta 497-538}$, respectively (Figure 18A, lower panel). This suggests that the interaction of TPL-2 with the p105 DD is not sufficient and residues 497-538 play a crucial role for the inhibition of TPL-2 MEK kinase activity by p105 *in vivo*.



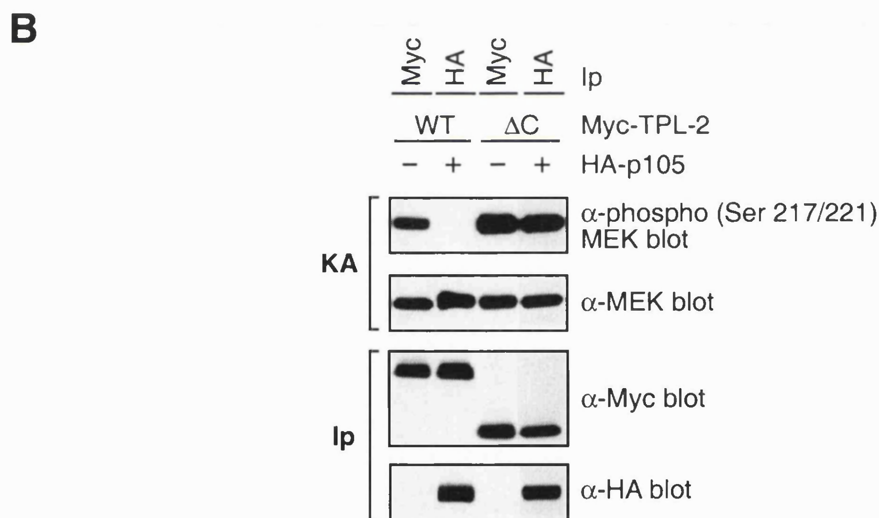


Figure 18. Both binding sites are essential for inhibition of TPL-2 MEK kinase activity by p105 in vivo. (A and B) 293 cells were co-transfected with vectors encoding Myc-TPL-2 or Myc-TPL-2ΔC and the indicated HA-p105 proteins or EV. The amounts of TPL-2 and TPL-2ΔC vector were adjusted so that similar levels of protein expression were obtained. MEK kinase activity of Myc-TPL-2 and Myc-TPL-2ΔC isolated by immunoprecipitation was determined as in Figure 16.

To directly test whether TPL-2, which solely binds to the p105 DD is active, the activity of wild type Myc-TPL-2 and Myc-TPL-2ΔC co-immunoprecipitating with HA-p105 was determined (Figure 18B). Wild type TPL-2, which co-immunoprecipitated with HA-p105, was inactive as expected. In contrast, Myc-TPL-2ΔC co-immunoprecipitated with HA-p105 had a similar activity as directly immunoprecipitated, free Myc-TPL-2ΔC. Therefore, inhibition of TPL-2 MEK kinase activity *in vivo* relies on simultaneous binding of the p105 DD and residues 497-538 of p105 to the TPL-2 kinase domain and the TPL-2 C-terminus, respectively.

In summary, these experiments indicate that the p105 DD inhibits TPL-2 MEK kinase activity and that this inhibitory effect is significantly increased if the TPL-2 C-terminus simultaneously interacts with residues 497-538 of p105. Thus, TPL-2ΔC is less sensitive to negative regulation by p105 *in vitro* than full length TPL-2 and insensitive to p105 negative regulation when expressed in cells. As a result, the MEK kinase activity of TPL-2ΔC is substantially increased (>40-fold) relative to wild type TPL-2 in cells expressing p105.

3. Discussion

3. Discussion

3.1. How does the p105 DD mediate p105 binding to IKK?

p105 proteolysis is triggered through phoshorylation of p105 serines 927 and 932 by IKK. In this study, it is demonstrated that p105 interacts with IKK via the DD motif in its C-terminus. The p105 DD is also required for the efficient phosphorylation and proteolysis of p105 induced by IKK or in response to TNFα stimulation. These data indicate that the p105 DD is a docking domain for IKK, facilitating phosphorylation and proteolysis of p105.

Protein kinases often form tight complexes with their substrates, which are crucial for their phosphorylation (Holland and Cooper, 1999). These complexes can involve a direct, high affinity interaction. It would be somewhat surprising if the p105 DD mediates the direct interaction of p105 with the IKK complex, since the DD motif is commonly involved in homotypic protein interactions, and IKKs contain leucine zipper and helix loop helix interaction motifs but no DD. However, even though the p105 DD is homologous to other DDs, it may be functionally different. Interestingly, the homology between the p105 DD and other established DDs is relatively low, yet a highly homologous region is found in the second NF-κB precursor protein NF-κB2 p100 (Feinstein et al., 1995).

Alternatively, binding of p105 to IKK may be indirect. As DDs commonly form homotypic interactions the p105 DD may directly bind to a DD-containing adapter protein, which bridges the interaction with IKK. The IKK complex interacts with the adapter proteins TRAF2 and RIP and is thereby recruited to the TNFR upon stimulation (Devin et al., 2000; Devin et al., 2001; Inohara et al., 2000; Zhang et al., 2000). It is tempting to speculate that p105 may also be recruited to activated TNFR via its DD, which may account for the marginal increase of p105 binding to IKK observed in

response to TNFα stimulation. Interestingly, the DD of NF-κB2 p100 was recently demonstrated to recruit NF-κB2 p100 to the TNFR upon stimulation (Wang et al., 2002).

Indeed, only a small fraction of IKK associated with p105 when co-expressed in 293 cells, suggesting that the interaction of p105 and IKK in cells may be facilitated via endogenous proteins. Preliminary results also show that HA-p105 does not interact with IKK1 or IKK2, when *in vitro* transcribed and translated together in a reticulocyte lysate system, which has a limited amount of endogenous proteins (data not shown). Furthermore, no association between purified GST-$p105_{497-968}$, fusion protein and recombinant IKK1/2 protein was observed (data not shown). Although the latter results could be explained by the lack of posttranslational modification (e.g. phosphorylation of p105 and/or IKK), which may be essential for the interaction, these data may indicate that the interaction of p105 and IKK is indirect.

