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Involvement of the low molecular weight G protein Rac1 in IL-1 signalling to NFκB activation

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Thesis submitted to the

University of Dublin

For the

Degree of Doctor of Philosophy

By

Caroline Jefferies

Department of Biochemistry

& Biotechnology Institute

Trinity College

Dublin

September 2000

Declaration

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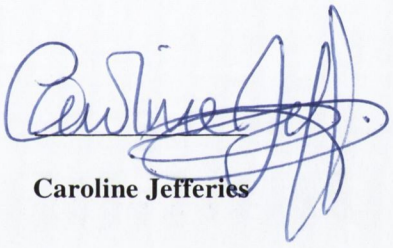

Caroline Jefferies

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Abbreviations

ABTS	2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid
AcP	Accessory protein
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AP-1	Activator protein 1
ATP	Adenosine triphosphate
Bcr	Break-point control region
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CAT	Chloramphenicol acetyl transferase
CBP	CREB binding protein
CKII	Casein kinase II
CMV	Cytomegalovirus
CREB	cAMP response element binding factor
DTT	Dithiothreitol
ECSIT	Evolutionarily conserved signaling intermediate in Toll/IL-1 pathways
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EMSA	Electrophoretic mobility shift assay
ERK1/2	Extracellular signal-regulated kinase 1/2
FAK	Focal adhesion kinase

FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAP	GTPase activating protein
GDI	Guanine dissociation inhibitors
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GSH	Glutathione
GST	Glutathione S transferase
GTP	Guanine triphosphate
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IKK	IκB kinase
IL	Interleukin
IL-1RacP	IL-1 receptor accessory protein
IL-1RI	IL-1 receptor type I
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRAK	Interleukin 1 receptor associated kinase
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LMWG	Low molecular weight G protein
LPS	Lipopolysaccaride
LTA	Lipoteichoic acid
MAPK	Mitogen activated protein kinase
MBP	Myelin basic protein
MCS	Multiple cloning site
MLK	Mixed lineage kinase

MyD88	Myeloid differentiation factor 88
NEMO	NFκB essential modifier
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
NIK	NFκB inducing kinase
NLS	Nuclear localisation sequence
Oct	Octamer factor
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PCAF	p300/CBP binding protein (CBP)-associated factor
PDGF	Platelet derived growth factor
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIX	PAK-interacting exchange factor
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
POR	Partner of Rac1
POSH	Plenty of SH_3 domains
PVDF	Polyvinylidene diflouride
RHD	Rel-homology domain
SDS	Sodium dodecyl sulphate
SH	Src homology
TAB	TAK1 binding protein

TAD	Transactivation domain
TAK	TGFβ-activated kinase
TBP	TATA binding protein
TCR	T cell receptor
TEMED	N, N, N', N',-Tetramethylethylenediamine
TIR	Toll/IL-1R region
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tollip	Toll/IL-1R interacting protein
TRAF	TNF-receptor associated factor
WASP	Wiscott-Alcott syndrome protein

Abstract

The involvement of the low molecular weight G protein Rac1 in IL-1 signal transduction has been suggested by studies that have demonstrated that a dominant negative mutant of Rac1 can inhibit IL-1-induced activation of both p38 and JNK mitogen activated protein kinase (MAPK) pathways. This prompted the investigation into the role of Rac1 in other IL-1-induced events in T lymphocytes, namely its ability to drive IL-2 expression, using the IL-1 receptor rich murine thymoma cell line EL4.NOB-1.

Initial studies in EL4.NOB-1 cells demonstrated that IL-1 stimulation resulted in activation of Rac1 and this result was confirmed in the human cervical carcinoma cell-line, HeLa, which are also highly IL-1 responsive. IL-1 also activated the downstream effector of Rac1, p21 activated kinase (PAK) in EL4.NOB-1. The role of Rac1 in the ability of IL-1 to drive IL-2 promoter activity, mRNA and protein production was next assessed. Results showed that constitutively active RacV12 potentiated all three responses in the presence of IL-1. This strong synergy between RacV12 and IL-1 in driving IL-2 expression was further investigated by studying the effects of Rac1 on the transcription factors critically important for regulating the IL-2 promoter – NFAT, AP1 and NFκB, using reporter genes linked to these elements. RacV12 but not IL-1 activated NFAT-CAT activity. While IL-1 weakly induced expression of AP1-CAT, transfection of cells with RacV12 alone strongly stimulated the AP-1 promoter. This activation was further potentiated in the presence of IL-1. Dominant negative RacN17 inhibited IL-1-induced activation of AP1-CAT suggesting that Rac1 plays an important role in IL1-induced activation of AP1. However, while RacV12 alone was unable to drive IL-2-dependent reporter gene expression, its ability to drive NFAT- and AP1-CAT in the absence of IL-1 suggests that RacV12-induced activation of these transcription factors is insufficient to drive IL-2- expression.

In contrast to NFAT-CAT and AP1-CAT, RacV12 alone was unable to activate NFκB-

CAT but in the presence of IL-1 synergised in driving NFκB-CAT activity. Importantly, dominant negative Rac1 inhibited IL-1-induced NFκB-luciferase. However neither RacV12 nor RacN17 were able to affect IκB degradation or nuclear translocation of NFκB in response to IL-1. Our results suggested a role for Rac1 in p65-mediated transactivation of gene expression by NFκB, a recently identified pathway regulating NFκB that is independent of IκBα regulation. IL-1 activated a pathway leading to increased p65 transactivation activity and transfection of cells with RacV12 alone could drive this response. In addition, RacN17 inhibited IL-1-driven p65-mediated transactivation, suggesting that Rac1 participates in this pathway. Using specific inhibitors of p38, p42/p44 and JNK MAPK it was concluded that both p38 and p42/p44, but not JNK, lay downstream of Rac1 on the IL-1 pathway leading to enhanced transactivation by p65.

Finally, the involvement of key components of the IL-1 signaling pathway in transactivation of gene expression by p65 was examined. Wild-type MyD88, IRAK-1 and TRAF-6 drove p65-mediated transactivation whereas IRAK-2 alone was unable to drive this response. In addition dominant negative forms of MyD88, IRAK-1 and TRAF-6 inhibited the IL-1-induced response. RacV12-mediated transactivation was not inhibited by dominant negative MyD88, while dominant negative RacN17 inhibited the MyD88-mediated response. This result indicated that MyD88 was not required for Rac1-induced activation of this pathway and placed Rac1 downstream of MyD88 on the pathway regulating p65-mediated transactivation. Dominant negative RacN17 inhibited wild-type IRAK-1 and TRAF-6 induced transactivation and, in turn, dominant negative IRAK-1 and TRAF-6 inhibited the RacV12-induced response. This suggested a mutual co-dependence of Rac1, IRAK-1 and TRAF-6 in regulating this pathway. Finally, Rac1 was found to associate with the receptor complex via interactions with both MyD88 and the IL-1 receptor accessory protein. A pathway emanating from MyD88 and involving IRAK-1, TRAF-6 and Rac1 is therefore involved in transactivation of gene expression by the p65 subunit of NFκB in response to IL-1.

Chapter One

General Introduction

1 Introduction

The pro-inflammatory cytokine interleukin-1 (IL-1) has been the subject of intense investigation since its initial characterisation as a co-mitogen of T lymphocytes in 1974 (61). Its involvement in the pathogenesis of inflammatory disease such as ulcerative colitis and rheumatoid arthritis has focused the attention of researchers into understanding how it mediates its effects on its target cells.

1.1 IL-1 family members

The IL-1 family includes four molecules, IL-1α, IL-1β, IL-18 and IL-1ra, which similar to the fibroblast growth factors (FGF), share a common β-barrel structure and act on target cells by binding to cell surface receptors with immunoglobulin-like folds. Human IL-1α, β and IL-1ra are transcribed from the IL-1 locus on chromosome 2. While IL-1α and β are biologically active, IL-1ra is a naturally occuring receptor antagonist which inhibits the pro-inflammatory effects of IL-1α and β (reviewed in (48)). Recently four additional members of the IL-1 family have been described that have been termed IL-1H1 to IL-1H4 (or alternatively FIL-1δ, ε, ζ and η, where FIL-1 denotes family of IL-1) (99, 171). Due to its sequence homology to the IL-1ra gene and similarities in their expression pattern it has been suggested that IL1HY1 (FIL-1γ) may be novel receptor antagonist gene (131), although evidence in support of such a function has so far been lacking. Although homologous to IL-1α and β, these novel IL-1 family members do not appear to signal via the same receptor complex and putative receptors and biological activities for these molecules remain to be characterised.

IL-1β is expressed as a 34 kilodalton (kDa) pro-protein which undergoes proteolytic removal of the pro-domains to generate the mature, active cytokine which is then secreted (shown in figure 1.1) (50). The enzyme reponsible for regulating IL-1β processing is the aspartate

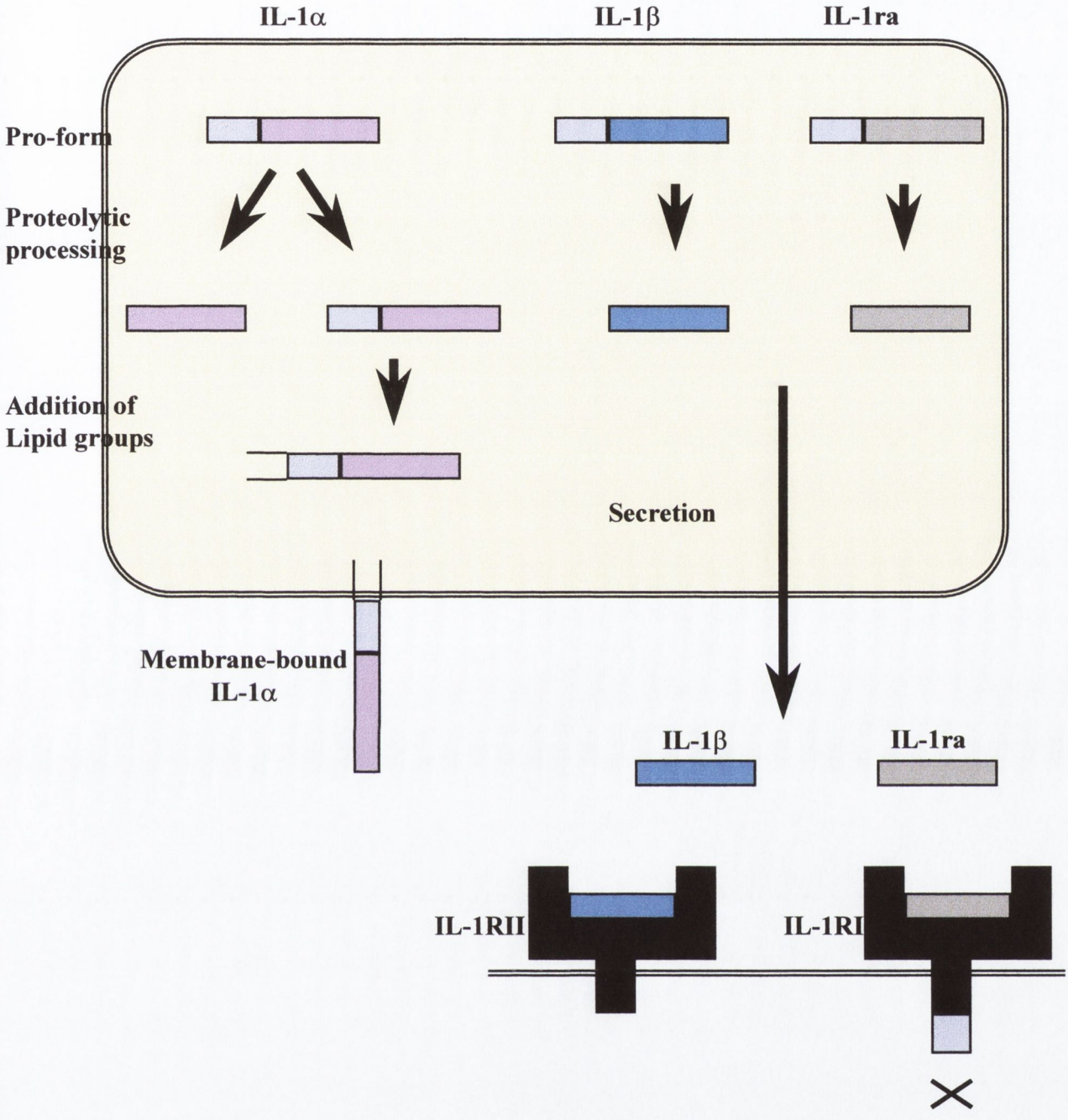


Figure 1.1: IL-1 isoforms and their expression.

IL-1 members are synthesised as 34kD pro-forms and are proteolytically processed by IL-1 converting enzyme (ICE) to 17kDa mature forms. IL-1α either remains in the cytosol in its pro- or mature form and is released following cellular damage. It can also be post-translationally modified by the addition of lipid groups by which it adheres to the cell membrane. IL-1α is active as both the pro- and mature form. IL-1β on the other hand is inactive until the prodomain is removed and it is secreted.

protease IL-1 concerting enzyme (ICE) which is also known as caspase-1. While IL-1β is only biologically active after removal of its prodomain, IL-1α is active in both its pro- and mature form. IL-1α is predominantly retained in the cytoplasm and is released following cell death. The prodomain is then removed by extracellular proteases following release. In addition IL-1α may be post-translationally modified by the addition of myristoyl groups to its pro-domain to generate a 21kDa membrane-anchored protein that is biologically active in cell-cell interactions (reviewed in (47)).

1.2 IL-1 receptor family

Both IL-1α and IL-1β mediate their effects on cells by binding to the type I IL-1 receptor (IL-1RI), an 80kDa glycoprotein with three immunoglobulin-like domains in its extracellular region with which it binds IL-1 (169, 174). The type II IL-1 receptor (IL-1RII) maps to the same chromosomal location in both mouse and humans (chromosomes 1 and 2, respectively) and is found as both a membrane-bound and soluble form but lacks the 81 amino acid cytoplasmic domain essential for signalling (122). Together with the naturally existing soluble form of IL-1RI, IL-1RII negatively regulates IL-1 responses by clearing IL-1 from the circulation prior to its binding IL-1RI on target cells (23, 36). Results have shown that IL-1α has a higher affinity for IL-1RI whereas IL-1β shows a greater affinity for IL-1RII (46). This difference in affinity is explained by the need for tighter control of the circulating form of IL-1 (IL-1β) compared with membrane-bound IL-1α, the rapid and efficient removal of IL-1β by IL-1RII (both soluble and membrane-bound forms) being essential in order to prevent detrimental effects on cells and tissues as a result of excessive IL-1 production. The anti-inflammatory effects of both IL-1ra and soluble forms of type I and II IL-1 receptors have been tested *in vivo* (110). Recombinant human IL-1ra has been used as a therapeutic tool for rheumatoid arthritis in clinical trials. Results

indicate that patients receiving subcutaneous injections of IL-1ra report significant reductions in the severity of the disease. In contrast, soluble IL-1RI therapy has not been as successful, no significant improvement having been observed following treatment of patients with soluble IL-1RI. This may be partially explained by the higher affinity of IL-1α for IL-1RI and it is hoped that soluble IL-1RII with its higher affinity for circulating IL-1β may be a better candidate as a therapeutic agent.

For IL-1 to signal, IL-1RI must form a complex with its accessory protein, IL-1RAcP, another member of the IL-1 receptor superfamily. IL-1RAcP was discovered by use of an antibody that blocked IL-1 binding to its receptor but which didn't recognise IL-1RI. The protein the antibody recognised was subsequently cloned and identified as IL-1RAcP, an essential component of the IL-1 receptor complex without which IL-1 is unable to signal (66, 194).

The IL-1 receptor family (the current members of which are shown in figure 1.2) shares a region of homology in its cytoplasmic region with a receptor called Toll (dToll) found in the fruit fly *Drosophila Melangaster.* The function of Toll in *Drosophila* is to mediate dorsal-ventral patterning during development and plays an important role in anti-fungal defences in the adult fly (57, 103). Homologues of Toll have been identified in most species and the term Toll-like receptors (TLR) has been given to the family. To date six members of the family have been identified in humans (hTLR1-6) and a role for them in mediating host responses to disease and infection (innate immunity) has been proposed. In particular the ligand for TLR4 has been identified as lipopolysaccaride (LPS) from gram negative bacteria, while TLR2 responds to products from gram positive bacteria such as lipoteichoic acid, peptidoglycan and bacterial lipoproteins (reviewed in (54)).

Together with the growing number of Toll-like receptors, the IL-1 receptor family has been termed the IL-1/Toll-like receptor superfamily. The homology between the two families does not extend to the extracellular region. As already mentioned the IL-1 receptor family

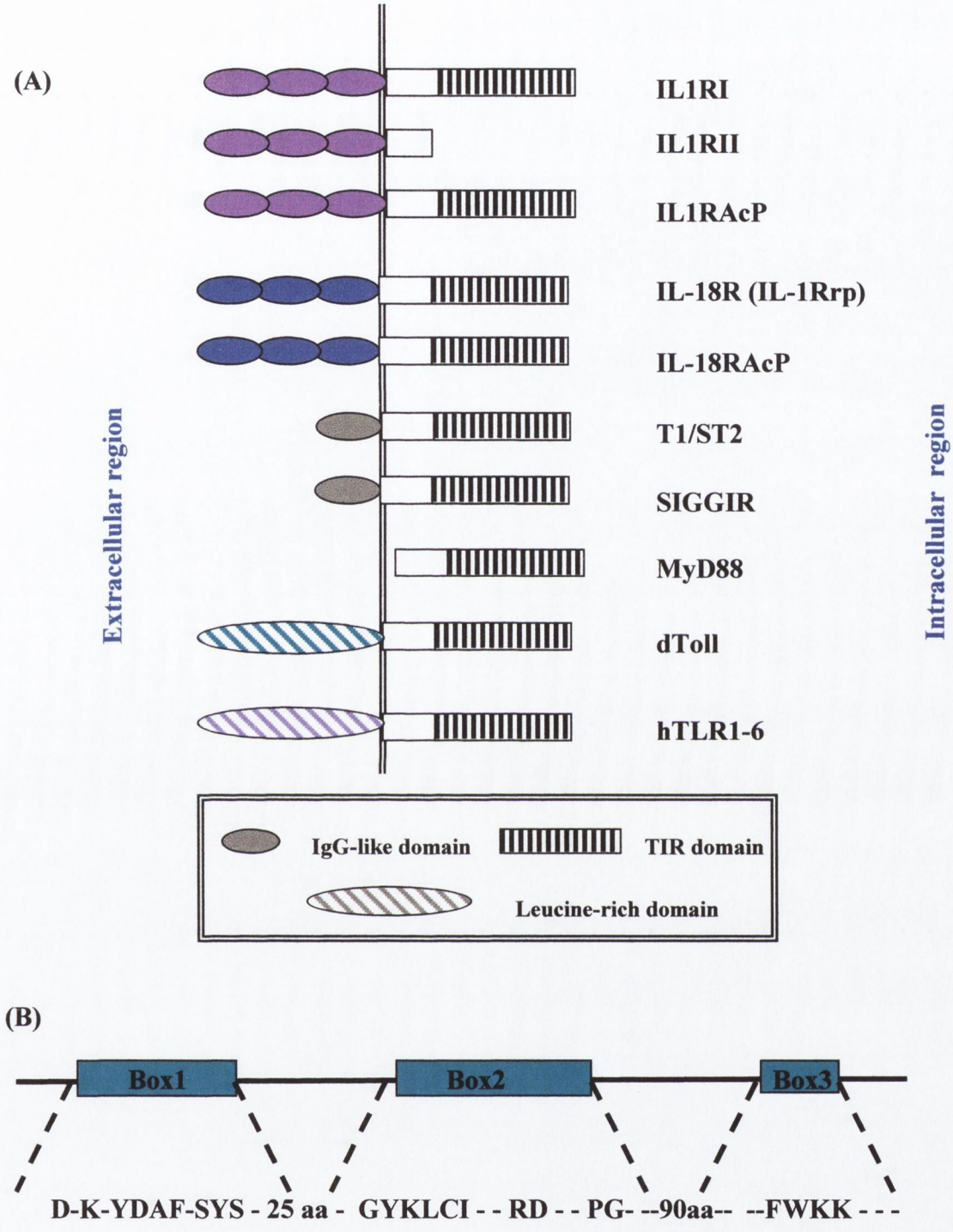


Figure 1.2: Toll/IL1 receptor super-family. (A) The IL-1 receptor family are typified by having multiple IgG-like domains in their extracellular domain whereas the Toll family (Drosophila and mammalian Tolls) have a leucine-rich repeat domain in its place. Both families share a region of homology in their intracellular domain termed the Toll/IL-1-like region (TIR). (B) Three regions of particular conservation within the TIR domain have been identified (Box 1-3) which play important roles in mediating signal transduction events.

characteristically possesses immunoglobulin-like domains extracellularly. In contrast, in TLRs these immunoglobulin-like domains have been replaced by leucine-rich repeats. The region of homology shared between the members of the IL-1/Toll-like receptor superfamily has been termed the Toll/IL-1R (TIR) domain and a consensus sequence has been identified which illustrates three regions of particular conservation within this region (figure 1.2b, boxes 1 to 3). These regions are critical for signal transduction from the receptor family as has been demonstrated by the discovery of naturally occuring mutations in TLR4 (proline to histidine substitution) which renders the receptor inactive. Although the members of this superfamily are quite diverse in both form and function, the degree of conservation within the TIR domain suggests that common signalling pathways emanate from this motif.

The most notable members of the IL-1 receptor family include IL-1RI, the IL-1R accessory protein (IL-1RAcP), and the IL-18 receptor (originally termed IL1Rrp) and its accessory protein (17, 145, 184). In addition, a key member of the IL-1/Toll-like receptor superfamily is the entirely cytosolic protein MyD88, an important mediator of IL-1 signal transduction in target cells. Possessing both an amino terminal death domain and a carboxy terminal TIR domain, MyD88 appears to act as an adaptor protein and is crucial for IL-1 receptor signalling (25). In addition two orphan receptors (receptors for which the ligand is not yet known), T1/ST2 (thought to be important in determining Th2-type responses) (129) and SIGGIR (181), have been identified.

1.3 Assembly of the IL-1 receptor complex

Dimerisation of IL-1RI and its accessory protein (IL-1RAcP) forms a high affinity ligand-binding receptor complex, which is responsible for transducing signals from the cell surface to the cytoplasm. IL-1RAcP on its own is incapable of binding IL-1 but its association with IL-1RI results in the formation of a high affinity binding site for IL-1. Studies examining the activity of

IL-1RAcP in cells found that its presence was essential for the formation of a fully functional IL-1 receptor complex that is capable of signalling (94, 195). Cell lines that do not express IL-1RAcP were found to be incapable of stress kinase activation, NFκB activation or to drive IL-2 production in response to IL-1, indicating the crucial importance of IL-1RAcP in IL-1 signal transduction (194).

Following binding of IL-1 to the receptor complex, the cytosolic adaptor protein MyD88 associates with the intracellular domain of the receptor complex via a homotypic interaction between the TIR domain of IL-1RAcP and the carboxy-terminal TIR domain of MyD88 (25, 193). The central importance of MyD88 in mediating signalling events downstream of certain members of the receptor superfamily is demonstrated by the failure of MyD88 negative cells to respond to IL-1, IL-18 or LPS (2, 86). The amino-terminal death domain of MyD88 serves as a means of recruiting downstream signalling molecules to the receptor complex via association with other death domain containing proteins (193).

Recent work has shown that a newly identified protein, Tollip (Toll/IL-1R interacting protein), mediates the association of IL-1 receptor associated kinase (IRAK) 1 with the receptor complex (24). IRAK-1 has an amino terminal death domain and a carboxy terminal kinase domain which is responsible for its autophosphorylating activity and resulting degradation (28, 200). Tollip was found to associate with IRAK-1 prior to treatment of cells with IL-1β and subsequent recruitment of Tollip-IRAK-1 complexes to the activated receptor complex following IL-1 stimulation occurred via the association of Tollip with IL-1RAcP (figure 1.3). It has been proposed that recruitment of Tollip-IRAK-1 complexes to the activated receptor complex allows the association of MyD88 and IRAK-1 via their death domains, which then stimulates the kinase activity of IRAK-1. Autophosphorylation of IRAK-1 results in the rapid dissociation of IRAK from Tollip and the receptor complex, thus allowing downstream signalling. Interestingly, it has been shown that the kinase activity of IRAK-1 is not required for IRAK-1-induced NFκB

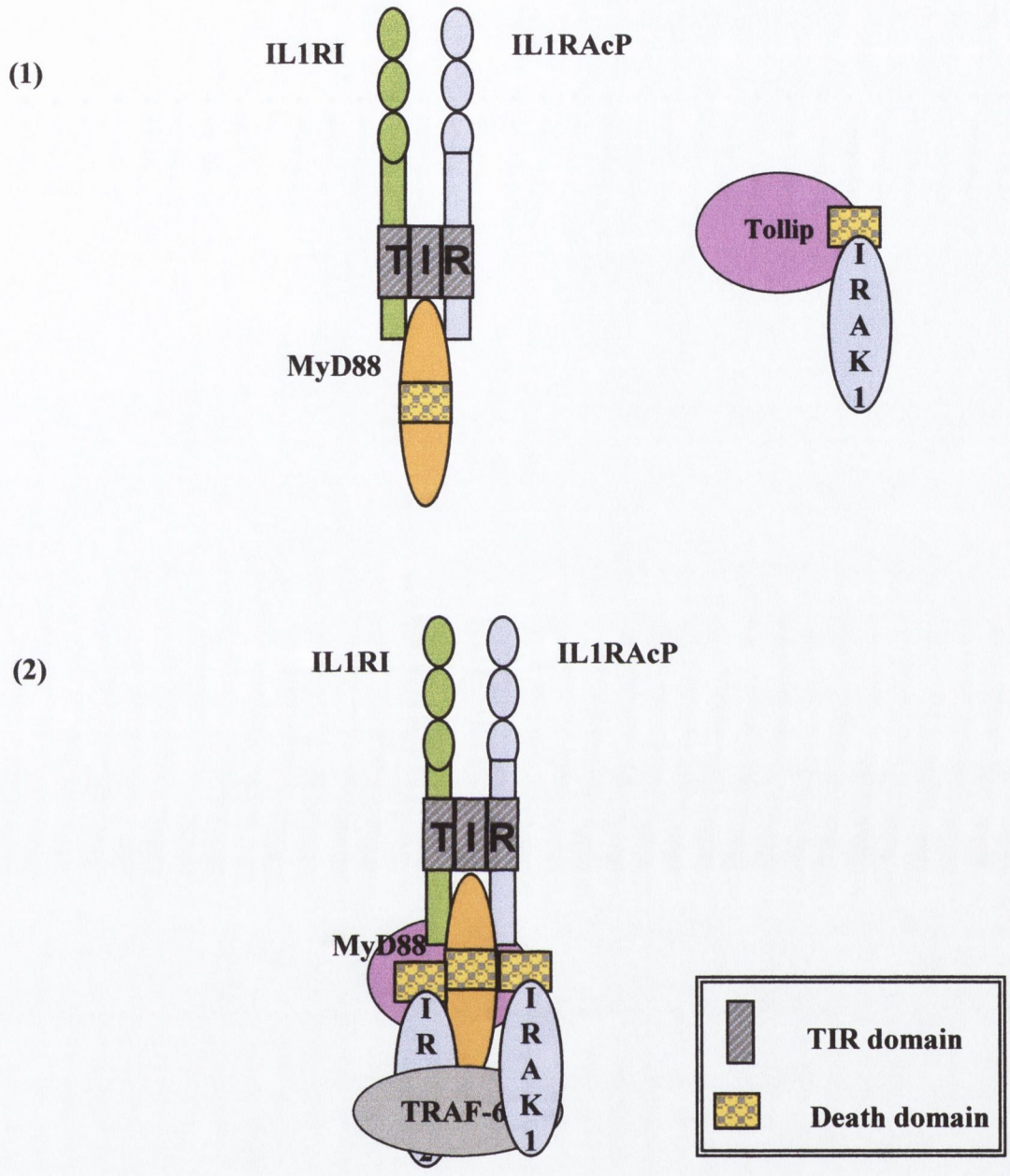


Figure 1.3: Recruitment of signalling mediators to the receptor complex.

(1) A high affinity IL-1 receptor complex is formed by the association of MyD88 with IL-1RI and IL-1RAcP via a homotypic interaction involving their TIR domains. In unstimulated cells Tollip associates with IRAK-1 in the cytoplasm. (2) Following stimulation with IL-1 Tollip-IRAK-1 complexes associate with IL-1RAcP, allowing the interaction between MyD88 and IRAK-1 to occur via their death domains. TRAF-6 is subsequently recruited to the activated multiprotein signalling complex.

activation and that kinase-deficient mutants are still able to drive NFκB activation when transfected into cells (119). An additional member of the IRAK family has been identified which also mediates NFκB activation in response to IL-1, IRAK-2 (132). IRAK-2 has been shown to associate with IL-1RAcP and MyD88 (via a death domain interaction) but not with IL-1RI.

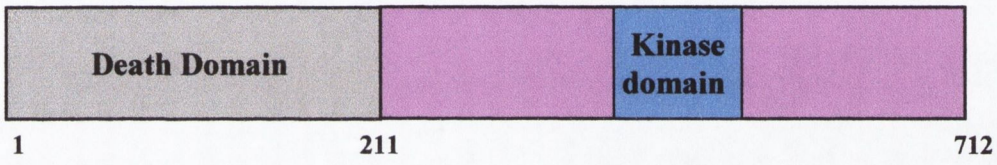
The association of IRAK-1 with the receptor complex promotes the subsequent recruitment of TRAF-6 (TNF receptor associated factor 6) which is an essential mediator of downstream signals in response to IL-1 (29). Antibodies to both IRAK-1 and -2 coprecipitate TRAF-6 and its recruitment to the activated receptor complex is said to be dependent on the association of MyD88 with IRAK-1 and -2. Figure 1.3 shows the sequential recruitment of MyD88, Tollip, IRAK-1 and -2 and TRAF-6 into a functional IL-1 receptor multi-protein complex which is responsible for mediating the activation of downstream signaling events which results ultimately in changes in gene expression associated with inflammation. A schematic representation of the key domains important for the activity of MyD88, IRAK-1 and TRAF-6, is shown in figure 1.4.

1.4 IL-1 receptor complex and the cytoskeleton

The IL-1 receptor complex has been shown to localise to focal adhesion complexes at the cell membrane in fibroblasts as shown in figure 1.5 (148). In addition, recent evidence has demonstrated a requirement for IRAK-1 recruitment to these complexes in order that IL-1 might activate the p42/p44 mitogen activated protein kinase pathway (108). Taken together this suggests an involvement of the actin cytoskeleton in regulating signalling pathways emanating from the IL-1 receptor complex. Focal adhesion complexes are integrin-dependent sites that link the extracellular matrix (ECM) to the actin cytoskeleton. Integrins form the transmembrane link to the focal complex, ligation of which results in recruitment of vincullin, talin, focal adhesion kinase (FAK), $p130^{Cas}$ and paxillin (reviewed in (74)). Both vincullin and talin are integral cytoskeletal

(A) MyD88

(B) IRAK-1



(C) TRAF-6

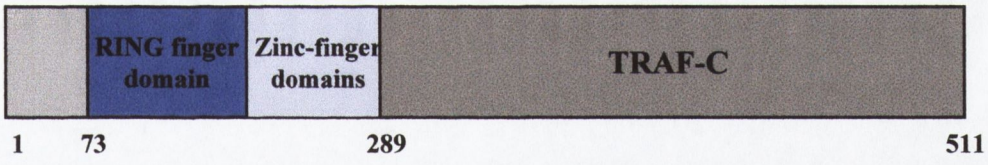


Figure 1.4: Key regulators of IL-1 receptor signalling (A) MyD88, (B) IRAK-1 and (C) TRAF-6.

A schematic representation of the key domains essential for signalling function of the above three proteins. The Toll/IL-1 receptor (TIR) domain of MyD88 is found in the carboxy terminal region of the protein.

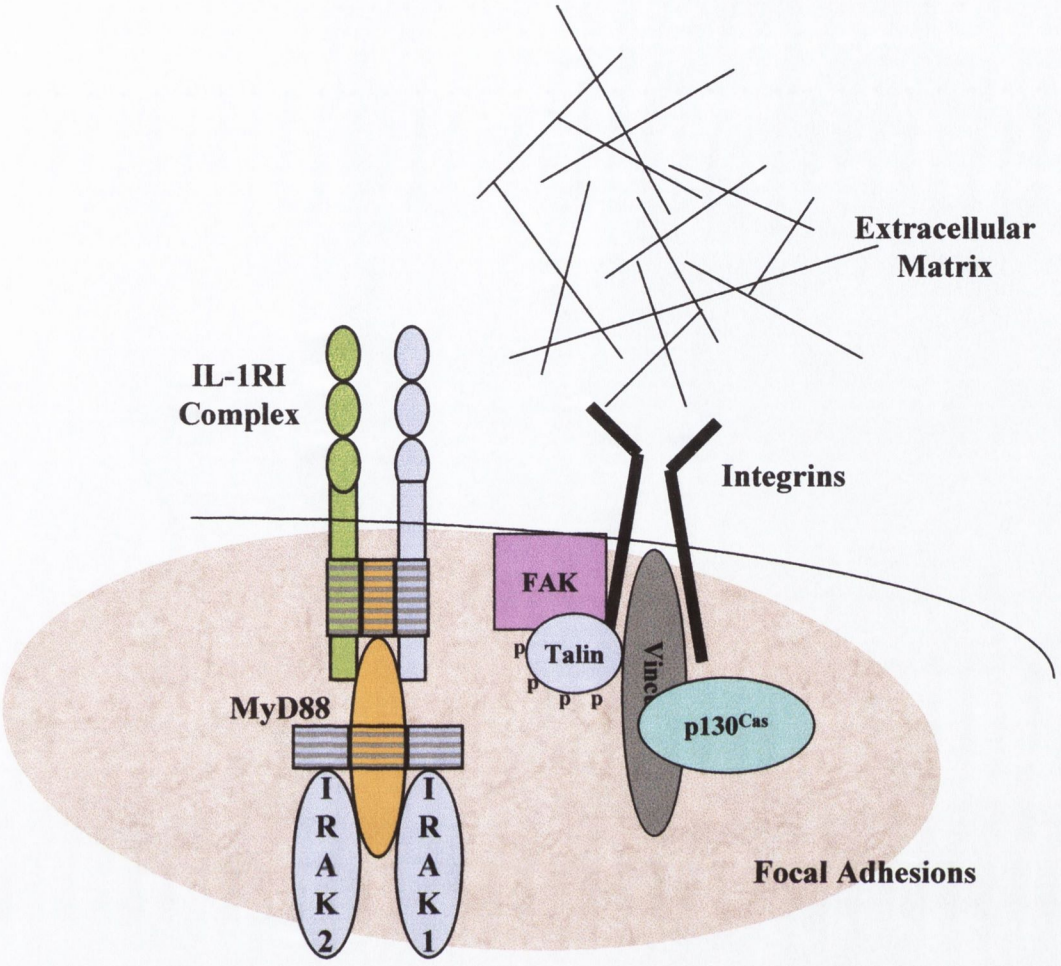


Figure 1.5: Localisation of the IL-1 receptor complex to focal adhesions

The high affinity IL-1 receptor complex is localised to regions at the cell membrane containing focal adhesion complexes. These complexes contain transmembrane receptors called integrins which link the extracellular matrix to the actin cytoskeleton and thereby provide access to signalling networks. The recruitment of actin filaments to the cytoplasmic domain of integrins is achieved via the formation of a multiprotein complex (which is represented here) containing talin (which is phosphorylated following IL-1 stimulation), vincullin, $p130^{cas}$ and the tyrosine kinase, focal adhesion kinase (FAK). Talin is responsible for FAK recruitment and interacting with the actin cytoskeleton. Vincullin interacts with both talin and F-actin and $p130^{cas}$ is a docking protein with multiple interaction domains which may mediate interactions between receptors, actin cytoskeleton and signalling networks.

proteins that are inducibly phosphorylated on tyrosine residues following activation of the tyrosine kinase FAK. While vincullin interacts with both talin and F-actin, talin is responsible for FAK recruitment and interaction with the actin cytoskeleton and is thus likely to play a key role in coupling membrane proteins to cytoskeletal structures and signalling pathways. In addition it has been demonstrated to become multiply phosphorylated following IL-1 stimulation, possibly leading to changes in membrane linkage of receptors and the cytoskeleton (147). $p130^{cas}$ is a docking protein with multiple interaction domains which may mediate interactions between receptors, the actin cytoskeleton and signalling networks. Localisation of the IL-1 receptor complex to these focal adhesion complexes in the cell membrane may therefore potentially provide an essential link between IL-1 signal transduction pathways and the actin cytoskeleton as a result of the close proximity of the activated receptor complex with signalling molecules such as paxillin and FAK.

1.5 IL-1 signal transduction in brief

A complex series of events are initiated following IL-1 binding to its receptor complex, resulting in the activation of signalling pathways that ultimately regulate the expression of genes involved in inflammation. How these pathways are regulated in response to IL-1 has been the focus of much research, although as yet the full details of how these pathways operate are unknown.

Activation of the transcription factor NFκB in response to IL-1 has been extensively documented and the pathway regulating its release from the cytoplasm well characterised (reviewed in (5)). A separate pathway regulating the transactivating potential of NFκB via phosphorylation of its p65 subunit has been recently described, adding another level of complexity to the regulation of NFκB-dependent gene expression by pro-inflammatory stimuli such as IL-1 and TNF (reviewed in (146)). In addition to NFκB activation, IL-1 activates each of the three mitogen activated protein kinases (MAPK) (p42/p44, p38, and JNK MAPK), key

pathways involved in regulating gene expression in response to diverse stimuli such as mitogens, growth factors, LPS, ultraviolet light and cytokines such as IL-1 and TNF. As important upstream regulators of MAPK pathways, it is not surprising that there is convincing evidence for the involvement of low molecular weight G proteins in IL-1 signal transduction, although the exact nature of their involvement has yet to be elucidated. IL-1 has also been reported to activate a myriad of different signalling pathways such as those regulating phospholipase A_2, phospholipase C, and protein kinase C activation (reviewed in (140)).

Ultimately, a consequence of activating any of these pathways is to change the pattern of gene expression. During inflammation IL-1 activates a wide array of genes responsible for establishing the inflamed phenotype. These include cytokines such as IL-2, IL-6, IL-8, G-CSF, and M-CSF; chemokines such as macrophage inflammatory protein-1α; acute phase proteins such as serum amyloid A, cyclooxygenase-2, inducible-nitric oxide synthase; and adhesion molecules such as ICAM-1, E-Selectin and ELAM-1, to name but a few (for a more comprehensive list see table 1) (reviewed in (47)).

1.6 NFκB

A common feature uniting many of the genes named above is their ability to be regulated by the pro-inflammatory transcription factor NFκB. The predominant form of NFκB is a heterodimer composed of the p50 and p65 subunits. In resting cells NFκB is retained in the cytoplasm by its association with an inhibitory protein IκB which masks the nuclear localisation sequence (NLS) on NFκB thus preventing translocation of the heterodimer into the nucleus (reviewed in (146)).

Cloning of the NFκB family members has revealed five distinct subunits of NFκB which share a 300 amino acid region of high homology encompassing an amino terminal DNA binding domain and the Rel homology domain (RHD). The RHD provides the dimerisation interface for NFκB subunits and is so called due to its high degree of homology with the viral oncogene v-Rel

Class	Protein
Cytokines	IL-1, IL-1ra, TNF, IL-2, IL-3, IL-6, IL-12, GM-CSF, TGF-β, G-CSF, M-CSF, stem cell factor, leukaemia inhibitory factor, IFNα, β and γ, IL-8 and other chemokines
Cytokine Receptors	Receptors for IL-2, IL-3, IL-5, GM-CSF, c-kit
Pro-inflammatory mediators	Cyclo-oxygenase type-2, inducible nitric oxide synthase, phospholipase A_2, endothelin-1
Growth Factors	Keratinocyte growth factor, nerve growth factor, fibroblast growth factor, platelet derived growth factor
Adhesion Molecules	ICAM-1, VCAM-1, E-selectin
Acute Phase Proteins	Serum amyloid A, complement C_2, xanthine oxidase, xanthine dehydrogenase

Table 1: Brief summary of some of the many genes whose expression is regulated by IL-1 (adapted from Dinarello (1981)).

(168). The NFκB family members shown in figure 1.6 can be divided into two subgroups: the first comprising p50 (62, 89) and p52 (20, 134), which are synthesised as the precursor protein p105 and p100 respectively; and the second containing p65 or RelA (referred to as p65 herein) (137, 155), RelB (156) and cRel (the proto-oncogene) (197). Both p105 and p100 require proteolytic processing to generate their DNA binding forms (p50 and p52) (143). The second group, RelA, RelB and cRel, do not require processing and possess transactivation domains in their carboxy terminal domains that are responsible for the transcription activating potential of NFκB and are essential for mediating interactions with the basal transcription apparatus (162). While the most common form of NFκB is the p50/p65 heterodimer, the subunits freely homo- and hetero-dimerise with the exception of RelB which will only efficiently form dimers with p50 and p52.

Similarly for IκB, cloning has demonstrated a number of different IκB genes all of which possess an ankyrin repeat domain in their carboxy terminus (as do the NFκB family members, p105 (also called IκBγ) and p100) (168). IκBα, β and ε have been demonstrated to preferentially target p65 and cRel containing complexes, masking their nuclear localisation sequence and thus preventing nuclear translocation. IκBα becomes inducibly phosphorylated on serines 32 and 36 which targets it for ubiquitination at lysine residues 21 and 22 and subsequent proteolytic degradation (143, 161). Both IκBβ and ε have similarly located serine residues (serines 19 and 23 for IκBβ) and are thought to be regulated in a similar fashion. The selective targeting of particular IκB proteins by various stimuli increases the means by which NFκB activation is regulated. For example IκBα is targeted by IL-1, TNF, LPS and the phorbol ester PMA, whereas IκBβ is only responsive to IL-1 and LPS. In addition the ability of the p50 and p52 precursor proteins p105 and p100 to inhibit nuclear translocation of NFκB by means of their ankyrin repeat domains prior to their proteolytic processing demonstrates the complex and varied means by which this pathway is regulated.

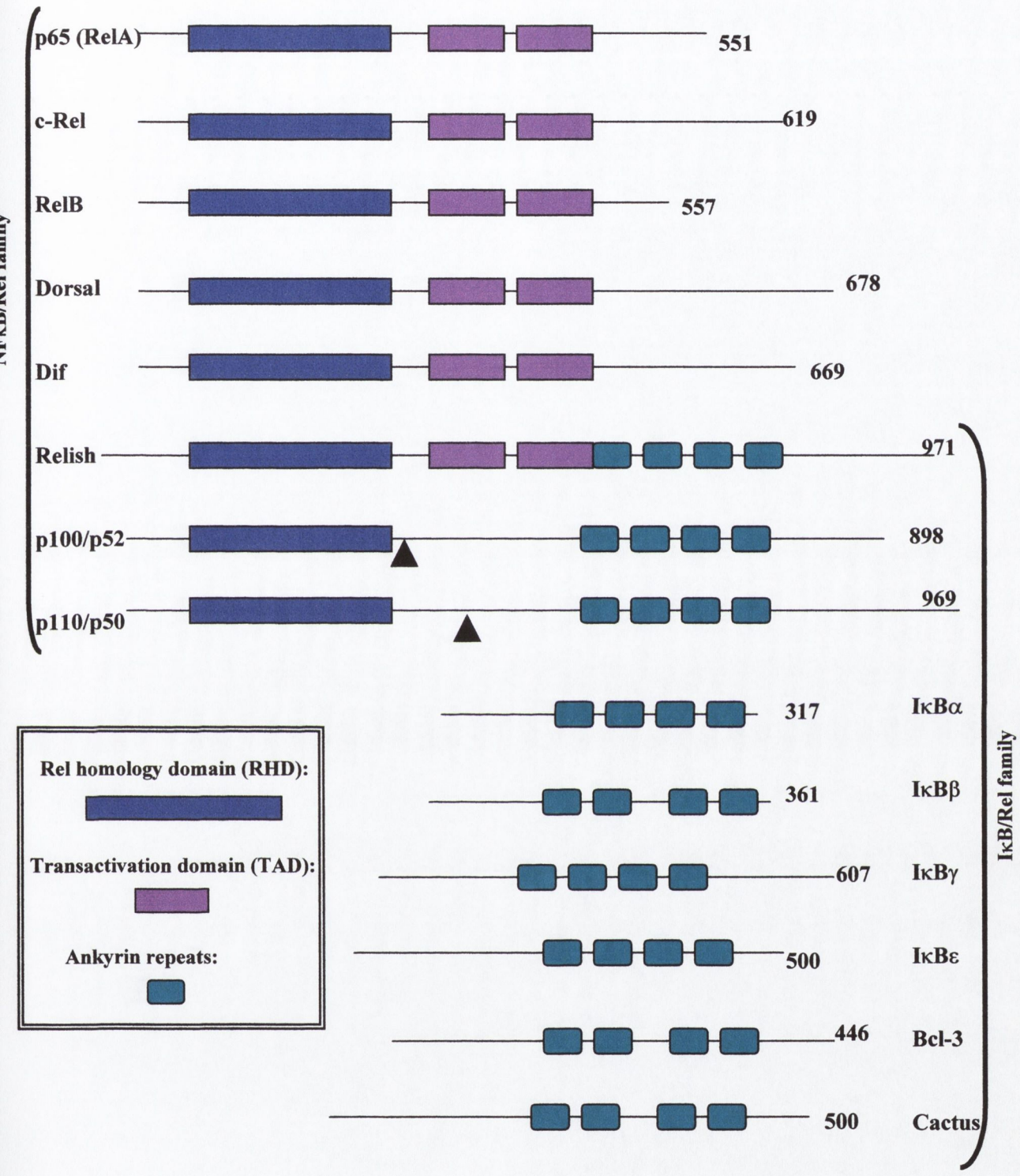


Figure 1.6: Schematic representation of members of the mammalian and Drosophila Rel/NFκB and IκB families of proteins. The numbering of the mammalian sequences corresponds to the human proteins and the approximate location of the processing sites for p100 and p105 are indicated with an arrow (▲). The key for the regions of homology is given in the box.

1.7 IL-1 signalling to NFκB

The kinases responsible for regulating IκB phosphorylation in response to IL-1 or TNF stimulation have recently been identified and described (reviewed in (85)). A high molecular weight multi-protein complex termed the signalsome with IκB kinase activity was isolated from TNF-treated HeLa cells (reviewed in (121)). Purification of this 900kDa multiprotein complex identified two proteins of 85 and 87kDa that demonstrated IκB kinase activity (43, 123). Microsequencing demonstrated that they were two closely related proteins that were subsequently called IKK1 (IKKα) and IKK2 (IKKβ) respectively. Each has a kinase domain at their amino termini, a leucine zipper region by which they homo- or hetero-dimerise and a helix-loop-helix motif at their carboxy terminus (shown in figure 1.7). The degree of homology between the IKKs suggests that the two kinases may have functionally redundant roles in the cell. Only by generation of IKK1 and 2 deficient mice have any differences between the two become evident. Briefly, $IKK1^{-/-}$ mice have been found to die within 4 hours after birth and show severe developmental defects consistent with a role for IKK1 in regulating epidermal differentiation. In addition studies on these mice showed that IKK1 was not essential for IKK complex activation by pro-inflammatory stimuli (79, 176). In contrast, IKK2 deficient mice are embryonic lethal as a result of massive liver apoptosis, exhibiting a phenotype identical to that of p65 knockout mice (9, 104, 105, 177). They also demonstrate very little NFκB activation in response to IL-1 or TNF, indicating that IKK2 is predominantly responsible for regulating IKK complex activation in response to IL-1.

Further analysis of the IKK complex reveals the presence of a scaffold protein, NEMO (NFκB essential modifier), also known as IKKγ, which interacts with IKK1 and 2 and is essential for allowing their activation (154, 199). It is thought that NEMO plays an important role in stabilising the interaction between the kinase domain of the IKKs and their helix-loop-helix

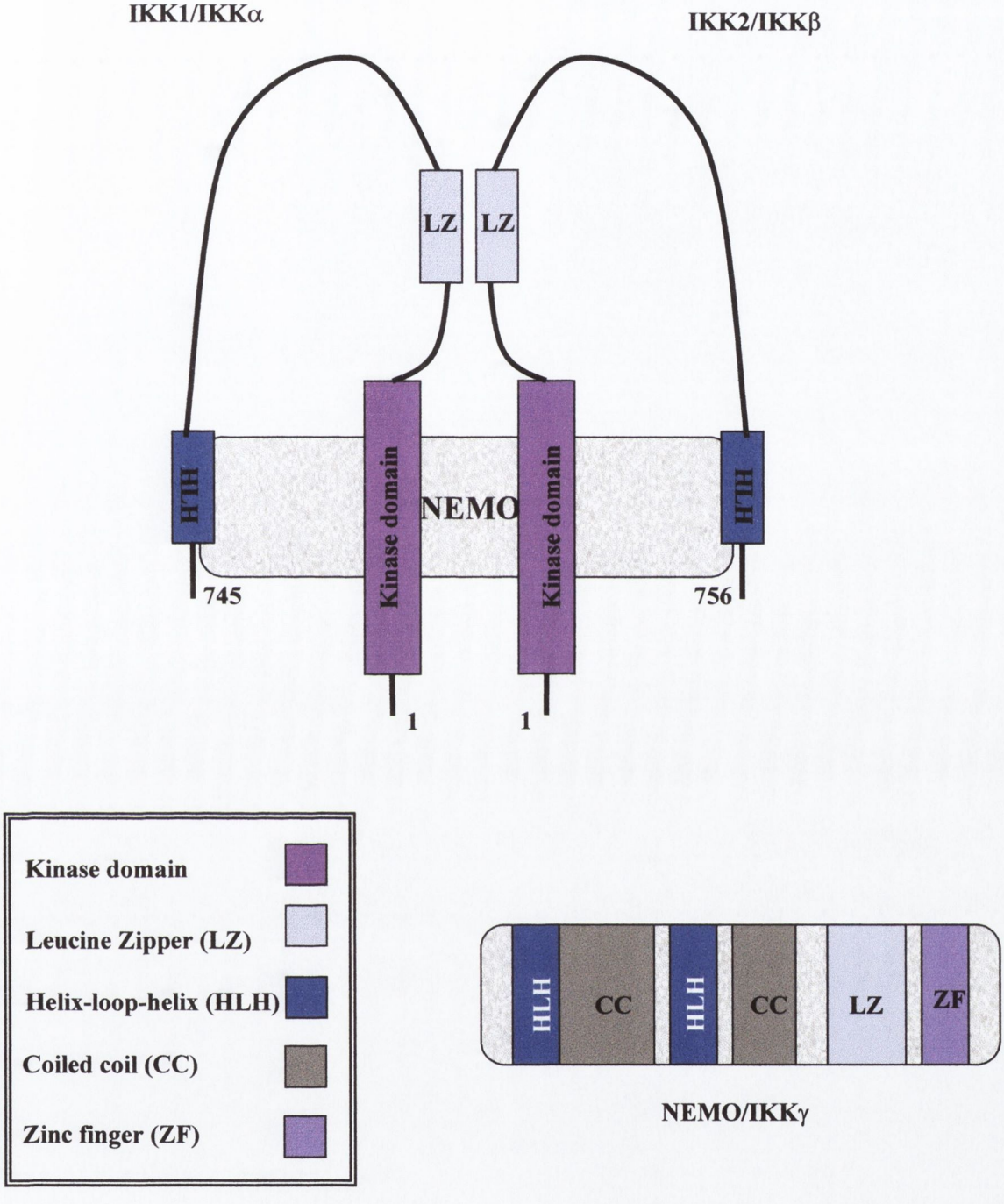


Figure 1.7: Schematic representation of IKK1, IKK2 and NEMO showing the domains important for their activation. IKK1 and 2 heterodimerise via their leucine zipper (LZ) domains and the interaction between their respective kinase domain and helix-loop-helix (HLH) domain is aided by the association of NEMO with the heterodimer. The association of NEMO is essential for the kinase activity of IKK1 and 2 *(adapted from May et al, 1999)*.

domain, which is essential for kinase activity (figure 1.7). Additional functional roles for NEMO have not yet been described.

The pathway leading from the activated IL-1 receptor complex leading to IKK activation requires the recruitment of IRAK-1 and TRAF-6 to the complex as previously described. Events downstream of this are less well defined although it is thought that activation of the IKK complex occurs as a result of phosphorylation of the IKKs by a kinase belonging to the MAP kinase kinase kinase (MAP3K) family. Candidates for this kinase include NIK (NFκB inducing kinase) and MEKK1, both of which belong to the MAP3K family and have been demonstrated to activate the IKKs and stimulate their IκB kinase activity (summarised in figure 1.8). Initial studies showed that NIK, via an interaction with TAK1 and TAB, associated with TRAF-6 and thus mediated activation of the IKK complex (although the physiological relevance of this interaction has been recently disputed) (111, 135). Very recently a link between TRAF-6 and MEKK1 has been described following the identification of a novel adaptor protein ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) which is specific for Toll/IL-1 pathways and is a regulator of MEKK1 processing (93). ECSIT interacts with TRAF-6 via its conserved carboxy terminal TRAF-C domain and also with MEKK1, thus bridging the gap between TRAF-6 and MEKK1.

1.8 DNA-binding of NFκB

As a result of phosphorylation and subsequent ubiquitin-mediated degradation of IκB, NFκB is released, allowing translocation to the nucleus where it binds to its consensus sequence upstream of target genes. Although the consensus sequence 5'GGGRNNYYCC 3' (where R is a purine, Y a pyrimidine and N any base) has been shown to preferentially bind p50/p65 heterodimers, there is certain degree of selectivity among the different NFκB subunits for various κB elements (reviewed in (146)). This ensures that promoters or enhancers containing variant κB elements

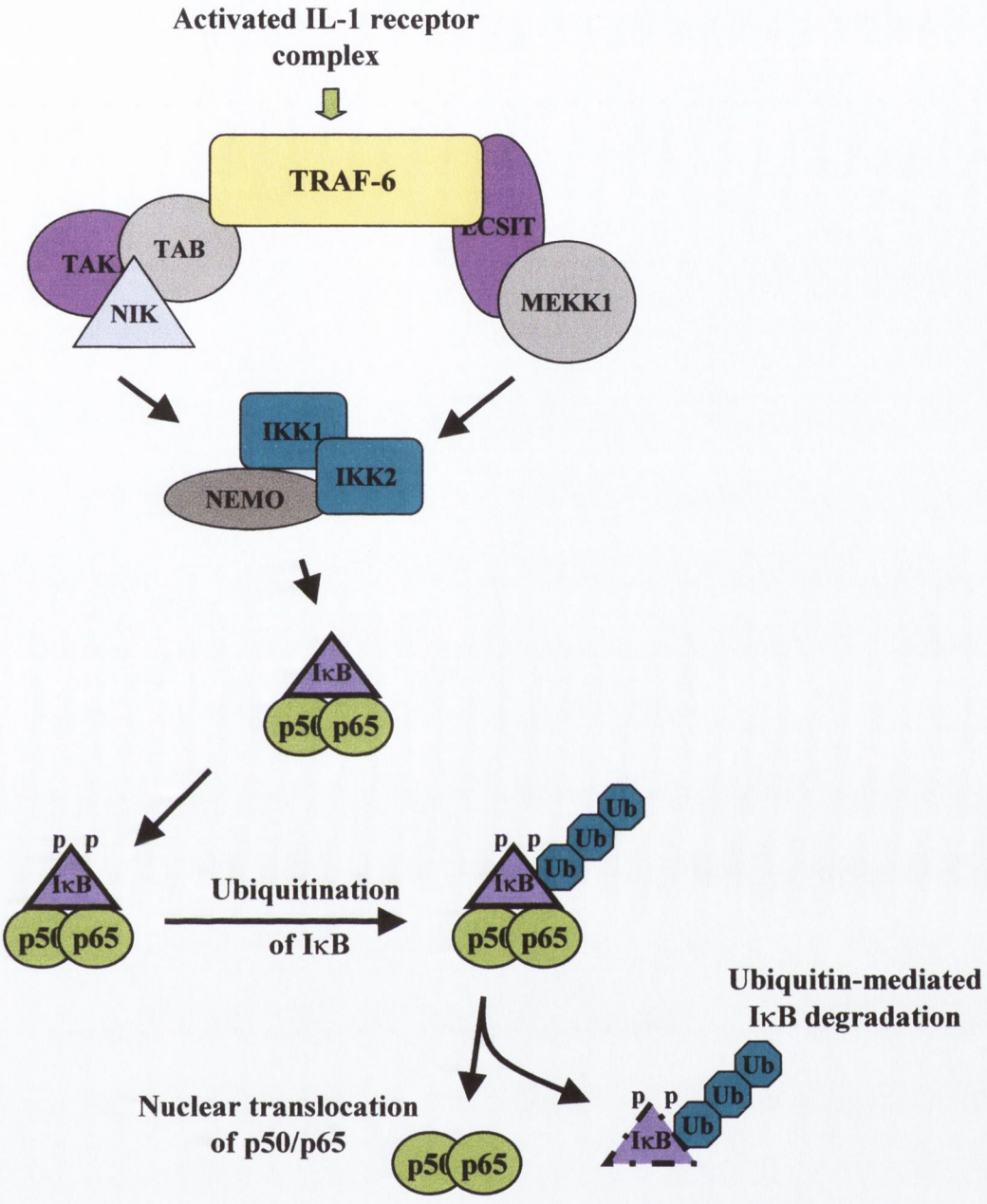


Figure 1.8: Schematic representation of events upstream of IKK activation and subsequent IκB phosphorylation and degradation. IKK1 and 2 are activated by phosphorylation by upstream kinases, two of which have been identified and are shown here, NIK and MEKK1. The interaction between NIK and the activated IL-1 receptor complex occurs via its association with TRAF-6, assisted by TAK1 and TAB, although doubt as to the involvement of NIK in IKK activation has been raised. MEKK1 associates with TRAF-6 via interaction with the adaptor protein ECSIT.

have the potential to be regulated by specific NFκB complexes. In addition to regulating the type of complexes that bind the κB sequences upstream of target genes, NFκB activity is also controlled once it is bound to its cognate DNA sequence. This level of regulation can be as a result of controlling the interaction of NFκB subunits with other transcription factors or by regulating the transactivating ability of the p65 subunit of NFκB by phosphorylation or interaction with the basal transcription machinery.

1.9 Regulation of Transactivation

p50/p65 heterodimers have been shown to interact with Interferon Regulatory Factor 1 (IRF-1) on the interferon β enhancer (180), and with AP-1 and C/EBP on the IL-8 promoter (172, 173). In addition to such interaction with other transcription factors on a promoter controlling transcription, phosphorylation of the p65 subunit of NFκB has also been demonstrated to regulate transactivation of gene expression. The earliest report that NFκB is inducibly phosphorylated described an unidentified serine kinase, approximately 43kDa, which associated with NFκB-IκB complexes in the cytoplasm and was responsible for its phosphorylation (73). Since then numerous findings have demonstrated that both p50 and p65 subunits of NFκB become phosphorylated in response to stimulation with IL-1 or TNF (reviewed in (146)). Early studies demonstrated that the p65 subunit of NFκB was shown to be responsible for the transcriptional activity of NFκB, the role of p50 being suggested to be to stabilise the interaction of p65 with the consensus sequence (162). Therefore attention has focused on the inducible phosphorylation of p65 in response to pro-inflammatory stimuli such as IL-1 and TNF.

p65 becomes phosphorylated on particular serine residues in both its amino terminal Rel homology domain (RHD) and either of its two carboxy terminal transactivation domains (TAD1 and 2) following stimulation of cells with TNF: Serine 276 (RHD) (204), Serine 529 (TAD1)

(190, 191) and Serine 536 (TAD1) (158) (figure 1.9). While the sites phosphorylated following IL-1 stimulation have not yet been mapped it is likely that a phosphorylation pattern similar to that seen with TNF would be observed. The kinases responsible for these particular phosphorylation events have been identified, with protein kinase A (PKA) responsible for serine 276 phosphorylation (204), casein kinase II (CKII) for serine 529 (12, 191), and the IKK complex for serine 536 (158). Phosphorylation of p65 enhances its ability to interact with coactivators such as CREB binding protein (CBP), p300 and pCAF (p300/CBP binding protein (CBP)-associated factor). Researchers have recently demonstrated that the interaction of p65 with a multiprotein complex of coactivators rather than CBP alone is responsible for transactivation of gene expression (167). In the case of PKA, phosphorylation of p65 on serine 276 releases an interaction between the N and C termini of p65 thus exposing its CBP binding site. Casein kinase II (CKII) appears to constitutively associate with IκB-NFκB complexes in the cytoplasm but is prevented from phosphorylating p65 by its association with IκB (191). Following IL-1 stimulation and the subsequent release of NFκB from IκB, CKII phosphorylates serine 529 in the transactivation domain of p65, thereby presumably promoting the interaction between p65 and the coactivator complex (figure 1.10). In addition to p65 transactivational ability being regulated by phosphorylation a recent report indicates that cRel is also phosphorylated on Ser-471 in transactivation domain 2 although the kinase responsible has yet to be identified (117).

1.10 IL-1 and MAPK activation

In addition to NFκB activation, IL-1 stimulation of cells has also been shown to activate members of the mitogen activated protein kinase (MAPK) pathways, namely the classical p42/p44 MAPK (also termed extracellular regulated kinase (ERK) 1 and ERK 2) and the stress activated protein kinases p38 MAPK and c-Jun N terminal kinase (JNK).

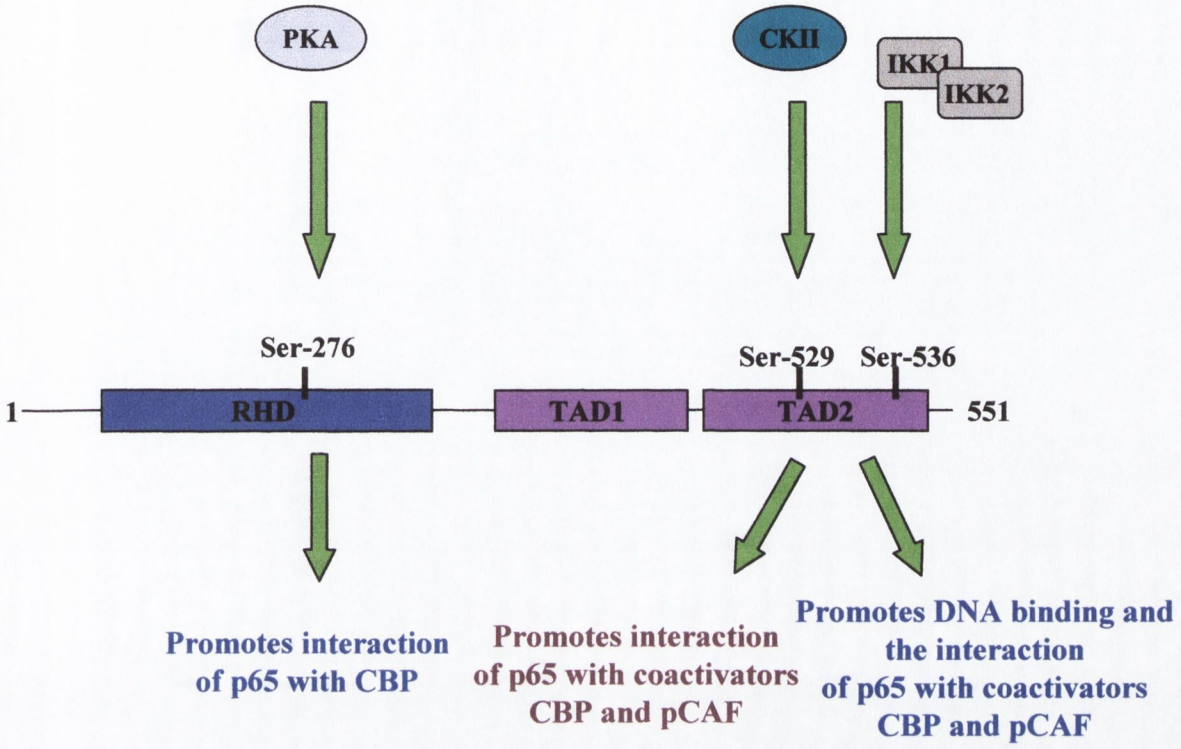


Figure 1.9: Representation of the serine residues known to be phosphorylated in response to TNF stimulation on the p65 subunit of NFκB. The kinases responsible for phosphorylating the various serines are shown: protein kinase A, PKA; casein kinase II, CKII; IκB kinase 1 and 2, IKK1 and 2. The effects of phosphorylation of these residues are also summarized.

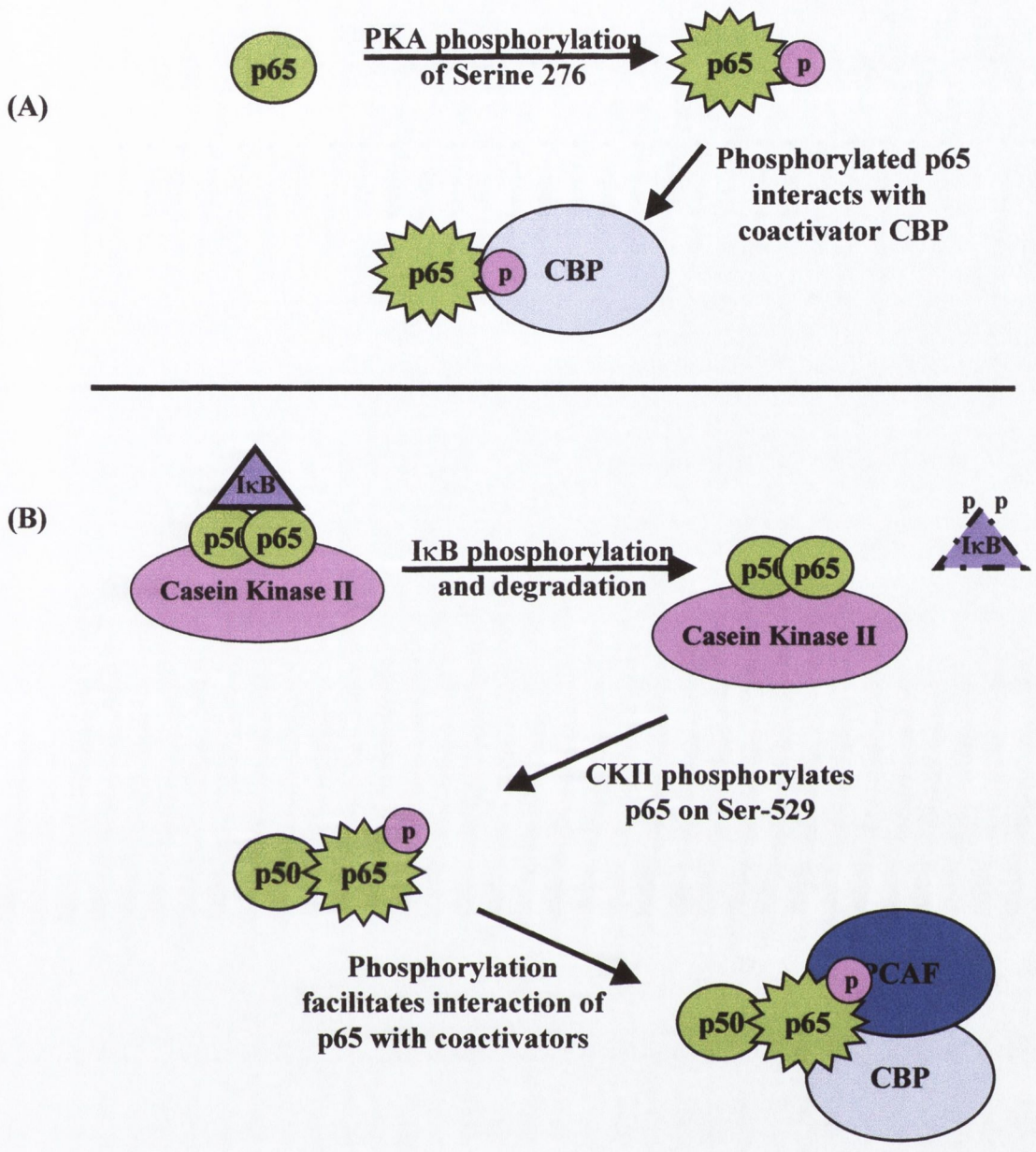


Figure 1.10: Proposed model for the role of (A) protein kinase A (PKA) and (B) casein kinase II (CKII) in p65 phosphorylation and subsequent transactivation of gene expression by p65. The coactivators CREB-binding protein (CBP) and pCAF are shown. Interaction of the coactivators with p65 facilitates interaction with the basal transcription machinery and hence promotes transactivation of gene expression.

While p42/p44 MAPK is activated by diverse stimuli such as mitogens, growth factors and IL-1, activation of p38 MAPK and JNK occurs in response to stress stimuli such as pro-inflammatory cytokines (IL-1 and TNF), UV-irradiation and osmotic shock. They are all activated by dual phosphorylation on nearby threonine and tyrosine sites by a dual specificity kinase known as a MAPK kinase (or MAP2K). The kinases at the MAP2K level are similarly activated by a MAPK kinase kinase (or MAP3K). Upstream of this lie the low molecular weight GTPases Ras, Rac1, Cdc42 and Rho, all of which have been reported to play a role in activation in one or more of the pathways (reviewed in (189)). The dual specificity kinases responsible for activating these pathways have very tightly defined specificities and their activation is highly regulated. The sequence around the phosphorylation site of p42/p44 MAPK, p38 MAPK and JNK specifies the kinase that will recognise it (27). For example, MEK1/2 will only recognise and phosphorylate a specific sequence of amino acids TEY on p42/p44 MAPK, MKK4/7 phosphorylates TPY on JNK and MKK3/6 phosphorylates TGY on p38 MAPK isoforms. While MAP2Kinases are specific for their respective downstream targets, a certain amount of cross-talk occurs between the kinases at the MAP3K level (figure 1.11).

The finding that IL-1 activated p42/p44 MAPK was initially somewhat surprising as this pathway is normally associated with growth factor action rather than with pro-inflammatory stimuli (13, 68). Further investigation demonstrated that IL-1 activated MEK1 (MAP kinase kinase 1), the kinase directly upstream of p42/p44 MAPK, which is responsible for its activation (157). A potent inhibitor of p42/p44 MAPK pathway, the MEK1 inhibitor PD98059, confirmed the involvement of p42/p44 MAPK in IL-1 signal transduction by inhibiting IL-1-induced IL-2 mRNA production and reporter gene expression in a murine thymoma cell line (120). Recently work in our laboratory has demonstrated that IL-1 activates the low molecular weight G protein Ras, which is an important regulator of this pathway (144).

IL-1 has also been demonstrated to activate the stress activated protein kinase, p38 MAPK. The five isoforms of p38 MAPK (α, β, $\beta 2$, γ, and δ) currently identified are activated

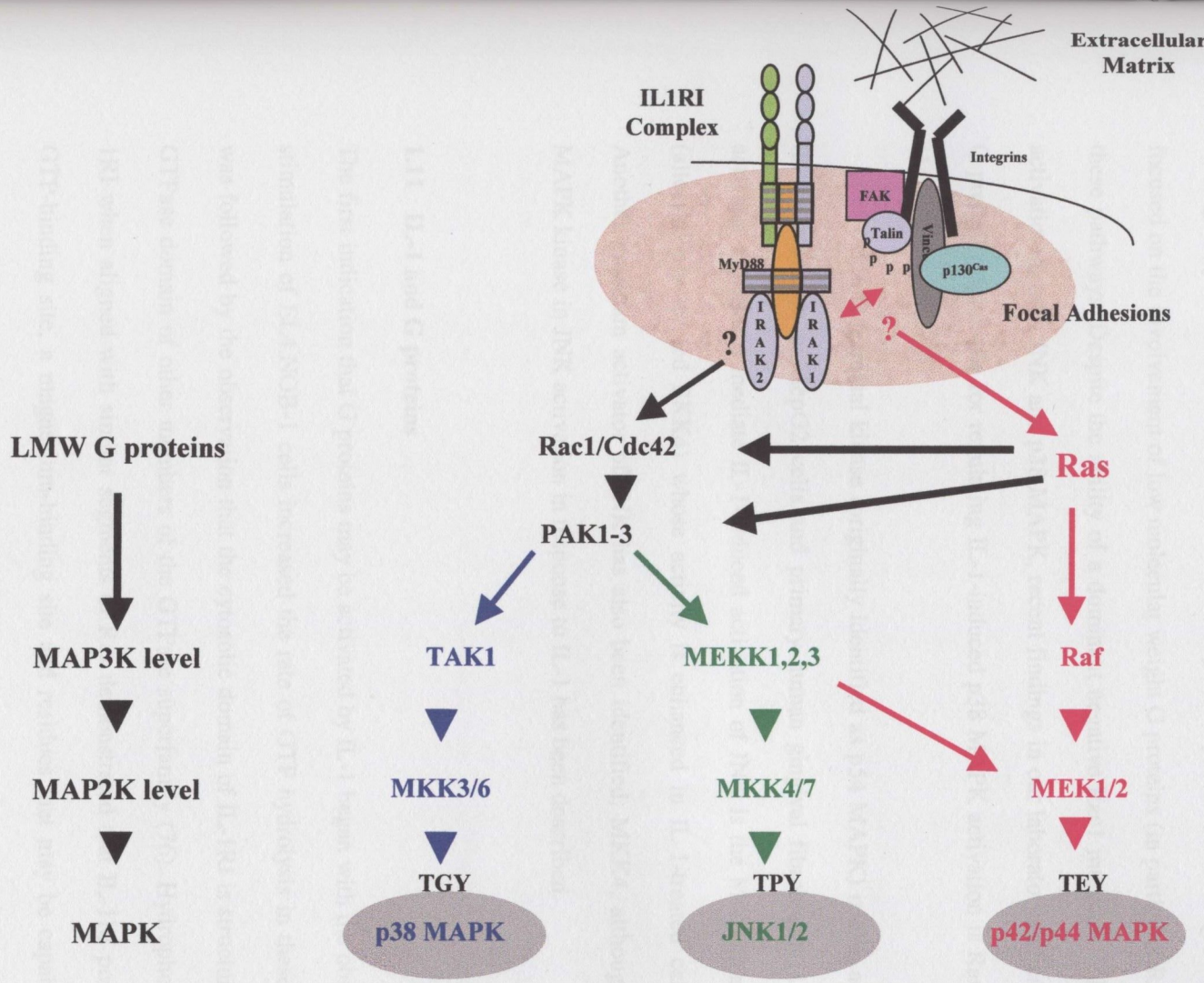


Figure 1.11: IL-1-induced activation of MAPK pathways. Schematic representation of the MAPK pathways and possible involvement of the IL-1 receptor complex in regulating their activation. Recent evidence has demonstrated that IRAK-1 localisation to focal adhesions is essential for p42/p44 MAPK activation. The exact molecular events responsible for regulating p38 MAPK and JNK activation in response to IL-1 stimulation have not yet been identified, although a role for Ras (possibly via Rac1) in IL-1-induced activation of p38 MAPk has been demonstrated. The dual phosphorylation sites on the respective MAPKs are shown.

selectively by the upstream kinases MKK 3 and 6. Both MKK3 and 6 have been demonstrated to be activated following IL-1 stimulation (38, 65, 82, 98, 192) (51, 83). A potent inhibitor of p38 MAPK, SB203580, has been shown to block multiple IL-1-induced responses including IL-2 and IL-8 induction, protease expression and prostaglandin production thus confirming the involvement of p38 MAPK in IL-1-activated signalling pathways (120, 152). The upstream events leading to p38 MAPK activation are not, as yet, fully defined although much attention has focused on the involvement of low molecular weight G proteins (in particular Rac1 and Cdc42) in these pathways. Despite the ability of a dominant negative Rac1 mutant to inhibit IL-1-induced activation of both JNK and p38 MAPK, recent findings in our laboratory indicate that the critical G protein responsible for regulating IL-1-induced p38 MAPK activation is Ras rather than Rac1 (144).

c-Jun N terminal kinase (originally identified as p54 MAPK) is also activated following IL-1 stimulation of HepG2 cells and primary human gingival fibroblasts (11). The upstream activator thought to mediate IL-1-induced activation of JNK is the MAP kinase kinase, MKK7 (alternatively termed SKK4) whose activity is enhanced in IL-1-treated cells (53, 95, 102). Another upstream activator of JNK has also been identified, MKK4, although no role for this MAPK kinase in JNK activation in response to IL-1 has been described.

1.11 IL-1 and G proteins

The first indication that G proteins may be activated by IL-1 began with the observation that IL-1 stimulation of EL4.NOB-1 cells increased the rate of GTP hydrolysis in these cells (138). This was followed by the observation that the cytosolic domain of IL-1RI is structurally related to the GTPase domain of other members of the GTPase superfamily (76). Hydrophobic regions of IL-1RI when aligned with similar segments of Ras demonstrated that IL-1RI possessed a possible GTP-binding site, a magnesium-binding site and residues that may be capable of hydrolysing

GTP. Although the functionality of this putative GTPase domain has not been demonstrated other evidence has been advanced that supports the role for G proteins in IL-1 signalling. In particular work in our laboratory using bacterial toxins which show inhibitory activity for defined groups of low molecular weight G proteins, has demonstrated their ability to inhibit IL-1-induced p38 MAPK activation thus confirming the importance of low molecular weight G proteins in IL-1-activated signalling pathways (144).

1.12 Low Molecular Weight G proteins

The Ras superfamily of low molecular weight G proteins (LMWG) are intracellular effectors of approximately 21kDa with a wide range of functions in cells such as regulating growth control, intracellular transport, cytoskeletal rearrangements and signalling to gene expression (reviewed in (42)). They cycle between an inactive GDP bound form and an active GTP bound form as represented in figure 1.12. The exchange of GDP for GTP and hence activation of the protein requires the activity of guanine nucleotide exchange factors (GEFs), many of which have been described, each with defined specificites for different classes of G protein. As low molecular weight G proteins have a low intrinsic GTPase activity, hydrolysis of GTP on the active protein is catalysed by associations with specific GTPase activating proteins (GAPs) depending on the class of G protein involved. It is therefore the relative activities of both GEFs (promoting activation of the G proteins) and GAPs (enhancing GTP hydrolysis and hence inactivation) that determines the activational state of the GTPase.

All G proteins are post-translationally modified with a lipid moiety being added to the carboxy terminus, which ensures its localisation at the plasma membrane (42). In addition to being regulated by the activities of both GEFs and GAPs, all classes of low molecular weight G proteins are negatively regulated by association within Guanine Dissociation inhibitors (GDIs).

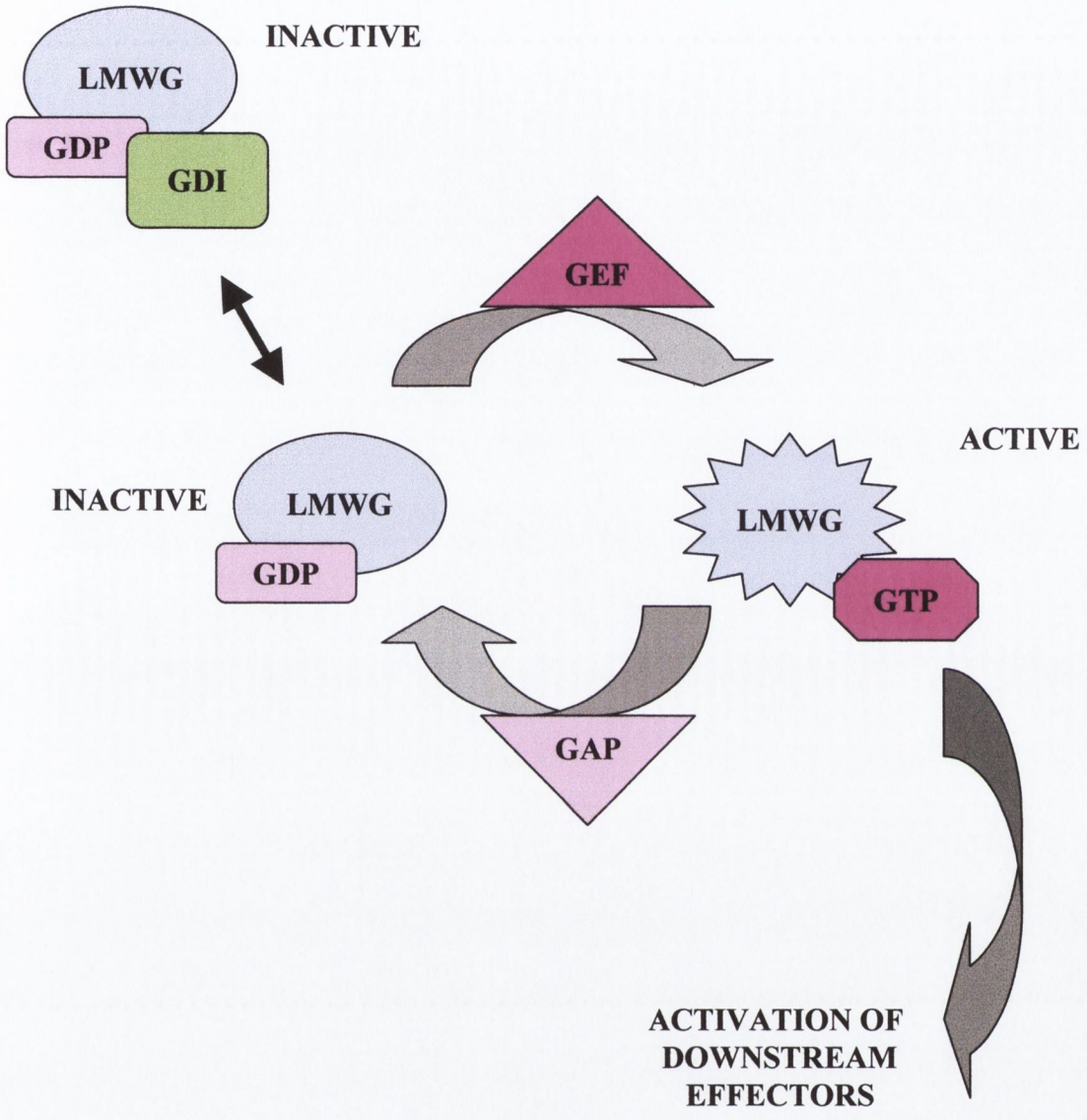


Figure 1.12: Regulation of low molecular weight G proteins (LMWG). Schematic representation of the regulation of LMW by (1) inactivating GTPase activating proteins (GAPs), which catalyse the hydrolysis of GTP to GDP, (2) guanine dissociation inhibitors (GDIs), which inhibit activation by sequestering GDP-bound LMWGs and preventing guanine nucleotide exchange and (3) activating guanine nucleotide exchange factors (GEFs), which catalyse the exchange of GDP for GTP on the protein. Once active (GTP-LMWG) the protein can interact with its downstream effectors and initiate signalling cascades.

These prevent GDP/GTP exchange by GEFs and also mask the palmitoyl group at the carboxy terminus thus preventing localisation to the plasma membrane (185).

Greater than 50 low molecular weight GTPases have been described to date and with the completion of the human genome project this number is most likely a conservative estimate of their number. They can be subdivided into five classes based on sequence homology: Ras, Rab, Arf, Ran and Rho. Ras family members (H-Ras, K-Ras, N-Ras, TC21, Rap1A, Rap1B, Rap2A, and Rap2B) are characterised by a high degree of homology in the effector-binding domain, the switch I region, and play important roles in cell growth and development (summarized in table 2 and reviewed in (19)). The proto-oncogene Ras was initially described for its transforming ability and has since been shown to be a crucial mediator of signalling events regulating growth control and proliferation (49, 72). The importance of Ras in these processes is dramatically illustrated by the fact that 90% of pancreatic adenocarcinomas have a mutation in Ras that renders it constitutively active and which thus promotes transformation (18). The principal role of Ras in cell signalling events is as a regulator of the classical p42/p44 MAPK pathway, with Ras lying upstream of Raf on the pathway (130). Ras has also been shown to play a role in phosphotidylinosital 3-kinase (PI3K) and phospholipase C activation (reviewed in (19)). As previously mentioned Ras is activated by IL-1 stimulation of a murine thymoma cell line and regulates IL-1-induced p38 MAPK activation in the same cell line (144). Ras is principally regulated via association with Sos, a GEF specific for Ras (32). Sos is recruited to activated receptors, such as the platelet-derived growth factor (PDGF) receptor, via association with the SH2/SH3 domain containing adaptor protein GRB2. Recruitment of Sos to the PDGF receptor catalyses the exchange of GDP for GTP on Ras thus activating it. Other GEFs such as CBL have been identified which also demonstrate guanine nucleotide exchange activity for Ras, although the involvement of any of the GEFs specific for Ras so far identified in IL-1-induced activation of Ras has yet to be identified (196). It may be that an as yet unidentified GEF or the GTPase activity of the IL-1 receptor itself is responsible.

Subfamily	Members	Function
Ras	H-Ras K-Ras Rap1A Rap1B Rap2A Rap2B Ral	Expressed in most cell types; plays important roles in cell proliferation and differentiation. Known activators of the p42/p44 MAPK cascade. Rap1 and 2 known to antagonise Ras signalling, also important for NADPH oxidase activation in myeloid cells.
Rho	RhoA-C RhoE RhoG Rac1-3 Cdc42 TC10	Shares 30% homology with the Ras subfamily and 50% within the family. Important roles in cytoskeletal changes (cell shape and motility) in response to growth factors and other stimuli. Also increasingly important in cell signalling events and regulating MAPK activation.
Rab	Rab1-11 Ram Smg-p25B Smg-p25C	Play an important role in regulating and directing intracellular traffic between compartments and to the plasma membrane. Also involved in regulating endocytosis and exocytocis
Arf	Arf 1-6	ADP-ribosylating factors (ARF) 1-6 are important for intracellular trafficking in cells.
Ran	Ran	Important regulators of nuclear import and export

Table 2: Ras superfamily of low molecular weight G proteins.
The members of the five families are listed together with a brief summary of their important functions within the cell.

Other members of the Ras sub-family of G proteins include Rap1A, Rap1B, Rap2A and Rap2B (reviewed in (19, 112)). They share approximately 50% homology with Ras and about 90% homology with one another. In polymorphonuclear lymphocytes Rap1 and 2 play an important role in NADPH oxidase activity responsible for generating the oxidative burst. Rap1 has also been shown to antagonise Ras-mediated signalling in certain cell types and in particular Ras-mediated activation of p38 MAPK pathway. This antagonism appears to be as a result of IL-1-induced activation of Rap, which then serves to down-regulate Ras-mediated signalling in the cells (144).

The Ral sub-family of low molecular weight G proteins is ubiquitously expressed and is found in abundance in the brain, testes and platelets (19, 112). Family members are normally localised to endocytic and exocytic vesicles, suggesting a role for Ral in vesicle trafficking within the cell. A role for Ral as a link between Ras and Rho-like GTPases in signalling has been suggested with Ral lying downstream of Ras on this pathway. Both Rab and Arf sub-families are also involved in vesicular trafficking in cells. All family members so far identified have been shown to associate with particular compartments and vesicles within the cell and thus control trafficking within the cell. Members of the Ran sub-family play an important role in regulating transport of proteins to and from the nucleus.

The Rho subfamily of G proteins, including RhoA, B, C, E and G, Rac1, 2 and 3, TC10 and Cdc42, were initially described for their ability to effect cytoskeletal rearrangements in cells (reviewed in (70)). Micro-injection of a constitutively active mutant of RhoA induced stress fibre and focal adhesion formation in Swiss 3T3 cells, whereas introduction of constitutively active mutant of Rac1 (constitutively active RacV12) into fibroblasts induced membrane ruffling (151). In addition to the importance of this family in regulating cytoskeletal dynamics, it is now recognised that Rho family of GTPases play an important role in the activation of signal transduction pathways in response to diverse stimuli (136, 150). The subfamily shares, on average, 30% homology with Ras, the main divergence of sequence being in the domain of the

protein that associates with downstream effectors, the switch I region (75). In the case of the Ras subfamily this region comprises hydrophobic residues whereas the equivalent domain in the Rho subfamily contains mostly acidic residues. Differences also lie in the regions that interact with the upstream activators, GEFs and the downstream inactivators, GAPs. Within the Rho sub-family Rho and Rac members share approximately 58% homology.

1.13 Rac1 and its regulation

Three highly homologous mammalian genes for *rac* have been identified to date, encoding Rac1, Rac2 and Rac3 (44, 69, 90). While cells of myeloid lineage express both Rac1 and Rac2, Rac2 expression is twenty-fold higher than Rac1. In contrast, non-myeloid cells exclusively express Rac1. The sequence divergence between Rac1 and 2 occurs at their carboxy terminus within the polybasic domain (figure 1.13) (84, 96, 97). In Rac1 this domain consists of 6 consecutive basic residues, while in Rac2 three of these have been converted to neutral amino acids. The importance of this divergence is obvious when the ability of Rac1 and 2 to activate p21 activated kinase (PAK) is compared, with Rac1 being a better activator of PAK than Rac2 (91). Domain swap experiments have shown that the difference in their ability to activate PAK lies in the polybasic tail domain (91). Rac3 expression differs from that of both Rac1 and 2 being confined mainly to brain, heart, placenta and pancreas. Also, it is more similar to Rac1 at its carboxy terminal domain, suggesting that it regulates similar downstream effectors (69). A hyperactive form of Rac3, which constitutively drives both PAK and JNK activation, has been demonstrated to be expressed in breast cancer-derived cell-lines and tumor tissues (128).

Due to its importance in regulating signalling events in many different cell types a lot of attention has focused on identifying the proteins involved in its regulation. In general Rho-GEFs are only weakly conserved within the Dbl-homology domain (10-30% identity), the domain responsible for gaunine nucleotide exchange activity, and outside of this domain the conservation

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Rac1            MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAG
Rac3            MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAG
Rac2            MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDSKPVNLGLWDTAG
                ********************************************.************

Rac1xx0         QEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLR
Rac3xx2         QEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPHTPILLVGTKLDLR
Rac2xx1         QEDYDRLRPLSYPQTDVFLICFSLVSPASYENVRAKWFPEVRHHCPSTPIILVGTKLDLR
                *****************************:*******:******** ***:*********

Rac1xx0         DDKDTIEKLKEKKLTPITYPQGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPP
Rac3xx2         DDKDTIERLRDKKLAPITYPQGLAMAREIGSVKYLECSALTQRGLKTVFDEAIRAVLCPP
Rac2xx1         DDKDTIEKLKEKKLAPITYPQGLALAKEIDSVKYLECSALTQRGLKTVFDEAIRAVLCPQ
                *******:*::***:*********:*:**.:*****************************

Rac1xx0         PVKKRKRKCLLL
Rac3xx2         PVKKPGKKCTVF
Rac2xx1         PTRQQKRACSLL
                *.::  : * ::
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*	-	complete conservation
A	-	highly conserved
B	-	weakly conserved
C	-	no similarity

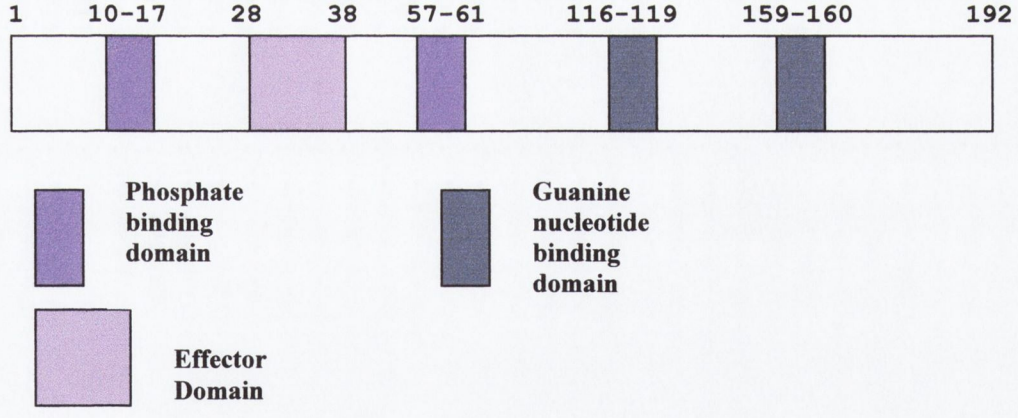


Figure 1.13: Rac family of G proteins.