Binding of p105 to IKK1/2 in cells may also depend endogenous proteins that form stable complexes with p105 and/or IKK. The prevalent IKK complex consists of IKK1 and 2 as well as the structural component NEMO. NEMO is required for TNFα-induced p105 proteolysis (Heissmeyer et al., 2001; Salmeron et al., 2001) and is likely to be a component of the IKK complex that associates with p105. It was demonstrated here that NEMO does not bind to p105 when co-expressed in 293 cells and is, therefore, unlikely to directly bridge the interaction of p105 and IKK1/2. However, it is possible that IKK1/2 binding to p105 is dependent on its complex formation with endogenous NEMO as NEMO also facilitates IKK2 binding to IκBα and IκBβ (Yamamoto et al., 2001).

It was also shown here that p105 forms a complex with TPL-2 via binding of its DD and residues 498-538 to the TPL-2 kinase domain and the TPL-2 C-terminus, respectively. This raises the question whether TPL-2 binding to the p105 DD is essential

to couple p105 to upstream signalling. TPL-2 could act as an adapter protein directly bridging the interaction of p105 and IKK. Alternatively, complexing with TPL-2 may confer a conformation to the p105 DD that can bind to upstream signalling proteins, while the free p105 DD may be in an unaccessible confirmation. Co-expression of TPL-2 did not affect binding of over-expressed IKK to p105 in 293 cells (data not shown). However, it is not possible to express all three proteins stoichiometrically. Therefore, over-expression experiment are insufficient to address this question and a potential function of TPL-2 binding to p105 in signal-induced p105 proteolysis remains to be evaluated in TPL-2 knockout experiments.

3.2. Significance of IKK binding to p105 for signal-induced p105 proteolysis

Data presented in section 2.1 indicate that IKK binding to p105 may be a crucial step in p105 proteolysis. The IKK complex has also been shown to bind to IκBα in a NEMO-dependent fashion, and this interaction facilitates IKK phosphorylation of IκBα (Yamamoto et al., 2001). p100 phosphorylation stimulated by the HTLV-encoded oncoprotein Tax involves the specific recruitment of IKK1 to p100 via the association of Tax with the p100 RHD and NEMO (Xiao et al., 2001a). Thus, binding of IKK to IκBs may be a general feature ensuring efficient target phosphorylation.

Binding of IKK to distinct IκBs could suggest that separate pools of IKK exist in cells, which may be differentially regulated and functionally separable. This model is also supported by the recent discovery that p100 processing in response to LTβ and BAFF is regulated by IKK1 independently of NEMO and IKK2, which suggests that an IKK complex consisting solely of IKK1 is upstream of p100 (Claudio et al., 2002; Dejardin et al., 2002; Yilmaz et al., 2003). Interestingly, in *Drosophila melanogasta* a complex equivalent to the mammalian IKK complex, which contains dmIKKγ and dmIKKβ,

regulates cleavage of the p105/p100 homologue Relish, but not degradation of the IκBα homologue Cactus (Silverman and Maniatis, 2001). Therefore, distinct IκBs are regulated by specific upstream kinases in *D. melanogasta.*

Alternatively, binding of p105 to the prevalent IKK complex, which regulates IκBα and is rapidly activated upon TNFα stimulation, may be important to facilitate p105 proteolysis. Interestingly, p105 serine 927 and 932 phosphorylation is detected rapidly after TNFα stimulation with similar kinetics as IκBα phosphorylation. p105 proteolysis also requires NEMO similar to IκBα (Heissmeyer et al., 2001; Salmeron et al., 2001). However, it is not entirely clear whether IKK1 or IKK2 regulates p105 proteolysis since both can phosphorylate p105 and substitute for each other in cytokine-induced p105 proteolysis in knockout cell lines (Salmeron et al., 2001). It will, therefore, be important to determine whether the IKK complex and the signalling pathway activating IKK is the same or different for p105 and IκBα.

Some of the endogenous p105 is shown to bind constitutively to IKK1 and 2. This pre-assembly of IKK with p105 may increase its specificity for this substrate and also shorten the time between kinase activation and p105 phosphorylation (Holland and Cooper, 1999). Phosphorylated p105 may then be released from IKK and replaced by another p105 protein, thereby increasing the V_{max} of p105 phosphorylation. The affinity of p105 and IKK may also increase upon stimulation, thereby recruiting the IKK complex to p105. However, although the total amounts of p105 associating with IKK increased in response to TNFα stimulation, the effect was only marginal. Since binding of p105 to IKK appears to be essential for its efficient phosphorylation, it is conceivable that only the fraction of p105 associated with IKK is phosphorylated when IKK is activated. Therefore, it is possible that inefficient recruitment of p105 to IKK may result in only a small fraction of p105 being phosphorylated in response to TNFα. This may contribute to the incomplete p105 proteolysis in response to TNFα in HeLa cells.

NF-κB1$^{\Delta C/\Delta C}$ and NF-κB1 knockout mice appear to develop normally, but have specific defects in innate and adaptive immune responses (Ishikawa et al., 1998; Sha et al., 1995). The function of p105/p50, therefore, is likely to be cell type and stimulus specific. Macrophages or B cells and T cells from NF-κB1$^{\Delta C/\Delta C}$ and NF-κB1 knockout mice display impaired responses to LPS and anti-CD3/anti-CD28 stimulation, respectively. Thus, it will be important to test whether these stimuli induce more efficient proteolysis of p105 in primary cells than observed in the cell lines tested so far.

It is also possible that there are other signals that induce the more pronounced proteolysis of p105. To date, there are more than a hundred stimuli known to induce NF-κB. Different members of the TNFR and IL-1R families mediate diverse biological functions in the development and activation of the immune system through the induction of NF-κB. In addition, distinct TLR family members trigger immune responses to different pathogens that require specific effector functions. This implies that activation the individual receptors results in specific gene expression profiles, to which the specific induction of individual IκBs and NF-κB dimers may contribute. However, only a small number of stimuli have been tested for the induction of p105 proteolysis, although IκBα has been intensively investigated. It will, therefore, also be important to test other stimuli for their potential to induce p105 proteolysis. Interestingly, one study has demonstrated that stimulation of monocytes with plasmin rapidly induces the degradation of p105, whereas degradation of IκBα is partial and delayed (Syrovets et al., 2001). It will be interesting to investigate whether more potent p105 stimuli induce stronger phosphorylation of p105 on serines 927 and 932 than TNFα and whether this coincides with the more pronounced induction of IKK association with p105.