(A) Alignment of the members of the Rac family (Rac1-3) performed using ClustalW alignment programme. Degree of conservation between the family members is highlighted. (B) Schematic representation of the GTP-binding, nucleotide binding and effector domains. Analysis of the 3D structure of the protein shows that the GTP and nucleotide binding domains associate to form GTP and guanine binding pockets respectively.

between GEFs is minimal (31). All Rho-specific GEFs are very large proteins, generally over 1000 amino acids containing multiple domains such as SH2/3 domains, zinc fingers and pleckstrin homology domains, that mediate protein-protein interactions. With respect to activation of Rac1 a number of GEFs have been identified which demonstrate specificity for Rac1. The first to be described was Vav, a proto-oncogene identified in fibroblasts with transforming capabilities (35). It has several distinct domains including: a cysteine-rich zinc finger domain, a phosphotyrosine-binding *src*-homology (SH) 2 domain which is flanked by two proline-binding SH3 domains, a pleckstrin homology (PH) domain and most importantly a Dbl-homology domain. This latter domain is responsible for the guanine nucleotide exchange activity associated with GEFs and is activated following tyrosine phosphorylation of Vav in response to diverse stimuli. In addition to Rac1, there is evidence for Vav having exchange activity for other members of the Rho sub-family (in particular Rho itself) and also the Ras sub-family (67), suggesting there may be cross talk betweeen these two groups of G proteins. This is supported by the ability of Ras-GRF2 and CDC25/Ras-GRF1, both Ras-specific GEFs, to catalyse GDP/GTP exchange on Rac1 (52, 80). A second GEF, Tiam1, with specificity for Rac1, has been identified that is responsible for promoting invasiveness in T lymphoma cell lines (124) (196). Tiam1 (and its close homologue Tiam2 (33)), like other Rho-specific GEFs, is a large multi-domain containing protein and is activated following stimulation of cells by kinases such as protein kinase C (PKC) and casein kinase II (CKII). It appears translocation of Tiam1 to the plasma membrane is contingent on phosphorylation of the protein by CKII. Similar to Vav, Tiam1 has several adaptor domains (SH2 and SH3) and also a Dbl homology domain that essential for GEF activity towards Rac1. Other GEFs reported to be specific for Rac1 include STEF (77), which is related to Tiam1 and Lfc, a Dbl family member (64). It is possible that it is the activation of the various GEFs specific for Rac1 that regulates the pathways that are activated downstream of Rac1 in response to diverse stimuli (205).

Several GTPase activating proteins (GAPs) have been reported which preferentially associate with Rac1 and stimulate its GTPase activity. These include p190 RhoGAP (166), Rac1-GAP, the carboxy-terminal domain of Bcr (break point cluster region) which act on both Rac1 and Cdc42 (45), a Bcr-related protein n-chimaerin which is predominantly expressed in the brain, and 3BP-1 (an SH3 domain containing protein) (34). Overexpression of Rac1-specific GAPs results in inhibition of Rac1 signalling and cytoskeletal rearrangements in cells. Figure 1.14 summarizes the known GEFs and GAPs specific for Rac1.

A certain amount of cross-talk between the different subfamilies occurs and of particular interest is the possibility that GEFs which activate Ras may also be involved in activating Rac1. This includes Ras-GRF1, for which researchers have shown that tyrosine phosphorylation of the latter by Src is essential for its activity towards Rac1 but not Ras (80). In addition two recently identified proteins EPS8 and E3B1 transduce signals from Ras to Rac1 by forming a complex with the Ras-specific exchange factor Sos and thereby activating the GEF activity of Sos towards Rac1 (163). There are also reports of cross-talk within the Rho-sub-family of GTPases, with PDGF-activated Rac1 reportedly down-regulating Rho activity and also cell migration in NIH3T3 fibroblast cells (159). Also, downstream effectors of Rac1 and Rho (PAK and MLCK, myosin light chain kinase) appear to have opposing effects on myosin light chain (MLC) phosphorylation, controlling the Rho-mediated response in cells (26).

Numerous studies have demonstrated phosphatidylinositol 3-kinase (PI3K) involvement in regulating Rac1 activation. Immunoprecipitation studies have shown direct interaction between Rac1-GTP and PI3K (183). In addition the PI3K inhibitor wortmanin inhibits growth factor-induced stress fibre formation in Swiss 3T3 cells, which is reversed by microinjection of a constitutively active mutant of Rac1. More recently PI3K has been demonstrated to regulate the guanine exchange activity of Vav by relieving inhibitory interactions between the pleckstrin homology and Dbl homology domains (71). Finally, there is evidence that Rac1 is negatively regulated by phosphorylation. A putative protein kinase B site (PKB) was found in Rac1 and *in*

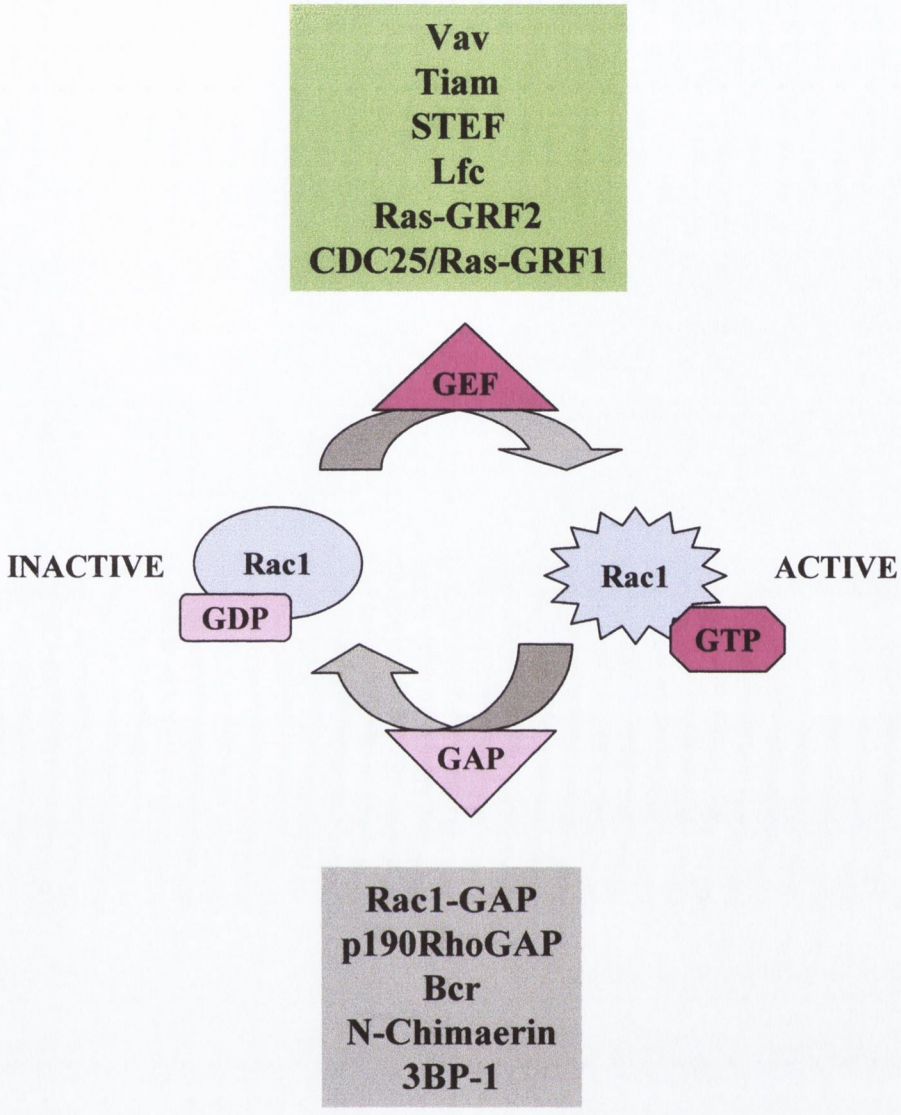


Figure 1.14: Summary of the GEFs and GAPs responsible for regulating Rac1 activity. Activating guanine nucleotide exchange factors (GEFs), which catalyse the exchange of GDP for GTP on Rac1 and inactivating GTPase activating proteins (GAPs), which catalyse the hydrolysis of GTP to GDP, specific for Rac1 are shown here.

vitro kinase assays demonstrated phosphorylation of Rac1 by PKB (101). Phosphorylation of Rac1 by PKB on serine 71 was found to inhibit its GTP-binding activity and suggests an additional mechanism by which Rac1 activity is regulated in response to PI3-kinase.

1.14 Downstream effectors of Rac1

A perplexing aspect of Rac1 signalling is the diversity of pathways it activates in response to diverse stimuli i.e. rearrangements of the actin cytoskeleton, activation of the stress activated protein kinase pathways (p38 MAPK and JNK), cell cycle regulation and control of apoptosis. Its ability to activate such a diverse array of pathways is as a result of the variety of downstream effectors with which it interacts (summarised in figure 1.15 and reviewed in (4)). The principal effector responsible for Rac1-induced actin reorganisation is IQGAP1, which has been demonstrated to bind directly to actin filaments and to cross link-them (100, 109). It also appears to be necessary for Rac1- (and Cdc42-) induced formation of cell-cell contacts which is regulated by the activity of E-cadherin and β-catenin. Two other Rac1-binding proteins, partner of Rac1 (POR1) (186) and p140Sra-1 (92) have been implicated in Rac-induced lamellipodia formation and a truncated form of POR1 was found to interfere with Rac-induced membrane ruffling.

p21-activated kinase (PAK) represents a group of Rac (and Cdc42) effectors with diverse biological roles, the three known isoforms PAK1-3 reportedly responsible for mediating both actin regulation and MAPK pathway activation (8). Structurally PAK consists of an N terminal regulatory domain and a C terminal kinase domain (reviewed in (6)). The regulatory domain contains four potential SH3 binding motifs (PXXP), the Rac1-binding domain (termed the p21-binding domain), an auto-inhibitory domain that overlaps with the PBD, and a domain which interacts with additional proteins such as PIX (PAK-interacting exchange factor). How Rac1 binding to PAK triggers its activation is not entirely clear, although it has been suggested that binding disrupts negative intramolecular interactions between the auto-inhibitory domain and the

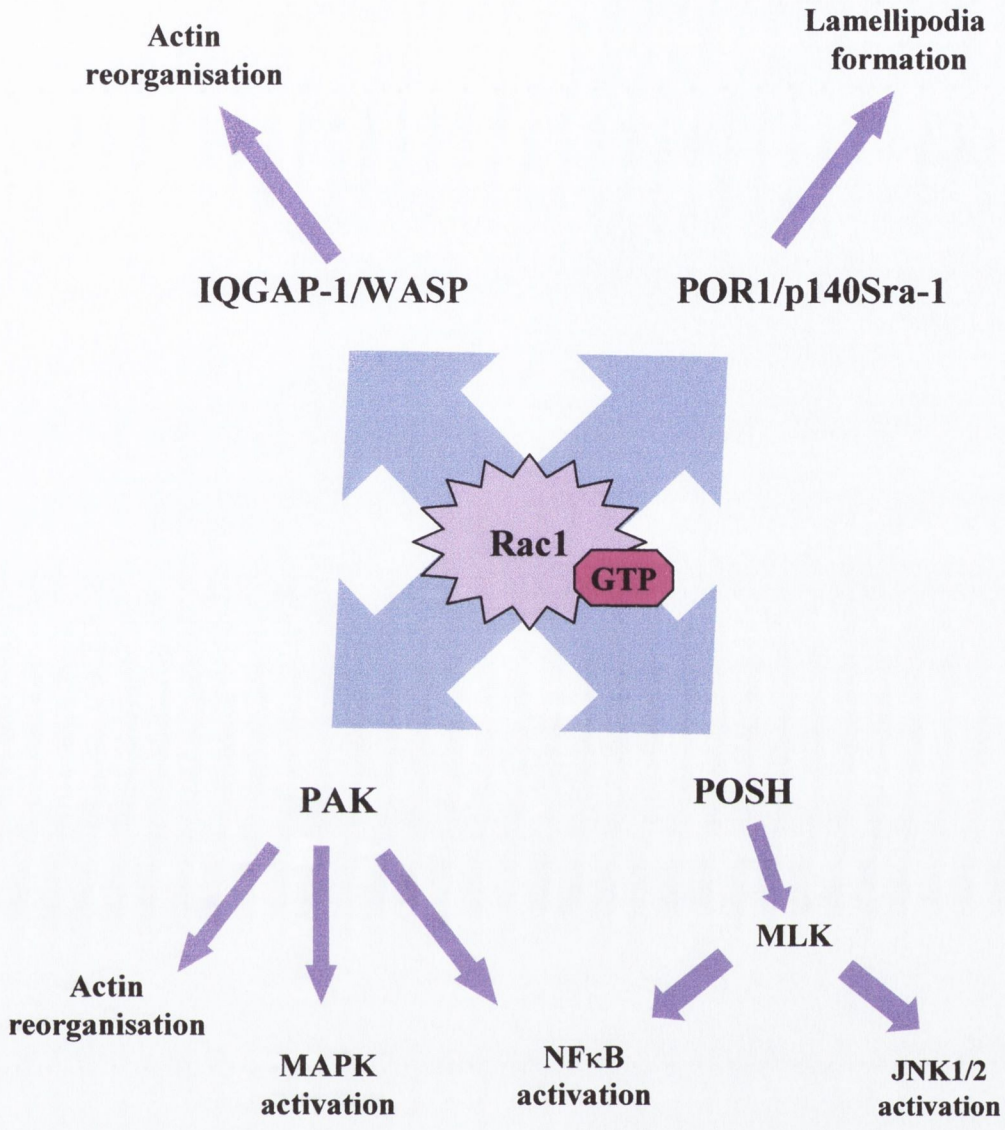


Figure 1.15: Downstream Effectors of Rac1. Summary of Rac1 effectors known to date and the downstream consequences of their activation. The abbreviations used are as follows: PAK, p21 activated kinase; POSH, plenty of SH_3 domains; WASP, Wisc-Alcott syndrome protein; POR-1, partner of Rac1; MAPK, mitogen activated protein kinase; NFκB, nuclear factor kappa B; MLK, mixed lineage kinase; JNK, c-Jun N terminal kinase.

carboxy terminal kinase domain (202). Once bound by Rac1-GTP the autophosphorylating activity of PAK is subsequently activated and downstream signalling initiated. Recently PAK binding partners such as PIX have been described which are localised to Rac1-stimulated focal complexes and recruit PAK from the cytoplasm to these sites (113). PIX also has a Dbl homology domain, although it shows weak GEF activity for Rac1 on its own. It is possible however that binding of PAK to PIX enhances this activity. Little information is available about the signalling components downstream of PAK although mounting evidence suggests a role for PAK in MAPK activation. Initially it was reported that PAK was the downstream effector of Rac1 responsible for activation of JNK and p38 MAPK (7). A role for PAK in Ras-induced transformation has been demonstrated by the ability of kinase deficient PAK to inhibit Ras-induced p42/p44 MAPK activation and thus transformation (178). Surprisingly, it appears that it is the activation of Rac1 by Ras that promotes PAK-induced activation of p42/p44 MAPK, again supporting the notion of crosstalk between Ras and Rac1 signaling pathways. Although Pak was initially thought to lie upstream of JNK, it is now believed that the true mediators of Rac1-induced JNK activation are the mixed-lineage kinases (MLK) 2 and 3 (26). A novel Rac1 effector POSH (plenty of SH3 domains) has been identified that acts as a link between Rac1 and the MLKs. POSH has also been implicated in Rac1-induced NFκB activation in Cos-1 cells where it was isolated using yeast two hybrid selection (179).

1.15 Regulation of the actin cytoskeleton by Rac1

Members of the Rho family of GTPases have emerged as key regulators of the actin cytoskeleton and through their interaction with multiple target proteins they ensure coordinated control of other cellular activities such as gene transcription. While Rho itself controls the formation of stress fibers and focal adhesions, Rac1 induces membrane ruffling and focal complex assembly. Another member of the family, Cdc42, is responsible for filopodia formation in addition to focal

complex assembly (reviewed in (70)). While each of the proteins induces very distinct changes in cell morphology, many of the features of these changes are shared such as an increase in polymerised actin and the assembly of large protein complexes containing vincullin, talin, focal-adhesion kinase and paxillin. The effectors downstream of Rac1 responsible for actin rearrangement have already been mentioned. These include PAK, IQGAP1, and POR1. WASP (Wiscott-Aldrich syndrome protein) has also been identified as a Rac1-binding protein that is essential for the formation of membrane ruffles (126). A homologue of WASP, WAVE, also functions downstream of Rac1 in regulating actin reorganisation, although its activity is reportedly dependent on phosphorylation by p42/p44 MAPK (125).

1.16 Role of Rac1 in regulating MAPK pathways

The low molecular weight GTPases Ras, Rac1, Cdc42 and Rho, have all been reported to play a role in activation in one or more of the MAPK pathways (reviewed in (189)). Rac1 has been specifically associated with activation of the stress activated protein kinase cascades, p38 MAPK and JNK. A constitutively active version of Rac1 (RacV12) was shown to activate both p38 MAPK and JNK (127). Importantly, dominant negative Rac1 (RacN17) inhibited IL-1-induced activation of both pathways thus indicating that Rac1 is required for IL-1-induced activation of both JNK and p38 MAPK (37). Activation of p38 MAPK and JNK is thought to be mediated by the downstream effectors PAK and MLK, respectively (as shown in figure 1.16) (7, 133). Support for a role for PAK in these pathways comes from its homology with a regulator of the yeast pheromone pathway, also a MAPK cascade. While there is experimental evidence to support a role for PAK in JNK activation, it is now believed that MLK is principally responsible for Rac1-mediated activation of JNK via association of POSH with active Rac1. As already mentioned, a role for Rac1 in p42/p44 MAPK activation has come from results which have shown

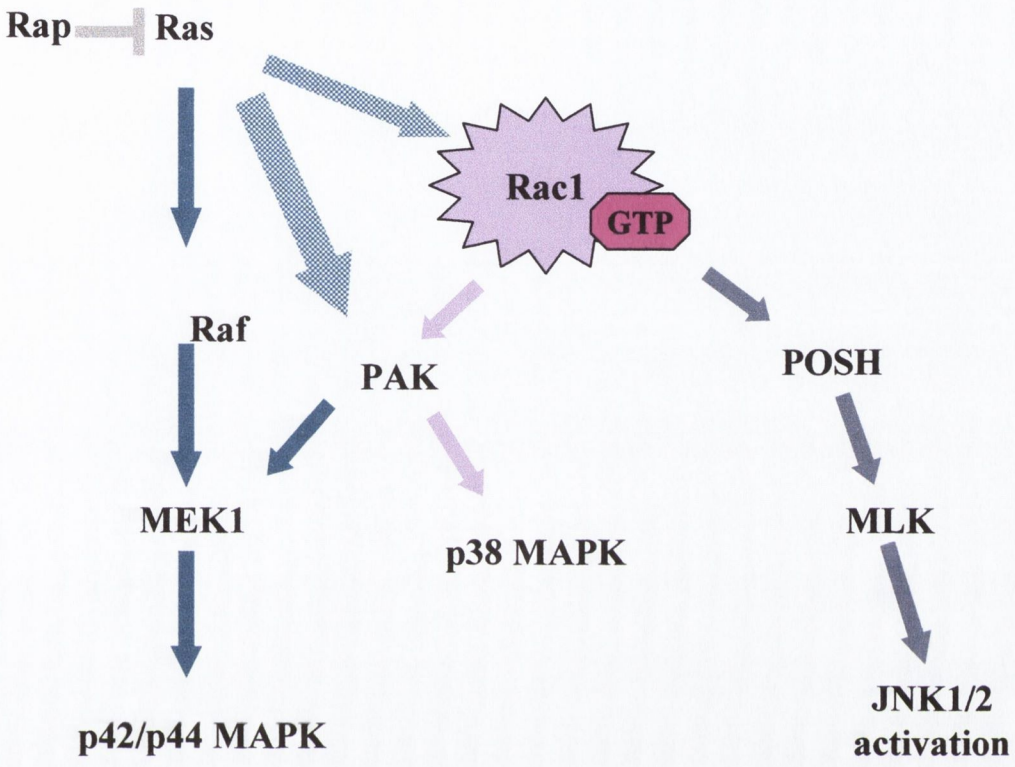


Figure 1.16: Rac1 involvement in MAPK activation. Rac1 has been demonstrated to regulate activation of p38 MAPK and JNK MAPK pathways in particular via activation of PAK and POSH/MLK respectively. Recent evidence has also implicated PAK in p42/p44 MAPK activation, either by directly activating Raf-1 or by phosphorylating MEK1 which results in increased association of Raf-1 with MEK1. Whether PAK-regulated activation of p42/p44 MAPK is via Ras or Rac1 (or Ras-induced activation of Rac1) has yet to be determined. The abbreviations used are as follows: PAK, p21 activated kinase; POSH, plenty of SH_3 domains; MAPK, mitogen activated protein kinase; MLK, mixed lineage kinase; JNK, c-Jun N terminal kinase.

that PAK can activate this pathway via both Raf and MEK1, and that this pathway appears to be mediated by Ras-induced activation of Rac1 (55) (178).

1.17 Rac1 and NFκB activation

NFκB activation in response to pro-inflammatory stimuli has been well documented as discussed previously. More recently however the role of low molecular weight GTPases in NFκB activation has been investigated. The GTPase Ras has been demonstrated to regulate both the pathway to IκB degradation and the separate pathway regulating transactivation of gene expression by the p65 subunit of NFκB. In addition a role for Rac in NFκB activation has been reported, with constitutively active RacV12 stimulating nuclear translocation of NFκB via a redox-dependent pathway in rabbit synovial fibroblasts (87). Similarly, the ability of RacV12 to stimulate NFκB-dependent reporter gene expression in HeLa cells was also demonstrated to be redox-dependent, as was the ability of dominant negative RacN17 to inhibit IL-1-induced reporter gene activation (175). However, as the generation of reactive oxygen species by Rac is highly cell type dependent, it is unlikely that this response is universally applicable. Of particular note for this study is the inability of Rac1 to generate reactive oxygen species in T lymphocytes, suggesting that any involvement of Rac1 in pathways regulating NFκB activation in these cells will involve alternative signalling mechanisms (15).

1.18 Aims and Objectives

The proposal that Rac1 is involved in IL-1 signalling in cells prompted investigation into the precise involvement of Rac1 in IL-1 signal transduction using the IL-1 receptor rich murine thymoma cell-line EL4.NOB-1 (58).

- The initial objective was to assess the ability of IL-1 to activate Rac1 using a novel assay based on the preference of its downstream effector PAK for binding activated GTP-Rac1 (59).
- Having established that IL-1 may or may not be able to activate Rac1, the aim was to assess the involvement of Rac1 in pathways regulating IL-2 production.
- This cytokine is regulated by three key inducible transcription factors: NFκB, AP-1 and NFAT. The involvement of Rac1 in activation of any of these three and its role in IL-1-induced activation of NFκB and AP-1 was next assessed.
- The results gained from these studies prompted more in depth investigation into the role of Rac1 in IL-1-induced activation of NFκB and its possible association with the activated IL-1 receptor complex.

Chapter Two

Materials and Methods

2 Materials and methods

2.1 Materials

Human recombinant IL1-α was a kind gift from Prof. J.Saklatvala (Kennedy Institute of Rheumatology, U.K.).The pyridinyl imidazole SB203580 was kindly provided by Peter Young, Smithkline Beecham Pharmaceuticals, King of Prussia, PA. PD98059 (2'-amino-3'-methoxyflavone) was originally a kind gift of Alan Saltiel, Parke-Davis Research Division, Warner Lambert Company, Ann Arbor, Michigan. Commercially PD98059 was available from Calbiochem, and SB203580 was obtained from Alexis Corporation. Cep-1347 was a kind gift of Dr. Donna Bozyczko-Coyne from Cephalon Incorporated, West Chester, Pennsylvania 19380. All inhibitors were prepared in dimethyl sulphoxide (DMSO).

The murine thymoma cell line EL4.NOB-1 (ECACC No. 87020408) was obtained from the European Collection of Animal cultures (Salisbury, U.K.). The porcine aortic endothelial cell-line was a kind gift from Dr. Bill Davis, as were the lines which were stably transfected with either constitutively active RacV12 or dominant negative RacN17. HeLa cells (human cervical carcinoma cells) were obtained from the European Collection of Animal cultures (Salisbury, U.K.). 293 cells (both parental and stably transfected cell lines) were obtained from Dr. Cao (Tularik, Inc., San Fransisco, CA, USA). All cell culture materials, RPMI 1640, Ham's F12 medium, Dulbeccos Modified Essential medium, penicillin, streptomycin, gentamycin, and foetal calf serum were obtained from Sigma (Poole, Dorset, U.K.). All solutions used for cell culture were autoclaved at 120°C for 20 minutes or filter sterilised (pore size 0.4μm) as appropriate.

The anti-Rac1 monoclonal antibody was purchased from Calbiochem and was used at a dilution of 1:1000 for western blot analysis. A polyclonal antibody to PAK1 was purchased from Santa Cruz Biotechnology and was used at a dilution of 1:1000 for western blot analysis. Anti-IκBα was a kind gift from Professor Ron Hay, (St. Andrews University, Fife, Scotland) and was used at a dilution of 1:100-1:200 for western blotting. Anti-flag M2 and anti-myc monoclonal

antibodies were purchased from Sigma (Poole, Dorset, UK). Anti-phospho JNK antibody was obtained from New England Biolabs (Beverly, MA, USA). Anti-phospho MBP (myelin basic protein) was purchased from Upstate Biotechnology (Lake Placid, New York). Purified mouse IgG, goat anti-mouse and anti-rabbit IgG peroxidase conjugates were from Sigma or New England Biolabs (Beverly, MA, USA).

The 22 base pair oligonucleotide containing the consensus sequence for NFκB (5'-AGTTGAGGGGACTTTCCCAGGC-3') and poly (dI.dC) were from Promega (Madison, WI, U.S.A). TRI-reagent used for RNA extraction was purchased from Sigma and the Titan RT-PCR kit was purchased from Boehringer Mannheim GmH, Mannheim, Germany. Plasmid purification kits were purchased from Qiagen (California) and Endotoxin-free kits were purchased from Promega (Madison, WI, U.S.A).

Anti-mouse IL-2 antibody, biotinylated anti-IL-2 antibody and murine IL-2 standard were obtained from Pharmingen (San Diego, California, U.S.A). Avidin peroxidase used to detect the secondary antibody in the IL-2 ELISA was purchased from Sigma (Poole, Dorset, U.K.).

[^{14}C]-labelled chloramphenicol and [^{32}P]-labelled ATP were purchased from Amersham (Nycomed Amersham plc, Amersham place, Little Chalfont, Buckinghamshire, U.K.). Acetyl CoA (sodium salt) was purchased from Sigma (Poole, Dorset, U.K.).

All other reagents used, including DEAE-dextran (for transfections), glutathione-agarose beads (affinity purification of GST-fusion proteins), protein A-sepharose beads (for immunoprecipitations), were of molecular biology or Analr grade and were purchased from Sigma (Poole, Dorset, U.K.).

2.2 Cell Culture

EL4.NOB-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum (FCS), 100U/ml penicillin, 100μg/ml streptomycin (or 100μg/ml gentamycin) and 2mM L-

glutamine and maintained at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 3-4 days. Cells were seeded at a density of $1x10^6$ ml^{-1} for experiments and pretreated with inhibitors prior to stimulation with IL-1α as indicated in the figure legends.

HeLa cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 100U/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine, maintained at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 3-4 days. Cells were seeded at a density of $1x10^5$/ml for experiments.

Parental 293 cells and IL-1RI/AcP-293 cells (stably expressing myc-tagged IL-1RI and flag-tagged IL-1RAcP) were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 100U/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine and maintained at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 3-4 days. Cells were seeded at a density of $1x10^5$/ml for experiments.

Parental porcine aortic endothelial (PAE) cells (designated Wt-PAE) were grown in F12 nutrient mixture (Ham F12; SIGMA) containing 15% FCS, 2mM L-glutamine, 50μg/ml gentamycin and 500nM puromycin at 37°C in a humidified atmosphere of 5% CO_2. V12Rac-PAE and N17Rac-PAE cell lines were maintained in F12 nutrient mixture as above with the addition of 0.1mM hygromycin B. 24 hours prior to the induction of Rac1 mutants, cells were cultured in serum free medium containing 0.2% FCS, 0.1% fatty acid free BSA, 2mM L-glutamine, 50μg/ml gentamycin, 500nM puromycin and 0.1mM hygromycin B. Expression of RacV12 and RacN17 was induced by the addition of 15mM filter-sterile isopropyl-β-D-thiogalactopyranoside (IPTG) to the starvation medium for the time periods indicated in the figure legends.

In all cases cell viability was determined using the dye Trypan blue which is excluded from healthy cells but is taken up by non-viable cells. Cells were counted using hemocytometer and a bright light microscope.

2.3 Plasmid constructs

The pEF expression vector encoding N terminal myc-tagged constitutively active RacV12 and dominant negative RacN17 were kind gifts from Dr. D. Cantrell (ICRF, London, U.K.) and have been described elsewhere (figure 2.1) (60). The AP-1 reporter plasmid (AP-1 chloramphenicol acetyl transferase, AP1-CAT), the NFAT-dependent reporter gene NFAT-CAT were also a gift from Dr. D. Cantrell (ICRF, London, U.K.) (figure 2.2a and b, respectively). The NFκB dependent reporter NFκB-CAT was a gift from Dr. Hofmeister (Universität Regensgurg, Regensburg, Germany) and is shown in figure 2.2c. The IL-2 dependent reporter (containing residues –293 to –5) was a kind gift from Dr. Werner Falk (Universität Regensgurg, Regensburg, Germany) (figure 2.2d). The κB-luciferase reporter gene (pGL3-5xκB-luc) was a kind gift of Dr. R. Hofmeister (Universität Regensgurg, Regensburg, Germany) (figure 2.3). Gal4-$p65^{1-551}$ plasmid encoding the full p65 subunit (amino acids 1-551) fused to the DNA-binding domain of Gal4 and the Gal4-dependent reporter construct, Gal4-luciferase, were obtained from Dr. Lienhard Schmitz (German Cancer Research Center, Heidelberg, Germany) and have been described previously (41, 162). IκBctag (figure 2.4) was constructed by cloning IκBα into the $pCDNA_3$ expression vector containing a 3' sequence encoding the viral SV5 Pk tag and was a kind gift from Prof. R.T. Hay (University of St. Andrews, Scotland). A plasmid encoding HA-PAK (figure 2.5) was obtained from Dr. D. Cantrell (ICRF, London). The $pCDNA_3$ expression vector encoding AU1-tagged wild-type (figure 2.6a) and dominant negative MyD88 (figure 2.6b) was a kind gift from Dr. Marta Muzio (Mario Negri Institute, Milan). IRAK-1 (wild-type and dominant negative versions, figure 2.7a and b, respectively) was a kind gift from Emma-Louise Cooke (Glaxo-Welcome, Stevenage, U.K.). IRAK-2 in $pCDNA_3$ was obtained from Dr. Marta Muzio (Mario Negri Institute, Milan, Italy). The $pCDNA_3$ expression vector encoding wild-type (figure 2.8a) and dominant negative (figure 2.8b) TRAF-6 was a kind gift from Tularik (San Francisco, USA).

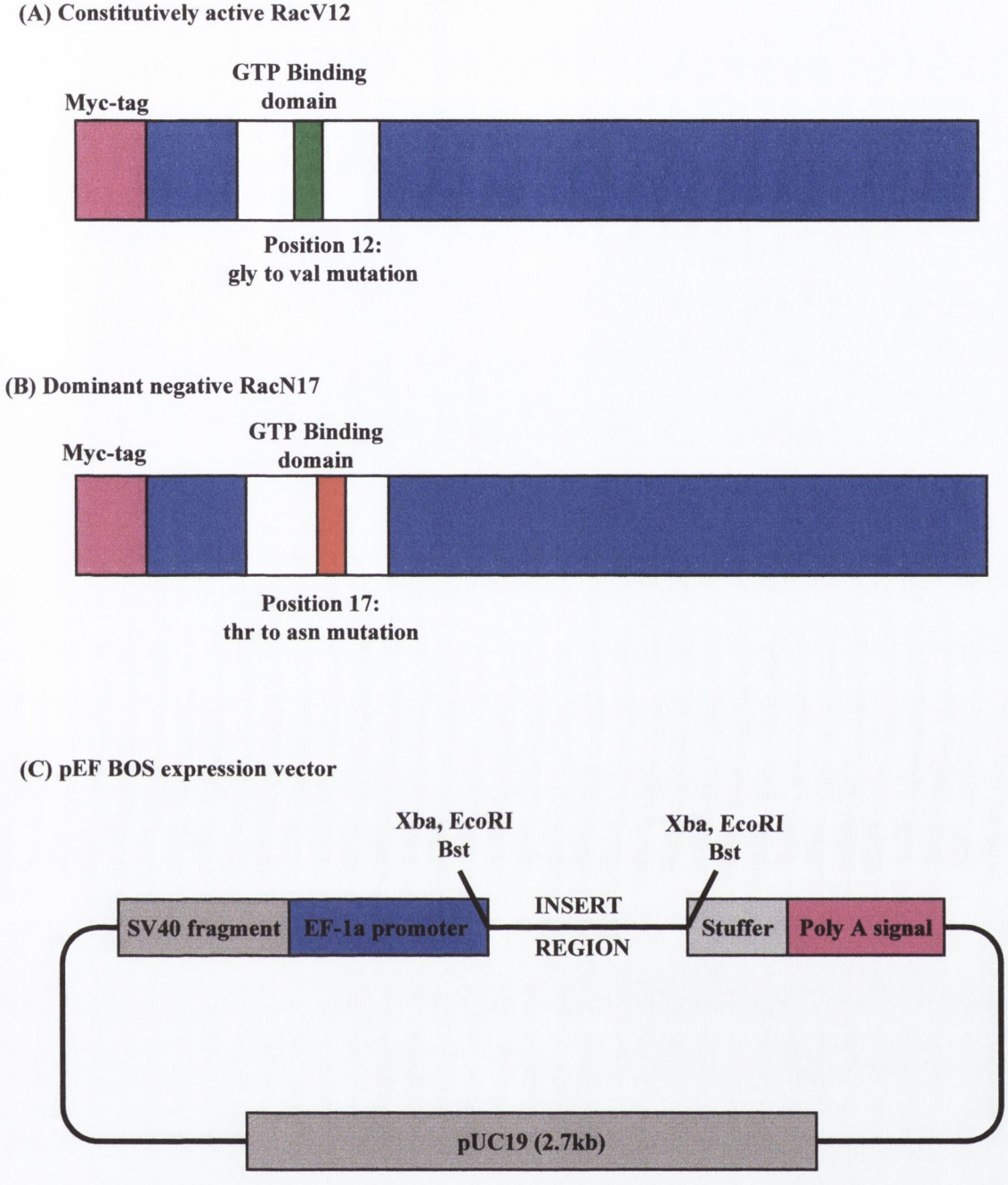


Figure 2.1: Schematic map of expression vectors for RacV12 and RacN17.

Diagrams represent (A) RacV12 and (B) RacN17, indicating N terminal Myc tag, GTP-binding domain and site of point mutation. RacV12 and RacN17 were cloned into the insert region of the mammalian expression vector pEF-BOS (C) which is shown here with elongation factor promoter region, restriction sites and insert region indicated.

(A) AP1-CAT: Five AP-1 consensus sites cloned into insert region

(B) NFAT-CAT: Three NFAT consensus sites cloned into insert region

(C) NFκB-CAT: Three NFκB consensus sites cloned into insert region

(D) IL2-293-CAT: IL-2 promoter region residues –293 to -5

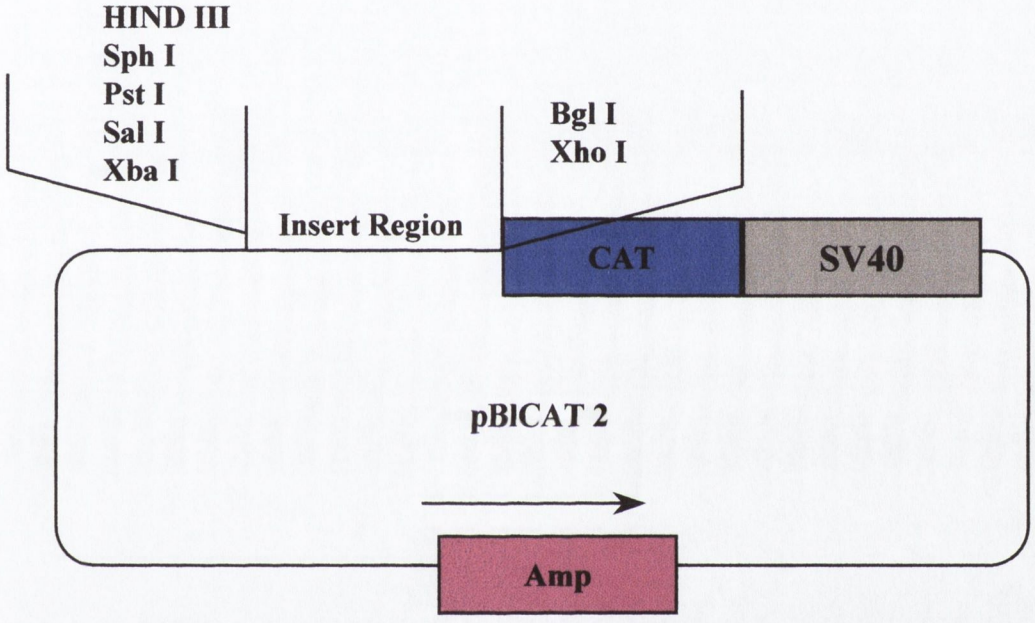


Figure 2.2: Graphical representation of pBlCAT 2 vector used to construct (A) AP1-CAT, (B) NFAT-CAT, (C) NFκB-CAT, and (D) IL2-293-CAT reporter plasmids. Consensus sites for the different transcription factors were cloned into the insert region. Restriction sites at either side of the insert region are shown as is the ampicillin resistance gene used to select for bacteria transformed with the plasmids.

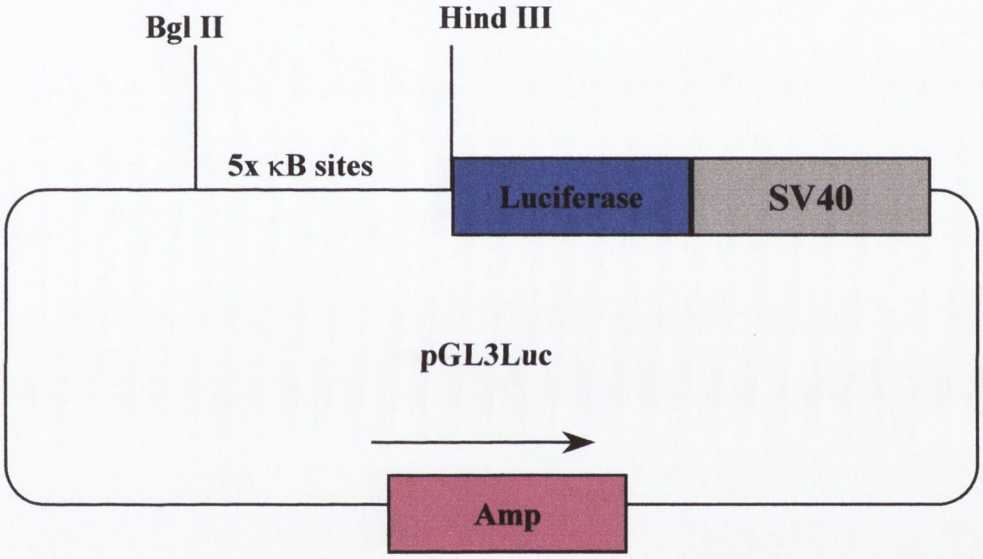
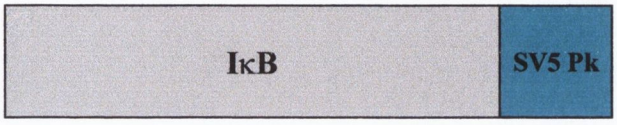


Figure 2.3: Graphical representation of pGL3Luc vector used to construct the NFκB-luciferase reporter plasmid.

Five copies of the canonical consensus site for the transcription factor NFκB were cloned into the insert region of the reporter vector, pGL3-Luc. The restriction sites used to insert the sequence are as shown.

(A) IκBctag



(C) pCDNA$_3$ expression vector with SV Pk epitope tag engineered in to be placed at the C terminus of proteins

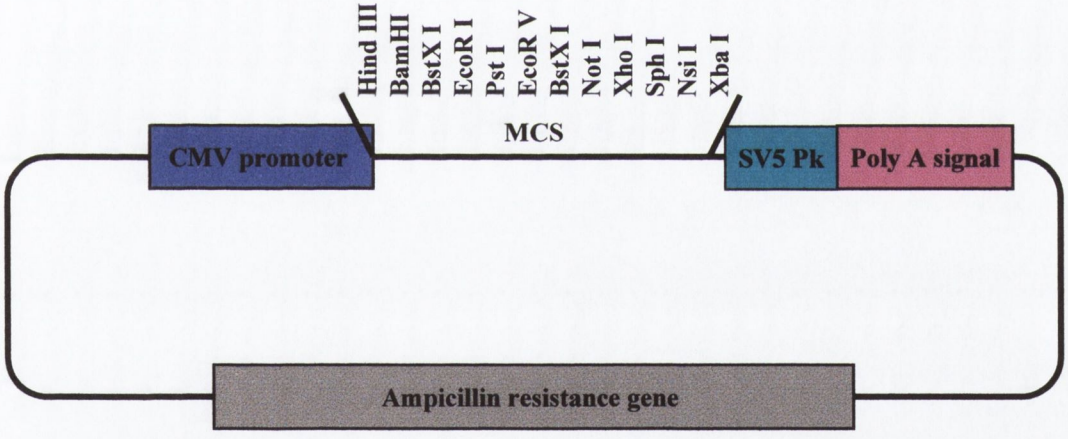
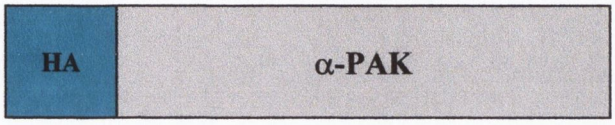


Figure 2.4: Schematic map of expression vector for IκBctag

(A) wild-type IκB was inserted into the mammalian expression vector pCDNA$_3$ which had been engineered such that the epitope SV5 Pk would be added to the carboxy terminus of the protein. (B)The modified vector into which IκBctag was inserted is shown here with the cytomegalovirus (CMV) promoter region, the epitope tag and the multiple cloning site illustrated.

(A) HA-PAK



(B) pCDNA$_3$ expression vector with HA epitope tag engineered in to be placed at the N terminus of proteins

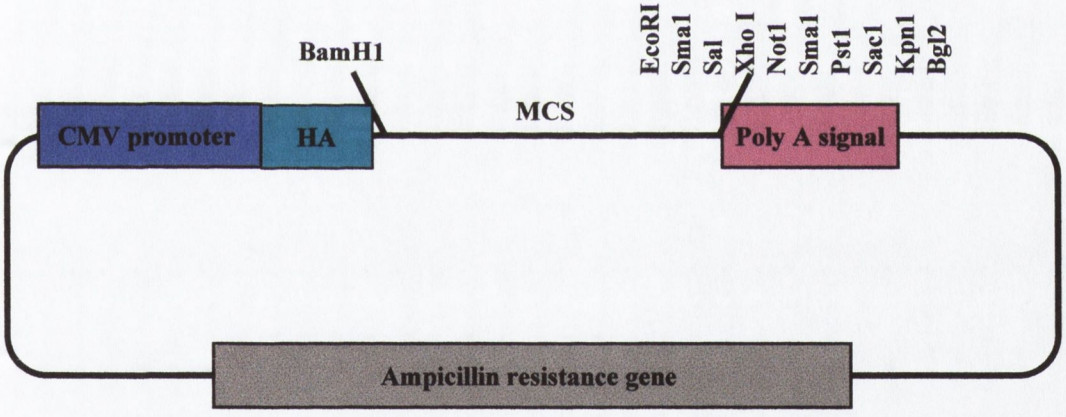


Figure 2.5: Schematic map of expression vector for HA-PAK

Diagrams represent (A) schematic representation of HA-tagged PAK which was inserted into the mammalian expression vector pCDNA$_3$ using BamH1 and EcoRI. (B) The modified vector is shown here with the cytomegalovirus (CMV) promoter region, the epitope tag and the multiple cloning site (MCS) illustrated.

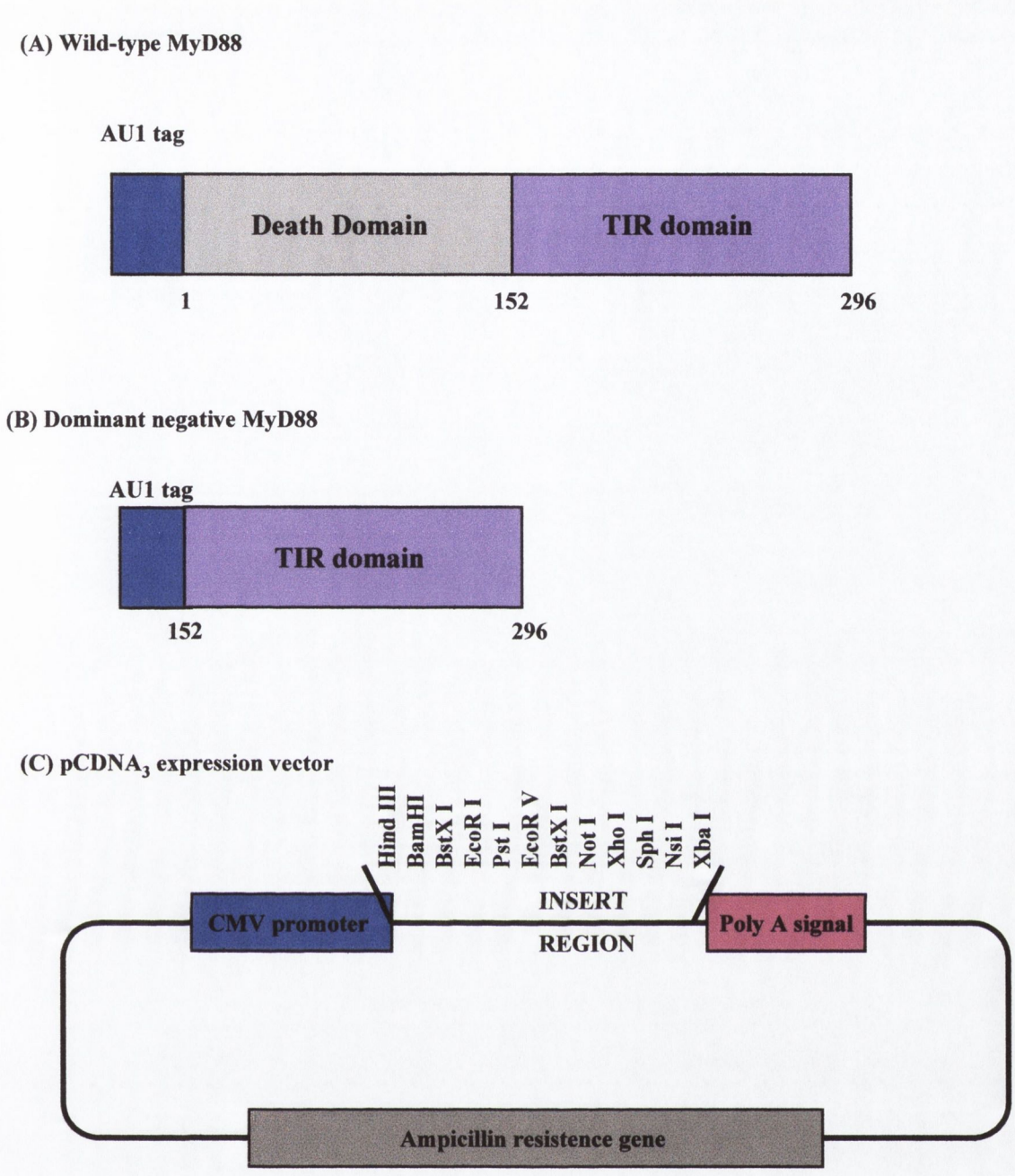
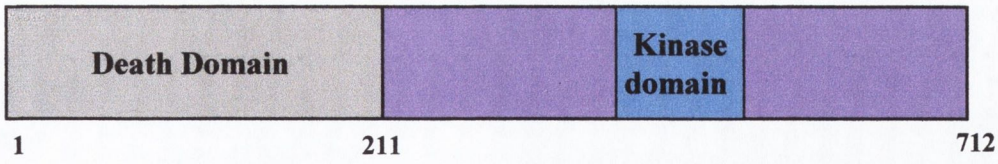


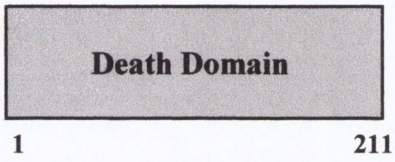
Figure 2.6: Schematic map of expression vectors for wild-type MyD88 and dominant negative MyD88

Diagrams represent (A) wild-type MyD88 and (B) dominant negative MyD88, indicating the N terminal AU1 tag, death domain and Toll/IL-1 receptor (TIR) domain. Dominant negative MyD88 has an amino terminal deletion removing the death domain. Both were cloned into the insert region of the mammalian expression vector $pCDNA_3$ (C) which is shown here with CMV promoter region, restriction sites and insert region indicated.

(A) Wild-type IRAK-1



(B) Dominant negative IRAK-1



(C) pRSV expression vector

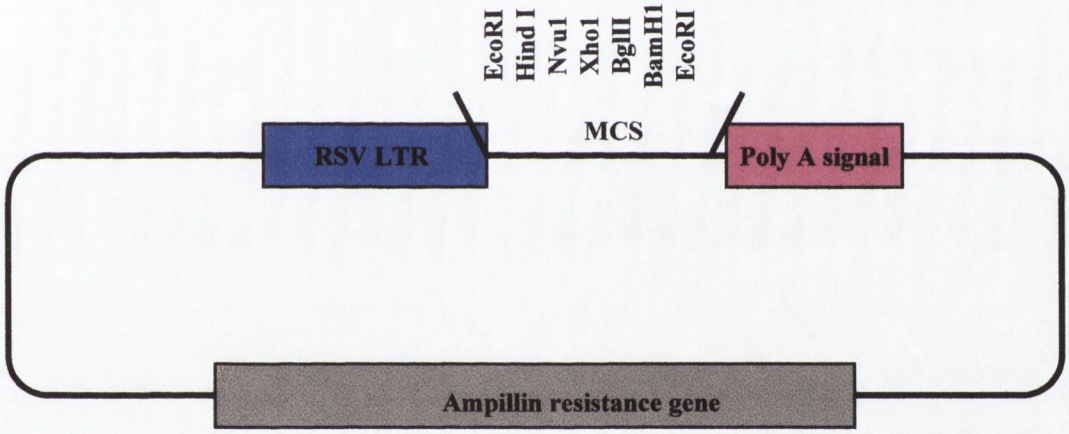
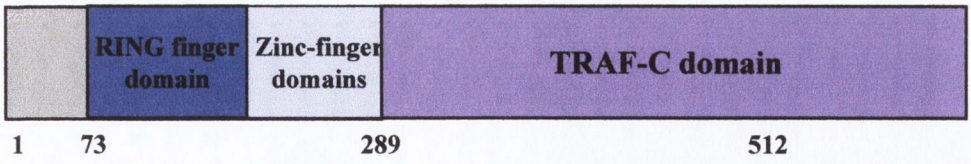


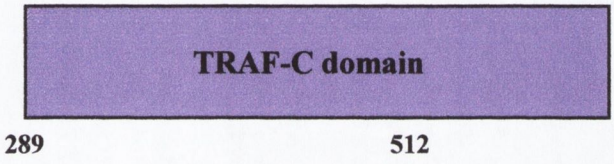
Figure 2.7: Schematic map of expression vectors for wild-type IRAK-1 and dominant negative IRAK-1

Diagrams represent (A) wild-type IRAK-1 and (B) dominant negative IRAK-1, indicating the N terminal death domain and the kinase domain. Dominant negative IRAK-1 encodes the death domain only of IRAK-1. Both were cloned into the insert region of the mammalian expression vector pRSV (C) which is shown here with the RSV long terminal repeat (RSV LTR) and the restriction sites in the multiple cloning site (MCS) shown.

(A) Wild-type TRAF-6



(B) Dominant negative TRAF-6



(C) pRK5 expression vector

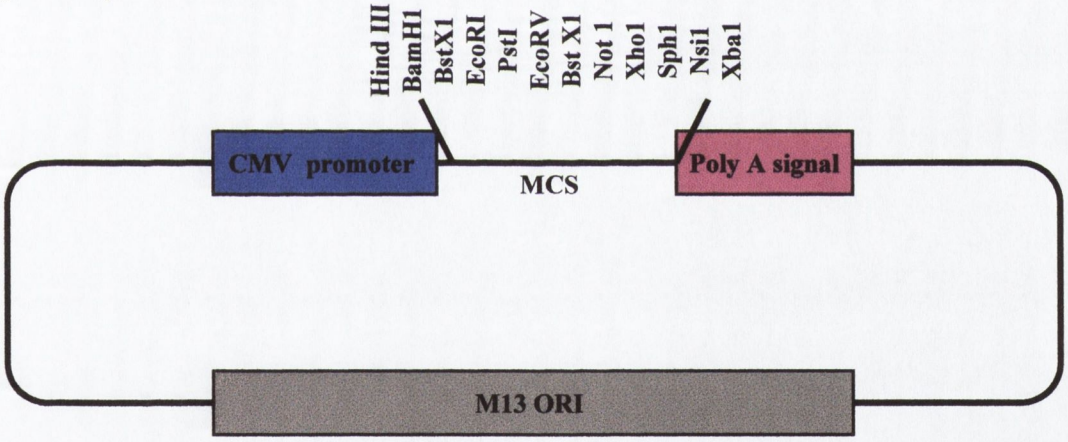


Figure 2.8: Schematic map of expression vectors for wild-type TRAF-6 and dominant negative TRAF-6

Diagrams represent (A) wild-type TRAF-6 and (B) dominant negative TRAF-6, indicating the N terminal AU1 tag, death domain and Toll/IL-1 receptor (TIR) domain. Dominant negative MyD88 has a carboxy terminal deletion removing the TIR domain. Both were cloned into the insert region of the mammalian expression vector $pCDNA_3$ (C) which is shown here with the cytomegalovirus (CMV) promoter region and the restriction sites in the multiple cloning site (MCS) shown.

2.4 Plasmid purification

2.4.1 Preparation of competent cells for transformation

Working aseptically, 1ml of an overnight 2ml culture of *E.coli* DH5α was added to 50ml of L-broth and grown at 37°C in an orbital incubator (200rpm) until OD_{500} reached 0.6. Cells were centrifuged at 6000xg at 4°C for 10 minutes and then resuspended in 500µl ice-cold 100mM $CaCl_2$. Cells were left on ice for 20 minutes.

2.4.2 Plasmid transformation

0.1µg of plasmid DNA was added to 100µl of competent cells and left on ice for 15 minutes. The cells were then heat-shocked for 2 minutes at 43°C and cooled on ice for 2 minutes. Cells were transferred to warm L-broth and incubated at 37°C for 1 hour. 100µl aliquots were plated out onto L-agar containing L-Ampicillin and grown 16-18 hours at 37°C. Transformed cells were then single colony purified and used to purify plasmids for transfection.

2.4.3 Plasmid purification procedure

Plasmids were purified using QIAGEN Midi- or Maxi-prep Plasmid purification protocol based on a modified alkaline lysis procedure. Volumes for Maxi kits are given in brackets. 25ml (100ml) of L-broth (amp) was inoculated with a single colony from single purification step above. Cells were grown to a density of OD_{600} 1-1.5 ($1x10^9$ cells) at 37°C in an orbital shaker (approximately 16 hours of growth). Cells were harvested by centrifugation at 6000xg for 10 minutes at 4°C. Pellets were resuspended in 4ml (10ml) chilled resuspension buffer (50mM Tris-HCL, pH 8; 10mM EDTA; 100µg/ml RNaseA). Cells were lysed for 5 minutes in 4ml (10ml) lysis buffer (200mM NaOH, 1% SDS) at room temperature and the reaction stopped using 4ml (10ml) neutralisation buffer (3M potassium acetate, pH 5.5), incubating the cells at 4°C for 15 minutes. To avoid localised potassium dodecyl sulfate precipitation, the solution was mixed

thoroughly immediately after the addition of neutralisation buffer by inverting the tube several times. The sample was then centrifuged at 20,000xg for 45 minutes at 4°C. The supernatant was removed immediately and applied to a QIAGEN column which had been equilibrated with 4ml (10ml) equilibration buffer (750mM NaCl; 50mM MOPS, pH 7; 15% ethanol). The column was washed 3 times with 4ml (10ml) wash buffer (1M NaCl; 50mM MOPS, pH 7; 15% ethanol) and the bound plasmid eluted using 2.5ml (15ml) elution buffer (1.25M NaCl; 50mM Tris-HCl, pH 8.5; 15% ethanol), passed twice through the column in the case of the Midi-prep kit. The DNA was precipitated using 0.7 volumes of room temperature isopropanol and collected by centrifugation for 30 minutes at 12,000xg. The pellet was washed twice with 70% ethanol and the DNA resuspended in a suitable volume of sterile H_2O (approx. 300µl for Midi and 1ml for Maxi-prep). The concentration and purity of the DNA was determined on a 0.8% agarose gel stained using ethidium bromide.

2.5 Transient transfection

2.5.1 Transfection of cells using DEAE-dextran

DEAE-dextran was used to transfect the murine thymoma cells EL4.NOB-1. Cells were seeded at a density of $2.5x10^5$ per ml in 100ml complete medium per transfection the day before to ensure that they were in log phase of growth prior to transfection. Aliquots of $1.4x10^7$ cells were used per transfection and washed twice in phosphate buffered saline (PBS: 1.45M NaCl, 39mM NaH_2PO_4, 0.23M Na_2HPO_4, pH 7.5) pre-warmed to 37°C. Plasmid DNA (concentrations and combinations of plasmids used in particular experiments indicated in the results section) was made up to 600µl with TBS (25mM Tris, pH 7.4, 137mM NaCl, 5mM KCl, 0.7mM $CaCl_2$, 0.5mM $MgCl_2$) and 40µg/ml Chloroquine added. In all cases the amount of DNA used per transfection was normalised using the appropriate amount of relevant empty vector control. DEAE-Dextran was made up to 10mg/ml concentration with H_2O and filter sterilised. 30µl of this mixture was added

to 570µl of TBS per transfection. These were then mixed and used to resuspend $1.4x10^7$ cells. Cells were incubated at room temperature for 30 minutes, shaking every 2 minutes. Cells were washed once with 10mls complete medium containing 5U/ml heparin followed by two washes with complete medium alone. Cells were resuspended and allowed to recover for 16-18 hours in complete medium supplemented with 20% FCS. Cells were then counted as described before and percentage recovery and cell viability determined. Smaller scale transfections were performed in eppendorfs using 50% the number of cells, 50% of all volumes given above and half the amount of DNA per transfection.

2.5.2 Transfection of cells using calcium phosphate

Calcium phosphate was used to transfect HeLa and 293 cell lines. Briefly, cells were set up 24 hours prior to transfection in 10cm tissue culture plates at a density that ensured that they were no greater than 70% confluent on the day of transfection ($1x10^5$/ml) as the transfection efficiency using calcium phosphate is critically dependent on the amount of surface area exposed to the calcium phosphate-DNA precipitate. Prior to transfection the medium was replaced with 9mls fresh medium. Solution I was prepared by adding 250µl 2x HBS (280mM NaCl, 10mM KCl, 1.5mM $Na_2HPO_4.2H_2O$, 12mM Dextrose, 50mM Hepes, final pH 7.1). In a second eppendorf the DNA to be transfected was made up to a volume of 200µl using sterile, double-distilled water. 50µl of $CaCl_2$ was added to the DNA (solution II). Solution II was added to solution I dropwise and the mixture incubated at room temperature for 30 minutes after which time it was added dropwise to the cells, ensuring that the total surface area of the plate was covered. Cells were incubated with the calcium phosphate-DNA mixture for 4-6 hours after which time the medium was replaced with 10mls of fresh medium. The cells were allowed to recover for 48 hours prior to treatment. The volumes and composition of solution I and II used in larger or smaller scale transfections are given in table 3.

Volumes of Solutions I and II to be used in smaller and larger scale transfections:

Tissue Culture Plate:	**10cm**	**6 well**	**24 well**	**96 well**
Diameter	10cm	3.5cm	1.5cm	0.75 cm
DNA	5-10µg	1-5µg	Upto 2µg	Upto 1µg
H_2O	200µl	80µl	31µl	16µl
$CaCl_2$	50µl	20µl	9µl	4µl
2xHBS	250µl	100µl	40µl	20µl
Total Volume	500µl	200µl	80µl	40µl

Table 3: Calcium Phosphate transfection components

2.6 Protein concentration determination

Protein determination was carried out by the method of Bradford (21). Samples were diluted 1:10 with Tris-HCL to give a final volume of 20µl and 200µl Bradford reagent (0.01% w/v Coomassie Brilliant Blue G-250, 4.7% v/v ethanol, 8.5% v/v orthophosphoric acid) was added to each. The reaction was allowed to develop for 5 minutes after which time the plates were read at OD_{570} using a Dynatech MR5000 plate reader. Protein concentrations were determined using a standard curve constructed using BSA concentrations in the range 0-20 µg/20µl. A typical standard curve is shown in figure 2.9.

2.7 Reporter gene assays

2.7.1 Determination of CAT reporter gene activity

Cells were seeded at a density of $1x10^6$ per ml in a 24 well plate and stimulated with IL-1 (10ng/ml) for 24 hours. Cells were centrifuged at 250xg and the supernatant removed for IL-2 determination by ELISA as described below. Cells were washed once with PBS and then resuspended in 1ml PBS and transferred to eppendorfs. All subsequent steps were performed on ice. Cells were pelletted, resupended in 80µl Tris-HCL and then lysed by freeze-thawing three times in liquid nitrogen. The lysates were then centrifuged for 5 minutes at 12,000xg for 5 minutes at 4°C and the supernatant removed for protein determination. For each sample 40µg of protein was made up to 79.5µl with 0.25M Tris-HCL (pH 8). 9µl of 10mM Acetyl CoA and 3µl [^{14}C]Chloramphenicol (0.3µCi) were added to each sample and vortexed briefly to mix. Samples were incubated at 37°C for 16-18 hours. The reaction was terminated by the addition of 350µl of ethyl acetate to each sample. Samples were vortexed for 1 minute to abstract all forms of chloramphenicol into the ethyl acetate layer and the layers were separated by centrifugation at 12,000xg for 1 minute at 4°C. 300µl of ethyl acetate was removed to fresh eppendorfs and the

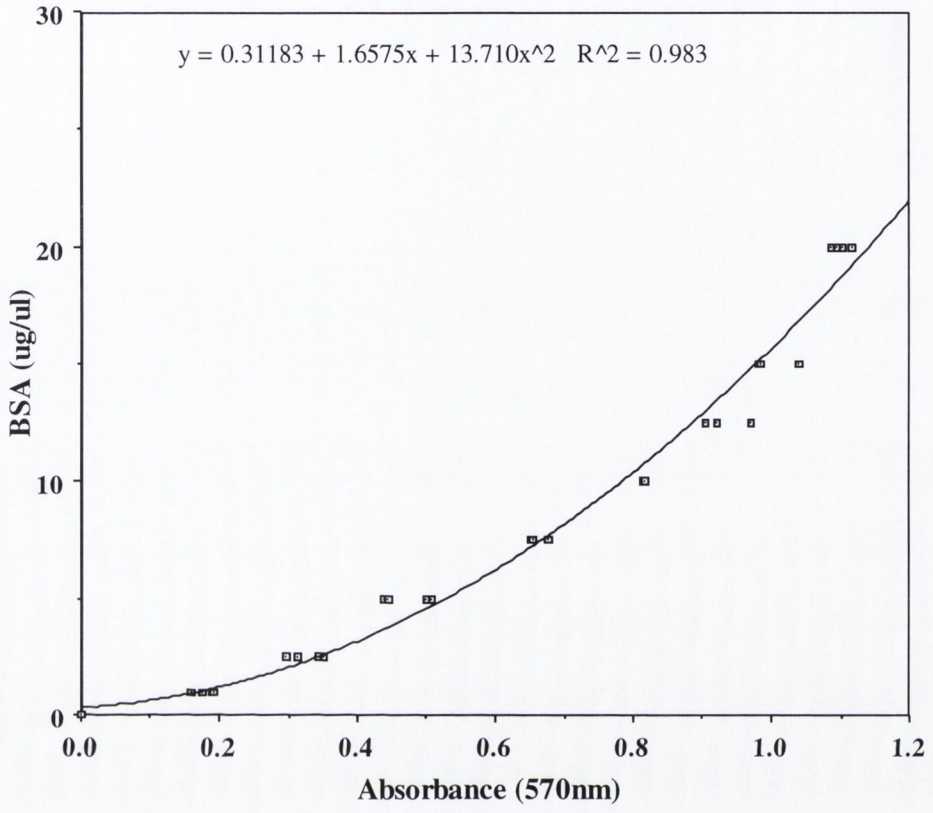


Figure 2.9: Bradford standard curve relating protein concentration to absorbance at 570nm.

Absorbance was measured 5 minutes following addition of Bradford reagent to concentrations of Bovine Serum Albumin (BSA) in the range 0.1 to 20µg/µl.

samples freeze-dried. The samples were resuspended in 12µl ethyl acetate and spotted onto a thin layer chromatography plate. The plate was resolved with 19:1 (v/v) chloroform/methanol and then analysed using a Packard Instant Imager which directly reads counts from TLC plates. Percentage acetylation was determined according to the following equation:

$$\% \text{ acetylation} = \frac{\text{Acetylated chloramphenicol (cpm)}}{\text{Total chloramphenicol (cpm)}}$$

2.7.2 Determination of luciferase reporter gene activity

Cells transfected with NFκB-luciferase or Gal4-luciferase were stimulated with IL-1 for 3 and 6 hours, respectively. Cells were centrifuged (1200xg) for 5 minutes at room temperature and washed once with PBS. After removal of PBS cells were lysed using the appropriate amount (50µl for 96 well plates, 200µl for 24 well plates) of a 1:5 dilution of passive lysis buffer (Promega) in water according to manufacturer's instructions and incubated at room temperature with vigorous shaking. Following centrifugation of plates for 10 minutes (2500xg) at room temperature, 50% of the supernatant was used to determine luciferase activity and an equivalent amount retained for either protein concentration determination or β-galactosidase activity. Luciferase activity was assayed by the addition of 40µl of luciferase assay mix (20mM tricine, 1.07mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT, 270mM coenzyme A, 470mM luciferin, 530mM ATP) to the sample and luminesence read using Mediators PHL luminometer. Luminesence readings were corrected for either protein concentration or β-galactosidase activity and expressed as fold increase over unstimulated control values.

2.7.3 Determination of β-galactosidase activity

β-galactosidase (β-gal) activity was assayed by the addition of 40µl O-nitrophenylgalactosidase (4mg/ml) to the sample volume retained above, followed by the addition of 140µl of β-gal buffer (23mM NaH_2PO_4, 77mM Na_2HPO_4, 0.1mM $MnCl_2$, 2mM $MgSO_4$, 40mM β-mercaptoethanol, pH 7.3) to each sample.

2.8 IL-2 ELISA

2.8.1 Preparation of samples

Cells were seeded at a density of $1x10^6$ ml^{-1} in a 24 well plate, and stimulated with IL-1 for 24 hours. The cells were centrifuged at 250xg and the supernatants removed for IL-2 determination.

2.8.2 IL-2 ELISA protocol

A 96 well plate was coated using a monoclonal rat anti-mouse IL-2 antibody diluted to 4µg/ml in coating buffer (0.1M $NaHCO_3$. pH 8.2). The plate was covered and incubated overnight at 4°C and then washed twice with PBS/0.5% polyoxyethylenesorbitan (Tween-20), allowing plates to stand for 1 minute prior to removing wash solution. Plates were pounded dry on paper towels as a final step. The plate was blocked with PBS/10% FCS at 200µl per well and incubated at room temperature for 2 hours. The plate was washed twice and dried as before. IL-2 standards (in the range 0.1 - 10ng/ml) and samples were added at 100µl per well and incubated for 4 hours at room temperature or overnight at 4°C. Plates were then washed four times with PBS/Tween and dried as above. Biotinylated rat anti-mouse IL-2 monoclonal antibody was diluted to 2µg/ml in PBS/10% FCS and 100µl added per well. The plate was incubated at room temperature for 45 minutes and then washed six times as before. Avidin-peroxidase (1mg/ml) was diluted 1:400 in PBS/10% FCS and 100µl added per well. Plates were covered and incubated at room temperature

for 30 minutes and subsequently washed eight times as before. 0.3mg ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) per ml of freshly made 0.1M citric acid buffer (pH 4.3) was prepared 5 minutes prior to use. Immediately before use, 1µl of 30% H_2O_2 per ml of substrate solution was added, the solution vortexed and 100µl added per well. The reaction was allowed to develop in the dark, the plate being read after 30 and 60 minutes at OD_{405} using a Dynatech MR5000 plate reader. A standard curve was constructed relating IL-2 concentration to absorbance at 405nm and was used to determine the concentration of IL-2 in unknown samples. A typical standard curve is shown in figure 2.10.

2.9 Total RNA isolation

Cells were seeded at a density of $1x10^6$/ml and stimulated with 10ng/ml IL1 for 6 hours prior to extraction of RNA. Total cell RNA was extracted from EL4.NOB-1 via the Triazole reagent (TRI-reagent) method. Cells were centrifuged at 1400xg for 5 minutes and the supernatant removed. Pellets were resuspended in 1ml TRI-reagent (250g guanidinium thiocyanate, 293ml 0.1% v/v aqueous diethylpyrocarbonate (DEPC) solution, 17.6ml 0.75M sodium citrate (pH 7), 26.3ml 10% sarcosyl) and lysed by repeat pipetting. 0.2ml chloroform was added and the solution mixed for 15 seconds before being allowed to stand at room temperature for 15 minutes. The mixture was then centrifuged at 12,000xg, 4°C, 15 min. The upper aqueous phase containing total RNA was removed to a fresh eppendorf and 0.5ml isopropanol added. The solution was mixed and allowed to stand at room temperature for 10 min prior to centrifugation at 12,000xg (4°C, 10 minutes). The resulting pellet was washed with 1ml 75% (v/v) ethanol (in 0.1% (v/v) aqueous DEPC solution) and allowed air dry. 50µl 0.1% v/v DEPC solution was used to resuspend the pellets. The absorption of 1µl of the RNA solution at 260nm and 280nm was used to quantify the amount of RNA present and to measure its purity (ratio of A260:A280). The solution was stored at –70°C until required.

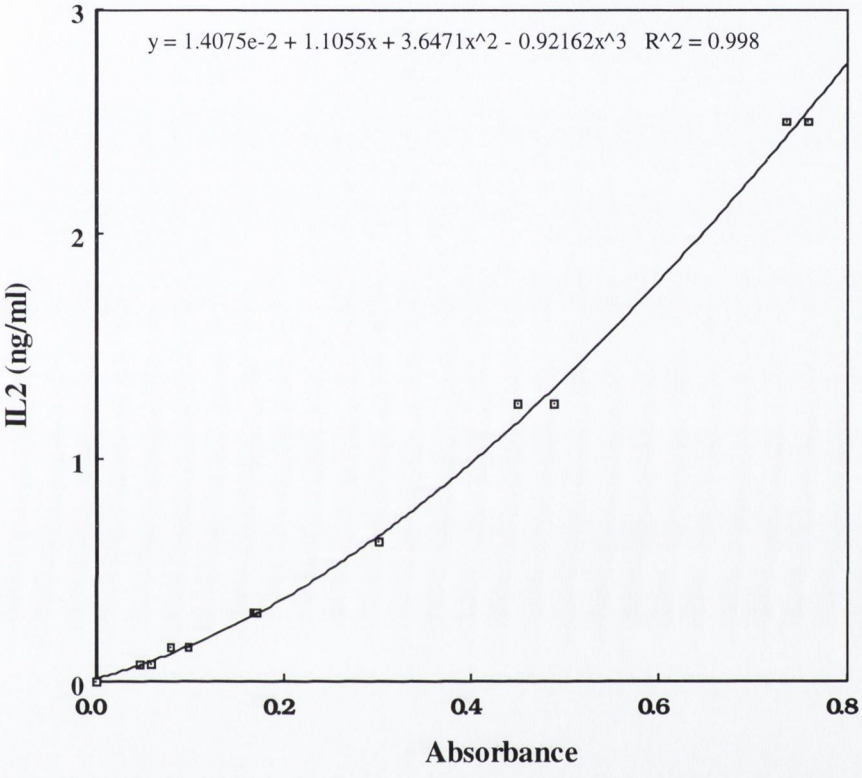


Figure 2.10: IL-2 standard curve relating IL2 protein concentration to absorbance at 405nm.

A standard curve was constructed as described in the methods section and was used to determine IL-2 concentrations from unknown samples. A representative standard curve is shown.

2.10 RT-PCR

One step RT-PCR was carried out using the Titan RT-PCR kit (Boehringer Mannheim GmbH, Mannhein, Germany) according to manufacturers instructions. The primers used were synthesised by MWG-Biotech (Milton Keynes, U.K.). The IL-2 primers chosen to amplify a 412 base pair product from the IL-2 transcript were as follows:

Forward: 5'-AAGCTCCACTTCAAGCTCTACAGCG-3'

Reverse: 5'-TTGACAGAAGGCTATCCTCTCCTC-3'

In addition the following primers were used to amplify a 464 base pair product from the house-keeping gene, aldolase, to ensure equal efficiency of reaction:

Forward: 5'-TTCCACGAGACACTGTACCAGAAGG-3'

Reverse: 5'-ACCATGTTGGGCTTCAGCAATG-3'

Two master mixes were made up in sterile 0.2ml PCR tubes for each of the samples as shown in table 4. Master mix 2 was added to master mix 1 and overlaid with 30µl mineral oil. The mixture was then thermocycled in a PTC-15016 Minicycler, purchased from MJ Research Inc, Watertown, Massachusetts, USA, according to the following program:

Step 1: **Denaturation of Template RNA**

1x 2 min at 94°C

Step 2: **Reverse transcription of template RNA to cDNA**

10x 30 sec at 94°C (denaturation)

30 sec at 55°C (annealing)

1 min at 68°C (elongation)

Master Mix 1:

Component	Volume	Final Concentration
ddH_20	Up to 25µl	
dNTPs	1µl	0.2mM
Forward primer (20µM)	0.2µl	0.3µM
Reverse primer (20µM)	0.2µl	5mM
DTT 100mM	2.5µl	0.4 Units/µl
RNasin (40U/µl)	0.25µl	25ng/µl
Template RNA	Vol. equal to 1µg	
Total Volume:	**25µl**	

Master Mix 2:

Component	Volume	Final Concentration
ddH_2O	Up to 25µl	
5x RT-PCR buffer (with Mg^{2+})	10µl	1.5mM $MgCl_2$
Enzyme mix (AMV reverse transcriptase, Taq DNA polymerase, Pwo DNA polymerase)	1µl	
Total Volume	**25µl**	

Table 4: The components of the RT-PCR reaction mixtures

Step 3: **PCR reaction**

25x 30 sec at 94°C (denaturation)

30 sec at 55°C (annealing)

1 min at 68°C (elongation)

The products were electrophoresed on a 1% w/v agarose gel containing 2.5µl ethidium bromide (with EcoR1/Hind III digest molecular weight markers), and visualised under UV light.

2.11 Electrophoretic mobility shift assay (EMSA)

2.11.1 Preparation of nuclear extracts

Cells ($5x10^6$) were treated with IL-1 as described in the figure legends. Stimulation was terminated by transferring cells to 5mls of ice-cold PBS. All subsequent steps were performed on ice using ice-cold buffers. Nuclear extracts were prepared as described by Osborn *et al* (142). Cells were pelletted by centrifugation (12,000xg) and then resuspended in 1ml hypotonic buffer (10mM Hepes buffer (pH 7.9) containing 1.5mM $MgCl_2$; 10mM KCl; 0.5mM Dithiothreitol (DTT); 0.5mM phenylmethylsulphonylflouride (PMSF)). Samples were centrifuged for 10 minutes at 4°C (13,000xg) and then lysed by resuspending the resulting pellets in 20µl hypotonic buffer containing 0.1% (v/v) Nonidet P-40. Samples were incubated on ice for 10 minutes. The nuclei were pelletted by centrifugation at 13,000xg for 10 minutes at 4°C. The nuclei were then resuspended in 20µl nuclear lysis buffer (20mM Hepes buffer (pH 7.9) containing 1.5mM $MgCl_2$; 420mM NaCl; 0.2mM EDTA; 25% v/v glycerol; 0.5mM PMSF) and incubated on ice for 15 minutes. Lysed nuclei were centrifuged (13,000xg, 10 minutes, 4°C) and the supernatant removed to 75µl storage buffer (10mM Hepes buffer (pH 7.9) containing 50mM KCl, 0.2mM EDTA, 20% v/v glycerol, 0.5mM DTT, 0.5mM PMSF). 5µl of each sample was diluted 1:4 using 0.25M Tris-HCl (pH 8) and protein content determined as described previously.

2.11.2 Electrophoretic mobility shift assay

Nuclear extracts were analysed for NFκB activity by electrophoresis using the method of Sen and Baltimore (1986). 4μg of nuclear extract protein was incubated in a final volume of 25μl at room temperature for 30 minutes with 10,000cpm [^{32}P]-labelled DNA probe in the presence of poly(dI.dC) as non-specific competitor DNA in a binding buffer (40% v/v glycerol; 10mM EDTA; 50mM DTT; 100mM Tris (pH 7.5); 1M NaCl; 1mg/ml BSA). The binding reaction was terminated by the addition of 2μl gel loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol; 15% Ficoll in water). Protein-DNA complexes were separated by electrophoresis in 5% acrylamide gels for 2 hours at 150 volts. The components of the gel are given in Table 5. The gel was pre-run for 30 minutes in running buffer (44.6mM Tris-borate; 1mM EDTA, pH 8). Gels were dried and then autoradiographed using Kodak X-OMAT diagnostic film at –70°C in an autoradiography cassette with intensifying screens.

2.12 Western blot analysis

2.12.1 Preparation of cell extracts

Cells were seeded at a density of 1x10^6 per ml and stimulated with IL1 (10ng/ml) for 30 minutes. Reactions were terminated by the addition of ice-cold PBS to the cells followed by centrifugation at 150xg for 5 minutes. Pellets were resuspended in 1ml PBS and transferred to eppendorfs. The samples were centrifuged at 12000xg (5 minutes, 4°C) and the pellets resuspended in 150-250μl of RIPA buffer (PBS buffer containing 1% v/v Nonidet P40, 0.5% w/v sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS)) containing 10μg/ml PMSF, 30μl/ml aprotonin and 10μl/ml sodium orthovanadate. Following gentle mixing, samples were incubated on ice for 30 minutes. The cells were further disrupted by passage through a 21-gauge needle (10 strokes). A further 10μg/ml Aprotonin was added to each sample at this point and the samples incubated on ice for an additional 30 minutes. The samples were then centrifuged at 13,000xg for 20 minutes at 4°C.

Component	Volume/Amount added
40% Acrylamide:bisacrylamide (29:1)	3.125ml
10x TBE (0.89M Tris, 20mM EDTA)	2.5ml
H_2O	19.4ml
1M DTT	5µl
TEMED	30µl
Ammonium persulphate	0.05g

Table 5: Constituents of native polyacrylamide gel used for EMSA

The supernatants (total cell lysate) were assayed for protein as described previously and aliquots containing equal amounts of protein were mixed with 5µl sample buffer ((5ml glycerol, 10ml 10% SDS, 10mg bromophenol blue, 6.25ml 1M Tris (pH 6.8), 28.75ml water) 250µl β-mercaptoethanol was added per ml of sample buffer). Samples were made up to 20µl with RIPA buffer and boiled for 3 minutes.

2.12.2 Preparation and running of gels

Samples were resolved by electrophoresis on a 10% SDS polyacrylamide gel. The resolving gel (10%) was prepared using 5ml 30% bisacrylamide mix (Protogel), 3.75ml 1.5M Tris (pH 8.8), 0.15ml 10% SDS, 0.15ml 10% ammonium persulphate and 6µl of TEMED made up to 15ml with water. The stacking gel (5%) was prepared using 1ml 30% Protogel, 0.75ml 1M Tris (pH 6.8), 0.06ml 10% SDS, 0.06ml 10% ammonium persulphate and 6µl of TEMED, made up to 6ml with water. The samples were electrophoresed at 25mA per gel. Table 6 shows the volumes of gel components required to make the various percentage gels.

2.12.3 Transfer of proteins to membrane

The resolved proteins were transferred to either nitrocellulose or polyvinylidene difluoride (PVDF) using a wet transfer system with all components soaked beforehand in transfer buffer (25mM Tris-HCl (pH 8.0), 0.2M glycine, 20% methanol). Briefly, the gel was placed on a layer of filter paper and sponge and overlaid with nitrocellulose paper. A second piece of filter paper was placed on top followed by the second sponge. The entire assembly was placed in a cassette and an electric current of 150mA was applied for 2 hours.

Running Gel

	6%	8%	10%	12%	15%
Acrylamide:Bisacrylamide	3ml	4ml	5ml	6ml	7.5ml
H_2O	7.9ml	6.9ml	5.9ml	4.9ml	3.4ml
1.5M Tris-HCL (pH 6.8)	3.8ml	3.8ml	3.8ml	3.8ml	3.8ml
10% SDS	0.15ml	0.15ml	0.15ml	0.15ml	0.15ml
10% APS	0.15ml	0.15ml	0.15ml	0.15ml	0.15ml
TEMED	6µl	6µl	6µl	6µl	6µl

Stacking Gel

Acrylamide:Bisacrylamide	1ml
H_2O	4.1ml
1M Tris-HCL (pH 8.8)	0.75ml
10% SDS	60µl
10% APS	60µl
TEMED	6µl

Table 6: Composition of stacking and resolving SDS-PAGE gels

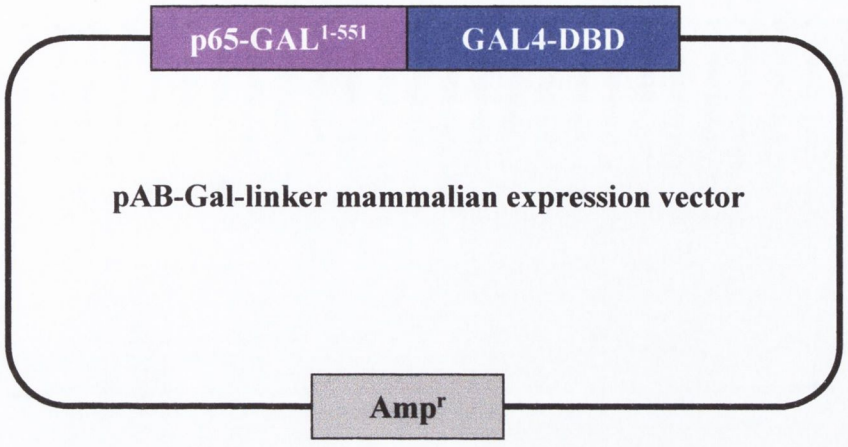
2.12.4 Detection of protein of interest

The membrane was incubated in blocking solution (PBS containing 5% w/v non-fat milk powder) overnight at 4°C. The membrane was then washed twice (5 minutes per wash) with TBS containing 0.5% (v/v) Tween-20 (TBS-T) and incubated with an antibody against the protein of interest (diluted appropriately in 5% non-fat dried milk in TBS-T) for 1 hour at room temperature. Following 3 washes with TBS-T (2x 5 minutes, 1x 15 minutes) the membrane was incubated with an anti-mouse IgG peroxidase or anti-rabbit IgG peroxidase conjugate as appropriate (1:1000 dilution prepared in TBS-T containing 1% non-fat dried milk powder) for 1 hour, room temperature. The membrane was subsequently washed twice with TBS-T (5 minutes each) and once with TBS (5 minutes). Blots were developed by enhanced chemiluminescence (ECL) according to manufacturers instructions (Amersham).

2.13 Gal4-p65$^{1-551}$ one-hybrid assay

This assay has been devised to examine transactivation of gene expression by the p65 subunit of NFκB. It comprises two components: firstly a mammalian expression vector encoding the p65 subunit of NFκB fused to the DNA binding domain of the yeast transactivator, GAL4 (figure 2.11), and secondly a plasmid containing the GAL4 consensus binding site (upstream activator sequence) linked upstream of the luciferase reporter gene (figure 2.12). Once transfected into cells (using the methods described above) Gal4-p65$^{1-551}$ was expressed and bound to its consensus site on the Gal4-luciferase reporter gene. However, the reporter gene was not expressed until the appropriate signal was received that activated the transcriptional activity of p65 (figure 2.12). Following transfection (by the methods described above) cells were allowed to recover for a period of 16-18 hours after which time they were stimulated with IL-1 as indicated for 6 hours. Luciferase activity was determined as previously described.

(A) p65-gal$^{1-551}$



(B) Gal-luciferase

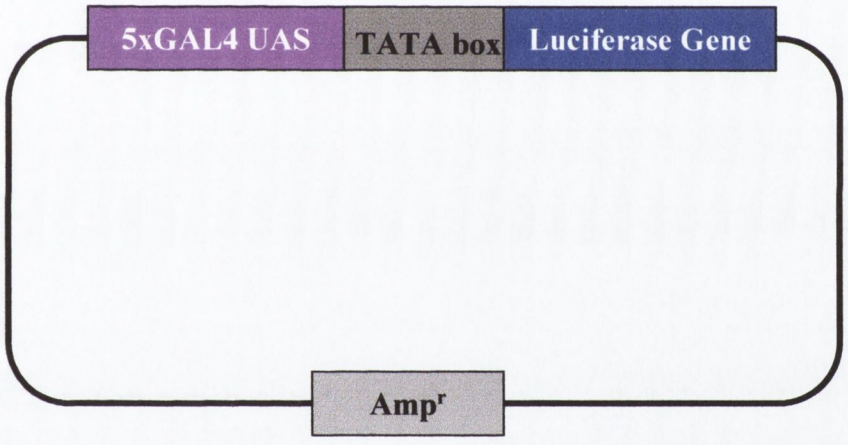


Figure 2.11: Schematic representation of the plasmids used in the p65 one hybrid assay

(A) The p65-GAL$^{1-551}$ expression plasmid was constructed by inserting p65 (1-551) into the pAB-Gal-linker mammalian expression vector. (B) The GAL4-responsive promoter contains 5 upstream activating sequences (UAS) upstream of the TATA box. The entire promoter region is placed upstream of the luciferase reporter gene.

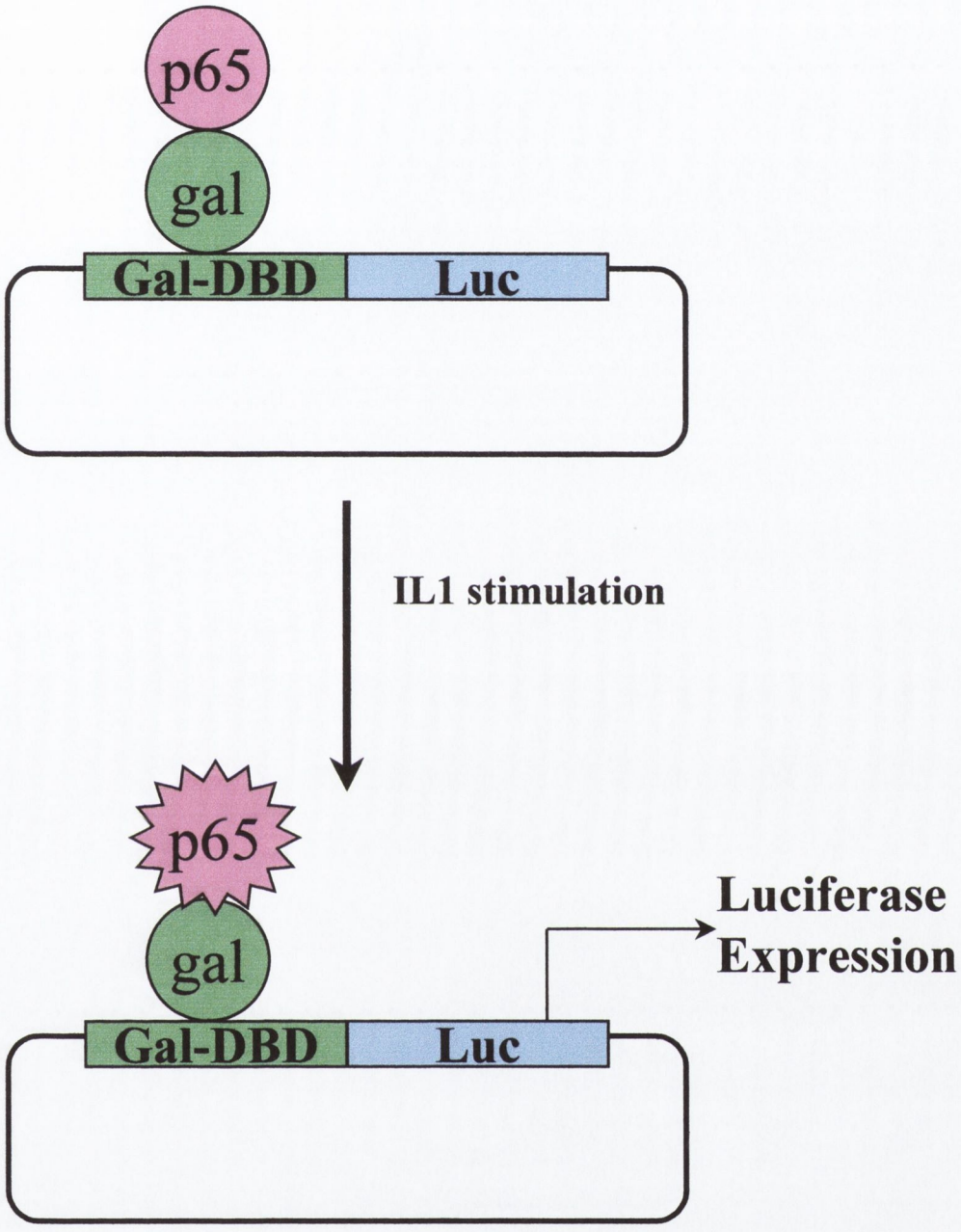


Figure 2.12: Schematic representation of p65 one hybrid assay.

2.14 Purification of fusion proteins

50ml of L-broth containing ampicillin (LB-Amp) was innoculated with a 5ml overnight culture which had been inoculated with BL21 strain of *E.coli* transformed with the GST-fusion protein to be purified and grown overnight at 37°C in an orbital incubator. The following day 50ml of the culture was added to 500ml of LB-Amp and incubated for 1 hour at 37°C after which time the fusion protein was induced by the addition of IPTG (100μM) to the culture. Following incubation of the culture for three hours the bacteria were pelleted by centrifugation (15 minutes, 4000xg). The pellet was resuspended in $1/50^{th}$ the original culture volume with ice-cold lysis buffer (20mM Tris, 100mM NaCl, 1mM EDTA, pH 8.0, 0.5% NP40, 1mg/ml lysozyme, 10μg/ml PMSF, 30μl/ml aprotonin and 10μg/ml sodium orthovanadate). The bacteria were lysed by sonication on ice and the debris pelleted by centrifugation (10,000xg, 5 minutes, 4°C). The supernatant was added to 1ml of glutathione-S-agarose beads (70% mixture in lysis buffer) and incubated with rotation at 4°C for 30 minutes. The beads were washed three times with lysis buffer and the GST-fusion protein coupled to the beads stored at 4°C as a 50% slurry in lysis buffer.

2.15 Rac1 activation assay

EL4.NOB-1 cells ($2x10^7$) were stimulated with IL-1 for various time points as outlined in the figure legends. Stimulation was terminated by washing cells with ice-cold PBS followed by lysis in 1ml ice-cold lysis buffer (25mM HEPES, pH 7.5, 1% nonidet P-40, 0.25% deoxycholate, 10% glycerol, 10mM $MgCl_2$, 150mM NaCl, 1mM phenylmethylsulphonyl flouride, 10mM Na_3VO_4, 2mg/ml aprotonin) incubated for 30 minutes at 4°C. Cells were cleared by centrifugation and the supernatants normalised for protein content using the method of Bradford to determine protein concentration. Lysates containing equal amounts of protein were incubated for 1 hour at 4°C with approximately 10μg of GST-PAK pre-coupled to glutathione agarose beads. The bead pellet was

washed three times in lysis buffer and finally resuspended in 25µl of Laemmli sample buffer. Proteins were separated by electrophoresis using a 15% SDS-PAGE gel. Following transfer of proteins to PVDF membrane, the association of activated Rac1 with GST-PAK was determined by western blotting using an anti-Rac1 antibody (Upstate Biotechnology, Lake Placid, N.Y.).

2.16 Immunoprecipitation

Cells were treated as described in the figure legends and treatment terminated by washing cells with ice-cold PBS. Cells were lysed on ice (30 minutes) in 1ml lysis buffer containing 25mM Tris-HCl, pH 7.4, 150mM NaCl, 2mM EDTA, 1% nonidet P-40, 1mM phenylmethylsulphonyl flouride, 10mM Na_3VO_4, 2mg/ml aprotonin. Lysates were cleared by centrifugation (12,000xg, 5 minutes, 4°C) and the protein concentration of the supernatant determined by the method of Bradford. Samples normalised for protein content were pre-cleared with 30µl of protein A-insoluble (30 minutes, 4°C) to remove any proteins that non-specifically bind to protein A. Following precipitation of protein A-insoluble, 2-5µg of antibody against the protein being immunoprecipitated was added to the supernatant and incubated at 4°C for 2 hours. Immunocomplexes were precipitated by the addition of 30µl protein A-sepharose beads and incubating samples for a further 45 minutes at 4°C. Pelletted beads were subsequently washed three times with lysis buffer and finally resuspended in 25µl of Laemmli sample buffer. Proteins were separated by SDS-PAGE electrophoresis and following transfer of proteins to PVDF membrane, the precipitated immunocomplexes were analysed by western blotting.