3.3. Is TPL-2 upstream of p105?

TPL-2 induces proteolysis of p105 when co-expressed in 3T3 cells, suggesting that TPL-2 may act as a kinase upstream of p105 in signal-induced p105 proteolysis (Belich et al., 1999). p105 proteolysis is mediated by the phosphorylation of its destruction box in the PEST region by the IKK complex. TPL-2 also activates the IKK complex when over-expressed, which may account for its stimulatory effect on p105 proteolysis (Lin et al., 1999).

Interestingly, it is demonstrated here that binding to p105 is important for TPL-2 to induce p105 proteolysis. However, TPL-2-induced p105 proteolysis does not require the p105 DD, which plays an important role for IKK binding to p105 and IKK-induced p105 proteolysis. Therefore, it is unlikely that activation of the endogenous IKK complex by over-expressed TPL-2 and its phosphorylation of the destruction box of p105 accounts for TPL-2-induced p105 proteolysis.

Consistent with this, TPL-2 can induce p105 proteolysis in IKK1/2 double-deficient and NEMO-deficient fibroblasts (J. Janzen and S.C Ley, personnel communication). However, TPL-2-induced p105 proteolysis also relies on phosphorylation of serines 927 and 932 of p105, which TPL-2 can directly phosphorylate *in vitro* (J. Janzen, V. Lang and S.C Ley, personnel communication). Together, these findings suggest that over-expressed TPL-2 can induce p105 proteolysis independently of IKK. Serines 927 and 932 of p105 reside in a sequence motif that is similar to the activation loop motif in MAP 2-kinases and IKK1 and 2 (S-X-X-X-S, see diagram 4). It is, therefore, possible that p105 phosphorylation and proteolysis induced by TPL-2 in transfected cells arise as an artefact of over-expression and *in vitro* phosphorylation of p105 by TPL-2 is unspecific. However, NIK, which is homologous to TPL-2 in its kinase domain (Belich et al., 1999), regulates NF-κB2 p100 processing to p52 (Pomerantz and

Baltimore, 2002). Therefore, it will be important to determine whether phosphorylation of p105 by TPL-2 is physiologically relevant for p105 proteolysis by more detailed analyses of TPL-2 knockout mice.

3.4. Regulation of TPL-2 - a novel function of p105

In vitro, binding of the p105 DD to the TPL-2 kinase domain inhibits TPL-2 MEK kinase activity, and this inhibition is significantly augmented by concomitant interaction of the TPL-2 C-terminus with residues 497-538 of p105. In co-transfected cells, both binding sites are required for inhibition of TPL-2 kinase activity by p105. These data indicate that p105 is a negative regulator of TPL-2 activity in addition to its role as a precursor for p50 and a cytoplasmic inhibitor of NF-κB.

It has previously been suggested that TPL-2 kinase activity is regulated by an intra-molecular interaction with its C-terminus (Ceci et al., 1997). This hypothesis was based on the observation that a GST fusion protein encoding the C-terminus of TPL-2 binds to recombinant TPL-2ΔC and inhibits TPL-2ΔC kinase activity *in vitro*. In the present study, in which both kinases were isolated in an effectively p105-free form from lysates of transfected 293 cells, Myc-TPL-2ΔC was indeed found to have somewhat higher MEK kinase activity than Myc-TPL-2 (2-3 fold). However, although TPL-$2_{397-467}$ peptide bound to TPL-2ΔC, it was not found to affect its *in vitro* MEK kinase activity (data not shown). It is, therefore, unclear whether the stimulatory effect of C-terminal deletion on p105-free TPL-2 kinase activity is due to abrogation of an intra-molecular interaction or simply the result of a gross structural change in the kinase. More importantly, it is apparent from this work that the majority of the physiologically relevant forms of TPL-2 and TPL-2ΔC exist in a complex with p105. Under these conditions, it is apparent that there is a far more pronounced difference in specific MEK kinase activity

between TPL-2 and TPL-2ΔC, because of the insensitivity of the latter protein to negative regulation by p105.

The molecular mechanisms for the activation of most MAP 3-kinases are poorly understood. Inhibition of kinase activity by an intra-molecular interaction of the kinase domain with a pseudosubstrate domain often located in the C-terminus has indeed been reported for many kinases, e.g. GSK3β (Huse and Kuriyan, 2002). However, as an entry point for MAP kinase signalling modules MAP 3-kinases feature complex regulation. RAF-1 for example is activated as a consequence of its interaction with Ras-GTP at the plasma membrane (Kolch, 2000). Ras binding is followed by a sequence of events that involve phosphorylation, protein – protein interactions and protein – lipid interactions, which result in the release of intrinsic kinase inhibition by its cysteine-rich domain (CRD). Interestingly, recent studies have shown that RAF-1 release from repression by the RAF kinase inhibitory protein (RKIP), which disrupts the physical interaction between RAF-1 and MEK, is another important event that facilitates its activation of MEK and ERK (Yeung et al., 2000; Yeung et al., 1999). An inhibitor protein for MAPK upstream kinase (MUK), a MAP 3-kinase in the JNK pathway, called MUK binding inhibitory protein (MBIP) was also recently cloned (Fukuyama et al., 2000), highlighting the potential widespread utilisation of this regulatory mechanism.

The finding that NF-κB1 p105 is a negative regulator for TPL-2 kinase activity establishes an unexpected link between an IκB protein and the regulation of a MAP 3-kinase. It is possible that different pools of p105 exist, and that the role of p105 in regulating TPL-2 may be functionally distinguishable from its role as a cytoplasmic inhibitor of NF-κB or a precursor of p50, depending on the cellular context. However, LPS induces both p105 proteolysis (data not shown) and MEK activation in mouse BMDMs (Dumitru et al., 2000). Linking NF-κB and TPL-2 regulation by the same pool of p105 may have important biological implications. If the induction of p105 proteolysis

simultaneously results in the release of negative regulation of TPL-2 by p105, this would couple the activation of MEK/ERK with a specific NF-κB response that is controlled by p105.

3.5. A rationale for the oncogenic potential of C-terminally truncated TPL-2

The C-terminally truncated form of TPL-2 was identified in Moloney murine leukemia virus-induced T cell lymphomas (Patriotis et al., 1993). A C-terminal truncated version of the human homologue of TPL-2, Cot, is the transforming gene for a human thyroid carcinoma cell line (Chan et al., 1993). Furthermore, transgenic experiments confirm that C-terminally truncation of TPL-2 is necessary to activate its oncogenic potential in T cell lineage cells (Ceci et al., 1997). More recently, the oncogenic potential of TPL-2 has been substantiated in two separate large scale retroviral tagging screens for oncogenes in mice, in which retroviral insertions in the TPL-2 gene were identified in multiple lymphoid and myeloid tumors (Lund et al., 2002; Mikkers et al., 2002).

This study reveals a mechanism by which deletion of the C-terminus could lead to aberrant TPL-2 activity. Although TPL-2ΔC binds to p105 when expressed in cells, its kinase activity is largely unaffected by this association, in contrast to the full-length protein. The C-terminus of TPL-2, therefore, appears to be essential for negative regulation of its kinase activity by the p105 DD and its removal generates an oncogenic form that has much higher specific activity in cells, which co-express p105, than wild type. Persistent phosphorylation of MEK by TPL-2ΔC is likely to be important for TPL-2ΔC to transform cells, as constitutively active MEK mutants will oncogenically transform tissue culture cells (Cowley et al., 1994; Mansour et al., 1994).

To verify this model for oncogenic activation of TPL-2ΔC, the effect of p105 on TPL-2-induced cell transformation needs to be investigated. This could be done using a cell culture based assay to analyse the transformation potential of TPL-2 over-expressed with or without of p105 (Aoki et al., 1991). Transgenic mice expressing C-terminally truncated TPL-2 under the control of a T cell specific promotor develop T cell lymphoblastic lymphomas, whereas mice expressing the wild type protein fail to show an oncogenic phenotype (T. Ahmed and S.C. Ley, personnel communication) (Ceci et al., 1997). Expression of endogenous p105 in T cell lineage cells (Gerondakis et al., 1993) could be sufficient to efficiently inhibit the kinase activity of over-expressed wild type TPL-2, while TPL-2ΔC would be expected to be insensitive to this inhibition. To test this hypothesis, tumor progression in transgenic TPL-2 wild type or TPL-2ΔC mice crossed onto a NF-κB1-/- background (Sha et al., 1995) will be analysed in future experiments.

3.6. Does p105 regulate TPL-2 activity in response to LPS?

TPL-2 is known to play a crucial role in the activation of MEK/ERK in response to LPS in BMDMs (Dumitru et al., 2000). *In vitro* studies suggest that TPL-2 may function as the MAP 3-kinase activating MEK in this pathway (Chiariello et al., 2000; Salmeron et al., 1996), but the activation of TPL-2 kinase activity in response to LPS remains to be demonstrated. Data presented above suggests that p105 is negative regulator of TPL-2 kinase activity. However, it remains to be established that p105, indeed, plays an important physiological role in the regulation of TPL-2 activity in response to LPS in macrophages. To analyse this, the effects of p105-deficiency on LPS-induced MEK1/2 activity should be investigated in BMDMs from NF-κB1 knockout mice. Stabilisation of TPL-2 by p105 may complicate the issue because it is likely that there is very little TPL-2 in these cells. To overcome this, similar TPL-2 steady-state

levels compared to wild type macrophages would need to be established in NF-κB1-deficient BMDMs by adenoviral transfer. This experiment may have three different possible outcomes: First, deregulated activity of TPL-2 that is not complexed with p105 may increase the basal activity of MEK. Second, LPS stimulation may induce increased or faster activation of MEK, which would suggest that other regulatory mechanisms for TPL-2 activity in addition to its association with p105 exist. Third, sustained LPS-induced MEK activation would indicate that the negative regulation of TPL-2 kinase activity by p105 is important for down-regulation of the signal.

3.7. What is the mechanism of TPL-2 activation?

A particularly important question that arises from this work is the mechanism by which LPS stimulation triggers phosphorylation of MEK1 and 2 in macrophages via TPL-2 (Dumitru et al., 2000). Since essentially all TPL-2 is complexed with p105 in these cells, TPL-2 would be expected to be subject to regulation by p105. It is possible that the p105 DD acts as a substrate inhibitor interfering with the interaction of for the TPL2 kinase domain with MEK as described for RKIP, RAF-1 and MEK (Yeung et al., 2000; Yeung et al., 1999). To test this a GST-MEK1 fusion protein could be used to analyse MEK binding to over-expressed TPL-2 with or without p105. LPS stimulation could presumably modify the interaction of TPL-2 with p105 to allow its binding with MEK, which could also be analysed by GST-MEK1 pull-down of endogenous TPL-2 from lysates of LPS stimulated BMDMs.

LPS-induced activation of TPL-2 MEK kinase activity could occur as a consequence of LPS-induced proteolysis of p105 (Donald et al., 1995), thereby releasing TPL-2 from negative regulation. This could be tested by analyses of MEK activation kinetics in p105-deficient macrophages reconstituted with HA-p105$_{S927/932A}$, which lacks

crucial phosphorylation sites and is not degraded in response to stimulation. However, preliminary kinetic experiments in BMDMs have indicated that LPS-induction of MEK phosphorylation precedes p105 proteolysis and actually occurs coincidentally with LPS-induced proteolysis of TPL-2 itself by the proteasome (data not shown). TPL-2 proteolysis after LPS stimulation presumably constitutes a down-regulatory mechanism.

An alternative possibility is that LPS may induce the disruption of one or both of the interactions between TPL-2 and p105, thus relieving p105 negative regulation. To investigate this possibility the interaction of TPL-2 with p105 in response to LPS stimulation could be analysed in BMDMs. Preliminary results indicate that slightly reduced amounts of p105 immunoprecipitate with TPL-2 after 30 min of LPS treatment (data not shown). However, this reduction is dependent on the stringency of the buffer used for washing. This is consistent with a model in which only one of the binding sites of TPL-2, presumably the TPL-2 kinase domain, dissociates from p105 thereby reducing the affinity of the interaction. However, TPL-2 would always remain bound to p105 in cells through the second binding site. Stably transfected p105 deletion mutants for either binding site could be used to test whether LPS stimulation indeed results in dissociation of one of the binding sites.