2.17 PAK kinase assay

2.17.1 Transfection of cells and Preparation of cell extracts

HeLa cells were seeded in 10cm tissues culture plate at a density of $1x10^5$/ml 24 hours prior to transfection. Cells were transfected with 10mg of a mammalian expression plasmid encoding HA-PAK per plate using calcium phosphate. Cells were allowed to recover for 48 hours after which time they were stimulated with IL-1 (10ng/ml) for various time points. Stimulation was terminated by washing cells with ice-cold PBS followed by lysis of the cells on ice for 30 minutes in 1ml lysis buffer (25mM Tris-HCl, pH 7.4, 150mM NaCl, 2mM EDTA, 1% nonidet P-40, 1mM phenylmethylsulphonyl flouride, 10mM Na_3VO_4, 2mg/ml aprotonin).

2.17.2 Immunoprecipitation of HA-PAK

Lysates were cleared by centrifugation (12,000xg, 5 minutes, 4°C) and the protein concentration of the supernatant determined by the method of Bradford. Samples containing equal amounts of protein were pre-cleared with 30µl of protein A-insoluble (30 minutes, 4°C) to remove any proteins that non-specifically bind to protein A. Following precipitation of protein A-insoluble, 2-5µg of anti-HA antibody was added to the supernatant and the samples incubated at 4°C for 2 hours. Immunocomplexes were precipitated by the addition of 30µl protein A-sepharose beads and incubating samples for a further 45 minutes at 4°C.

2.17.3 Kinase assay

Pelletted beads were subsequently washed three times with lysis buffer and twice in kinase buffer (25mM HEPES, pH 7.5, 5mM β-glycerophosphate, 2mM DTT, 0.1mM Na_3VO_4, 10mM $MgCl_2$). The kinase reaction comprised protein A sepharose-bound HA-PAK, 10µl kinase buffer, 5µg myelin basic protein (MBP), 60µM ATP (final volume of 15µl). The kinase reaction was performed for 30 minutes with mixing at 37°C and was terminated by the addition of 5µl of

sample buffer to the beads. Phosphorylated MBP was detected by western blot analysis using an anti-phospho MBP antibody.

2.18 Affinity purification of complexes using GST-fusion proteins

Cells were treated as described in the figure legends and treatment terminated by washing cells with ice-cold PBS. Cells were lysed on ice (30 minutes) in 1ml lysis buffer containing 25mM Tris-HCl, pH 7.4, 150mM NaCl, 2mM EDTA, 1% nonidet P-40, 1mM phenylmethylsulphonyl flouride, 10mM Na_3VO_4, 2mg/ml aprotonin. Lysates were cleared by centrifugation (12,000xg, 5 minutes, 4°C) and the protein concentration of the supernatant determined by the method of Bradford. Lysates containing equal amounts of protein were incubated for 1 hour at 4°C with approximately 10µg of GST-fusion protein (or 10µg of GST alone as a control) pre-coupled to glutathione agarose beads. The bead pellet was washed three times in lysis buffer and finally resuspended in 25µl of Laemmli sample buffer. Proteins were separated by SDS-PAGE electrophoresis and following transfer of proteins to PVDF membrane, the precipitated immunocomplexes were analysed by western blotting.

Chapter Three

Activation of the GTPase Rac1 by IL-1 and its involvement in regulating IL-2 gene expression

3 Chapter three

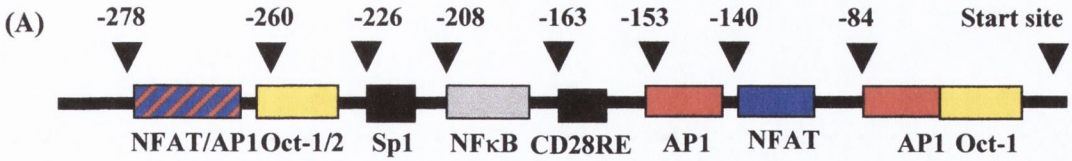
3.1 Introduction

Upon stimulation of cells with IL-1 a number of signalling pathways are activated, ultimately resulting in changes in gene expression. Of particular interest to us was the suggestion of a role for low molecular weight G proteins in IL-1 signal transduction. In particular a role for the low molecular weight G protein Rac1 has been suggested from work which has shown that dominant negative Rac1 inhibits IL-1-induced activation of both p38 and JNK mitogen activated protein kinase (MAPK) pathways (37, 127, 203).

In its active form Rac1 is bound to GTP, inducing a conformational change that facilitates interaction with its downstream effectors such as p21 activated kinase (PAK) (reviewed in (42)). Inactivation of Rac1 occurs following hydrolysis of GTP to GDP which is catalysed by the association of Rac1 with GTPase activating proteins (GAPs). Conversion of GDP-bound inactive Rac1 to its activated state occurs through the activity of guanine nucleotide exchange factors (GEFs), in particular Tiam1 and Vav, which catalyse the exchange of GDP for GTP. Two mutants of Rac1 have been described which have been extensively used as tools to elucidate the involvement of Rac1 in various signalling pathways (151). Constitutively active RacV12 has a glycine to valine mutation at position 12 of the protein in the domain that interacts with the gamma phosphate of GTP. This mutation prevents the hydrolysis of GTP thus rendering RacV12 constitutively active. RacV12 was initially shown to promote membrane ruffling in fibroblasts via effects on the actin cytoskeleton following microinjection (151). Since this initial observation, constitutively active RacV12 has been demonstrated to activate both JNK and p38 MAPK, thus implicating Rac1 in the pathways regulating these kinases (37, 203). The mutation of threonine 17 to asparagine in the phosphate binding loop of Rac1 inhibits the exchange of GDP with GTP and hence Rac1 remains permanently GDP-bound. The resulting dominant negative mutant, RacN17,

has been demonstrated to prevent both membrane ruffling in cells (151) and IL-1-induced activation of JNK and p38 MAPK (37, 127, 203).

IL-1 was initially described for its ability to act as a co-mitogen for T-cells (61). A primary consequence of IL-1 co-stimulation of T lymphocytes is induction of the cytokine interleukin 2 (IL-2), a crucial regulator of T cell activation (63). As such much work has gone into mapping the promoter region of the IL-2 gene and identifying the transcription factors that bind to it (for review see (165)). The IL-2 promoter contains sites for the inducible transcription factors NFκB, AP-1 and NFAT (figure 3.1a). The pathways regulating these transcription factors are briefly summarized in figure 3.1b. The pathway regulating IL-1-induced activation of NFκB is well characterised and briefly involves a kinase cascade resulting in the phosphorylation and subsequent degradation of the inhibitor of NFκB, IκB, thus allowing entry into the nucleus and DNA-binding of NFκB. In addition to IL-1, ligation of the T cell receptor has been demonstrated to activate NFκB-dependent gene-expression, although the pathway involved is unclear. AP-1 is an inducible transcription factor activated in response to growth factors, hormones, cytokines such as IL-1 and neurotransmitters (reviewed in (160)). It comprises a group of structurally related, bZip containing transcription factors that belong to the cJun and cFos families. In order to be active AP-1 components must dimerise prior to DNA binding. c-Jun proteins can either bind as homodimers or as heterodimers of the various Jun family members wherease c-Fos proteins must dimerise with c-Jun members to be sufficiently stable to bind their consensus site (TRE; triphorbol ester response element). c-Jun is the most potent transcriptional activator and c-Jun/c-Fos heterodimers give rise to highly stable and transcriptionally active complexes. Combinatorial interactions between the different members of c-Jun and c-Fos give rise to dimers with varying activities. Activation of AP-1 occurs as the result of the activation of protein kinase cascades involving Ras and the p42/p44 MAPK pathway, PKC and JNK pathway. In addition oxidative stress has been shown to regulate AP-1 activation. Activation occurs on two levels: the first



(B)

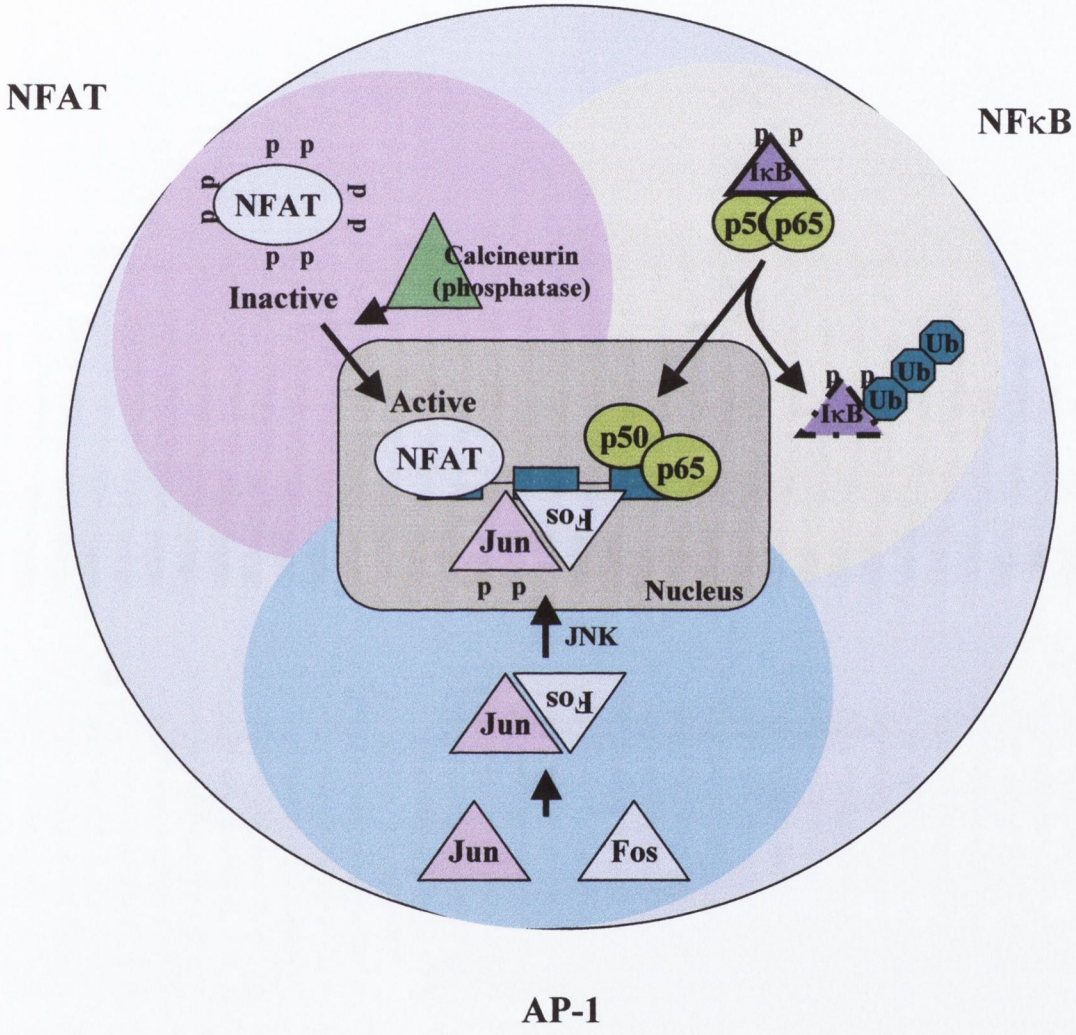


Figure 3.1: Regulation of the transcription factors involved in IL-2 transcription.

(A) Schematic representation of the murine IL-2 promoter and the transcription factors that bind to it. (B) Schematic interpretation of the basic steps involved in regulating NFAT, NFκB and AP-1 activation, all three being key transcription factors responsible for the transcription of IL-2.

involves transcription of c-Fos, an immediate early gene product following growth factor stimulation, involving the transcription factors ELK1 and TCF which bind the serum response element upstream of *c-fos*. The second level of regulation of AP-1 activity occurs following dimerisation of c-Jun/c-Fos and DNA-binding and involves dual phosphorylation of c-Jun on serine residues within its amino terminal transactivation domain that stimulates transcriptional activity. The kinase responsible for this event has been identified as JNK (also termed p54 stress activated protein kinase (SAPK)) which phosphorylates ser-63 and ser-73 in the transactivation domain. p42/p44 MAPK has also been demonstrated to exhibit kinase activity for c-Jun.

The NFAT family of proteins contains four family members (NFAT1-4) that are regulated by the calcium-dependent serine/threonine phosphatase calcineurin (reviewed in (88)). A fifth atypical member, NFAT5, is regulated by osmotic shock rather than by calcineurin and won't be discussed here. NFAT activity is regulated by a cycle of dephosphorylation (activating) and rephosphorylation (inactivating). Increased calcium levels in the cell in response to activation of enzymes such as phospholipase C results in activation of calcineurin. The amino terminal regulatory domain of NFAT, with which it interacts with calcineurin, is hyperphosphorylated in its inactive form and becomes dephosphorylated in a calcineurin dependent manner thus allowing nuclear translocation of NFAT. Once in the nucleus NFAT binds its consensus sequence and may be further regulated by phosphorylation, acetylation or by interactions with other transcription factors or coactivators. In T cells NFAT is activated following T cell receptor ligation and as such much attention has focused on understanding the pathways regulating its activation. A role for Rac1 in regulating NFAT in response to T cell receptor stimulation has been proposed, involving its upstream activator Vav (60).

As IL-1 was initially described for its ability to act as a co-mitogen for T cells, much attention has focused on the signalling events regulating IL-2 production in these cells. An ideal model system for studying IL-1-induced IL-2 production is the murine thymoma cell line EL4.NOB-1 (58). These cells have been demonstrated to respond to IL-1 stimulation alone

without the need for a co-stimulus due to the high level of constitutive expression of IL-1RI on their cell surface thus making them ideal for studying IL-1 signal transduction.

The suggestion that Rac1 is involved in IL-1-induced signal transduction prompted our investigation into the possible role of Rac1 in these pathways. Our initial aim was to establish whether IL-1-stimulation of EL4.NOB-1 cells resulted in Rac1 activation. Having determined that Rac1 is activated following stimulation of cells with IL-1, we next examined its role in IL-1-induced expression of IL-2 in EL4.NOB-1 cells. Constitutively active RacV12 was found to potentiate IL-1-induced IL-2 production and, importantly, the ability of Rac1 to potentiate this response appeared to involve the transcription factor NFκB.

3.2 Results

3.2.1 Activated Rac1 binds its downstream effector PAK1

Studies on IL-1-induced activation of p38 and JNK MAPK pathways suggested that the low molecular weight G protein Rac1 lies downstream of IL-1 on these pathways. To investigate whether IL-1 could activate Rac1 in IL-1 receptor rich murine thymoma cells, EL4.NOB-1, an assay was used which is based on the fact that only in its active form (Rac1-GTP) does Rac1 bind to its downstream effector p21-activated kinase (PAK) (115, 118, 164). GTP-bound Rac1 activates PAK by binding to the p21-binding domain located in the N-terminal regulatory part of the protein (91, 182). This domain contains a highly conserved 14-amino acid CDC42/Rac1 interaction/binding (CRIB) domain (residues 74-88) found in many proteins that interact with active Rac1- or Ccd42-GTP. Whereas the minimal CRIB domain is sufficient for interaction with Rac1-GTP a larger sequence is required for high affinity interaction (59, 91, 182). A GST-fusion protein containing residues 1-252 of PAK will therefore recognise and bind GTP-bound Rac1 and can be used to pullout any active Rac1 in cells. This method has been previously used successfully to determine the activational state of Rac1 in pro-myelocytic leukemic HL-60 cells and human neutrophils (10, 59). The GST-PAK fusion protein was expressed in the BL21 strain of *Escherichia coli* as described in the methods section and the resulting purified fusion protein analysed by gel electrophoresis (figure 3.2.1a). As indicated in figure 3.2.1a, a protein of the expected molecular weight of GST-PAK (65kDa) was expressed following induction of the cells (lane 2) compared with uninduced cells (lane 1) and following purification of GST-PAK from the lysates a single band corresponding to GST-PAK was observed (lane 3).

The ability of GST-PAK to bind activated Rac1 was subsequently verified. Purified GST-PAK was used as a probe in an affinity precipitation assay with over-expressed constitutively active RacV12. To do this EL4.NOB-1 cells were transfected with 10μg of a plasmid encoding constitutively active RacV12 and following a period of recovery cells were lysed and the lysates

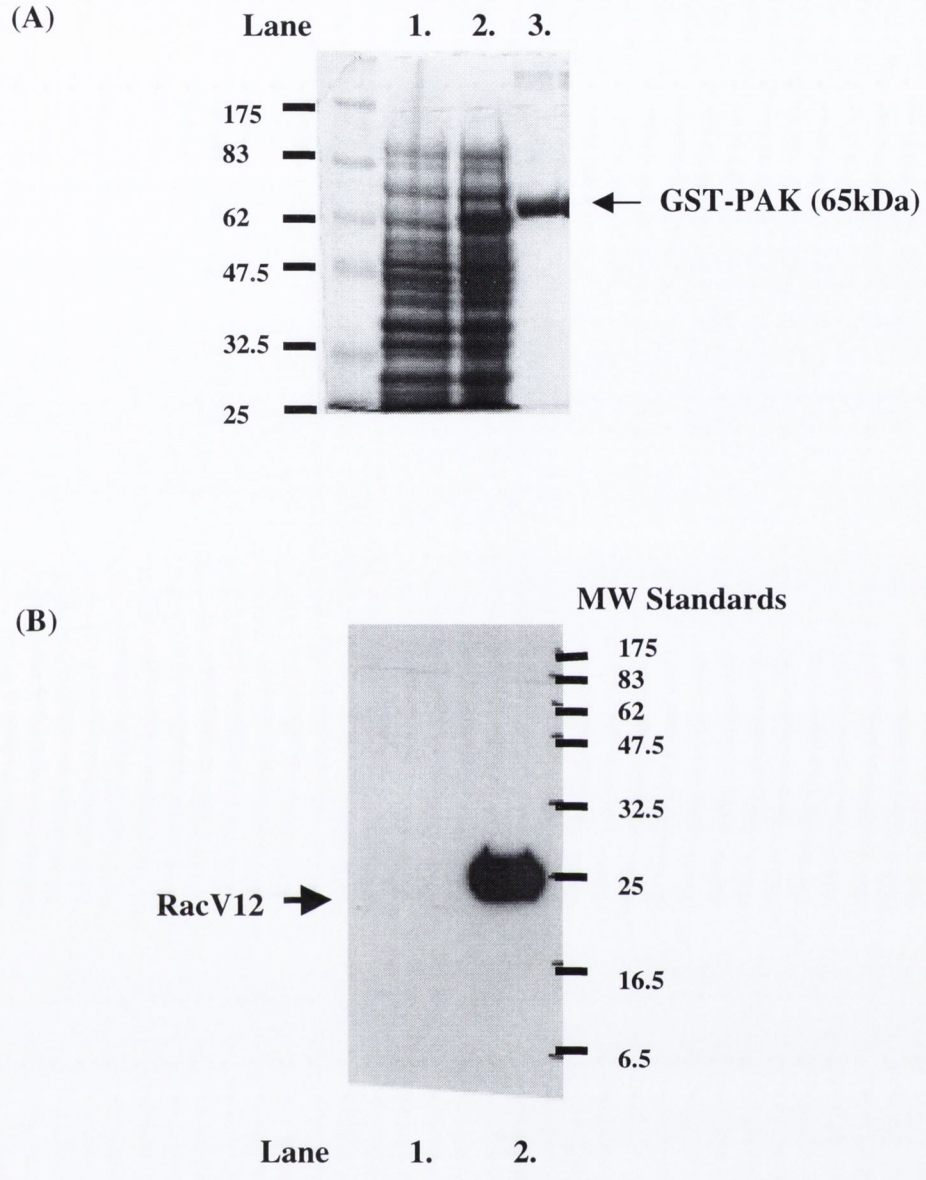


Figure 3.2.1: Activated Rac1 binds to GST-PAK

(A) The GST-PAK fusion protein was purified from the BL21 strain of *Escherichia coli* as described in the methods section. Lane 1 represents the uninduced culture, lane 2 whole cell lysates from induced culture (3 hours) and lane 3 GST-PAK purified from induced cells. (B) EL4.NOB-1 cells were transiently transfected with 10μg of RacV12 and following recovery (16-18 hours) cell lysates were incubated with 10μg GST-PAK. RacV12 association was detected by western blot analysis using an anti-Rac antibody

incubated with 10μg of GST-PAK coupled to glutathione-agarose beads for 2 hours at 4°C. The beads were washed three times with lysis buffer after which time RacV12 binding to GST-PAK was assessed by western blot analysis using an anti-Rac antibody. Figure 3.2.1b demonstrates that constitutively active RacV12 associates with GST-PAK in transfected cells (lane 2) compared with empty vector transfected cells (lane 1), a strong band corresponding to RacV12 (molecular weight 21kDa) being detected.

3.2.2 IL-1 stimulation of EL4.NOB-1 cells activates Rac1.

Having established that activated Rac1 binds GST-PAK I next determined whether IL-1 stimulation was able to activate endogenous Rac1 in EL4.NOB-1. Cells ($2x10^7$) were stimulated with IL-1 (10ng/ml) for the indicated time points and lysed. Cell lysates were normalised for total protein content following determination of protein concentration using the Bradford assay (21) and incubated with 10μg of GST-PAK coupled to glutathione-agarose beads for 2 hours. The association of Rac1 with GST-PAK was detected by western blot analysis. As shown in figure 3.2.2a IL-1-stimulation of EL4.NOB-1 cells activated Rac1 in a time dependent manner with Rac1 association with GST-PAK being observed at 5 minutes (lane 2) post stimulation and increasing to 60 minutes (lane 4) post stimulation. Following transfer the gel was stained with coomassie brilliant blue to demonstrate that equivalent amounts of GST-PAK were added to each sample (figure 3.2.2b).

3.2.3 Endogenous Rac1 coprecipitates with PAK1 in IL-1-stimulated cells.

The ability of IL-1 to activate Rac1 was confirmed by immunoprecipitating endogenous Rac1 in EL4.NOB-1 cells treated with IL-1 and then assessing the ability of endogenous Rac1 to co-precipitate its downstream effector PAK by western blot analysis using an anti-PAK1 antibody. In keeping with our previous results I found that stimulation of EL4.NOB-1 cells with IL-1 potently

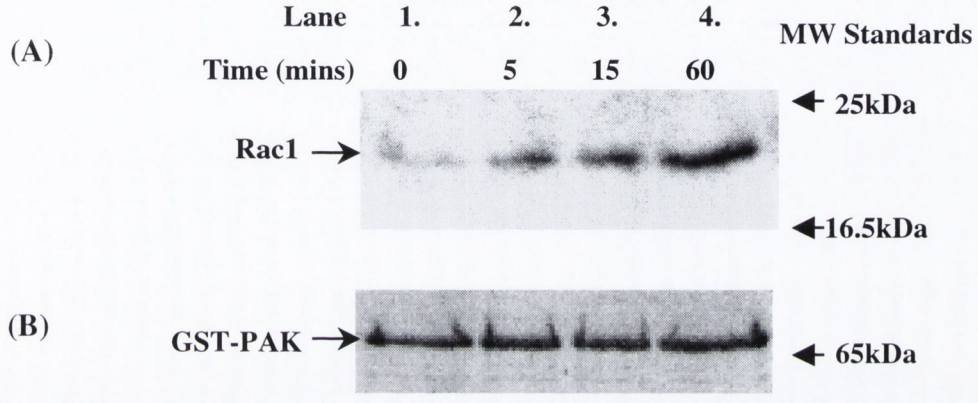


Figure 3.2.2: IL1 stimulation of EL4.NOB-1 activates Rac1

(A) EL4.NOB-1 ($2x10^7$) cells were stimulated with IL1 (10ng/ml) for various time points as indicated. Activated Rac1 was affinity purified from cell lysates, which had been normalised for protein concentration, following incubation with 10μg GST-PAK. Rac1 association with GST-PAK was determined by western blot analysis using an anti-Rac antibody. (B) After transfer, gels were stained with coomassie brilliant blue in order to show that equivalent amounts of GST-PAK were added to each sample.

enhanced the ability of Rac1 to coprecipitate PAK (Figure 3.2.3a, compare lane 1 (untreated) with lane 2 (stimulated with IL-1 for 15 minutes)). To ensure that the amount of Rac1 immunoprecipitated in both samples was equivalent the blot was stripped and re-probed with anti-Rac1. As shown in figure 3.2.3b the amount of endogenous Rac1 immunoprecipitated was identical in both samples.

3.2.4 IL-1 activates the kinase activity of PAK.

The ability of IL-1 to activate the kinase activity of PAK using an *in vitro* kinase assay was next assessed. For this assay I used another IL-1-responsive cell line, Hela, which are routinely used for assessing kinase activity of proteins in the laboratory and which also transfect to a high degree of transfection efficiency. I initially confirmed that IL-1 stimulated Rac1 activation in these cells using GST-PAK in a pulldown assay and demonstrated that stimulation of HeLa cells ($1x10^5$/ml) with IL-1 activated Rac1 in a time dependent manner (figure 3.2.4a). HeLa ($1x10^5$/ml) cells were then transfected with an expression plasmid encoding tagged PAK1 (HA-PAK) and the ability of IL-1 stimulation to activate its kinase activity was assessed. HA-PAK was immunoprecipitated from transfected cells which had been normalised for protein content using a HA-specific polyclonal antibody (Santa Cruz) and the ability of HA-PAK to phosphorylate an exogenous substrate (myelin basic protein (MBP)) determined. Stimulation of HeLa cells with IL-1 resulted in lysates which demonstrated an increased ability to phosphorylate MBP. This was determined by western blot analysis using an antibody raised against the phosphorylated form of residues 89-105 of MBP. The level of phosphorylation of MBP increased with time, with IL1 stimulation for 15 minutes resulting in maximal activation of the ability of HA-PAK to phosphorylate MBP (figure 3.2.4b, lane 4) compared with unstimulated cells (figure 3.2.4b, lane 1), indicating that the kinase activity of PAK was activated by IL-1. Following stimulation of cells with IL-1 for 60 minutes the kinase activity of of HA-PAK had returned to basal levels (Lane 5).

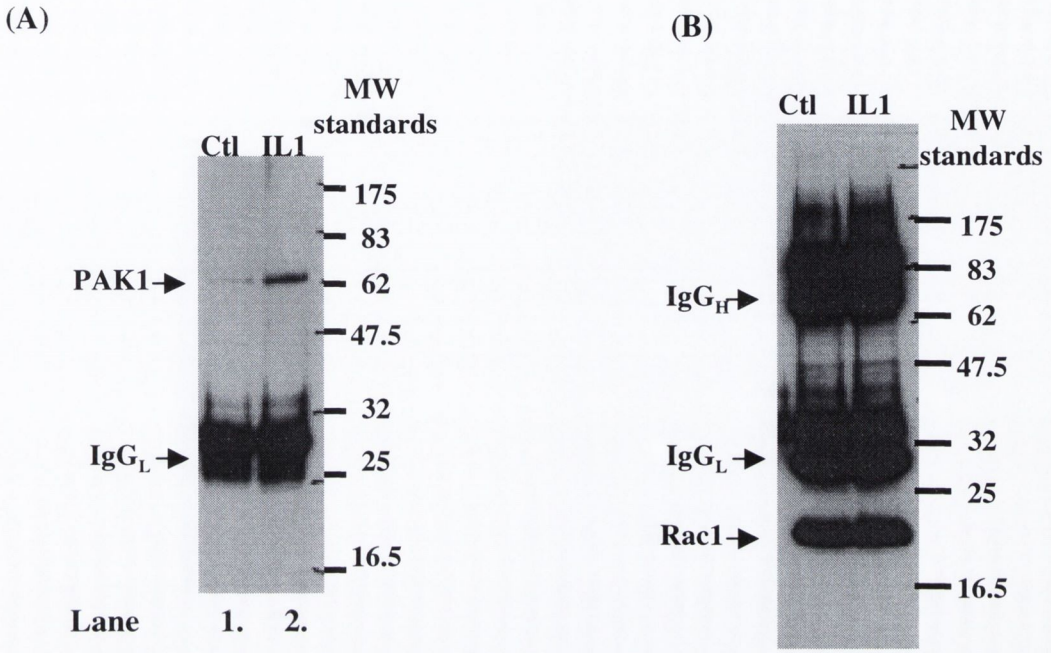


Figure 3.2.3: Rac1 co-precipitates endogenous PAK1 in IL1-treated cells.
EL4.NOB-1 ($1x10^7$cells) were stimulated with IL1 (10ng/ml) for 15 minutes as indicated. (A) Total endogenous Rac1 was immunoprecipitated from cell lysates, which had been normalised for protein content, using 4μg of anti-Rac. Association of PAK1 with Rac1 was assessed by western blotting using an anti-PAK antibody. (B) Blots were stripped using 50mM glycine buffer (pH 2) and reprobed using anti-Rac antibody to demonstrate that equal levels of Rac were immunoprecipitated in both samples.

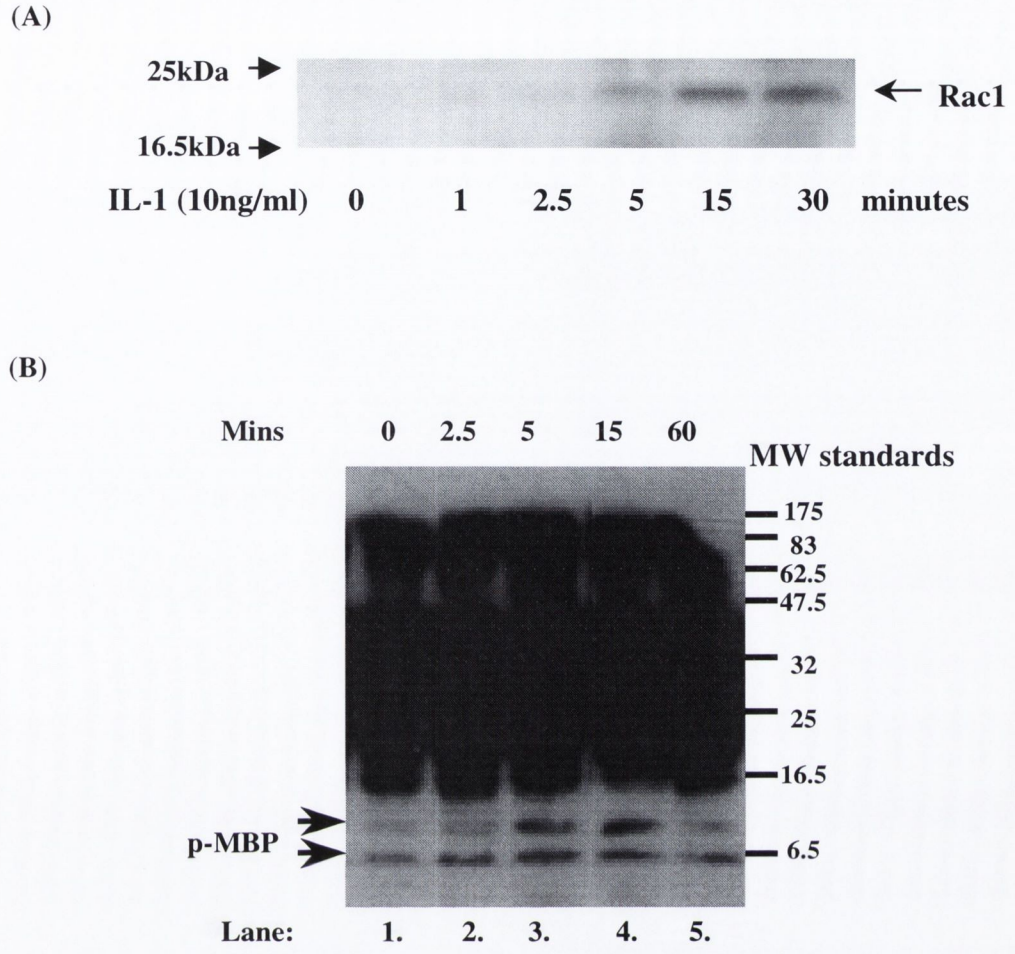


Figure 3.2.4: IL1 stimulation of HeLa cells activate Rac1 and PAK

(A) The ability of IL1 to activate Rac1 was confirmed in HeLa cells. HeLa cells were seeded at a density of $1x10^5$/ml 24 hours prior to stimulation with IL1 (10ng/ml). Active Rac1 was affinity purified from cell lysates (normalised for protein content) using GST-PAK and associated Rac1 detected by western blot analysis using an anti-Rac antibody. (B) HeLa cells ($1x10^5$/ml) were transfected with 10µg of a plasmid encoding HA-PAK using Ca_2PO_4. 48 hours post-transfection, samples were stimulated with IL1 (10ng/ml) for the indicated time points and HA-PAK was immunoprecipitated from cell lysates. The kinase activity of HA-PAK was assessed using myelin basic protein (MBP) as an exogenous substrate in a kinase assay. Phosphorylation of MBP was assessed using a phospho-specific MBP antibody. The multiple phosphorylated forms of MBP are indicated.

3.2.5 IL-1 stimulation activates EL4.NOB-1 cells to drive IL2 protein production.

Having demonstrated that IL-1 stimulation of both EL4.NOB-1 and HeLa cells results in activation of the GTPase Rac1, my next aim was to investigate the role of Rac1 in IL-1 signalling to gene expression using IL-2 expression as a readout in EL4.NOB-1 cells. EL4.NOB-1 cells were initially tested for their ability to drive IL2 production in response to IL-1 stimulation alone. Stimulation of cells with 1ng/ml IL-1 resulted in a potent increase in the level of IL-2 detectable in the supernatant following stimulation of cells for 24 hours (figure 3.2.5). No further increase in IL-2 levels was detectable following stimulation of cells with 10ng/ml IL-1.

3.2.6 IL-1 stimulation of EL4.NOB-1 cells activates the IL-2 promoter.

The effects of IL-1 stimulation on IL-2 promoter activation was next examined. For this assay an IL-2-dependent reporter gene, IL-2-CAT, which has the IL-2 promoter (-7 to -293) fused upstream of the bacterial enzyme chloramphenicol acetyl transferase, was used in transient transfection assays. IL-1 stimulation of cells was shown to result in a 2.5 fold increase in IL-2-CAT activity compared with control levels (figure 3.2.6a). In order to confirm the effects of IL-1 on IL-2-CAT, we examined its ability to activate the native IL-2 promoter by RT-PCR. Following stimulation of cells with IL-1 (10ng/ml, 6 hours) total mRNA was extracted from the cells. Using IL-2-specific primers, RT-PCR analysis of IL-2 mRNA expression in cells following IL-1 stimulation demonstrated that IL-1 strongly increased the level of IL-2 mRNA in cells (figure 3.2.6b, lane 2) compared with levels in unstimulated cells (figure 3.2.6b, lane 1). In contrast no increase in the level of control mRNA, aldolase, was observed following stimulation of cells with IL-1 (figure 3.2.6b, lower panel).

3.2.7 IL-1 stimulation activates both AP1- and NFκB-dependent reporter genes.

The ability of IL-1 to activate key transcription factors involved in regulating the expression of IL-2 was subsequently addressed. Figure 3.2.7a illustrates the key elements found in the murine

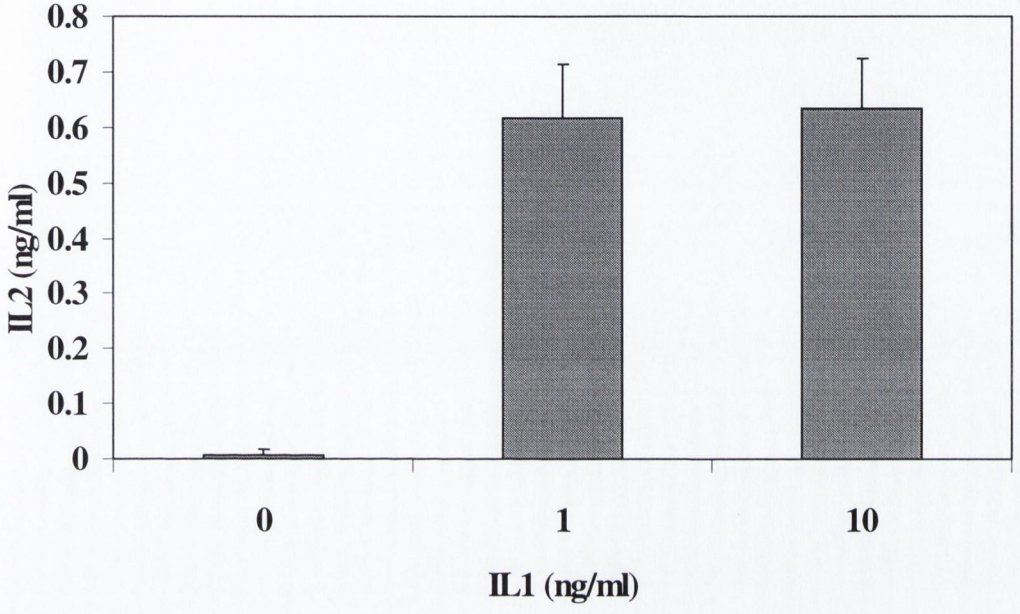


Figure 3.2.5: IL-1 stimulation activates EL4.NOB-1 cells to drive IL-2 protein production.

EL4.NOB-1 cells were seeded at a density of $1x10^6$ ml^{-1} and stimulated with IL-1 at the concentrations indicated for 24 hours. Cells were briefly centrifuged and the supernatant analysed for IL-2 concentration by ELISA as described in the methods section. Results represent mean $\pm$ S.D. from triplicate determinations.

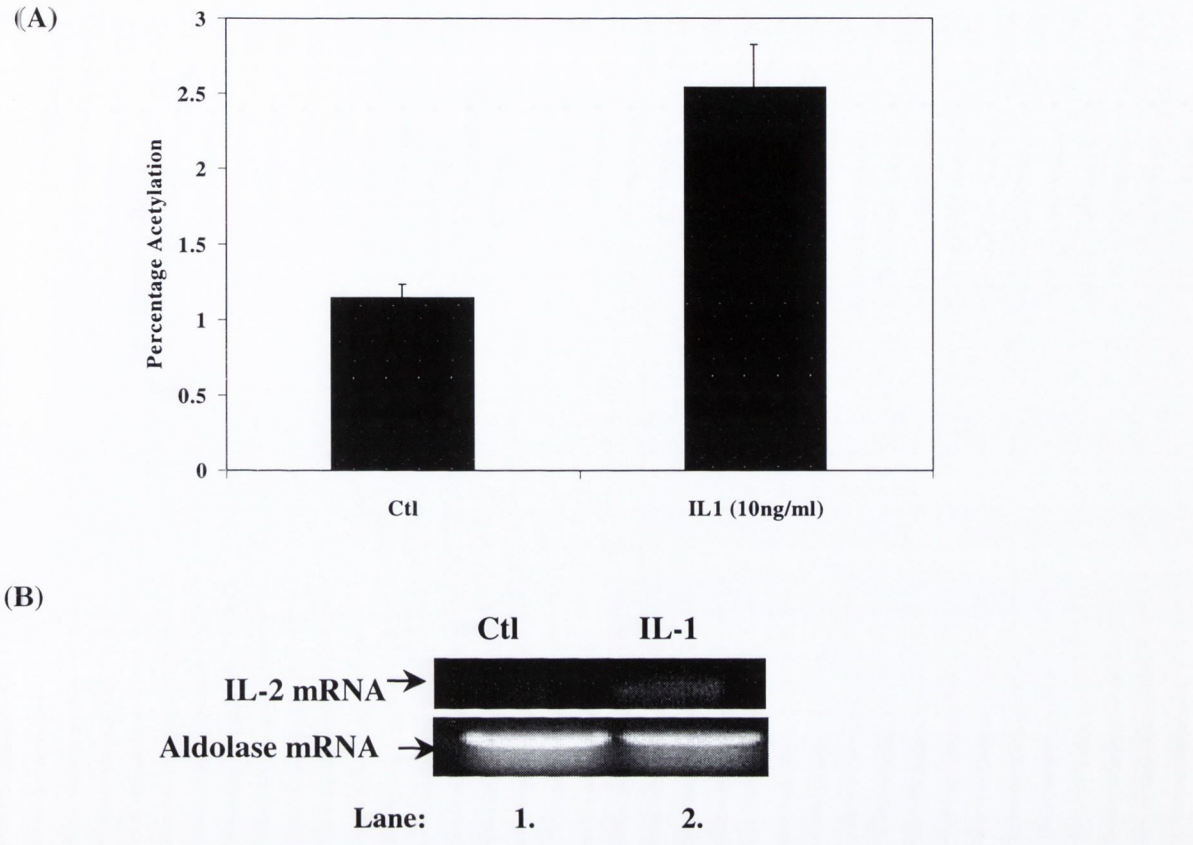


Figure 3.2.6: IL-1 stimulation of EL4.NOB-1 cells activates the IL-2 promoter

(A) EL4.NOB-1 cells ($1.4x10^7$/transfection) were transiently transfected with the IL-2-CAT reporter gene (10μg). Following 16-18 hours recovery cells were seeded at a density of $1x10^6$ ml^{-1} and stimulated with IL-1 (10ng/ml) as indicated for 24 hours after which time reporter gene activity was analysed by assessing CAT activity in the cells. Results (mean ± S.D.) shown are from triplicate determinations and are representative of at least three separate experiments. (B) EL4.NOB-1 cells were seeded at a density of $1x10^6$/ml and stimulated with IL-1 (10ng/ml, 6 hours) in a final volume of 5mls. Total cellular RNA was isolated post-stimulation using TRI-reagent according to the manufacturers protocol (Sigma, Poole, Dorset, U.K.). Reverse transcription and PCR amplification were performed in a one step reaction according to the manufacturer's instructions (Titan, Boehringer Mannheim, East Sussex, U.K.). Primers for PCR amplification were chosen from the 5' and 3' ends of exon 2 for detection of IL-2 mRNA expression. Primers to amplify a 464bp product from the aldolase transcript were also used as a control.

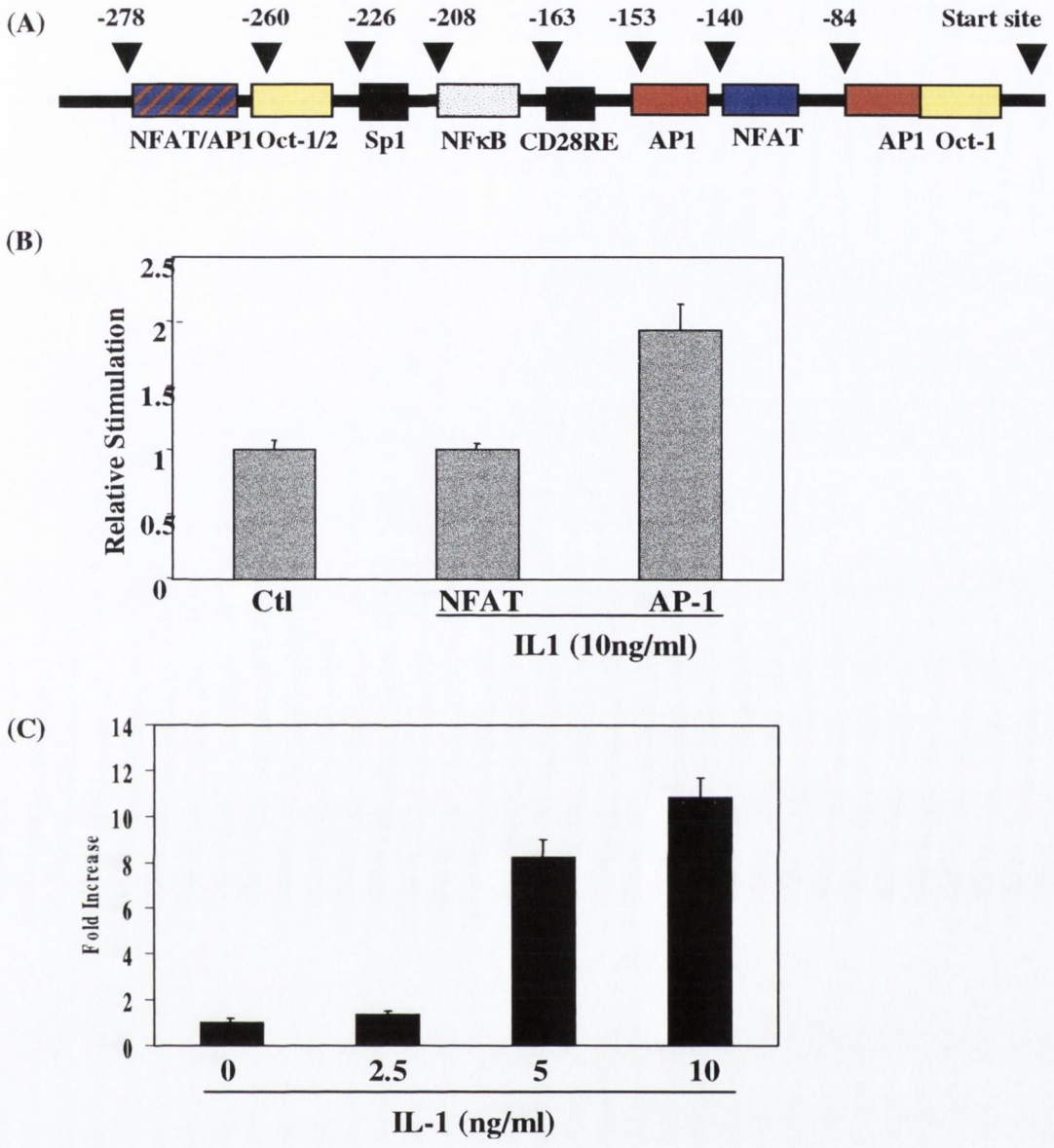


Figure 3.2.7: IL-1 stimulation activates both AP-1- and NFκB-dependent reporter genes. (A) A schematic representation of the murine IL-2 promoter region is shown above with consensus sequences for the various transcription factors illustrated. EL4.NOB-1 ($1.4x10^7$) were transfected with reporter plasmids encoding (B) 10μg NFAT-CAT and AP-1-CAT and (C) 2.5μg κB-luc reporter gene. Following a recovery period of 16-18 hours cells ($1x10^6$) were stimulated with IL-1 as indicated and reporter gene activation determined as a function of CAT or luciferase activity. Results are represented as fold increase over empty vector control and are presented as Mean±S.D. from triplicate determinations and are representative of at least three separate experiments.