Inhibition of TPL-2 kinase activity is mediated by the interaction of the TPL-2 kinase domain with the p105 DD. DDs commonly form inducible homo-typic interactions between DD containing proteins involved in signalling pathways leading to the induction of NF-κB, AP-1 or apoptosis. Results presented in section 2.1 indicate that the p105 DD mediates the interaction with the IKK complex and plays an important role for signal-induced p105 proteolysis. SODD and TOLLIP, which do not contain DDs, have been reported to be constitutively associated with the DDs of the TNFR and IRAK, respectively (Burns et al., 2000; Jiang et al., 1999). Upon stimulation both proteins dissociate from their binding partners. Therefore, dissociation of the TPL-2 kinase

domain from the p105 DD may occur as a consequence of a conformational change of the p105 DD induced by binding to upstream signalling proteins upon stimulation.

Alternatively, dissociation of the TPL-2 kinase domain from the p105 DD could also occur as a consequence of either TPL-2 or p105 phosphorylation. Interestingly, auto-phosphorylation of IRAK in its DD was implicated in the dissociation of IRAK/TOLLIP complexes (Burns et al., 2000). It has been reported that the p105 DD is phosphorylated by IKK *in vitro* (Heissmeyer et al., 1999), and it will be important to determine whether this modification alters the inhibition of TPL-2 activity by p105.

The interaction of the C-terminus of TPL-2 with residues 497-538 of p105 is required for efficient negative regulation of TPL-2 kinase activity additionally to the interaction of the TPL-2 kinase domain with the p105 DD. It is, therefore, also conceivable that modification of this binding site modulates TPL-2 kinase activity. Interestingly, the TPL-2 C-terminus contains multiple potential serine and threonine phosphorylation sites (Ceci et al., 1997).

3.8. Implications of TPL-2 stabilisation by p105

TPL-2 stability relies on its association with p105 and all TPL-2 is complexed with p105 in HeLa cells (Belich et al., 1999) and BMDMs (this study). No detectable TPL-2 remains in NF-κB1-deficient 3T3 fibroblasts despite the fact that they synthesise similar levels of TPL-2 mRNA as wild type 3T3 fibroblasts, suggesting that free TPL-2 is degraded in cells. Therefore, p105 is an obligate partner for TPL-2 *in vivo* that is required to maintain its metabolic stability. The regulation of TPL2 stability by p105 may have evolved to ensure that unstimulated cells do not contain p105-free, unregulated TPL-2 activity.

It will clearly be important to re-examine the molecular phenotype of NF-κB1$^{\Delta C/\Delta C}$ and NF-κB1 knockout mice, which may arise in part because of defective ERK activation caused by TPL-2-deficiency. Impaired B and T cell function in NF-κB1$^{\Delta C/\Delta C}$ and NF-κB1 knockout mice is probably due to a lack of p105/p50, as TPL-2-/- splenocytes respond normally to LPS and anti-CD3/anti-CD28 antibody treatment. Macrophages from NF-κB1$^{\Delta C/\Delta C}$ mice produce reduced amounts of TNFα compared to wild type after stimulation with LPS and interferon (Ishikawa et al., 1998). This may be due to an increase in nuclear p50 homodimers, which bind to the TNFα promoter and negatively regulate TNFα gene transcription (Bohuslav et al., 1998; Udalova et al., 2000). However, TPL-2 is essential for LPS induction of TNFα in these cells, and TPL-2 levels are presumably severely reduced in NF-κB1$^{\Delta C/\Delta C}$-deficient macrophages (Dumitru et al., 2000). Therefore, it is not clear whether this phenotype specifically results from an altered NF-κB response due to p105-deficiency. Interestingly, macrophages from NF-kB1 null mice produce normal levels of TNFα after LPS stimulation (Bohuslav et al., 1998; Sha et al., 1995). It is possible that the absence of inhibitory p50 homodimers in NF-kB1-deficient macrophages compensates for the relatively low levels of TPL-2 protein. However, it remains important to analyse whether TPL-2 protein levels are diminished and LPS-induced MEK activation is affected in NF-κB1$^{\Delta C/\Delta C}$ and NF-κB1-deficient macrophages.

3.9. Implications for p105 and TPL-2 as therapeutic targets

Analyses of TPL-2 knockout mice demonstrated that TPL-2 mediates the production of TNFα in response to LPS in macrophages by activating the MEK/ERK MAP kinase cascade (Dumitru et al., 2000). Consequently, TPL-2 is essential for LPS-induced septic shock and the development of inflammatory bowl disease, illustrating the

importance of TPL-2 for inflammatory responses (Dumitru et al., 2000; Kontoyiannis et al., 2002). Thus, TPL-2 is a potential target for the pharmacological intervention in inflammatory immune disorders, in which the production of TNFα plays a critical role, e.g. septic shock, inflammatory bowl disease, rheumatoid arthritis, lupus erythematosus, or multiple sclerosis (Kollias et al., 1999). Furthermore, the C-terminally truncated form of TPL-2 has been identified as an oncogene (Chan et al., 1993; Patriotis et al., 1993), and it will be important to establish whether mutations that impair TPL-2 regulation occur physiologically in human malignant cell transformation. In this case, targeting of TPL-2 kinase activity may be important as a specific anti-cancer treatment of these tumors.

NF-κB1 or NF-κB1$^{\Delta C/\Delta C}$ knockout mice display specific defects in innate and adaptive immune responses (Ishikawa et al., 1998; Sha et al., 1995). Analyses of NF-κB1-/- mice indicate that p105/p50 is critical for B-cell function and immune responses to bacterial infection as well as the induction of asthma. However, in T cells and macrophages, p50 seems to have a negative regulatory function, as the production of cytokines, such as TNFα, is impaired in NF-κB1$^{\Delta C/\Delta C}$ mice, which have increased nuclear p50 levels. Therefore, targeting of p105/p50 in a cell type specific manner may allow the modulation of unwanted immune responses.