IL-2 promoter (165). Using NFAT- and AP-1-dependent reporter genes in transient tranfection experiments the results demonstrate that while IL-1 was unable to activate the NFAT-specific reporter gene NFAT-CAT, IL-1 elicited a two-fold increase in AP1-CAT activity (figure 3.2.7b). In contrast, IL-1 strongly stimulated the NFκB-dependent reporter gene NFκB-CAT, the degree of stimulation increasing as IL-1 concentration increased (figure 3.2.7c), with 10ng/ml IL-1 resulting in an eleven-fold increase in NFκB-CAT activation compared with unstimulated controls. This data confirms previous reports on the ability of IL-1 to activate both AP-1 and NFκB but not NFAT.

3.2.8 Constitutively active RacV12 potentiates IL-1-induced IL-2 production.

Having demonstrated that IL-1 activates Rac1 and that EL4.NOB-1 cells are highly responsive to IL-1 stimulation in driving IL-2 expression, the involvement of the GTPase Rac1 in signal transduction events regulating IL-2 expression was next determined. Transient transfection of cells with a plasmid encoding constitutively active RacV12 alone only weakly drove IL-2 production as shown in figure 3.2.8. Stimulation of RacV12 transfected cells with IL-1 however resulted in a strong potentiation of the ability of IL-1 to drive IL2 production in these cells (figure 3.2.8).

3.2.9 Constitutively active RacV12 potentiates the ability of IL-1 to activate the IL-2 promoter

Transient transfection of cells with a plasmid encoding constitutively active RacV12 and the IL-2 promoter construct, IL-2-CAT, in the absence of IL-1 stimulation showed no effect on reporter gene expression (figure 3.2.9a). However stimulation of RacV12-transfected cells with IL-1 (10ng/ml, 24 hours) resulted in potentiation of the IL-1-induced response (figure 3.2.9a), an eight-fold increase in IL-2-CAT activity being observed compared with the weak response seen in cells

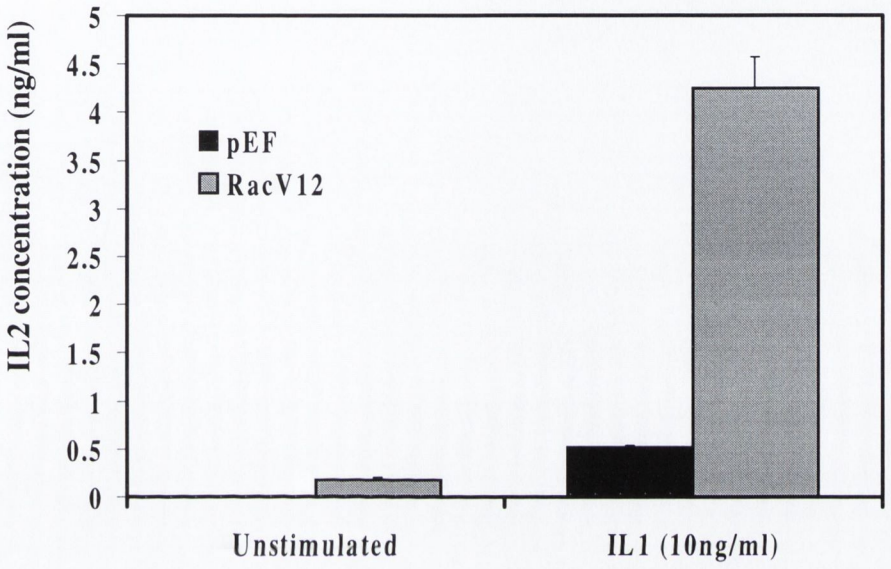


Figure 3.2.8: Constitutively active RacV12 potentiates the ability of IL-1 to drive IL-2 production in EL4.NOB-1 cells.

EL4.NOB-1 cells ($1.4x10^7$/transfection) were transiently transfected with 8μg of pEF or constitutively active RacV12 as indicated. Following 16-18 hours recovery, cells were seeded at a density of $1x10^6$ ml^{-1} and stimulated with IL-1 (10ng/ml) as indicated for 24 hours after which time IL-2 levels in the supernatant was measured by ELISA. Results are represented as mean ± S.E.M from three separate experiments, each carried out in triplicate.

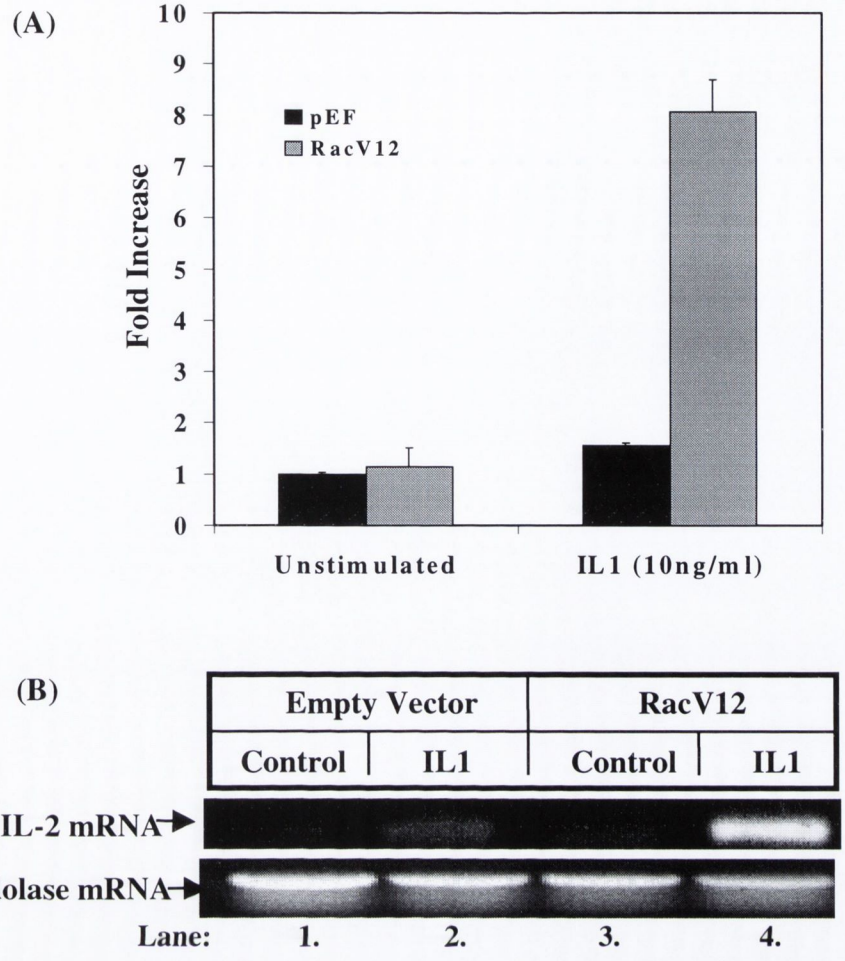


Figure 3.2.9: Constitutively active RacV12 potentiates IL-1-induced IL-2-CAT activity and IL-2 mRNA production in EL4.NOB-1 cells.

(A) EL4.NOB-1 cells ($1.4x10^7$/transfection) were transiently co-transfected with the IL-2-CAT reporter gene and either 8μg of pEF or RacV12 as indicated. Following 16-18 hours recovery cells were seeded at a density of $1x10^6$ ml^{-1} and stimulated with IL-1 (10ng/ml) as indicated for 24 hours after which time reporter gene activity was analysed by assessing CAT activity in the cells. Results are represented as mean±S.E.M from three separate experiments, each carried out in triplicate. (B) EL4.NOB-1 cells were transfected with empty vector or RacV12 (8μg/$1.4x10^7$ cells). Following recovery, cells were seeded at a density of $1x10^6$/ml and stimulated with IL-1 (10ng/ml, 6 hours) in a final volume of 5mls. Total cellular RNA was isolated post-stimulation using TRI-reagent according to the manufacturers protocol (Sigma, Poole, Dorset, U.K.). Reverse transcription and PCR amplification were performed in a one step reaction according to the manufacturer's instructions (Titan, Boehringer Mannheim, East Sussex, U.K.). Primers for PCR amplification were chosen from the 5' and 3' ends of exon 2 for detection of IL-2 mRNA expression. Primers to amplify a 464bp product from the aldolase transcript were also used as a control.

treated with IL-1 alone. This ability of constitutively active RacV12 to potentiate IL-2 expression was confirmed by examining the effect of transfecting cells with RacV12 on IL-2 mRNA expression (figure 3.2.9b). While IL-1 alone resulted in increased expression of IL-2 mRNA as determined by RT-PCR (lane 2), transient transfection of cells with RacV12 (10μg) was unable to induce IL-2 mRNA expression (lane 3). However, IL-1 stimulation of RacV12 transfected cells potently enhanced the level of mRNA in EL4.NOB-1 cells as determined by RT-PCR (lane 4).

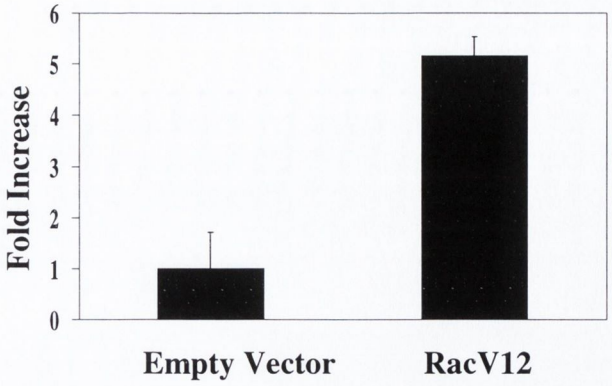
3.2.10 The effect of Rac1 on the IL-2 promoter.

The ability of RacV12 to potentiate IL-1-mediated induction of both IL-2-CAT and IL-2 mRNA expression suggested that the effects of constitutively active RacV12 on IL-2 expression were at the level of the promoter. I therefore examined the effects of Rac1 mutants on NFAT-, AP1-, and NFκB-dependent reporter genes (NFAT-CAT, AP1-CAT and NFκB-CAT, respectively) in transient transfection experiments. In contrast to the IL-2 promoter we found that cotransfection of EL4.NOB-1 cells with constitutively active RacV12 alone resulted in a five-fold increase in NFAT-dependent reporter gene expression compared with empty vector transfected cells (figure 3.2.10a). In addition transient transfection of cells with RacV12 alone resulted in activation of AP1-CAT and furthermore IL-1 stimulation of RacV12 transfected cells potentiated the AP1-CAT response. Importantly, transient transfection of cells with a dominant negative mutant of Rac1, RacN17, resulted in a partial inhibition of the IL-1-induced response suggesting that Rac1 lie downstream of IL-1 on the pathway regulating AP-1 activation (figure 3.2.10b).

3.2.11 Rac1 is a critical regulator of IL-1-signalling to NFκB activation.

In contrast to AP1-CAT it was observed that, as with IL-2-CAT, constitutively active RacV12 alone was insufficient to drive NFκB-dependent reporter gene expression and that the effects of RacV12 on the system were only seen in the presence of IL-1, with RacV12 potentiating the IL-1-

(A)



(B)

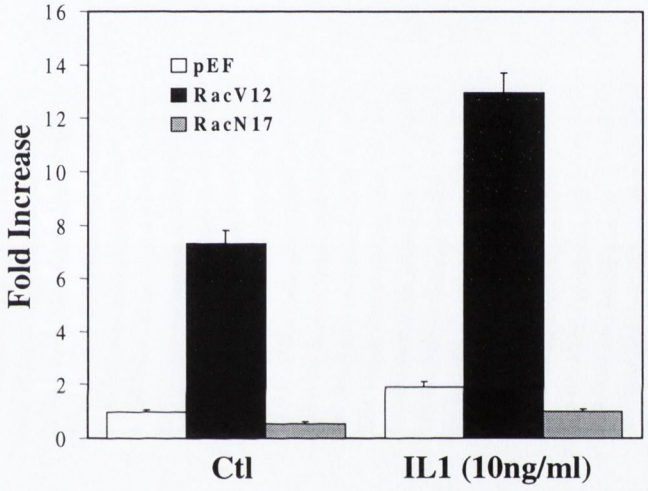


Figure 3.2.10: RacV12 activates NFAT- and AP-1-driven reporter genes.

EL4.NOB-1 ($1.4x10^7$) were co-transfected with 10μg of plasmids encoding (A) NFAT-CAT and (B) AP1-CAT reporter genes and 8μg of plasmid encoding empty vector (as control), constitutively active RacV12, or dominant negative RacN17 ((B) only). Following a recovery period of 16-18 hours cells ($1x10^6$/ml) were stimulated with IL-1 (10ng/ml) for 24 hours. Reporter gene activity was determined as a function of CAT activity. Results are represented as fold increase over empty vector control and are the mean±S.E.M. from three separate experiments each carried out in triplicate.

induced response (figure 3.2.11a). When the effects of dominant negative RacN17 on IL-1-induced activation of NFκB-CAT were examined however it was observed that RacN17 inhibited the IL-1-induced response in a dose dependent manner (figure 3.2.11b). The ability of RacN17 to inhibit κB-dependent gene expression was found to be concentration dependent and correlated with the level of expression of RacN17 in the cells as determined by western blot analysis (Figure 3.2.11c). This result suggested that even though a direct effect on NFκB-dependent reporter gene expression by RacV12 was not evident, Rac1 was required for this IL-1 response.

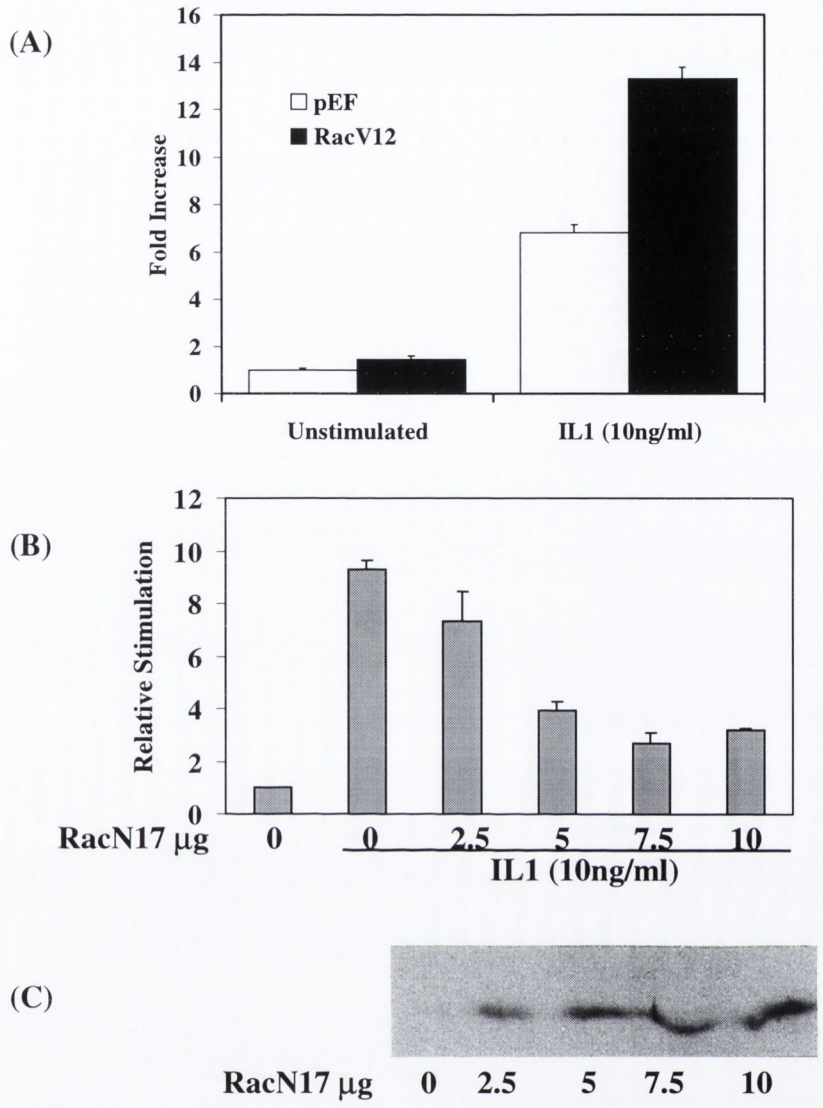


Figure 3.2.11: Rac1 is a critical regulator of IL-1-signalling to NFκB activation

(A) EL4.NOB-1 ($1.4x10^7$) were co-transfected with 10µg NFκB-CAT reporter gene and 8µg of plasmid encoding empty vector (as control) or constitutively active RacV12. Following a recovery period of 16-18 hours cells ($1x10^6$) were stimulated with IL-1 (10ng/ml) for 24 hours. NFκB activity was determined as a function of CAT activity. Results are represented as fold increase over empty vector control and are the mean±S.E.M. from three separate experiments, each carried out in triplicate. (B) EL4.NOB-1 ($1.4x10^7$) were co-transfected with 2.5µg κB-luciferase reporter gene and dominant negative RacN17 in the range 0-10µg. Following a recovery period of 16-18 hours cells ($1x10^6$) were stimulated with IL-1 (10ng/ml, 3 hours) and NFκB activity determined as a function of luciferase activity. Results are represented as fold increase over empty vector control and are presented as Mean ± S.D. (C) RacN17 expression was determined by western-blot analysis of transfected cells using an anti-myc antibody.

3.3 Discussion

A number of studies using dominant negative RacN17 have placed Rac1 downstream of IL-1 on the pathways regulating both p38 MAPK and JNK activation (37, 127, 203). In keeping with these findings we showed that stimulation of EL4.NOB-1 and HeLa cells with IL-1 resulted in strong activation of Rac1 as assessed by its binding to its downstream effector p21 activated kinase (PAK). IL-1-induced activation of Rac1 was also demonstrated by the ability of Rac1 to coprecipitate PAK1 following IL-1 stimulation of EL4.NOB-1 cells. Both results are the first demonstration that IL-1 activates the low molecular weight G protein Rac1 and furthermore strongly supports an involvement of Rac1 in IL-1 signalling.

Research into the role of Rac1 in the cell has largely focused on its ability to effect actin cytoskeletal changes as opposed to its involvement in various signal transduction pathways (70, 150). In addition to regulating membrane ruffling, Rac1 also plays a key role in mediating the assembly of focal complexes at the cell surface. These multiprotein complexes are integrin-dependent sites linking the extracellular matrix to the actin-rich cytoskeleton. Formation of focal complexes results in the recruitment of talin, vincullin and the tyrosine kinase, focal adhesion kinase and may provide the link between the actin cytoskeleton and signal transduction processes in cells. Increasingly, integral members of signal transduction pathways are been identified as scaffold proteins whose primary role is to position a multiprotein complex correctly in the cytoplasm. Direct evidence for the involvement of focal complexes with IL-1-induced signal transduction pathways has been recently given by the discovery that localisation of IL-1 receptor associated kinase (IRAK) to these sites is essential prior to IL-1-induced p42/p44 MAPK activation (108). As an important regulator of focal complex assembly the role of Rac1 in IL-1-induced signal transduction in cells may be crucially linked with its effects on the actin cytoskeleton. PAK associates with focal complexes in cells and this association appears to be independent of its Cdc42/Rac1-binding domain (114). Reports have placed PAK as a key

mediator of both cytoskeletal and signal transduction events in cells (6). This discrepancy may be linked to the central role that Rac1 and PAK play in mediating focal complex assembly, further underlining the importance of the actin cytoskeleton for signal transduction networks in cells.

Initially the investigation into the role of Rac1 in IL-1-signal transduction focused on its effects on IL-2 production in EL4.NOB-1 cells and the results demonstrated a strong synergistic effect of RacV12 on IL-2 mRNA expression, promoter activity and protein production. While stimulation of cells with IL-1 alone resulted in induction of all three readouts, transient transfection of cells with constitutively active RacV12 in the absence of IL-1 had no effect. However when IL-1 was applied to this system it resulted in strong potentiation of all three responses. The ability of Rac1 to synergise with IL-1 to such a high level, plus its inability to drive IL-2 expression alone, suggested either that Rac1 does not lie directly on the signal transduction pathway regulating IL-2 expression or that additional IL-1 signals are needed in order to see the effects of constitutively active RacV12 on the system.

The ability of constitutively active RacV12 to synergise with IL-1 in driving IL-2 mRNA expression and the expression of an IL-2 dependent reporter gene suggested that the synergistic effects of Rac1 may be as a result of effects on the transcription factors regulating the IL-2 promoter. The transcription factor NFAT has been shown to be activated following ligation of the T cell receptor in T lymphocytes and is an important transcription factor regulating the expression of many genes during T cell activation. Rac1 has previously been shown to regulate NFAT activation in response to TCR ligation, and plays an important role in regulating co-stimulatory signals such as those emanating from CD28 (60). While the results confirm a role for Rac1 in regulating NFAT activation with constitutively active RacV12 driving NFAT-CAT activation in transfected cells, IL-1 stimulation of cells does not result in increased NFAT-CAT expression. This suggests that the co-stimulatory properties of IL-1 on T cells in driving IL-2 production are not mediated by the transcription factor NFAT.

In addition to NFAT, consensus sites for NFκB and AP-1, both inducible transcription factors regulated by IL-1, have been found in the IL-2 promoter (165). Using NFκB- and AP-1-dependent reporter genes the involvement of Rac1 in regulating transactivation of gene expression in response to these transcription factors was examined. While IL-1 stimulation resulted in a two-fold activation of AP-1-CAT activity in EL4.NOB-1 cells, unlike the effects of RacV12 on the IL-2 promoter, transient transfection of cells with constitutively active RacV12 induced a strong AP-1-CAT response (6-8 fold increase compared with unstimulated cells) in the absence of IL-1 stimulation. In addition a dominant negative mutant of Rac1, RacN17, was able to inhibit IL-1-induced AP-1-CAT activation, implying that Rac1 lies downstream on the IL-1 pathway to AP-1 activation. This result is in keeping with reports that demonstrate that dominant negative RacN17 inhibits IL-1-induced activation of JNK, the upstream kinase responsible for regulating AP-1 activity (37, 127). However, the strength of the RacV12 response compared with stimulation of cells with IL-1 suggests that RacV12 may be mimicking additional signals, independent of IL-1 stimulation, which drive AP-1 activation. This is supported by reports which show that constitutively active RacV12 can activate JNK MAPK in the absence of any exogenous signals (37, 127). What is evident from our results is that AP-1 activation by constitutively active RacV12 is insufficient to induce IL-2 expression in the absence of IL-1.

Investigation into the role of Rac1 in NFκB activation has clearly demonstrated that transient transfection of EL4.NOB-1 cells with constitutively active RacV12 is unable to mimic the ability of IL-1 to induce strong activation of NFκB-dependent reporter gene expression. As with IL-2 expression, only in the presence of IL-1 is the effect of RacV12 evident, potentiating the IL-1-induced response. While this result argues against a role for Rac1 in IL-1-induced activation of NFκB due to its inability to mimic IL-1-induced NFκB-CAT activity, it may also be explained by the requirement for IL-1 to activate additional signals which are required before the effects of RacV12 are observed. In keeping with this latter conclusion our data shows that

dominant negative RacN17 inhibited IL-1-induced NFκB-dependent gene expression, clearly indicating that Rac1 is necessary for IL-1-induced NFκB activation. This ability of RacN17 to inhibit κB-dependent gene expression correlated with the level of expression of RacN17 in the cells as determined by western blot analysis. Together these results clearly implicate Rac1 in IL-1-induced activation of NFκB but suggest that additional signals are required in order that the effects of RacV12 on the system are seen. How Rac1 may be involved in regulating NFκB activation in response to IL-1 is unclear although certain of its downstream effectors, PAK and POSH, have been shown to regulate NFκB (56) (179).

The ability of constitutively active RacV12 to drive AP-1-dependent gene expression in the absence of IL-1, coupled with its inability to drive IL-2-dependent reporter gene expression, argues against AP-1 as being the key, determining factor regulating IL-2 gene expression. Instead our results clearly show the similarity in effect of RacV12 on both IL-2-CAT and NFκB-CAT, neither being activated by RacV12 alone. In light of our results the strong potentiation observed between RacV12 and IL-1 in driving IL-2 production can be seen as a combination of IL-1-induced activation of NFκB, the potentiating effect of RacV12 on NFκB-dependent reporter gene expression in the presence of IL-1 and also the ability of RacV12 to activate AP-1 in the absence of IL-1. Clearly however the effect of RacV12 on AP-1 is not a sufficient stimulus to activate the IL-2 promoter and it would seem that IL-1 induced activation of NFκB is the limiting step in regulating IL-2 expression. The clear involvement of Rac1 in NFκB activation in response to IL-1 prompted further investigation in to the role of Rac1 in this pathway.

Chapter Four

Rac1 regulates the ability of the p65 subunit to transactivate gene expression in response to IL-1.

4 Chapter 4

4.1 Introduction

The inhibition of IL-1-induced NFκB-dependent gene expression by dominant negative RacN17 clearly demonstrates a role for Rac1 downstream of IL-1 on this pathway. However, the inability of constitutively active RacV12 to independently drive NFκB-dependent gene expression suggests that the effects of Rac1 on the system are complex. Two scenarios presented themselves for our consideration. The first was that Rac1 lay downstream of IL-1 on the pathway to IκB degradation and the second was that the involvement of Rac1 was independent of this pathway, regulating instead the ability of NFκB to transactivate gene expression. This latter explanation would explain the ability of RacN17 to inhibit IL-1-dependent NFκB-dependent reporter gene expression and also explain the need for IL-1 on the system in order to initiate NFκB activation before the effects of constitutively active RacV12 were seen.

In resting cells NFκB exists as a heterodimer in the cytoplasm (typically comprising the p50 and p65 subunits) complexed to an inhibitory protein IκB (inhibitory protein kappa B). Regulation of IκB degradation and the subsequent release of NFκB is a crucial control point in the pathway to NFκB activation. In addition, recent evidence has emerged that supports the existence of an IκB-independent pathway, which when activated results in the enhanced transactivation potential of the p65 subunit of NFκB once it is bound to its consensus sequence. Inducible transcription factors such as NFκB and AP-1 regulate transcriptional activation in two ways: firstly, by post-translational modification (phosphorylation or acetylation) either prior to entry into the nucleus or once bound to its consensus sequence; and secondly via its interaction with either other transcription factors or with coactivators. Post-translational modification of transcription factors enhances their ability to interact with the basal transcription machinery usually via increased interactions with coactivators such as CBP/p300 and PCAF. It may also

release inhibitory intra- or inter-molecular interactions that would prevent engagement with coactivators or basal transcription factors such as TBP. Interactions between transcription factors have been demonstrated to either enhance (NFκB and AP-1 interaction) or repress (steroid hormone receptors and NFκB, for example) transcriptional activation. These interactions may result in either increased accessibility to or competition for coactivators essential for mediating interaction with the basal transcription apparatus.

The p65 subunit of NFκB is principally responsible for NFκB-dependent transactivation of gene expression, having two transactivation domains at its carboxy terminus which mediate this effect. In contrast to the pathway activated by IL-1 leading to IκB degradation, little is known regarding the signalling components involved in the pathway regulating the transactivating activity of the p65 subunit of NFκB. Several reports have demonstrated that upon stimulation of cells with tumour necrosis factor (TNF) the ability of the p65 subunit of NFκB to transactivate gene expression is enhanced possibly as a result of phosphorylation of multiple serine residues on p65 (12, 188). In particular, phosphorylation of serine 279 of p65 by protein kinase A (PKA) has been demonstrated to enable association of p65 with the transcriptional coactivator and histone acetylase CBP/p300 (204). The upstream kinases regulating these events have yet to be identified, although recent evidence suggests an involvement of p42/p44 MAPK and p38 MAPK in mediating the signal regulating transactivation of gene expression by p65 in response to stimulation of cells with TNF.

Where Rac1 might participate in either pathway regulating IκB degradation or p65-mediated transactivation of gene expression is not known. We have investigated the involvement of Rac1 in both pathways. Constitutively active RacV12 was unable to drive either IκB degradation or DNA-binding activity of NFκB. Instead, our data clearly demonstrates a role for Rac1 downstream of these events at the level of enhancing the transactivating potential of the p65 subunit of NFκB. Our results show that IL-1 activates two pathways in the NFκB system: the

first, and well characterised, one leading to IκB phosphorylation, and the second involving Rac1, which regulates transactivation of gene expression by p65.

4.2 Results

4.2.1 Rac1 is not involved in IL-1-induced degradation of endogenous IκB.

It is estimated that over 80% of cellular IκB is represented by the IκBα isoform, which regulates transient activation of NFκB in response to IL-1, TNF, LPS and PMA. The mechanism regulating degradation of IκBβ is poorly understood in comparison and appears to be degraded in response to only LPS and IL-1 (reviewed in (146)). For these reasons the majority of studies examining NFκB activation focus on the effects of various stimuli and proteins on IκBα regulation as opposed to IκBβ. Based on our previous results implicating Rac1 in IL-1-induced activation of NFκB-dependent gene expression it was decided to examine whether Rac1 might be involved in IκBα degradation, a crucial control point on the pathway to NFκB activation. This was achieved by analysing the effects of constitutively active RacV12 and dominant negative RacN17 on IκBα degradation by western blot analysis using an anti-IκB antibody. While IL-1 stimulation (10ng/ml, 30 minutes) of EL4.NOB-1 cells resulted in rapid and total degradation of IκBα (figure 4.2.1, comparing lanes 1 and 2), transient transfection of cells with constitutively active RacV12 was unable to induce IκBα degradation (figure 4.2.1, lane 3). This effect is consistent with the failure of RacV12 to induced NFκB-dependent reporter gene expression in the absence of IL-1. Most importantly however we found that dominant negative RacN17 was unable to inhibit IL-1-induced IκBα degradation in RacN17-transfected cells (figure 4.2.1, comparing lanes 5 and 6) which strongly indicates that Rac1 is not involved in the pathway to IκBα degradation and the release of NFκB.

4.2.2 RacV12 does not induce degradation of IκBctag when co-transfected into cells.

One concern which was raised about the interpretation of these results was based on the possibility that the effects of the Rac1 mutants on the system may not be seen if the transfection

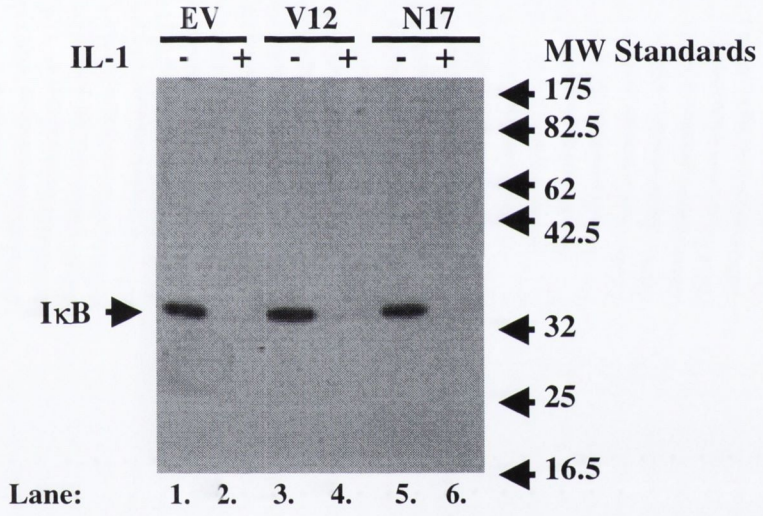


Figure 4.2.1: Rac1 is not involved in IL-1-induced degradation of endogenous IκB degradation in EL4.NOB-1.

Cells were transfected with plasmids encoding either empty vector (EV), RacV12 (V12) or RacN17 (N17) (10μg/1.4x10^7 cells), seeded at a density of 1x10^6/ml and stimulated with IL-1 (10ng/ml, 30minutes). IκB degradation was detected by immunoblot analysis using a mouse anti-human monoclonal antibody (1:100 dilution) directed against IκBα. No other bands were detected. The results shown are representative of at least three separate experiments.

efficiency of our cells was low. In order to address this question cells were co-transfected with either Rac1 mutant (RacV12 or RacN17) together with an expression plasmid encoding a tagged version of IκBα (IκBctag). IκBctag was easily distinguishable from endogenous IκBα due to its higher molecular weight (figure 4.2.2, upper band on gel) and analysing the effects of the Rac1 mutants on IκBctag ensured that the transfected population of cells were being studied. The results shown in figure 4.2.2 demonstrates that IL-1 stimulation of cells (10ng/ml, 30 minutes) transfected with IκBctag induced degradation of the tagged protein in a manner identical to that of endogenous IκB (comparing lanes 1 and 2). Cotransfection of cells with constitutively active RacV12 was unable to mimic the effects of IL-1 on the system (figure 4.2.2, lane 3) nor did dominant negative RacN17 inhibit IL-1-induced degradation of IκBctag (lanes 5 and 6), both results consistent with the effects of the Rac1 mutants on endogenous IκB. This result confirms the lack of involvement of Rac1 in IL-1-induced IκB degradation.

4.2.3 Effects of Rac1 on DNA-binding activity of NFκB.

Having shown that Rac1 was not involved in the IL-1-induced IκB degradation we next examined its involvement in regulating DNA-binding activity of NFκB in response to IL-1. EL4.NOB-1 cells were transfected with either constitutively active RacV12 or dominant negative RacN17 (10μg/1.4x10^7 cells). Following IL-1 stimulation of cells (10ng/ml, 1 hour) nuclear extracts of the variously treated samples were prepared. The DNA-binding activity of NFκB was assessed by electrophoretic mobility shift assay (EMSA) using a radiolabelled consensus sequence for NFκB. The result shown in figure 4.2.3 demonstrates a lack of involvement of Rac1 in the pathway regulating DNA-binding activity of NFκB. While IL-1 stimulation of empty vector transfected cells potently enhanced the amount of NFκB bound to its consensus sequence (figure 4.2.3, comparing lane 1 and lane 2), transfection of cells with constitutively active RacV12 was unable to mimic the effects of IL-1 on the system (figure 4.2.3, compare lane 3 with lane 2).

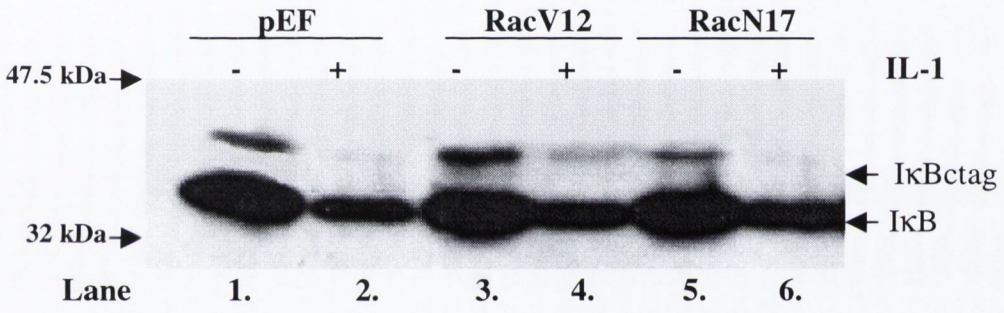


Figure 4.2.2: Effect of Rac1 mutants on IL-1-induced degradation of IκBctag.

Cells were cotransfected with 10μg IκBctag and either empty vector (pEF), RacV12 (V12) or RacN17 (N17) (10μg/1.4x10^7 cells). Following recovery period of 18 hours cells were stimulated with IL-1 (10ng/ml) for 30 minutes. IκBctag degradation was detected by immunoblot analysis using a mouse anti-human monoclonal antibody (1:100 dilution) directed against IκBα. No other bands were detected.

Figure 4.2.3: Rac1 is not involved in regulating IL-1-stimulated DNA-binding activity of NFκB

Cells were transfected with empty vector, constitutively active RacV12 or dominant negative RacN17 (10μg/1.4x10^7 cells). Cells were seeded at a density of 1x10^6/ml and stimulated with IL-1 (10ng/ml) for 1 hour. Following stimulation nuclear extracts were prepared and equal amounts of each sample analysed for NFκB binding ability by electrophoretic mobility shift assay (EMSA) as described in the methods section. DNA-binding of NFκB to its consensus sequence was visualised by autoradiography. The autoradiograph shown is representative of at least three separate experiments.

Importantly, dominant negative RacN17 was unable to inhibit IL-1-induced DNA-binding activity of NFκB (figure 4.2.3, lanes 5 and 6), indicating that the effects of Rac1 on NFκB-dependent gene expression lie downstream of this point.

4.2.4 RacV12- and RacN17-expressing Porcine Aortic Endothelial (PAE) cells.

A concern throughout this study was the reproducibility of these results both in a different cell line and also in a stably transfected cell line. We addressed this question by obtaining porcine aortic endothelial (PAE) cells, which had been stably transfected with either constitutively active RacV12 (V12Rac1-PAE) or RacN17 (N17Rac1-PAE) under the control of an IPTG-inducible promoter (40). These cell lines were then used to repeat the observations made in EL4.NOB-1 cells regarding the lack of involvement of Rac1 in either IκB degradation or DNA-binding activity of NFκB. The induction of Rac1 mutants in these cells was performed after incubation of both cell lines in starvation medium (0.5% FCS, 0.2% BSA) for 24 hours, followed by the addition of IPTG (100μM) to the medium for the time points indicated. As both Rac1 mutants were tagged with glutamic acid residues at their N terminus their expression was readily determined by western blot analysis using an anti-Rac1 antibody (Upstate Biotechnology) due to their higher molecular weight compared with endogenous Rac1. Figure 4.2.4 indicates that expression of the mutants was induced as early as 6 hours by the addition of IPTG (lane 5), while 24 hours induction achieved optimal expression (lane 6). Of note here is the low level of basal expression of RacN17 in N17Rac-PAE cells in the absence of IPTG (figure 4.2.4b, lane 1).

4.2.5 Stimulation of PAE cells with IL-1 activates NFκB.

We next tested the responsiveness of the parental PAE cell line (and by extension V12Rac-PAE and N17Rac-PAE cells) to IL-1 by assessing DNA-binding activity of NFκB by EMSA. Cells were seeded at a density of $1x10^5$/ml, stimulated with IL-1 at the concentrations and time points

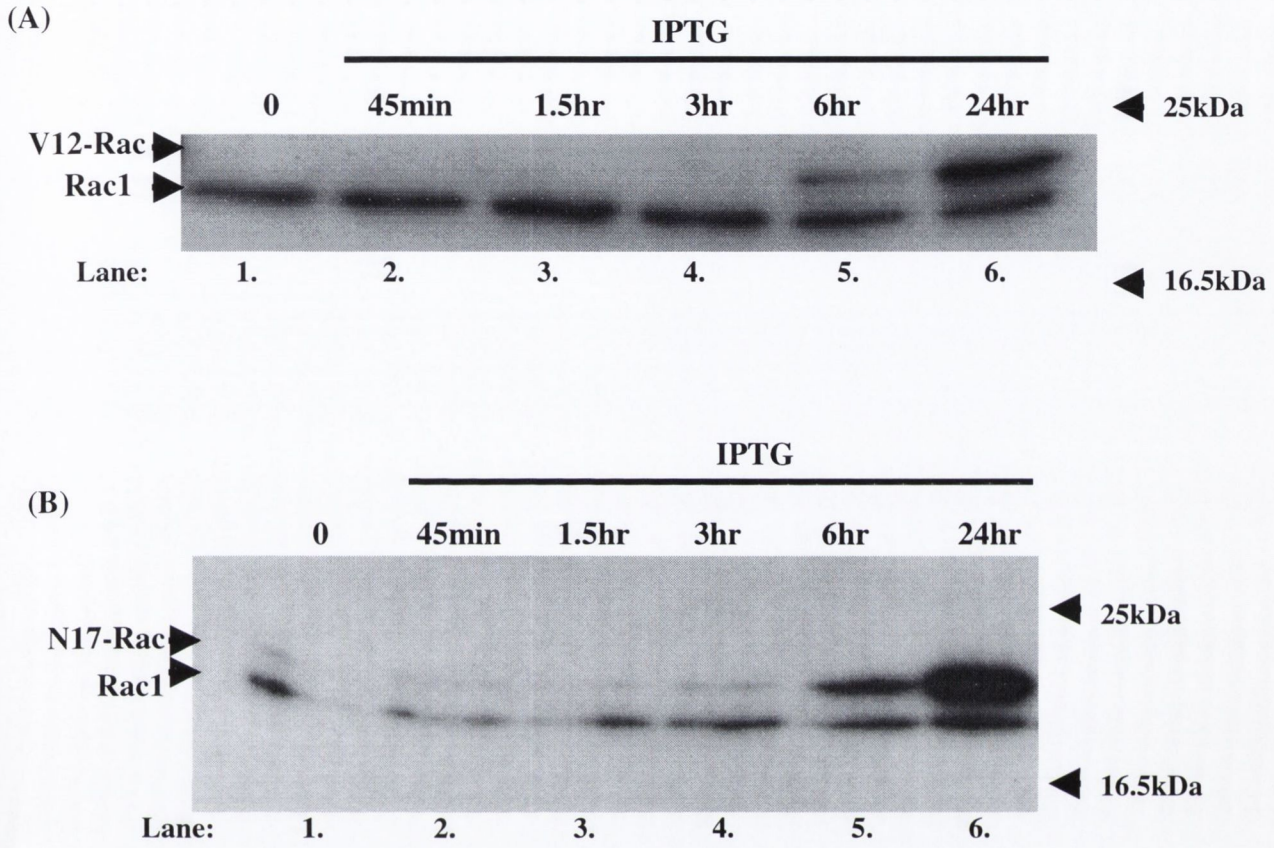


Figure 4.2.4: Addition of IPTG to V12Rac-PAE or N17Rac-PAE cells results in the induction of RacV12 expression

PAE cells stably transfected with either (A) RacV12 (V12Rac-PAE) or (B) RacN17 (N17Rac-PAE) under the control of an inducible promoter were cultured in serum-free medium containing 0.2% fetal calf serum, 0.1% fatty acid-free bovine serum albumin, 2mM L-glutamine, 50µg/ml gentamycin, 500nM puromycin and 0.1mM hygromycin B. Expression of RacV12 and RacN17 was induced by the addition of 15mM filter sterile isopropyl-β-D-thiogalactopyranoside (IPTG) to the starvation medium for the time periods indicated. (A) EE-tagged RacV12 and (B) EE-tagged RacN17 were detected by western blot analysis of whole cell lysates following induction. No other bands were detected apart from those shown.

indicated in figure 4.2.5a and 4.2.5b and nuclear extracts assessed for DNA-binding activity of NFκB by EMSA. The cells were found to be highly IL-1 responsive with 1ng/ml IL-1 being sufficient to stimulate DNA-binding (figure 4.2.5a) and this activity was found to be time dependent, with optimal activation observed following 1 hour stimulation of cells with IL-1 (figure 4.2.5b).

4.2.6 Induction of either RacV12 or RacN17 does not effect either IκB degradation or DNA-binding activity of NFκB in PAE cells.

Having demonstrated that porcine aortic endothelial cells are IL-1 responsive, the effect of induction of RacV12 or RacN17 on IκB degradation and DNA-binding activity of NFκB was assessed. V12Rac-PAE and N17Rac-PAE cells were induced with IPTG (24 hours) to express constitutively active RacV12 (figure 4.2.6a, lanes 3 and 4) or dominant negative RacN17 (figure 4.2.6a, lanes 7 and 8). Figure 4.2.6 demonstrates that IL-1 treatment of non-induced cells results in both IκB degradation (figure 4.2.6b, compare lanes 2 with 1 and 6 with 5) and DNA-binding activity of NFκB (figure 4.2.6c, compare lanes 2 with 1 and 6 with 5) in both cell lines. In keeping with our results in EL4.NOB-1 cells, induction of RacV12 was unable to induce either IκB degradation (figure 4.2.6b, lane 3) or DNA-binding of NFκB (figure 4.2.6c, lane 3). Importantly dominant negative RacN17 was unable to inhibit either response (figure 4.2.6b and c, comparing lanes 7 and 8 with 5 and 6). These results confirm our finding in EL4.NOB-1 cells that Rac1 is not involved in regulating IL-1-induced IκB degradation or DNA-binding activity of NFκB. This argues against a role for Rac1 in regulating the IκB kinase complex and instead suggests its involvement in regulating events downstream of DNA-binding of NFκB, possibly at the level of regulating the transactivation of gene expression by the p65 subunit of NFκB.

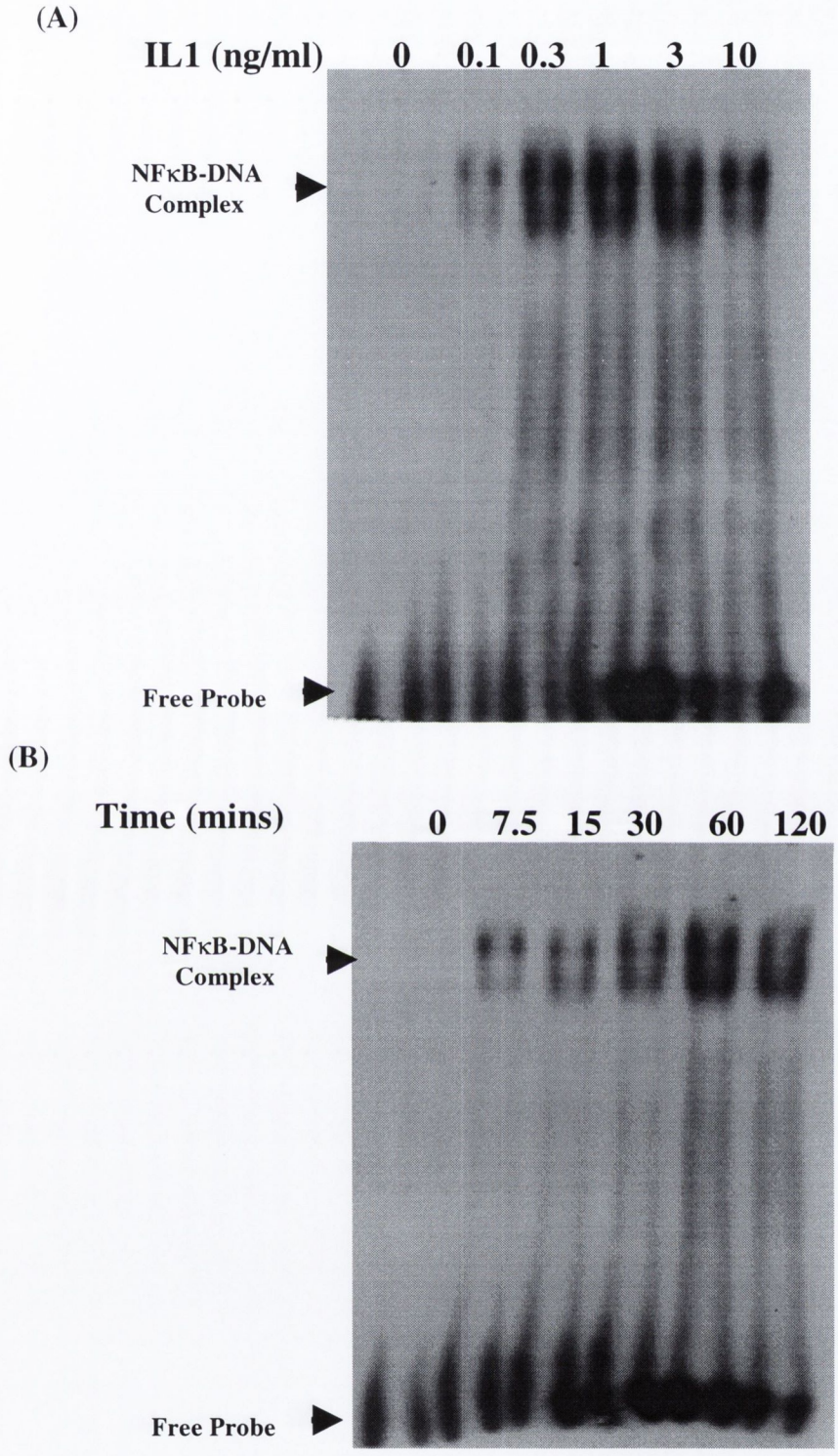


Figure 4.2.5: Stimulation of parental PAE cells with IL-1 activates NFκB.

Parental PAE cells were seeded at a density of $1x10^5$/ml 24 hours prior to treatment with IL-1. (A) Cells were treated with IL-1 for 1 hour at the IL-1 concentrations as indicated and (B) with 10ng/ml for the time points indicated. Nuclear extracts of the samples were prepared and DNA-binding activity of NFκB determined by electrophoretic mobility shift assay as described in the methods section.

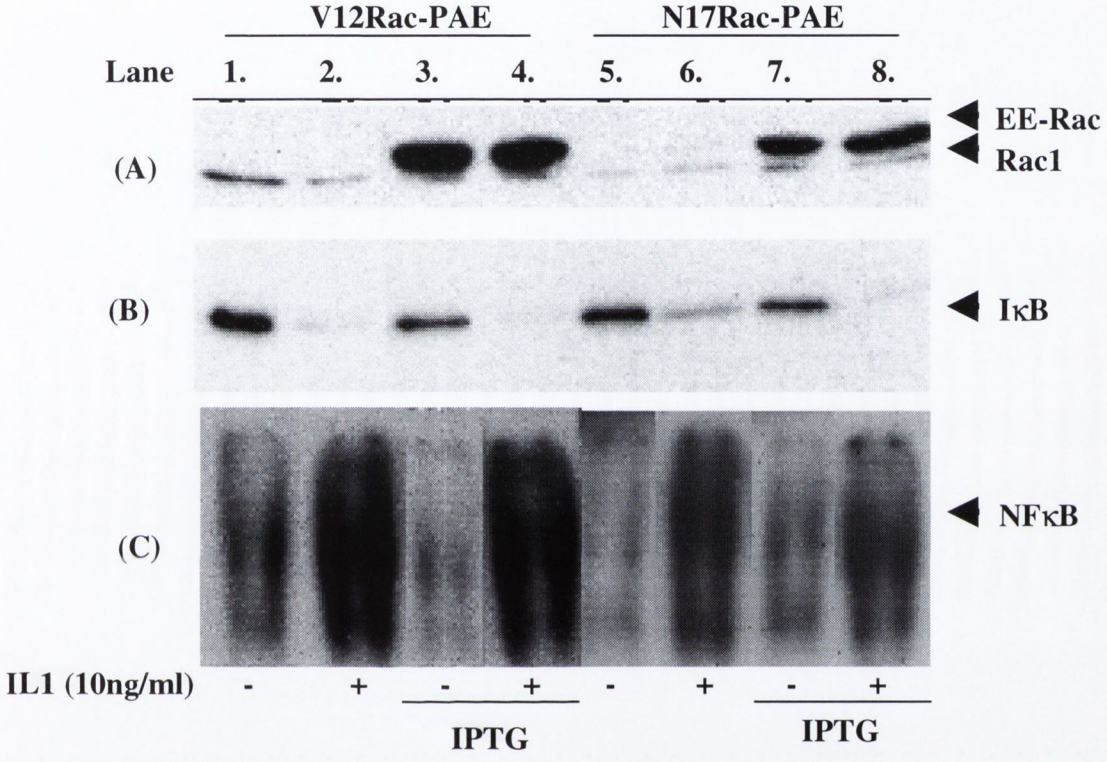


Figure 4.2.6 Induction of RacV12 or RacN17 in PAE cells fails to affect IκB degradation or DNA-binding activity of NFκB.

V12Rac-PAE cells and N17Rac-PAE cells were seeded at a density of $1x10^5$ cells/ml in starvation medium 24 hours prior to induction of the mutants. (A) 24 hours post-addition of IPTG to the medium the expression of the tagged mutants was determined by western blot analysis using an anti-Rac1 antibody. (B) The effect of induction of the mutants on IκB degradation was determined by western blot analysis (C) DNA-binding activity of NFκB was also examined on nuclear extracts of samples by EMSA.

4.2.7 IL-1 activates a pathway resulting in the enhanced transactivation of gene expression by p65.

To test our hypothesis that Rac1 regulates transactivation of gene expression by p65 in response to IL-1 we employed a technique that allows us to examine the transactivating activity of p65 independent of its DNA-binding properties as described in the methods section (41, 162). We initially assessed the ability of IL-1 to enhance p65-mediated transactivation by co-transfecting EL4.NOB-1 cells with Gal4-$p65^{1-551}$ (in the range of 0-10μg/1.4x10^7 cells) and Gal4-luciferase (either 5 or 10μg/1.4x10^7 cell). Following 16-18 hours recovery, cells were stimulated with IL-1 (0-10ng/ml, 3 hours) at the indicated concentrations and luciferase activity measured. The response of transfected cells varied according to the amount of both plasmids used in the transfection. The luciferase activity measured increased dose dependently with the amount of Gal4-$p65^{1-551}$ transfected with 10μg Gal4-luciferase giving higher luciferase readings than 5μg (figure 4.2.7a). We selected 5μg of Gal4-$p65^{1-551}$ as the amount to use in subsequent transfections as it gave sufficient activation of Gal4-luciferase with the advantage of keeping the overall amount of DNA used in subsequent transfections low. Most importantly figure 4.2.7b demonstrates that IL-1 activates p65-mediated transactivation of gene expression. IL-1 stimulation of cells cotransfected with either 5 or 10μg of Gal4-luciferase and 10μg Gal4-$p65^{1-551}$ drove Gal4-$p65^{1-551}$ activity in a dose dependent manner. The best results were achieved in cells transfected with 5μg Gal4-luciferase and 5μg of Gal4-$p65^{1-551}$, as stimulation of these cells with 10ng/ml IL-1 induced a 2.5 fold increase in luciferase activity compared with unstimulated controls (figure 4.2.7c). Additional experiments indicated that 2.5μg of Gal4-$p65^{1-551}$ gave similar results to 5μg and was therefore used in subsequent experiments (data not shown).

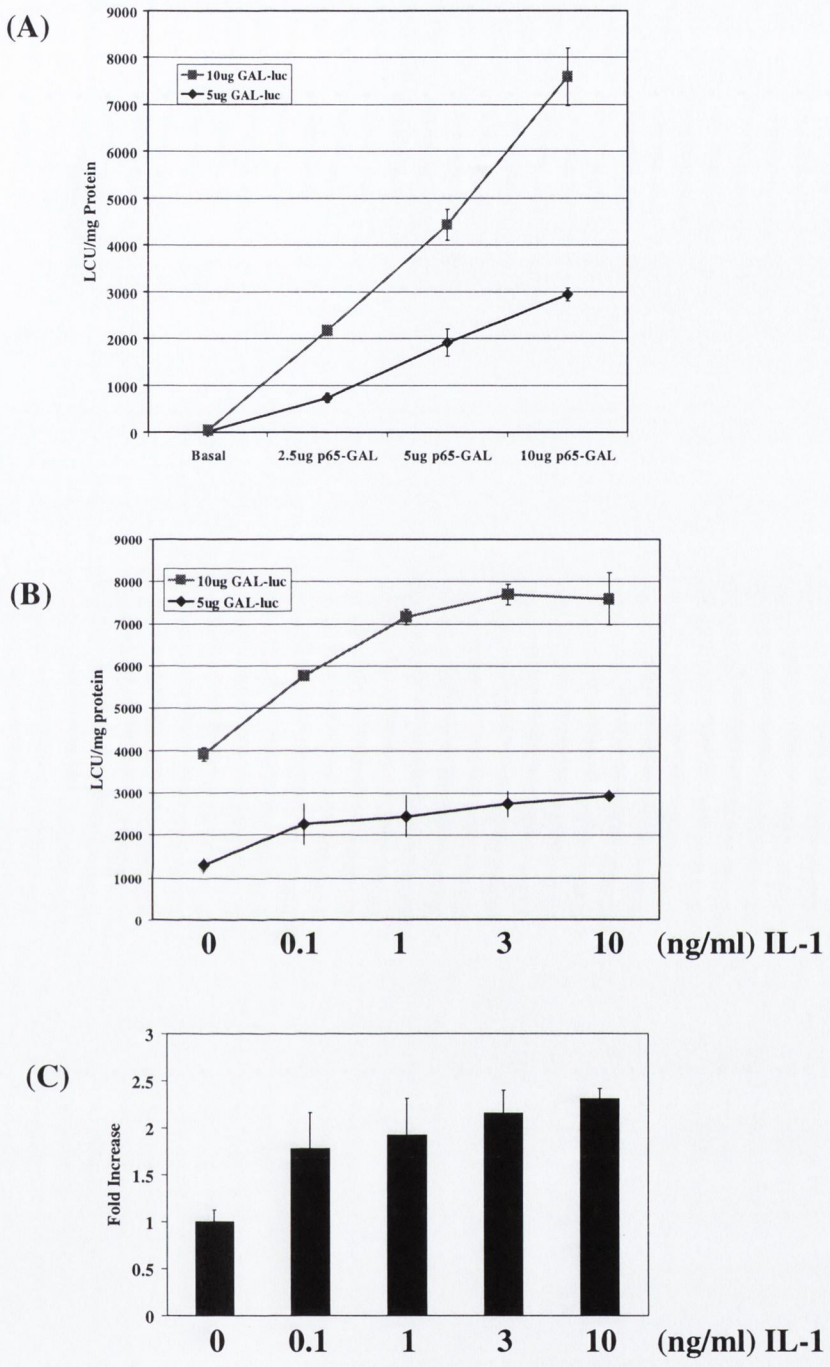


Figure 4.2.7: IL-1 stimulates Gal4-p65$^{1\text{-}551}$ mediated GAL-luciferase activity.

(A) EL4.NOB-1 cells were transfected with 5 or 10μg of Gal4-luciferase reporter gene as indicated and concentrations of Gal4-p65$^{1\text{-}551}$ ranging from 0 to 10μg and the ability of Gal4-p65$^{1\text{-}551}$ to induce basal expression of Gal4-luciferase assessed. (B) Cells transfected with 5μg Gal4-p65$^{1\text{-}551}$ and either 5 or 10μg of gal-luciferase were compared for their responsiveness to IL-1 stimulation. (C) Cells were transfected with 5μg of Gal4-p65$^{1\text{-}551}$ and 10μg of Gal4-luciferase and stimulated with IL-1 in the range 0-10ng/ml. Results are shown as mean $\pm$ S.D. from triplicate determinations.

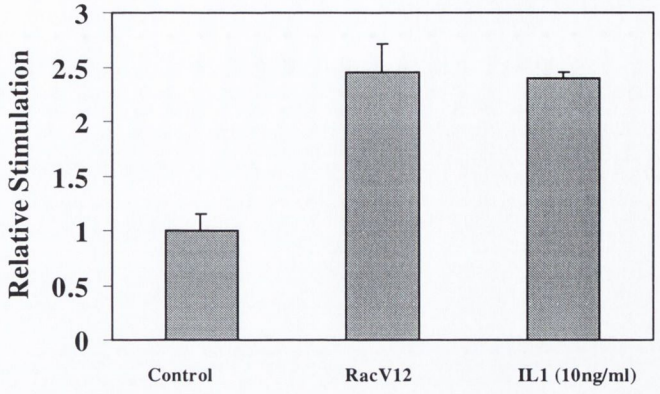
4.2.8 Constitutively active RacV12 drives p65-mediated transactivation.

The role of Rac1 in IL-1-induced transactivation by p65 was next examined. The result represented in figure 4.2.8 demonstrates the ability of constitutively active RacV12 to drive Gal4-$p65^{1-551}$ activity, independent of IL-1 stimulation (figure 4.2.8). Expression of constitutively active RacV12 alone in either EL4.NOB-1 (figure 4.2.8a) or V12Rac-PAE (figure 4.2.8b) cells resulted in enhanced activation of the transactivation response in these cells. IL-1-stimulation of constitutively active V12Rac-PAE cells resulted in a further potentiation of the Gal4-$p65^{1-551}$ response (figure 4.2.8b). This result suggests that Rac1 is involved in regulating the pathway leading to enhanced transactivation of gene expression by p65 and is in keeping with previous results suggesting a role for Rac1 downstream of NFκB binding.

4.2.9 Rac1 lies downstream of IL-1 on the pathway regulating p65-mediated transactivation of gene expression.

To place Rac1 downstream of IL-1 on the pathway regulating transactivation of gene expression by p65 EL4.NOB-1 cells were cotransfected with increasing amounts of dominant negative RacN17 and examined its effects on IL-1-induced Gal4-$p65^{1-551}$ activity. As shown in figure 4.2.9a, RacN17 dose dependently inhibited the ability of IL-1 to transactivate gene expression by p65. Due to the high level of constitutive expression (figure 4.2.9b) of RacN17 in N17Rac-PAE cells (possibly due to a leaky promoter) we were unable to induce Gal4-$p65^{1-551}$ activation in these cells in response to IL-1 or to test the effects of induction of RacN17 on the system (data not shown). Our data with EL4.NOB-1 cells however clearly demonstrates a role for Rac1 in transactivation by p65 and definitively places Rac1 downstream of IL-1 on this pathway.

(A)



(B)

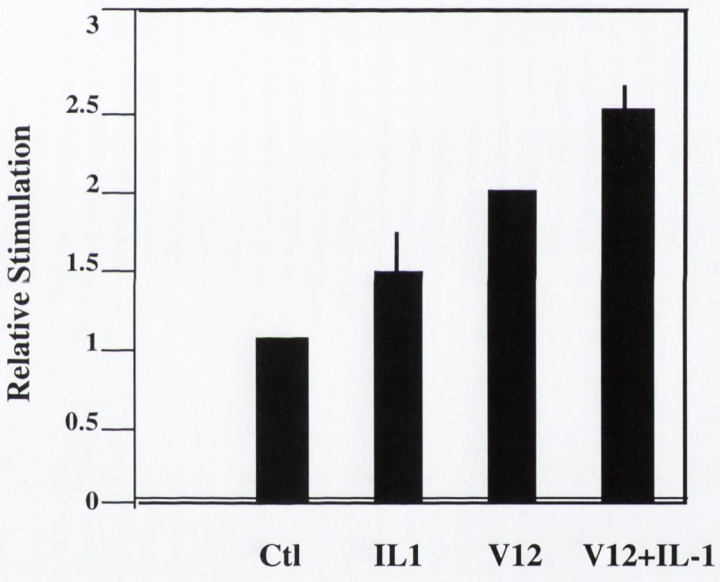
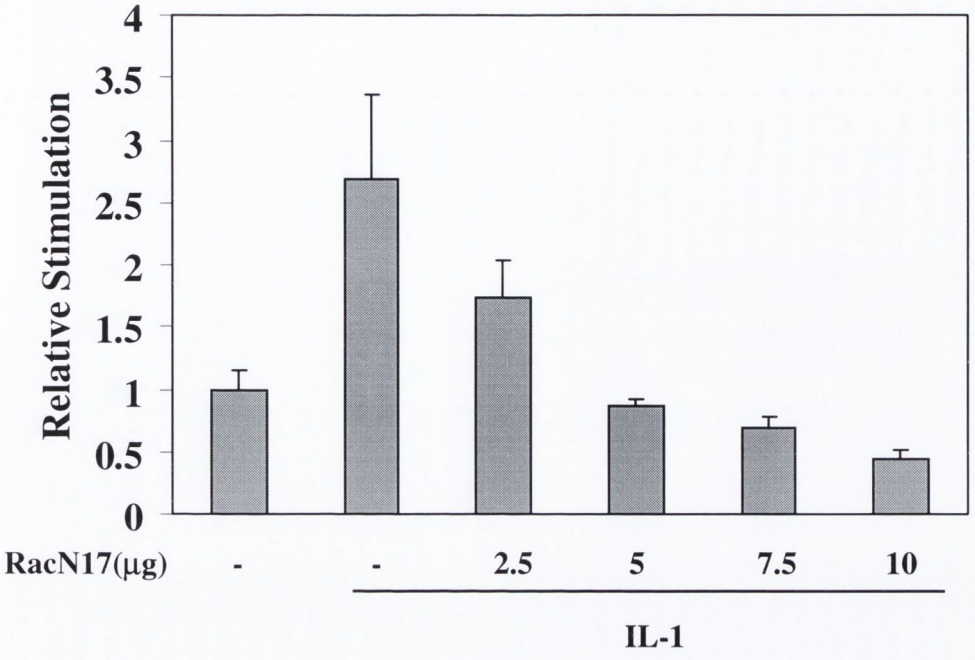


Figure 4.2.8: Constitutively active RacV12 drives p65-mediated transactivation.

(A) EL4.NOB-1 cells ($1.4x10^7$) and (B) V12Rac-PAE cells ($4x10^4$) were transfected with Gal4-luciferase (5μg and 350ng respectively) and Gal4-$p65^{1-551}$ (2.5μg and 350ng, respectively). In addition, EL4.NOB-1 cells were transfected with RacV12 (10μg) as indicated. RacV12 expression was induced 6 hours post-transfection in V12Rac-PAE cells and both cell types were stimulated with IL-1 (10ng/ml, 6 hours) 18 hours post-transfection. Cell extracts were subsequently analysed for luciferase activity. Results are expressed relative to unstimulated controls and represent the mean $\pm$ S.D from triplicate experiments. Results shown are representative of at least three separate experiments.

(A)



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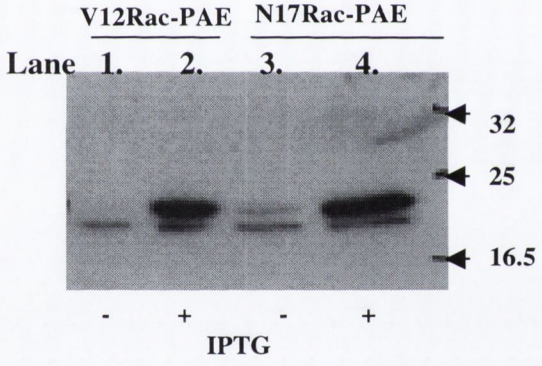


Figure 4.2.9: RacN17 inhibits IL-1-induced p65-gal activation

(A) EL4.NOB-1 cells ($1.4x10^7$) were transfected with 2.5μg Gal4-$p65^{1-551}$, 5μg of Gal-luciferase and increasing amounts of RacN17. Following a period of recovery (16-18hours) cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control (mean±S.D. from triplicate determinations). Similar results were seen in three independent experiments. (B) N17Rac-PAE cells were found to express a low level of RacN17 in the absence of induction with IPTG. The above blot shows both lysates from uninduced N17Rac- (lane 3) and V12Rac- (lane 1) PAE cells. EE-Rac1 is detected following western blot analysis of lysates in N17Rac-PAE cells.

4.2.10 p42/p44 MAPK lies downstream of IL-1 in regulating p65-mediated transactivation of gene expression.

The next question that was addressed was the possible involvement of the mitogen activated protein kinase (MAPK) pathways in regulating p65-mediated transactivation. Using the MEK1 inhibitor PD98059 the inhibition of IL-1-induced Gal4-p65$^{1-551}$ activity in EL4.NOB-1 cells was demonstrated. Briefly, cells co-transfected with 2.5μg Gal4-p65$^{1-551}$ and 5μg Gal4-luciferase were pre-treated with increasing concentrations of PD98059 (in the range 0-30μM) for 1 hour, after which cells were stimulated with IL-1 as indicated. Determination of luciferase activity demonstrated that PD98059 dose dependently inhibited the IL-1-induced response (figure 4.2.10) with 30μM PD98059 resulting in 80% inhibition. As the IC_{50} of PD98059 lies between 2-7μM there is a possiblility that the effects seen at this high dose are due to non-specific inhibitory effects at 30μM. However, the inhibition of IL-1-induced activity by approximately 40% at 3μM demonstrates an involvement of p42/p44 MAPK in this pathway. Previous work in our laboratory has demonstrated the inhibitory effect of PD98059 on p42/p44 MAPK activation in EL4.NOB-1 cells (120). This result indicates that p42/p44 MAPK lies downstream of IL-1 on the pathway regulating p65-mediated transactivation.

4.2.11 p38 MAPK is involved in IL-1 signalling to p65-mediated transactivation.

The p38 MAPK inhibitor SB203580 was tested against IL-1-induced Gal4-p65$^{1-551}$ activation in EL4.NOB-1 cells. As with the p42/p44 MAPK inhibitor, we found that pretreatment of cells with increasing concentrations of SB203580 (0-30μM) inhibited IL-1-induced transactivation of gene expression by p65 (figure 4.2.11). Both 0.3 and 3μM SB203580 only weakly inhibited the IL-1-induced response whereas pretreatment of cells with 30μm resulted in 70% inhibition of IL-1-induced Gal4-p65$^{1-551}$ activation. The possibility that the inhibitory effect seen at this high dose of SB203580 maybe due to non-specific inhibition of other pathways must be considered.

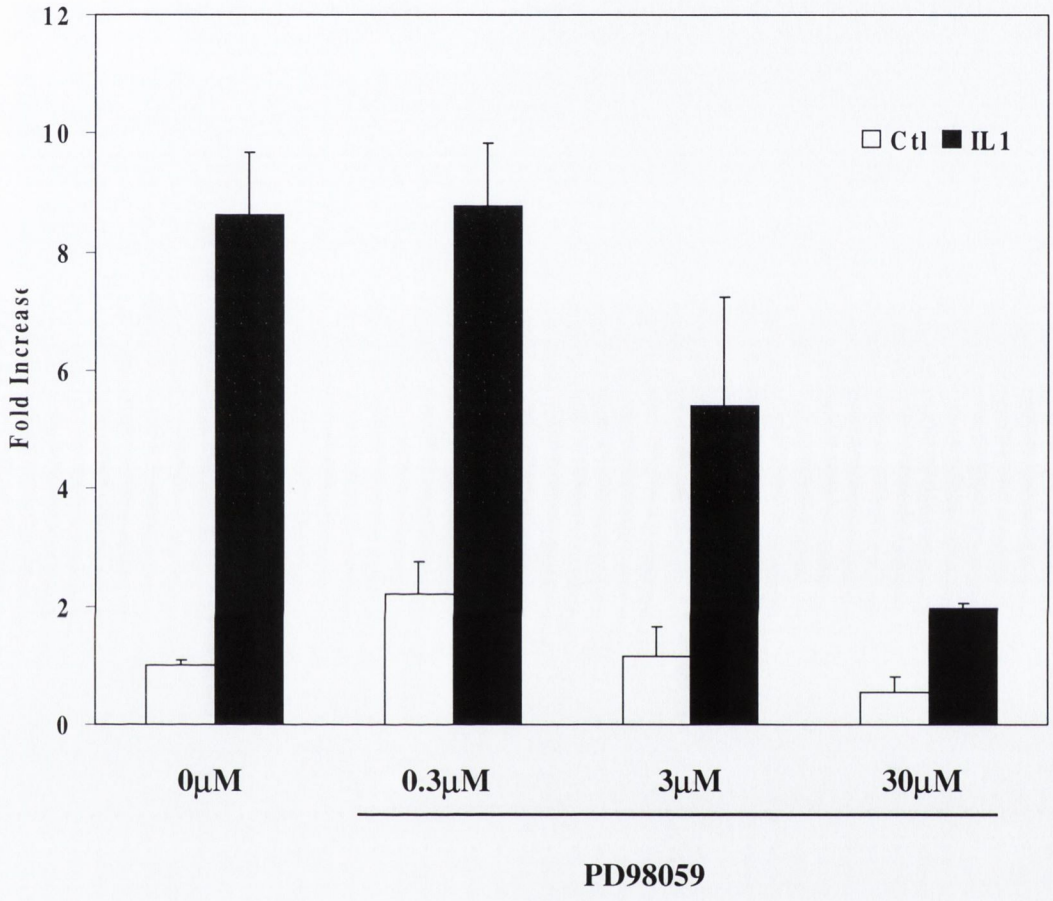


Figure 4.2.10: Inhibition of IL-1-induced Gal4-p65$^{1-551}$ activation by the p42/p44 inhibitor PD98059

EL4.NOB-1 cells (1.4x10^7) were transfected with 2.5μg Gal4-p65$^{1-551}$ and 5μg of Gal4-luciferase. Following a period of recovery (16-18hours) cells were pre-treated with the indicated concentrations of PD98059 and then stimulated with IL-1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal4-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations. Similar results were seen in three independent experiments.

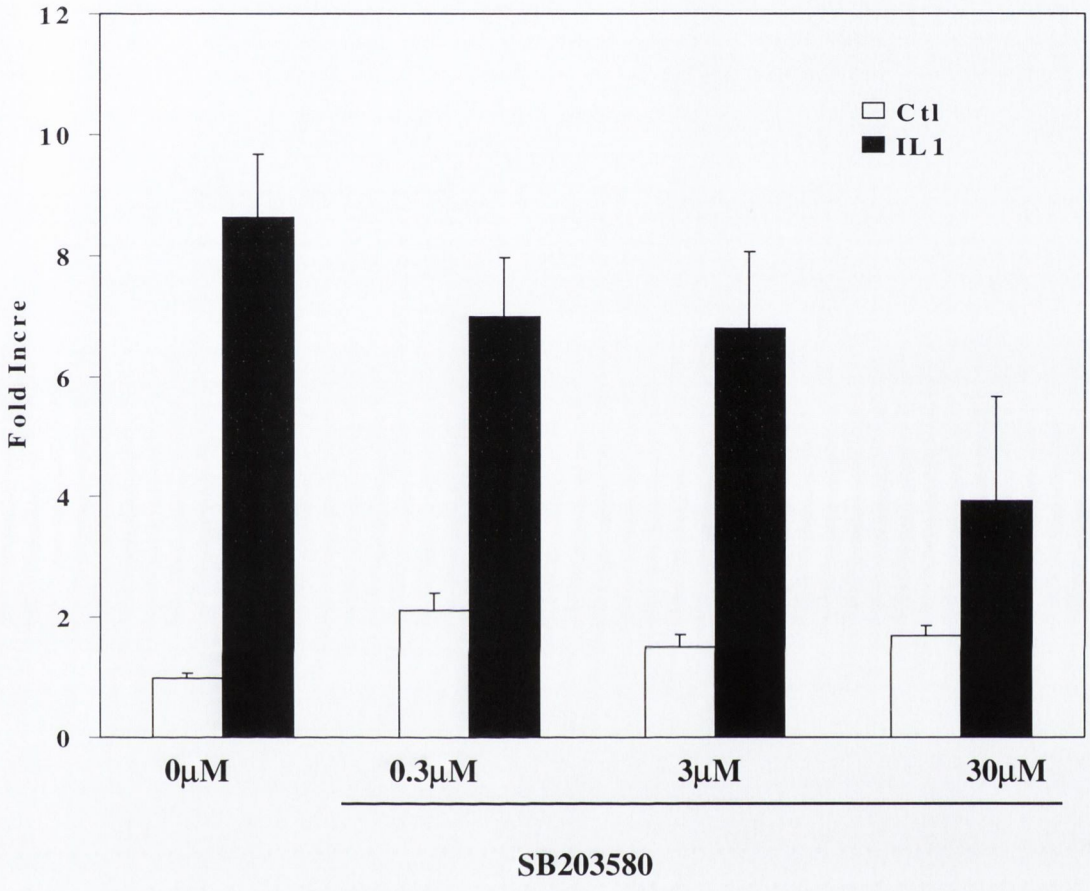


Figure 4.2.11: Inhibition of IL-1-induced Gal4-p65$^{1-551}$ activation by the p38 inhibitor SB203580

EL4.NOB-1 cells ($1.4x10^7$) were transfected with 2.5μg Gal4-p65$^{1-551}$ and 5μg of Gal4-luciferase. Following a period of recovery (16-18hours) cells were pre-treated with the indicated concentrations of SB203580 and then stimulated with IL-1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal4-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations. Identical results were obtained in three independent experiments.

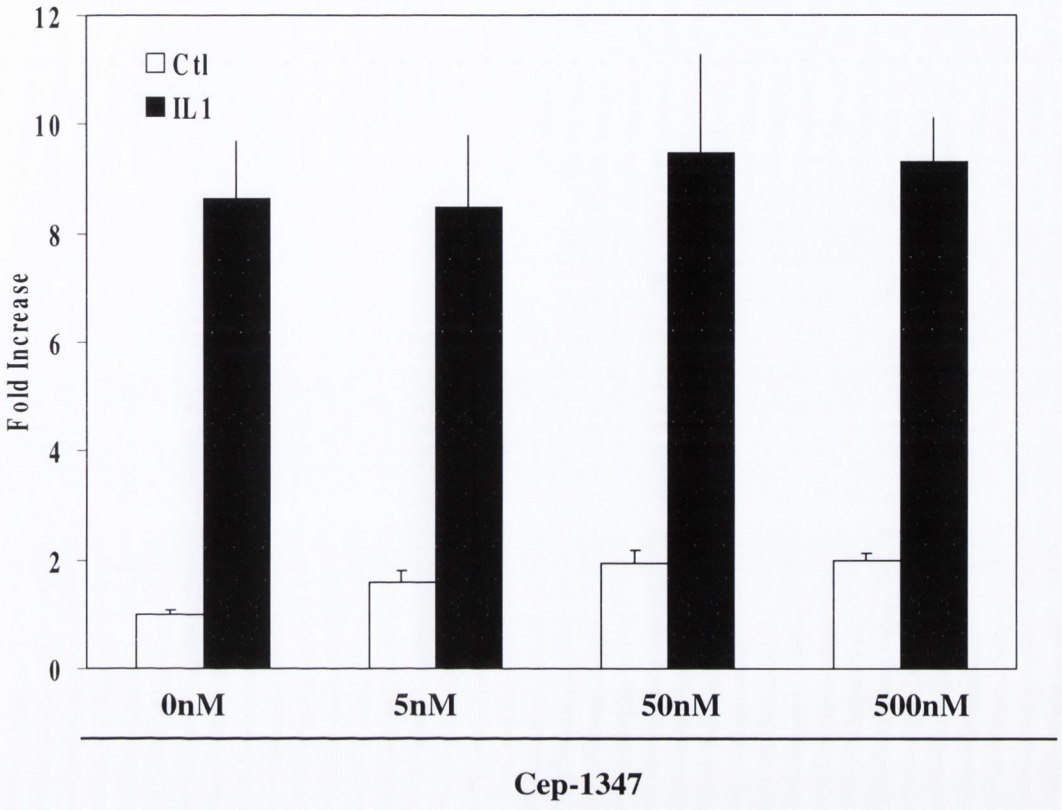
4.2.12 JNK MAPK is not involved in IL-1-induced p65-mediated transactivation of gene expression.

Finally, the possibility that JNK may be involved in our system was tested using a JNK specific inhibitor Cep-1347. Pretreatment of cells with increasing concentrations of Cep-1347 (0-500nM) failed to inhibit IL-1-induced Gal4-p65$^{1-551}$ activity (figure 4.2.13a). The ability of Cep-1347 to inhibit IL-1-induced JNK activation was tested using a phospho-JNK antibody that specifically recognises activated JNK. Western blot analysis of whole cell lysates prepared from EL4.NOB-1 cells pretreated with increasing concentrations of Cep-1347 prior to IL-1 stimulation demonstrated that 500nM Cep-1347 inhibited IL-1-induced JNK activation, although the inhibition observed was incomplete (figure 4.2.12b, compare lane 2 with lanes 8). Our results clearly indicate an involvement of p42/p44 and p38 MAPK but not JNK in IL-1-induced transactivation of gene expression by p65.

4.2.13 p42/p44 MAPK and p38 MAPK but not JNK lie downstream of constitutively active RacV12-induced transactivation by p65.

Having shown that p42/p44 and p38 MAPK but not JNK regulate transactivation of gene expression in response to IL-1 we next determined whether any of these MAPK pathways lie downstream of Rac1 on this pathway. As with IL-1-induced Gal4-p65$^{1-551}$ activation, pre-treatment of constitutively active RacV12-transfected cells with PD98059 and SB203580, but not Cep-1347, resulted in inhibition of RacV12-induced Gal4-p65$^{1-551}$ activity (figure 4.2.13). This result clearly confirms the involvement of both p42/p44 MAPK and p38 MAPK downstream of IL-1 and Rac1 in regulating p65-mediated transactivation, but shows no involvement of JNK in this pathway.

(A)



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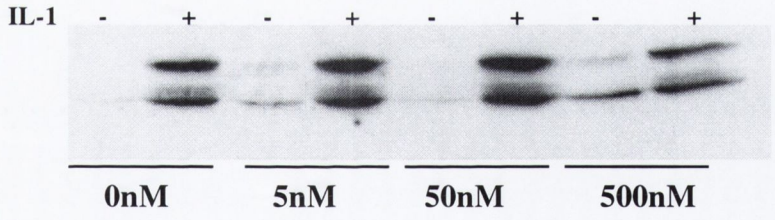


Figure 4.2.12:Lack of inhibition of IL-1-induced p65-gal activation by Cep-1347, a JNK inhibitor

(A) EL4.NOB-1 cells ($1.4x10^7$) were transfected with 2.5μg p65-gal and 5μg of Gal-luc. Following a period of recovery (16-18hours) cells were pre-treated with the indicated concentrations of Cep-1347 and then stimulated with IL-1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations.(B) EL4.NOB-1 cells were pretreated with Cep-1347 and stimulated with IL-1 (10ng/ml) for 15 minutes. Whole cell lysates were prepared and tested for JNK activation using an anti-phospho-JNK antibody.

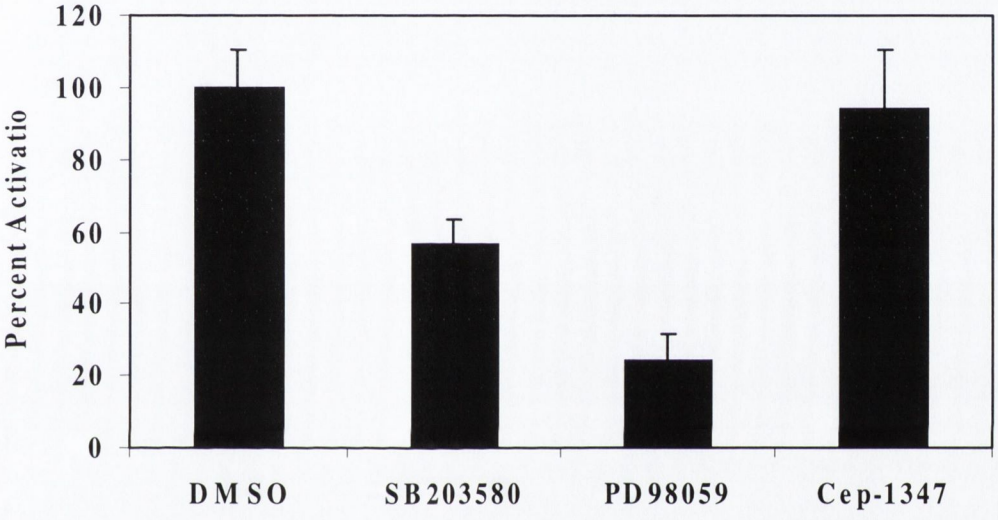


Figure 4.2.13: PD98059 and SB203580 but not Cep-1347 inhibit RacV12-induced activation of Gal4-p65$^{1-551}$.

EL4.NOB-1 cells ($1.4x10^7$) were transfected with 2.5µg Gal4-p65$^{1-551}$, 5µg of Gal4-luciferase and 2.5µg RacV12. Following a period of recovery (16-18 hours) cells were pre-treated with the relevant inhibitors for 1 hour and then stimulated with IL-1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal4-luciferase activity using standard protocols. Results are shown relative to the RacV12-induced response (100%) and are represented as Mean$\pm$S.D. from triplicate determinations. Identical results were obtained from three independent experiments.

4.3 Discussion

This data provides the first direct evidence that IL-1 stimulation of cells results in enhanced transactivation of gene expression by the p65 subunit of NFκB due to the activation of a signalling pathway not involved in IκB regulation. We have investigated the involvement of Rac1 in either of these two pathways using two well characterized mutants of Rac1, constitutively active RacV12 and dominant negative RacN17. Our results demonstrate that Rac1 does not lie on the IL-1-induced signalling pathway leading to IκBα phosphorylation and degradation, but instead lies downstream of IL-1 on the second pathway responsible for regulating transactivation of gene expression by p65.

Unlike IL-1 stimulation, expression of constitutively active RacV12 in either EL4.NOB-1 cells or V12Rac-PAE cells was unable to drive degradation of IκBα. In addition dominant negative RacN17 expression in either cell type failed to inhibit IκBα degradation in response to IL-1 stimulation. Similarly, we found that RacV12 expression was unable to mimic IL-1 stimulation of EL4.NOB-1 or PAE cells in activating the DNA-binding ability of NFκB. In keeping with its effects on IL-1-induced IκB-degradation, expression of dominant negative RacN17 did not inhibit IL-1-induced DNA-binding activity of NFκB. These results clearly indicate that Rac1 is not involved in the pathway regulating either IκB degradation or DNA-binding activity of NFκB. Instead we found that constitutively active RacV12 expression in either EL4.NOB-1 or V12Rac-PAE cells activated the ability of p65 to transactivate gene expression. Importantly, expression of dominant negative RacN17 in EL4.NOB-1 cells dose dependently inhibited IL-1-induced p65-mediated transactivation, thus placing Rac1 downstream of IL-1 on this pathway.

The involvement of Rac1 in NFκB activation has been previously suggested with constitutively active RacV12 driving NFκB-dependent reporter gene expression when transfected

into HeLa cells (175). Sulciner *et al* (1996) provide evidence that Rac1 regulates the production of reactive oxygen species (ROS) in HeLa cells and that Rac1 therefore functions as part of a redox-dependent signal transduction pathway leading to NFκB activation. In addition Rac1 has been shown to regulate NFκB activation in response to IL-1 via the production of reactive oxygen species when transfected into rabbit synovial fibroblasts (RSV) (87) with nuclear translocation and DNA-binding activity marginally enhanced in RacV12-transfected cells, but only in the presence of integrin ligation. While these results appear to suggest an involvement for Rac1 in NFκB activation via the production of ROS in both HeLa and RSV cells, recent evidence has clearly shown no involvement of Rac1 in regulating ROS production in lymphocytes (16). This supports our view that, in our system, Rac1 lies on an alternate pathway regulating NFκB activation, specifically, the pathway mediating the transactivation of gene expression by the p65 subunit. In addition we have been unable to find a role for ROS in NFκB activation by IL-1 in EL4.NOB-1 cells (22).

A key question concerns how IL-1 and Rac1 might mediate transactivation of gene expression by p65. Recent studies have pointed to a role for both p42/p44 and p38 MAPK pathways in TNF-mediated transactivation by p65 (187). Using specific inhibitors of p42/p44, p38 and JNK MAPK (PD98059, SB203580, and Cep-1347, respectively), the role of these MAPK in this pathway was examined. The p42/p44 MAPK inhibitor PD98059 has been demonstrated to be a highly selective inhibitor of MEK1 (IC_{50} = 2-7μM) in Swiss 3T3 cells (3) with no inhibitory effect on p42/p44 MAPK itself or a range of other kinases such as p38 MAPK and JNK. The pyridinyl imidazole SB203580 has been shown to be a highly selective inhibitor of p38 MAPK in a number of different systems (39) and has previously been demonstrated to inhibit p38 MAPK activation in our laboratory (120). The JNK-specific inhibitor Cep-1347 was selected for its ability to induce choline acetyl transferase activity in cultures prepared from embryonic spinal cord and hence promote neural survival. It was demonstrated that its anti-apoptotic activity

corresponded to its inhibitory activity on JNK at a level proximal to JNK itself (116). Using these highly selective inhibitors a role for p42/p44 and p38 MAPK but not JNK downstream of IL-1 on this pathway is suggested. However, the inability of SB203580 to inhibit IL-1-induced p65-mediated transactivation at doses lower than 30μM raises the possibility that its effects may due to non-specific inhibition of other signalling molecules in the cell. Similarly, RacV12-mediated Gal4-p65$^{1-551}$ activity was inhibited by PD98059 and SB203580 but not Cep-1347, implicating p42/p44 and p38 MAPK (but not JNK) downstream of RacV12-induced transactivation of gene expression by p65. The possible mechanism by which these MAPK pathways regulate p65-mediated transactivation in response to IL-1 is unclear. Recently however it has been shown that while SB203580 inhibited LPS-induced NFκB-dependent reporter gene expression, it was unable to inhibit NFκB DNA-binding or IκB degradation. Instead a role for p38 MAPK in transactivation has been suggested from studies that have demonstrated that SB203580 inhibits phosphorylation of the TATA-binding protein (TBP), thus preventing interaction of TBP with p65 and thereby blocking transactivation (30).

Several reports have indicated that upon stimulation of cells with either IL-1 or TNF the p65 subunit of NFκB becomes phosphorylated upon multiple serine residues which potentially acts to enhance its transactivating potential. Although p42/p44 and p38 MAPK may be involved in regulating these phosphorylation events, they are unlikely to directly phosphorylate p65 due to a lack of potential consensus sites on the subunit. The p65 subunit contains two transactivation domains at its C terminal end and a Rel homology domain at its N terminus, both regions undergoing phosphorylation following TNF stimulation of cells. The kinases directly responsible for phosphorylating p65 have recently been the subject of intense investigation although as yet only a few have been identified. These include casein kinase II, which has been proposed to phosphorylate the transactivation domain of p65 in response to TNF stimulation on serine 529, possibly resulting in enhanced transactivation of gene expression by p65. More recently, serine

536 (also in the transactivation domain) has been identified as being important for regulating transactivation, and the kinase responsible for its phosphorylation has been identified as IKK1 and 2 (156). Protein Kinase A (PKA) has been shown to regulate phosphorylation of serine 276 in the Rel homology domain of p65 which is crucial for its binding to the consensus sequence. This domain has also been shown to play a role in regulating transactivation of gene expression by p65 with protein kinase C zeta (activated by Ras) via phosphorylation of this domain on a site other than serine 276. How phosphorylation of these sites promotes transactivation is unclear although, as with other signalling cascades, the likelihood is that phosphorylation of p65 will increase its ability to interact with members of the basal transcription machinery and with coactivators such as CBP/p300. This has been shown to be the case where phosphorylation of serine 276 by PKA enhances the ability of p65 to interact with CBP/p300. In addition, as was already mentioned, phosphorylation of TBP via activation of p38 MAPK facilitates an interaction between TBP and p65. We would speculate that Rac1 is required for some or all of these events in our system, possibly via the activation of both p42/p44 and p38 MAPK pathways. It is possible, due to the requirement for IRAK-1 localisation to focal adhesion complexes in order that p42/p44 MAPK might be activated by IL-1, that the role of Rac1 in this pathway is to provide a link between the actin cytoskeleton, the receptor complex and the proteins involved in regulating the pathway (108).

As to where Rac1 acts on the IL-1-activated signalling pathway culminating in enhanced transactivation of gene expression by p65 is as yet unclear. A recent report indicates that Rac1 may interact with the IL-1 receptor complex via an interaction with the type I IL-1-receptor (168). This suggests that the means by which IL-1 stimulation of cells activates Rac1 is through an interaction with the receptor complex, possibly via an as yet unidentified exchange factor.

Our results clearly show that IL-1 activates two separate signalling pathways that, combined, regulate the activity of NFκB. We have demonstrated a role for Rac1 in IL-1-induced enhancement of gene expression by the p65 subunit of NFκB, independent of both IκB

degradation and DNA binding of NFκB. Furthermore, both p42/p44 and p38 MAPK pathways are required for p65-mediated transactivation of gene expression by p65 in response to IL-1 stimulation, with Rac1 being placed firmly downstream of IL-1 on this pathway as shown in figure 4.3.