The regulation of inhibitory p50 homodimers as well as the TPL-2/MEK/ERK MAP kinase casade by p105 may represent tight control unit that regulates the transient induction of pro-inflammatory mediators such as TNFα. Whereas the induction of ERK by TPL-2 positively regulates TNFα production, concomitant induction of p50 homodimers may ensure the immediate down regulation of this response. The model of TPL-2 kinase regulation by p105, proposed in this study, represents an important step in the understanding of the function of this kinase. As the inhibition of TPL-2 kinase activity seems to be directly mediated by the interaction of the TPL-2 kinase domain with

the p105 DD, more detailed biochemical and biophysical analysis of this mechanism may allow the development compounds that mimic this regulation and inhibit TPL-2.

It was also demonstrated here that the p105 DD has additionally a crucial function in coupling p105 to signal-induced proteolysis. This identifies an important regulatory mechanism, which contributes specificity of the IKK complex towards p105. Targeting of the interaction of the p105 DD with upstream signalling proteins could specifically uncouple p105 proteolysis without affecting the induction of other IκBs.

The p105 DD appears to be a molecular switch, which not only couples p105 to signal-induced proteolysis, but also regulates the activity of TPL-2. Therefore, the dissection of molecular mechanisms underlying these individual functions represents a challenging task for future research.

4. Materials and Methods

4. Materials and Methods

4.1. cDNA constructs, recombinant proteins and antibodies

HA epitope-tagged wild type p105 (HA-p105), deletion and point mutants of HA-p105, N-terminally Myc epitope-tagged p105 (Myc-p105), HA-p100 and a panel of N-terminal deletion mutants of Myc-TPL-2ΔC cDNAs were generated using the polymerase chain reaction (PCR) and verified by DNA sequencing. cDNAs were cloned into the pcDNA3 expression vector (Invitrogen) for transient expression experiments and into the pMX-1 expression vector (Ingenius) for generation of stably transfected HeLa cell lines. Expression vectors encoding HA epitope-tagged NEMO (HA-NEMO) and wild type or kinase-inactive Flag epitope-tagged IKK1 and IKK2 (FL-IKK1/2) have been described (Mercurio et al., 1997; Nakano et al., 1998; Yamaoka et al., 1998) and were kindly donated by Frank Mercurio (Signal Pharmaceuticals, USA), Hirano Nakano (Juntendo University Tokyo, Japan) and Alain Israel (Pasteur Institute, France), respectively. Myc-tagged and untagged versions of TPL-2 and TPL-2ΔC were also described previously (Belich et al., 1999). Myc-Raf1(CAAX) in the pEXV expression vector was kindly provided by Chris Marshall (Cancer Research UK) (Leevers et al., 1994).

To generate recombinant p105 protein, p105 cDNA sequences were cloned into pGEX-2T or pGEX-6P-1 (Amersham Pharmacia Biotech). GST fusion proteins were expressed at 30°C in the BL21DE3 *E. coli* strain and purified by affinity chromatography on GSH Sepharose 4B (Amersham Pharmacia Biotech). The p105 fragments were cleaved from GST with PreScission Protease (Amersham Pharmacia Biotech) and further purified by gel filtration on a Superdex column (Amersham Pharmacia Biotech). The resulting proteins, which were over 95% pure as judged by Coomassie Brilliant blue (Novex) staining of SDS-PAGE gels, were concentrated by ultrafiltration and stored at

–80°C. Expected masses of all proteins were confirmed by mass spectrometry (Steve Howell, NIMR).

12CA5 monoclonal antibody was used for immunoprecipitation of HA-p105, whereas a high affinity anti-HA monoclonal antibody 3F10 (Roche Molecular Biochemicals) was used for Western blot detection of HA-tagged proteins. Myc-p105 was immunoprecipitated using 9E10 monoclonal antibody and detected in Western blots using a commercial anti-Myc antibody (Santa Cruz). M2 monoclonal antibody (Sigma-Aldrich) was used for Western blotting of Flag-tagged proteins. Anti-p105C, anti-phospho Ser927 p105 and anti-phospho Ser927 p105 antibodies have been described (Salmeron et al., 2001; Lang et al., 2003). IκBα was Western blotted using polyclonal anti-IκBα serum (Santa Cruz). The anti-IKK1/2 antibody was raised against a peptide sequence present in both IKK1 and IKK2 (Salmeron et al., 2001) and therefore directly recognises both proteins. Endogenous p105 was immunoprecipitated and Western blotted with a polyclonal rabbit antibody raised to a synthetic peptide corresponding to the C-terminal 17 amino acids of murine NF-κB1 p105 (Salmeron et al., 2001). Endogenous TPL-2 was immunoprecipitated with polyclonal anti-TPL-2 antibody, which was raised in rabbits to a synthetic peptide corresponding to the C-terminal 70 amino acids of TPL-2, and Western blotted with TSP3 anti-TPL-2 antibody (Salmeron et al., 2001). Anti-MEK1/2 and anti-phospho Ser217/221 MEK1/2 antibodies were purchased from Cell Signaling Technology (USA). Nuclear Sam68 was detected using commercial anti-Sam68 antibody (Santa Cruz). Tubulin and actin were used as loading controls for cell lysates and were detected on Western blots with TAT-1 anti-tubulin monoclonal antibody (kindly provided by Keith Gull, University of Manchester, UK) and a commercial anti-actin monoclonal antibody (Sigma-Aldrich), respectively.

4.2. Cells and transfection

HeLa cells (Ohio subline from ECACC) and 293 cells (QIB293A cells, Quantum Biotechnologies Inc.) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, penicillin (100U/ml) and streptomycin (50U/ml). For NIH-3T3 (ECACC) fibroblasts, 10% new born calf serum was substituted for fetal calf serum.

Bone marrow-derived macrophages (BMDMs) were isolated and cultured essentially as described (Warren and Vogel, 1985). In brief, bone marrow was flushed from femurs of Balb/c mice with 5ml of complete medium comprising RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cell pellets were plated in 10ml complete medium plus 50ng/ml murine M-CSF (R & D Systems) at 2x 10^6 cells/ml in 25cm^2 tissue culture flasks (Life Technologies, Inc.) for 24h. The non-adherent cells were then removed and transferred to an 80cm^2 tissue culture flask (Nunc) and another 10ml of complete medium plus M-CSF added. Flasks were then incubated for 3 days at 37°C, at which time a further 10ml of complete medium plus M-CSF was added. After a total of 7days culture, adherent macrophages were harvested, plated in 90mm dishes (Life Technologies, Inc.) and re-cultured in RMPI 1640 + 0.5% FBS and antibiotics for a further 24h, prior to use in experiments. Over 95% of the resulting cell populations corresponded to macrophages as judged by flow cytometric analysis using two macrophage-specific markers (data not shown). All cells were maintained in a rapid growth phase for experiments.

3T3 cells (2x 10^5 per 60mm dish; Life Technologies, Inc.) and 293 cells (5x 10^5 per 60mm dish; Life Technologies, Inc.) were transiently transfected using LipofectAMINE (Life Technologies, Inc) after 18h of culture following the supplier instructions. In brief, 1ug DNA in 100ml Optimem/Glutamax (Life Technologies, Inc)

was mixed with 10ul lipofectAMINE in 100ml Optimem/Glutamax and incubated at room temperature for 30min before it was added to cells in a total volume of 1.8ml Optimem/Glutamax. After 5-6h at 37°C, the transfection mix was replaced with DMEM plus supplements and cultured for 24-48h.

To stably transfect HeLa cells with HA-p105 constructs, 7x 10^5 cells were plated in a 90mm dish (Life Technologies, Inc.) and, after 18h in culture, transfected using LipofectAMINE (Life Technologies, Inc.) as described above. Transfected cells were cultured for a further 48h and then selected for neomycin resistance with 1mg/ml G418 (Life Technologies, Inc.). After 3-4 weeks, clones were picked manually and then expanded. Expression of HA-p105 was determined by Western blotting.

4.3. Immunoprecipitation and Western blotting analysis

Cells were washed free of serum with phosphate-buffered saline (PBS) and then lysed with 400-1000μl of ice-cold buffer A (1% NP-40, 50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 20mM NaF, 1mM Na_3VO_4, 2mM $Na_4P_2O_7$ plus a cocktail of protease inhibitors (Roche Molecular Biochemicals)) with scraping. Cell lysates were cleared of insoluble debris or aggregated over-expressed proteins by centrifugation at 13 000g or 100 000g, respectively, for 10min at 4°C and then pre-cleared once by incubation with 10μl of protein A-Sepharose or protein G-Sepharose (Pharmacia) for 1h at 4°C. For immunoprecipitation, 10μg of purified monoclonal antibody or 5-10μl of polyclonal antiserum were coupled covalently to 10 μl of protein A-Sepharose or protein G-Sepharose with dimethylpimelimidate (Schneider et al., 1982) and incubated with pre-cleared cell lysate for 4h or overnight. Following extensive washing (5x) with ice-cold buffer A, isolated proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membrane were blocked

with 5% milk or bovine serum albumin (BSA) in PBS supplemented with 0.05% TWEEN 20 (Sigma) for 1h and then probed with the indicated antibodies in 1% milk or BSA in PBS/0.05% TWEEN 20 (Sigma) for 2h to over night. After extensive washing with PBS/0.05% TWEEN membranes were probed with horse raddish peroxidase (HRP) linked secondary antibodies or protein A for 1h. Following further washing with PBS/0.05% TWEEN signals were revealed using chemoluminesence (ECL, Amersham Pharmacia). In experiments in which blots were probed for multiple antigens PVDF membranes were stripped of bound antibody by incubation with 0.2M glycine pH2.5 for 15min.

4.4. Analyses of protein degradation

To analyse HA-p105 proteolysis in stably transfected HeLa clones, cells were plated at 6x 10^5 per well of a 6-well plate (Life Technologies, Inc.). After 18h in culture, cells were washed with PBS and cultured in methionine/cysteine-free minimal essential Eagle's medium (Sigma) for 1h. Cells were pulse-labelled with 2.65MBq of [^{35}S]-methionine/[^{35}S]-cysteine (Pro-Mix, Amersham Pharmacia Biotech) for 30min and chased for the indicated times in complete medium (Dulbecco's modified Eagle's medium plus 2% FCS) alone or complete medium supplemented with TNFα (20ng/ml; Amersham Pharmacia Biotech). Cells were lysed in buffer A supplemented with 0.5% deoxycholate and 0.1% SDS (RIPA buffer). HA-p105 was isolated by immunoprecipitation with 12CA5 mAb, resolved by 10% SDS-PAGE and revealed by fluorography. Labelled bands were quantified by densitometric imaging (BioRad). TNFα-induced IκBα degradation in stable HeLa cell lines was analysed by Western blotting of whole cell lysates.

To analyse IKK2 or TPL-2-induced p105 proteolysis or stability of transiently transfected Myc-TPL-2, 3T3 or 293 cells were pulse-chase metabolic labelled after 24h in culture as described above. Myc-TPL-2 or HA-p105 were isolated by immunoprecipitation, resolved by 10% SDS-PAGE and revealed by fluorography. Labelled bands were quantified by densitometric imaging (BioRad) and normalised against the amount of HA-p105 immunoprecipitated as determined in a parallel Western blot.

4.5. Statistics

A two-tailed Student's t-test was used to determine whether TNFα stimulation (HeLa stables) or IKK2/TPL-2 over-expression (3T3 cells) induced significant increases in p105 proteolysis compared with the appropriate control cells. The half-life of p105 determined in pulse-chase metabolic labelling experiments was calculated from an extrapolated trend-line of mean values ($n = 3$) using the Microsoft Excel programme.

4.6. Analyses of protein interaction

In experiments analysing the interaction of p105 with IKK1/2 or TPL-2, transiently transfected 293 cells were lysed after 24-48h culture with buffer A, which was supplemented with 0.25% deoxycholate and 0.05% SDS to assay the p105 / IKK interaction. Immunoprecipitation and Western blotting of transfected proteins was carried out as described using the indicated antibodies.

In vitro co-precipitation experiments were carried out using GST-p105 fusion proteins and TPL-2 that was synthesised *in vitro* and labelled with [^{35}S] methionine /

[^{35}S] cysteine (Amersham-Pharmacia Biotech) by cell-free translation (TNT-coupled rabbit reticulocyte system, Promega). Translated proteins were diluted with *in vitro* precipitation (IVP) buffer (250mM NaCl, 0.01% NP-40, 50mM HEPES – pH7.9, 50mM EDTA, 0.1mg/ml BSA) prior to incubation with 1ug GST fusion proteins for 1h at 4°C. 15ml Glutathione-sepharose beads were then added to the protein mixture and incubated further 30min at 4°C before washing the beads 4x with IVP buffer. The components of the resulting complexes were resolved by SDS-PAGE and revealed by fluorography.

To analyse the interaction of TPL-2 with p105 when synthesised together *in vitro* by cell-free translation (TNT-coupled rabbit reticulocyte system, Promega), proteins were diluted in IVP buffer and immunoprecipitated with the indicated antibodies. Immunoprecipitates were resolved by 10%SDS-PAGE and revealed by fluorography.

4.7. Cell fractionation

Cytoplasmic and nuclear localisation of transiently transfected p105 proteins was determined using a published methodology (Alkalay et al., 1995). Briefly, 293 cells were lysed in cytoplasmic lysis buffer (10mM HEPES pH7.6, 1.5mM $MgCl_2$, 0.1mM EGTA, 10mM KCl, 1mM DTT, 20mM NaF, 1mM $Na_4P_2O_7$, 1mM Na_3VO_4 plus protease inhibitor mix (Roche Molecular Biochemicals)) for 15min. NP-40 was then added to a final concentration of 0.6% and, after 2min, lysates cleared of nuclei by centrifugation. Pellets were washed once with cytoplasmic lysis buffer and then extracted by addition of nuclear lysis buffer (20mM HEPES pH7.6, 1.5mM MgC12, 0.1mM EGTA, 25% glycerol, 0.42M NaCl, 1mM DTT, 20mM NaF, 1mM $Na_4P_2O_7$, 1 mM Na_3VO_4 plus protease inhibitors) and three cycles of freeze thawing. Nuclear extracts were cleared by centrifugation. Cytoplasmic and nuclear extracts were analysed by Western blotting with the indicated antibodies.

4.8. Analysis of protein phosphorylation

To analyse *in vivo* phosphorylation of p105 on serine 927 after TNFα stimulation (Figure 9B), 5x 10^6 HeLa cells were plated per 100mm dish. After 18h in culture, cells were pre-treated with 20μM MG132 proteasome inhibitor (Biomol Research Labs) for 30min and then stimulated for 15min with TNFα (20ng/ml; Amersham-Pharmacia). Cells were lysed in RIPA buffer and transfected HA-p105 immunoprecipitated with 12 CA5 anti-HA monoclonal antibody. After extensive washing, immunoprecipitates were re-suspended in 100μl of buffer A supplemented with 1% SDS and heated to 100°C for 5min. The supernatant was then aspirated and diluted to 1.5ml in RIPA buffer. HA-p105 was re-immunoprecipitated with 12CA5 anti-HA monoclonal antibody and Western blotted with anti-phospho Ser927 p105 antibody. This two-step immunoprecipitation procedure disrupted the interaction of transfected HA-p105 with endogenous p105.

To determine serine 927 phosphorylation of transiently transfected HA-p105, 3T3 or 293 cells were treated with 20μM MG132 for 4h before lysis and immunopreciptation with 12CA5 anti-HA monoclonal antibody. Western blots of immunoprecipitates were probed with anti-phospho Ser927 p105 antibody.

For *in vitro* experiments analysing p105 phosphorylation by IKK, wild type or point mutated HA-p105 was transiently expressed in 293 cells and isolated by immunoprecipitation. Immunoprecipitates were washed four times in Buffer A and twice in kinase buffer (25mM Tris, pH 7.5, 5mM β-glycerophosphate, 2mM dithiothreitol, 0.1mM Na_3VO_4, 10mM $MgCl_2$). Immunoprecipitates were then incubated at room temperature for 30min in 50μl of kinase buffer containg 100μM ATP plus or minus 100ng of recombinant His_6-IKK1 or His_6-IKK2 protein (Salmeron et al., 2001). The reaction was stopped by washing immunoprecipitates twice with buffer A. Isolated HA-

p105 was eluted by boiling in 100μl of Laemmli sample buffer ( ) and then revealed by 8% SDS-PAGE, Western blotted and probed with anti-phospho Ser927 p105 antibody.

For the MEK *in vitro* kinase assay, Myc-TPL-2 or Myc-TPL-2DC was isolated by immunoprecipitation from lysates of transiently transfected 293 cells. Immunoprecipitates were washed four times in buffer A, followed by two washes in kinase buffer (50mM Tris – pH7.5, 150mM NaCl, 5mM b-glycerophosphate, 2mM dithiothreitol, 0.1mM Na_3VO_4, 10mM $MgCl_2$, 1mM EGTA, 0.01% Brij35). Beads were then re-suspended in 50ml of kinase buffer plus the indicated amounts of recombinant p105 protein and incubated at 4°C for 30min. 1mg kinase inactive GST-MEK1(K207A) protein (Upstate Biotechnology) was then added in 50ml kinase buffer plus 2mM ATP and incubated a further 30min at RT. The supernatant was removed, mixed with an equal volume of 2x Laemmli sample buffer and subjected to 8% SDS-PAGE and Western blotting. MEK1 phosphorylation was revealed using anti-phospho Ser217/221 MEK1/2 Ab. Immunoprecipitated Myc-TPL-2 or Myc-TPL-2DC was eluted with Laemlli sample buffer from the remaining anti-Myc mAb beads, resolved by 8% SDS-PAGE and revealed by Western blotting.

4.9. RNA isolation and RT-PCR analysis

Total RNA from NF-kB1-deficient or wild type 3T3 fibroblasts was isolated using Trizol reagent (Invitrogen). Semi-quantitative RT-PCR was performed utilising the QIAGEN OneStep RT-PCR kit. Primer pairs used were as follows: (5') primer 5'-CATTGCTGATTCATCATGC-3' and (3') primer 5'-ACTGGGCTCATACACTGC-3' for TPL-2; (5') primer 5'-GGCGGCTTGGTGACTCTAGATA-3' and (3') primer 5'-GCTCGGGCCTGCTTTGAACAC-3' for 18SrRNA. The lengths of the TPL-2 and 18SrRNA amplicons were 1169 and 560 bp, respectively. PCR products were visualised

on 1.5% agarose gels and TPL-2/18SrRNA ratios determined using a densitometric imager (Kodak ID 3.5).

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