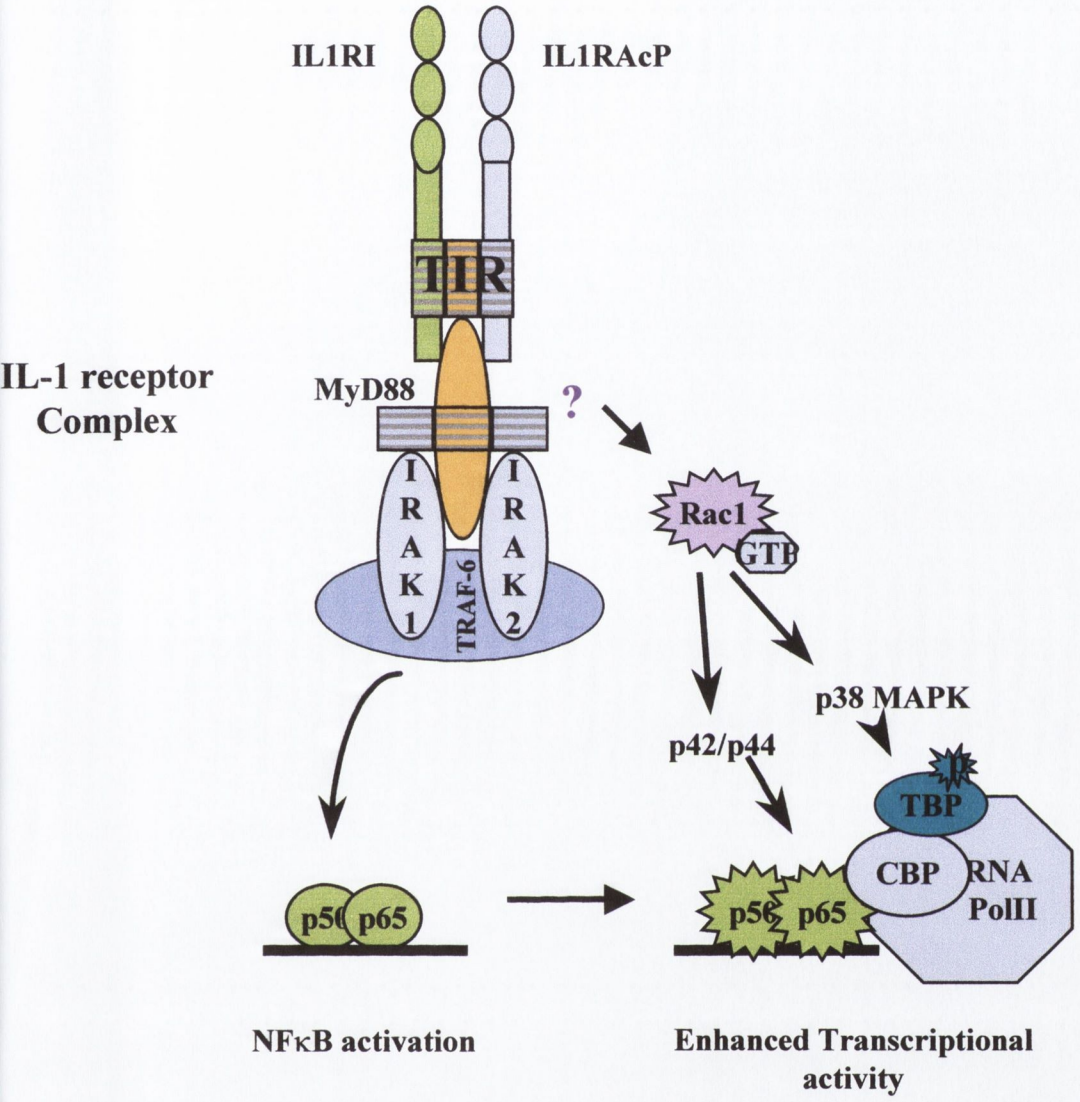


Figure 4.3: Proposed model for the role of Rac1 in IL-1 signalling to NFκB activation

Rac1 is activated following IL-1 stimulation of cells. Rac1 involvement in NFκB activation in response to IL-1 is independent of the pathway regulating IκB degradation. Instead Rac1 regulates the ability of the p65 subunit of NFκB to transactivate gene expression in response to IL-1. Both p38 MAPK and p42/p44 MAPK have been found to lie downstream of Rac1 on this pathway. While the role of p42/p44 MAPK in regulating this pathway is unknown, p38 MAPK has been demonstrated to regulate TBP, an integral protein of the RNA polymerase II holoenzyme (RNA polII). Phosphorylation of p65 has been demonstrated to enhance interaction with the coactivator CREB-binding protein (CBP).

Chapter Five

Signalling from the IL-1-receptor complex to transactivation of gene expression by p65 involves MyD88, IRAK-1, TRAF-6 and Rac1

5 Chapter 5

5.1 Introduction

Having demonstrated that IL-1 activates Rac1 and that Rac1 lies downstream of IL-1 on a pathway, independent of IκB degradation, which regulates transactivation of gene expression by the p65 subunit of NFκB, two key questions were immediately raised. Firstly, what the role of key regulators of IL-1 signal transduction (i.e. MyD88, IRAK-1 and TRAF-6) might be in this recently described pathway and secondly, how Rac1 mediates the signal from the IL-1-receptor complex which results in enhanced transactivation potential of the p65 subunit of NFκB.

The signal transduction pathway initiated as a result of IL-1 binding to its receptor complex has been the subject of intense investigation due the key role of IL-1 in regulating NFκB activation. Formation of the high affinity receptor complex recruits the key signalling molecules MyD88, IRAK and TRAF-6 to its intracellular domain (reviewed in (141)). The exact nature of the formation of the complex is not clear although recent observation have described a novel protein Tollip as been involved in IRAK recruitment to MyD88-containing receptor complexes (24). Formation of the signalling complex enables downstream signalling, resulting in activation of the IκB kinase (IKK) complex, responsible for IκB phosphorylation and subsequent ubiquitin-mediated degradation (43, 201).

In addition to regulating IKK activation and subsequent IκB degradation, MyD88, IRAK-1 and TRAF-6 have also been implicated in signalling pathways regulating activation of the transcription factor AP-1. Over-expression of MyD88 in HEK-293T cells resulted in activation of JNK (an upstream kinase responsible for activation of AP-1) as determined by western blot analysis of immunoprecipitated HA-JNK using an antibody that specifically recognises the active, phosphorylated form of JNK (25). In this paper the authors also demonstrate that MyD88 overexpression activated the kinase activity of JNK. Work in our laboratory has also shown that both IRAK-1 and TRAF-6 activates an AP-1-dependent reporter gene in EL4.NOB-1 cells and

that transfection of cells with dominant negative TRAF-6 inhibits IL-1-induced activation of AP-1-CAT. This suggests that the point of divergence of the pathways regulating NFκB and AP-1 activation lies downstream of TRAF-6 (139). Recently IRAK-1 has been implicated in regulating p42/p44 MAPK activation via recruitment of IRAK-1 to focal adhesion complexes where the IL-1 receptor complex has been previously demonstrated to localise (108, 148). While there is evidence for the involvement of MyD88, IRAK-1 and TRAF-6 in regulating signal transduction pathways resulting in MAPK activation, the molecular mechanisms governing these pathways are unclear.

Previous observations that IL-1 activates a pathway regulating the ability of p65 to transactivate gene expression, prompted further investigation into how signals are transduced from the IL-1 receptor complex in regulating this pathway. To this end the involvement of key regulators of IL-1 signal transduction, MyD88, IRAK-1 and -2, and TRAF-6, in this pathway was assessed. While MyD88, IRAK-1 and TRAF-6 were all shown to regulate p65-mediated transactivation in response to IL-1, no role for IRAK-2 in this pathway was demonstrated. Our results have shown that Rac1 lies downstream of IL-1 on the pathway regulating transactivation of gene expression by IL-1. It was therefore determined how Rac1 might relate to MyD88, IRAK-1 and TRAF-6. While Rac1 was found to lie downstream of MyD88 on this pathway, the ability of dominant negative RacN17 to inhibit IRAK-1 and TRAF-6 induced Gal4-p65$^{1-551}$ activity and vice versa suggested that Rac1, IRAK-1 and TRAF-6 form part of a complex downstream of MyD88 which regulates this pathway.

5.2 Results

5.2.1 MyD88 drives p65-mediated transactivation in EL4.NOB-1 cells.

The role of MyD88 in IL-1-induced p65-mediated transactivation of gene expression was initially assessed. EL4.NOB-1 cells were transiently transfected with increasing amounts of a plasmid encoding wild type MyD88 along with Gal4-p65$^{1-551}$ and Gal4-luciferase. As shown in figure 5.2.1a, transfection of EL4.NOB-1 cells with MyD88 alone activated p65-mediated transactivation, with activation increasing as MyD88 concentration increased. Stimulation of MyD88-transfected cells with IL-1 (10ng/ml) resulted in potentiation of the MyD88-induced response (figure 5.2.1b).

5.2.2 IRAK-1, but not IRAK-2, drives p65-mediated transactivation in EL4.NOB-1 cells.

To assess the effects of both IRAK-1 and -2 on our system EL4.NOB-1 cells were transiently transfected with increasing concentrations of plasmids encoding either wild-type IRAK-1 or IRAK-2. While IRAK-1 potently drove Gal4-p65$^{1-551}$ activity (figure 5.2.2a), IRAK-2 was unable to drive this response (figure 5.2.2b), although it potentiated the IL-1-induced response at the lowest concentration. Similar to MyD88, IL-1 stimulation of IRAK-1-transfected cells resulted in a potentiation of IRAK-1-induced Gal4-p65$^{1-551}$ activity (figure 5.2.2c).

5.2.3 TRAF-6 drives p65-mediated transactivation of gene expression

EL4.NOB-1 cells were transiently transfected with a plasmid encoding wild-type TRAF-6 in the range 1.25-5μg, and its effects on Gal4-p65$^{1-551}$ activity assessed. As shown in figure 5.2.3a TRAF-6 transfection resulted in a dose dependent increase in Gal4-p65$^{1-551}$ activity compared with control. IL-1 stimulation of TRAF-6 transfected cells resulted in a further increase in TRAF-6-mediated Gal4-p65$^{1-551}$ activity (figure 5.2.3b).

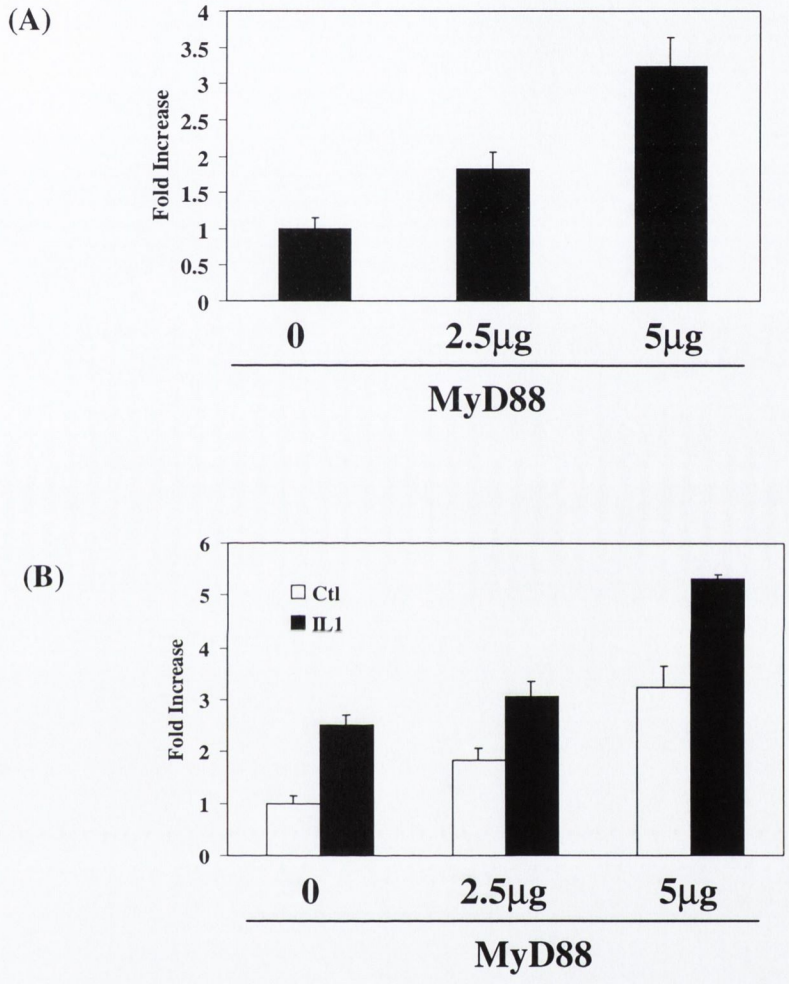


Figure 5.2.1: MyD88 drives p65-mediated transactivation

(A) EL4.NOB-1 cells ($7x10^6$) were transfected with 1.25µg Gal4-$p65^{1-551}$, 2.5µg of Gal-luciferase and increasing amounts of MyD88 as indicated. Following a period of recovery (16-18hours) cells were lysed and subsequently analysed for Gal-luciferase activity using standard protocols. (B) Transfected cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean$\pm$S.D. from triplicate determinations. Results are representative of at least three separate experiments.

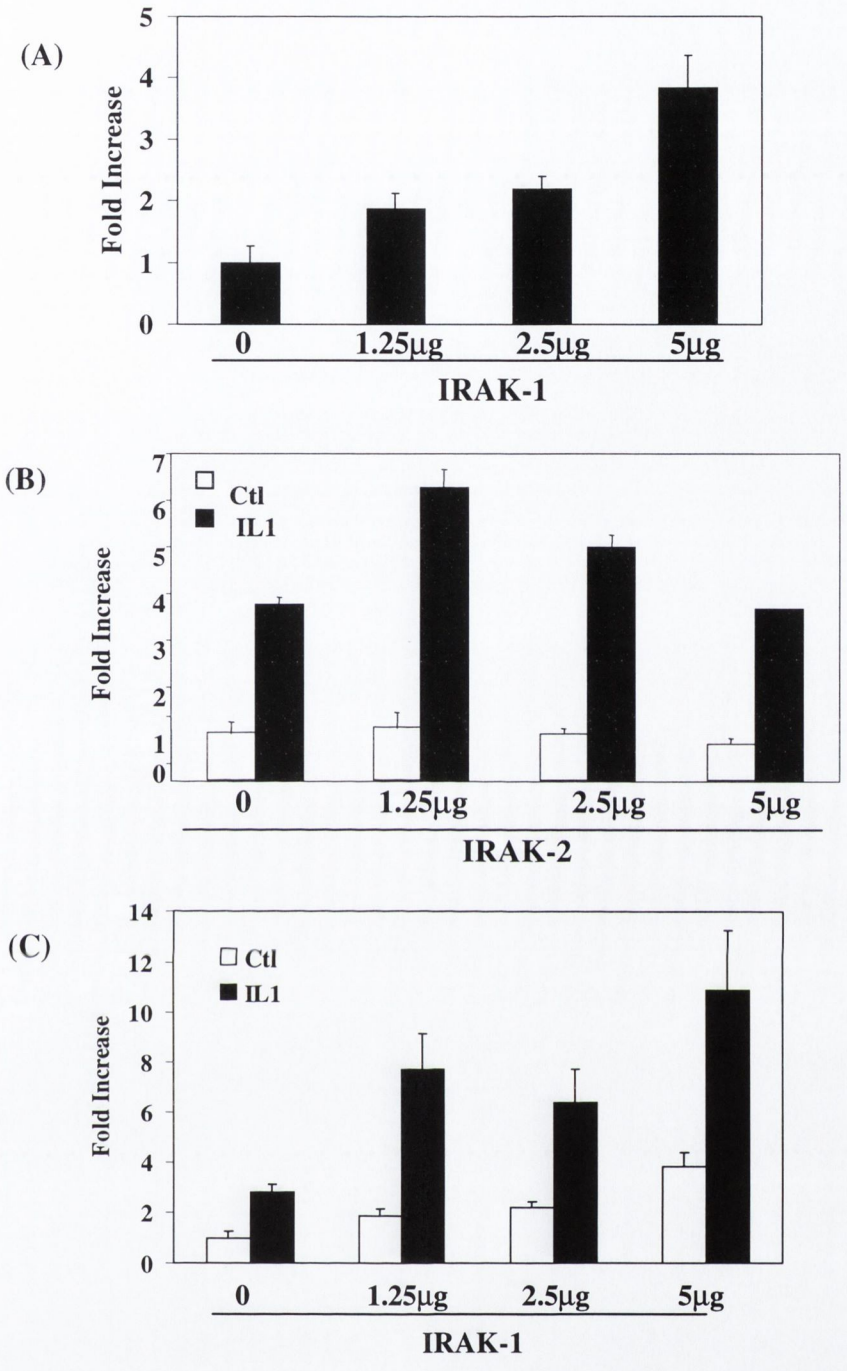


Figure 5.2.2: IRAK-1 drives p65-mediated transactivation

EL4.NOB-1 cells ($7x10^6$) were transfected with 1.25µg Gal4-$p65^{1-551}$, 2.5µg of Gal-luciferase and increasing amounts of (A) IRAK-1 or (B) IRAK-2 as indicated. Lysates were subsequently analysed for Gal-luciferase activity using standard protocols. (B) and (C) Transfected cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations. Results are representative of at least three separate experiments.

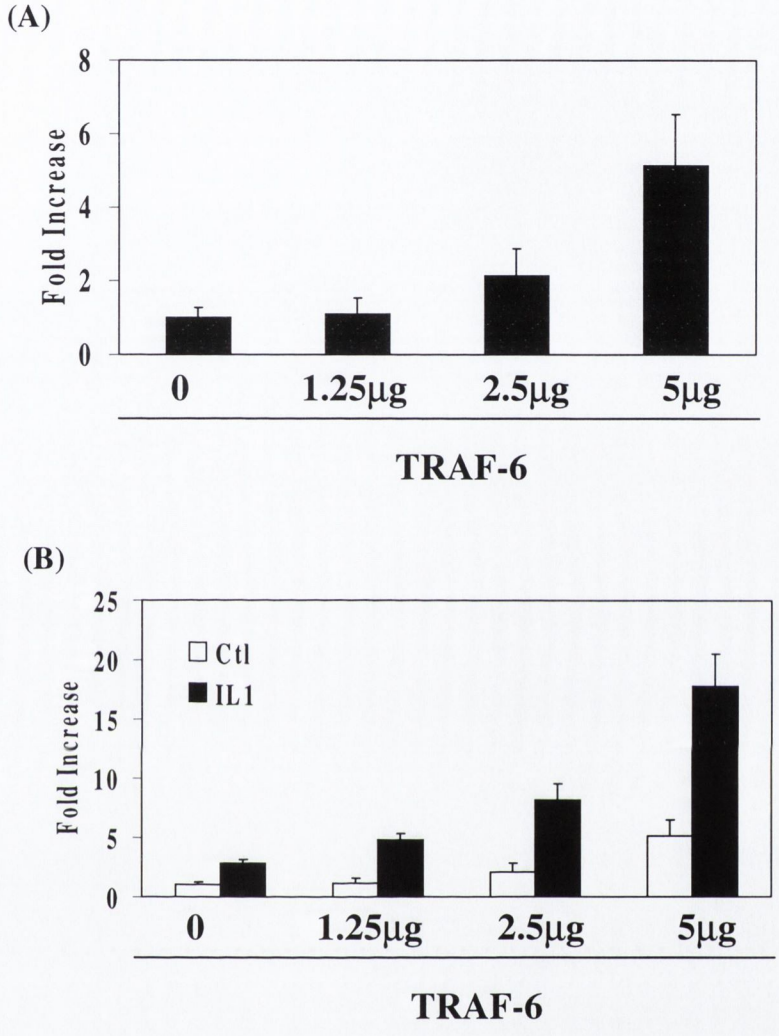


Figure 5.2.3: TRAF-6 drives p65-mediated transactivation

(A) EL4.NOB-1 cells ($7x10^6$) were transfected with 1.25µg Gal4-$p65^{1-551}$, 2.5µg of Gal-luciferase and increasing amounts of TRAF-6 as indicated. Following a period of recovery (16-18 hours) cells were lysed and subsequently analysed for Gal-luciferase activity using standard protocols. (B) Transfected cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean$\pm$S.D. from triplicate determinations. Results are representative of at least three separate experiments.

5.2.4 Dominant negative MyD88 inhibits IL-1-induced p65-mediated transactivation

Having shown MyD88, IRAK-1, TRAF-6, but not IRAK-2, can activate p65-mediated transactivation of gene expression, I set out to determine whether MyD88, IRAK-1 or TRAF-6 are involved in IL-1-induced activation of this pathway. The involvement of MyD88 in IL-1-induced activation of transactivation by p65 was determined using a dominant negative mutant of MyD88, a deletion mutant encoding the TIR domain only of MyD88 (amino acids 152-296). Transient transfection of EL4.NOB-1 cells with increasing amounts of dominant negative MyD88 resulted in a dose dependent inhibition of IL-1-induced Gal4-p65$^{1-551}$ activity (figure 5.2.4), thereby demonstrating the requirement for MyD88 for the effect of IL-1 on this pathway.

5.2.5 Dominant negative IRAK-1 potently inhibits IL-1-induced Gal4-p65$^{1-551}$ activity

Using a dominant negative mutant of IRAK-1, encoding the death domain only (amino acids 1-211), we assessed its role in IL-1-induced transactivation of gene expression by p65. Transient transfection of EL4.NOB-1 cells with as little as 1.25μg of dominant negative IRAK-1 potently inhibited IL-1-induced Gal4-p65$^{1-551}$ activation (figure 5.2.5), indicating that IRAK-1 is required for IL-1-induced p65-mediated transactivation of gene expression.

5.2.6 Dominant negative TRAF-6 inhibits Gal4-p65$^{1-551}$ activity in response to IL-1 in EL4.NOB-1 cells

Having established that MyD88 and IRAK-1 lie downstream of IL-1 on the pathway regulating p65-mediated transactivation of gene expression, we next determined whether TRAF-6 plays a role in IL-1-induced Gal4-p65$^{1-551}$ activity. To do this we used a dominant negative mutant of TRAF-6 (amino acids 289-522) in which the ring finger and zinc finger domains have been deleted. Transient transfection of as little as 1.25μg of dominant negative TRAF-6 resulted in

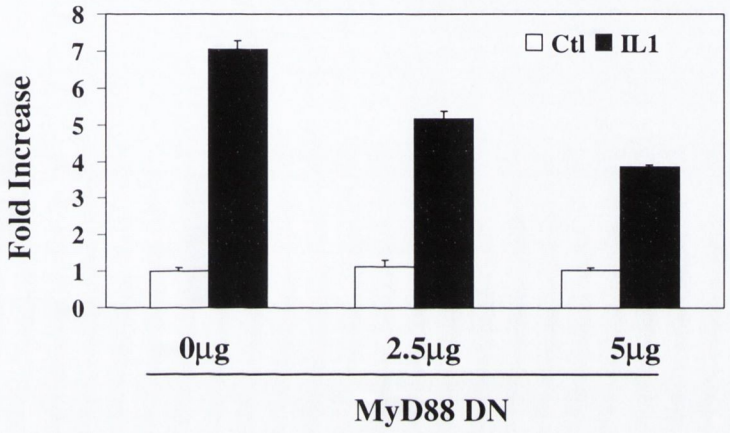


Figure 5.2.4: Dominant negative MyD88 inhibits IL-1-induced Gal4-p65$^{1-551}$ activation

EL4.NOB-1 cells (7x10^6) were transfected with 2.5μg Gal4-p65$^{1-551}$, 5μg of Gal-luciferase and increasing amounts of dominant negative MyD88 as indicated. Following a period of recovery (16-18hours) cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations.

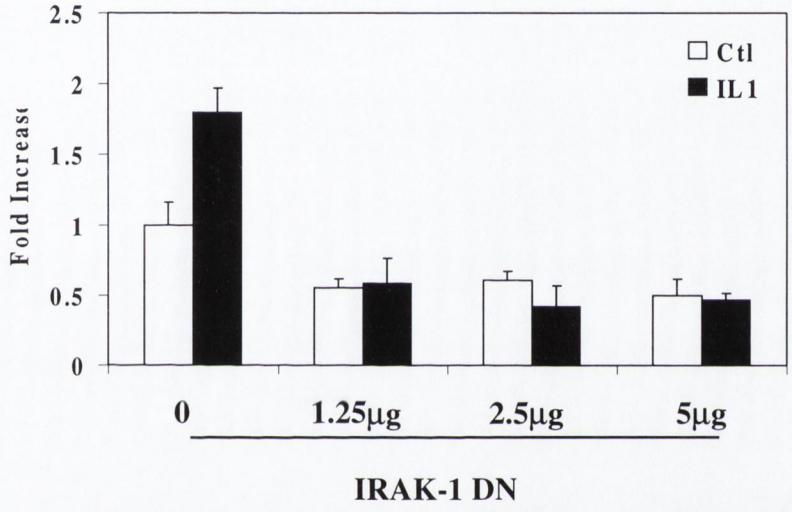


Figure 5.2.5: Dominant negative IRAK-1 inhibits IL-1-induced Gal4-p65$^{1-551}$ activation

EL4.NOB-1 cells ($7x10^6$) were transfected with 2.5µg Gal4-p65$^{1-551}$, 5µg of Gal-luciferase and increasing amounts of dominant negative IRAK-1 as indicated. Following a period of recovery (16-18hours) cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations.

complete inhibition of IL-1-induced Gal4-p65$^{1-551}$ activity, suggesting a crucial role for TRAF-6 in IL-1-induced activation of this pathway (figure 5.2.6).

5.2.7 IRAK-1 and TRAF-6 inhibit MyD88-induced Gal4-p65$^{1-551}$ activation

To position MyD88, IRAK-1 and TRAF-6 with respect to each other on this pathway we cotransfected our cells with wild type versions of each, together with dominant negative MyD88, IRAK-1 and TRAF-6. The effect seen on Gal4-p65$^{1-551}$ activity when cells were transfected with MyD88 was inhibited by cotransfection of cells with increasing concentrations of IRAK-1 (figure 5.2.7a). Similarly, cotransfection of cells with MyD88 and dominant negative TRAF-6 resulted in inhibition of the MyD88 response (figure 5.2.7b).

5.2.8 Dominant negative MyD88, TRAF-6 and IRAK-1 inhibit wild type IRAK-1 and TRAF-6-induced Gal4-p65$^{1-551}$ activity, respectively.

Having shown that both IRAK-1 and TRAF-6 are essential for MyD88-mediated transactivation by p65, their possible relationship with respect to one another in regulating this pathway was next determined. As shown in figure 5.2.8a dominant negative TRAF-6 was found to abolish the stimulatory effect of IRAK-1 and likewise dominant negative IRAK-1 inhibited TRAF-6-induced effect. This result suggests a possible co-dependency for both IRAK-1 and TRAF-6 in regulating this pathway, and confirms the importance of these proteins in p65-mediated transactivation of gene expression. Surprisingly, dominant negative MyD88 inhibited both IRAK-1- and TRAF-6-induced Gal4-p65$^{1-551}$ activation, suggesting that MyD88, IRAK-1 and TRAF-6 form part of a signalling complex essential for IL-1-induced activation of transactivation by p65 (figure 5.2.8b).

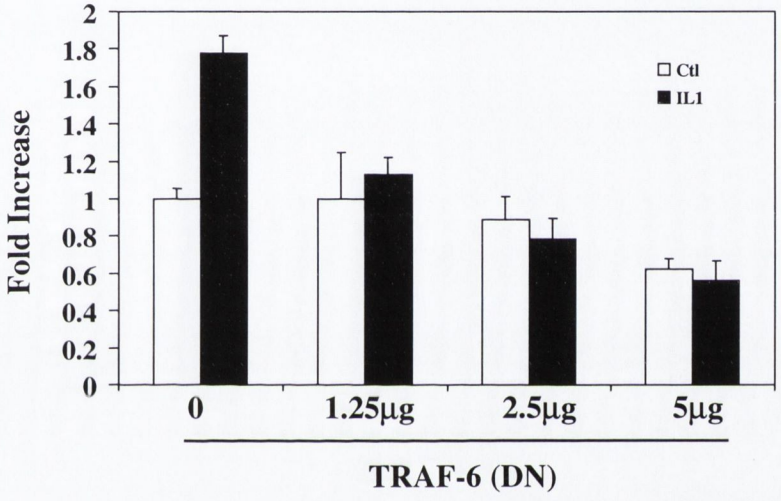
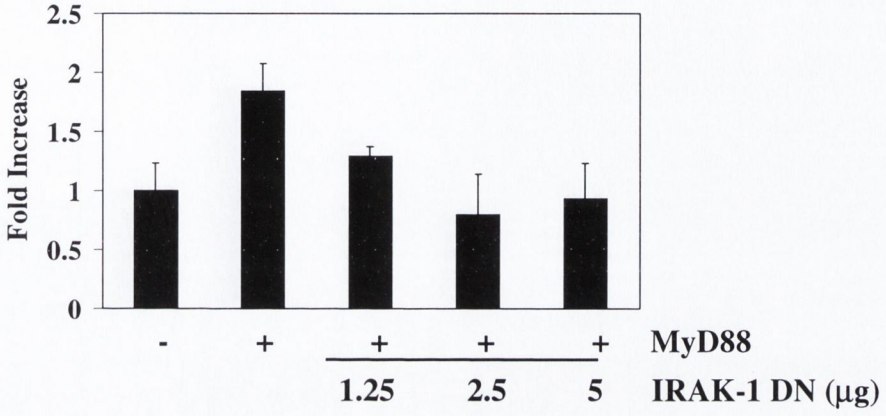


Figure 5.2.6: Dominant negative TRAF-6 inhibits IL-1-induced Gal4-p65$^{1-551}$ activation

EL4.NOB-1 cells (7x10^6) were transfected with 2.5µg Gal4-p65$^{1-551}$, 5µg of Gal-luciferase and increasing amounts of dominant negative TRAF-6 as indicated. Following a period of recovery (16-18hours) cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean$\pm$S.D. from triplicate determinations.

(A)



(B)

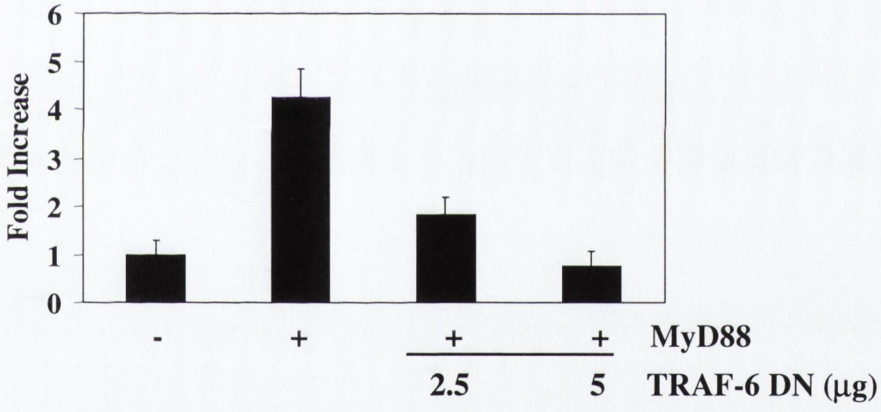


Figure 5.2.7: Dominant negative IRAK-1 and TRAF-6 inhibit MyD88-induced Gal4-p65$^{1-551}$ activation

EL4.NOB-1 cells ($7x10^6$) were co-transfected with 2.5μg Gal4-p65$^{1-551}$, 5μg of Gal-luciferase, 2.5μg of plasmids encoding wild-type MyD88 and either (A) dominant negative IRAK-1 or (B) dominant negative TRAF-6 as indicated. Following a period of recovery (16-18hours) cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations.

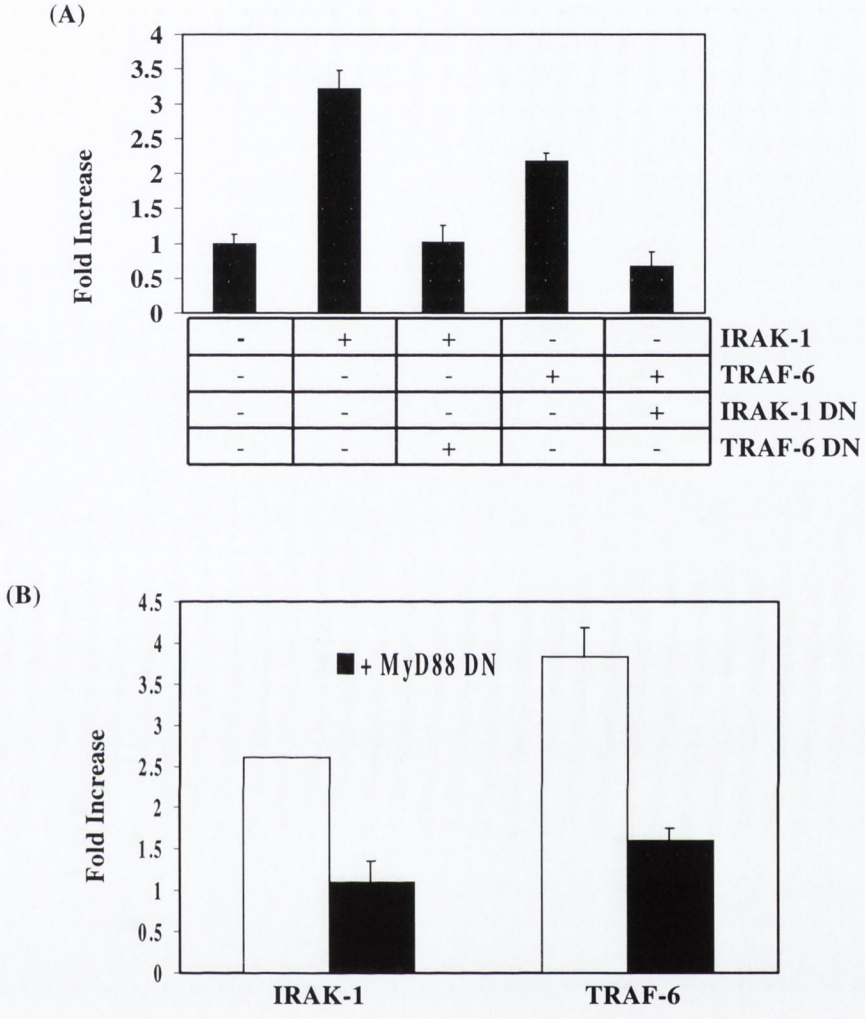


Figure 5.2.8: Dominant negative MyD88, IRAK-1 and TRAF-6 inhibit wild-type IRAK-1 and TRAF-6 induced Gal4-p65$^{1-551}$ activation

EL4.NOB-1 cells (7x10^6) were co-transfected with 2.5µg Gal4-p65$^{1-551}$, 5µg of Gal-luciferase, 2.5µg of plasmids encoding either wild-type IRAK-1 or TRAF-6 with (A) dominant negative IRAK-1 and TRAF-6 or (B) dominant negative MyD88 as indicated. Following a period of recovery (16-18hours) cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations.

5.2.9 Dominant negative RacN17 inhibits MyD88-, IRAK-1-and TRAF-6-induced Gal4-p65$^{1-551}$ activation.

The possible involvement of Rac1 in MyD88, IRAK-1 and TRAF-6 driven Gal4-p65$^{1-551}$ activity was next examined. To this end EL4.NOB-1 cells were transiently transfected with plasmids encoding either wild type MyD88, IRAK-1 or TRAF-6 together with dominant negative RacN17 and the effects on p65-mediated transactivation examined. The ability of wild type MyD88 to transactivate p65-mediated gene expression was inhibited by cotransfection of cells with dominant negative RacN17 (figure 5.2.9a). In a similar manner both IRAK-1 and TRAF-6-induced Gal4-p65$^{1-551}$ activity was inhibited by RacN17 (figure 5.2.9b and c, respectively). This result indicates that Rac1 is required for a pathway, regulated by MyD88, IRAK-1 and TRAF-6, which mediates the transactivation of gene expression by p65.

5.2.10 IRAK-1 and TRAF-6 but not MyD88 inhibit RacV12-induced Gal4-p65$^{1-551}$ activity.

To confirm the involvement of Rac1 and to position it on this pathway, the ability of dominant negative MyD88, IRAK-1 and TRAF-6 to inhibit constitutively active RacV12-induced transactivation by p65 was tested. Consistent with Rac1 being downstream of MyD88 on this pathway, dominant negative MyD88 failed to inhibit RacV12-induced Gal4-p65$^{1-551}$ activity in EL4.NOB-1 cells (figure 5.2.10a). In contrast both dominant negative IRAK-1 and TRAF-6 potently inhibited the RacV12 response (figure 5.2.10b and c, respectively). These results indicate that both IRAK-1 and TRAF-6, but not MyD88, play a crucial role in regulating the effects of RacV12 on this pathway.

5.2.11 Rac1 association with the IL-1 receptor complex.

Having determined that Rac1 plays an important role in IL-1-induced transactivation of gene expression by p65, involving MyD88, IRAK-1 and TRAF-6, the question as to whether Rac1 associates with the receptor complex was examined. Lysates from EL4.NOB-1 cells treated with

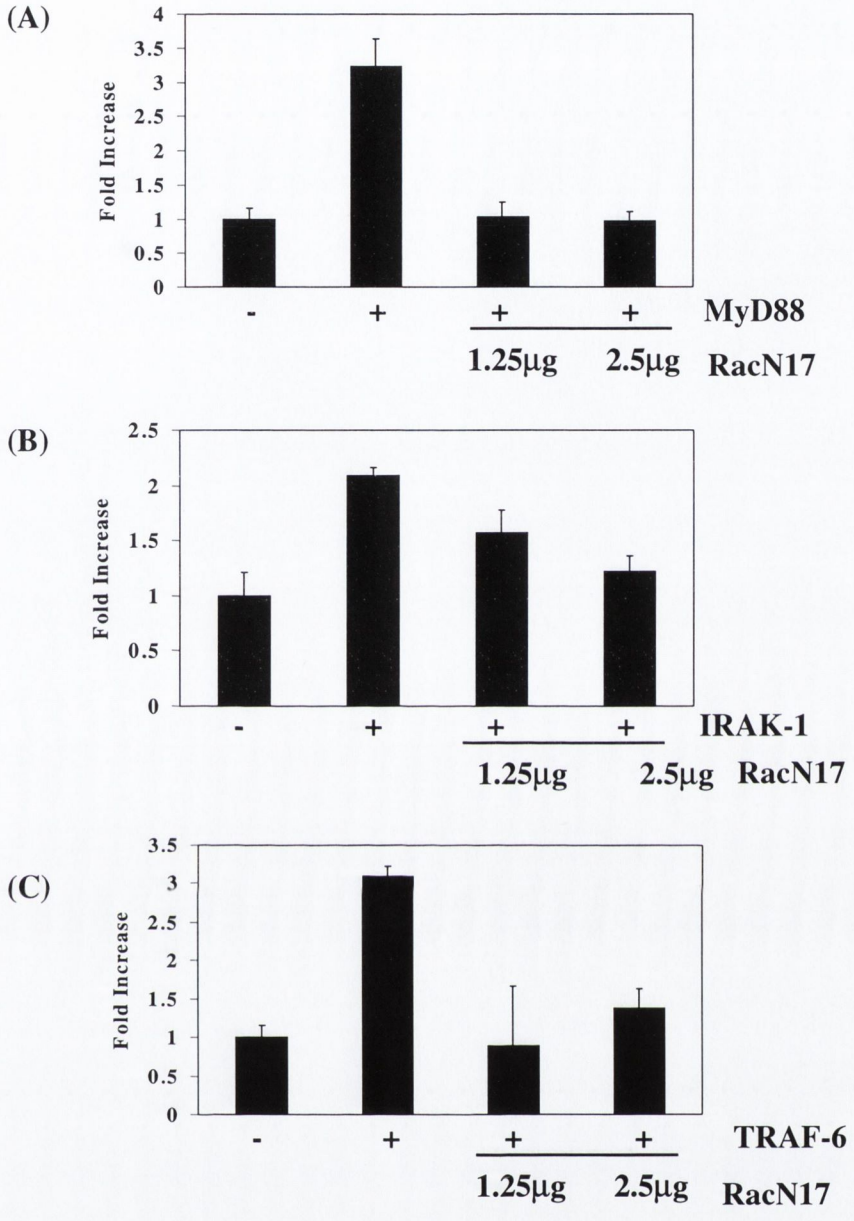


Figure 5.2.9: Dominant negative RacN17 inhibits wild-type MyD88, IRAK-1 and TRAF-6 induced Gal4-p65$^{1-551}$ activation

EL4.NOB-1 cells (7x10^6) were co-transfected with 2.5µg Gal4-p65$^{1-551}$, 5µg of Gal-luciferase, 2.5µg of plasmids encoding dominant negative RacN17 with wild-type (A) MyD88, (B) IRAK-1 and (C) TRAF-6 as indicated. Following a period of recovery (16-18hours) cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean$\pm$S.D. from triplicate determinations.

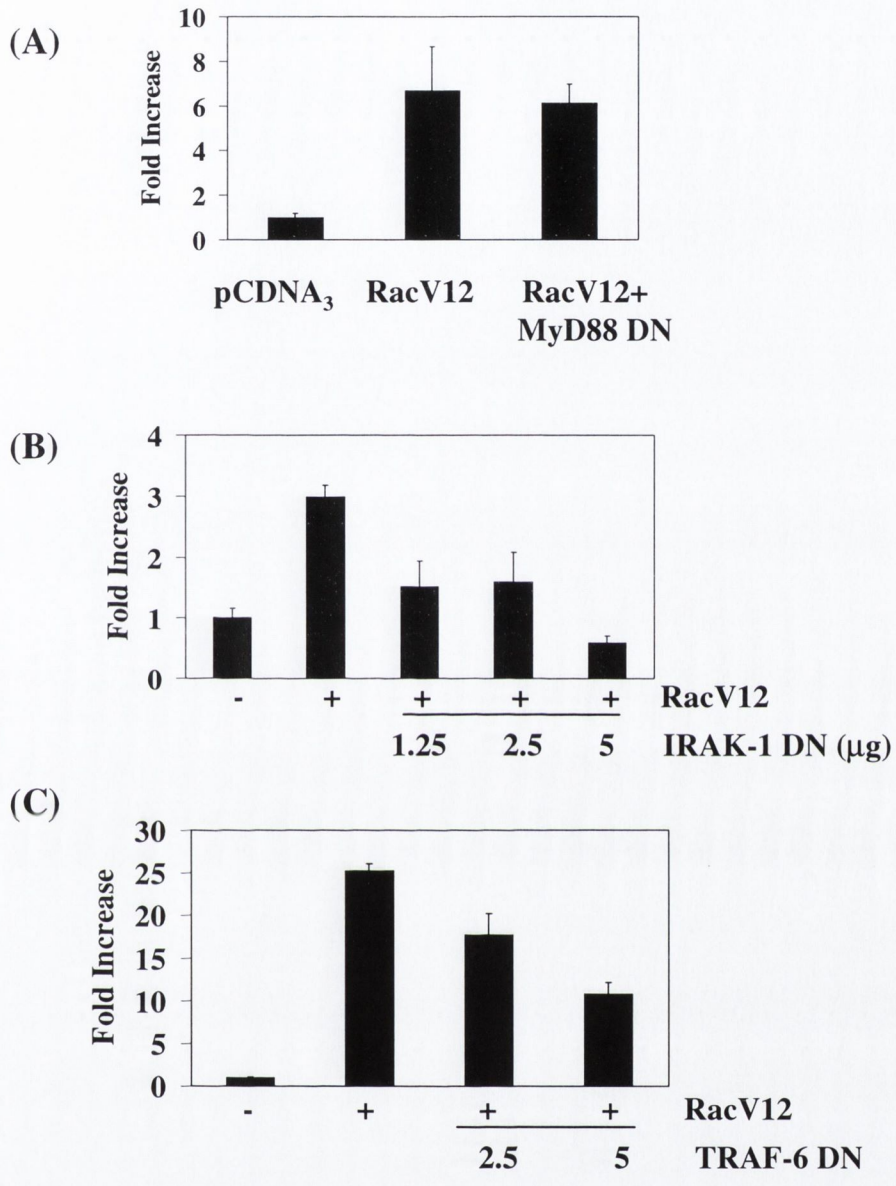


Figure 5.2.10: Dominant negative IRAK-1 and TRAF-6, but not MyD88, inhibit RacV12 induced Gal4-p65$^{1-551}$ activation

EL4.NOB-1 cells ($7x10^6$) were co-transfected with 2.5μg Gal4-p65$^{1-551}$, 5μg of Gal-luciferase, 2.5μg of plasmids encoding RacV12 with dominant negative (A) MyD88, (B) IRAK-1 and (C) TRAF-6 as indicated. Following a period of recovery (16-18hours) cells were stimulated with IL1 (10ng/ml) for 5-6 hours and subsequently analysed for Gal-luciferase activity using standard protocols. Results are represented as fold increase over unstimulated control and are represented as Mean±S.D. from triplicate determinations.

IL-1 (10ng/ml) for various time points were incubated with 10μg of GST-MyD88 fusion protein which had been immobilised on glutathione sepharose beads. The ability of Rac1 to bind GST-MyD88 determined by western blotting using a Rac1 specific antibody. While GST-MyD88 interacted weakly with Rac1 in unstimulated cells (figure 5.2.11, lane 2), the amount of Rac1 associated with GST-MyD88 increased following stimulation of cells with IL-1 (10ng/ml), with a strong association being detected at 15 minutes (figure 5.2.11, lane 4). As Rac1 has been previously shown to be maximally activated following 15 minute stimulation of EL4.NOB-1 cells with IL-1 (10ng/ml), this result indicates that the association between Rac1 and MyD88 may increase with the activation of Rac1.

5.2.12 Rac1 associates with over-expressed MyD88 in HEK 293T cells.

The ability of Rac1 to associate with MyD88 was confirmed by overexpressing AU1-tagged wild type MyD88 in human embryonic kidney 293T cells that are routinely used in these studies. Immunoprecipitation of AU1-MyD88, followed by western blotting for Rac1, confirmed the ability of Rac1 to associate with MyD88 as shown in figure 5.2.12. Importantly no Rac1 was detected in samples where a control antibody was used in place of the anti-AU1 antibody during the immunoprecipitation stage, confirming the specificity of this interaction (figure 5.2.12, lane 1).

5.2.13 Rac1 associates with IL-1RAcP in the receptor complex.

The ability of Rac1 to associate with the IL-1 receptor complex was also demonstrated in HEK-293 cells stably transfected with both myc-tagged IL-1RI and flag-tagged IL-1RAcP. Immunoprecipitation of flag-AcP, followed by western blotting for Rac1, demonstrated that Rac1 associates with the IL1-RAcP (figure 5.2.13a, compare lanes 1 and 2). In contrast however no Rac1 was detected in immune complexes isolated using an anti-myc antibody to immunoprecipitate myc-IL-1RI (figure 5.2.13b, compare lanes 1 and 2). These results clearly

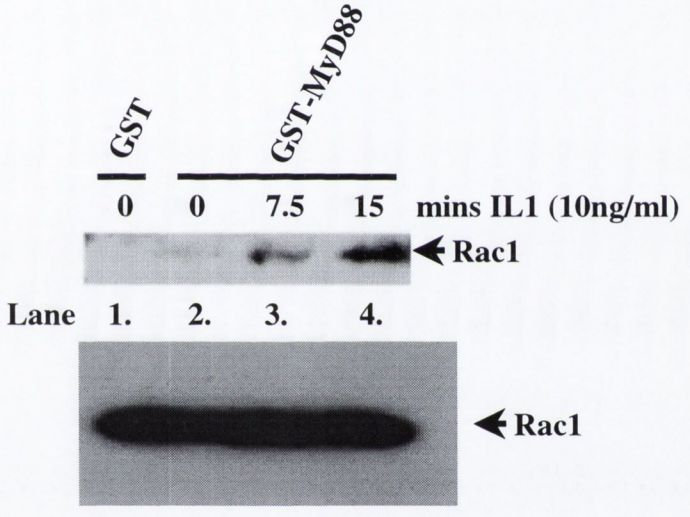


Figure 5.2.11: Rac1 associates with MyD88 in EL4.NOB-1 cells.

Lysates of EL4.NOB-1 cells ($2x10^7$) treated with IL-1 (10ng/ml) as indicated were normalised for protein and incubated with 10μg of either GST as a control or GST-MyD88 (2 hours, 4°C) as shown which had been pre-coupled to glutathione-agarose beads. Rac1 association was detected by western blot analysis using an anti-Rac antibody (upper panel). The lower panel shows a western blot of cell lysates to indicate that the total amount of Rac1 present per sample was equivalent.

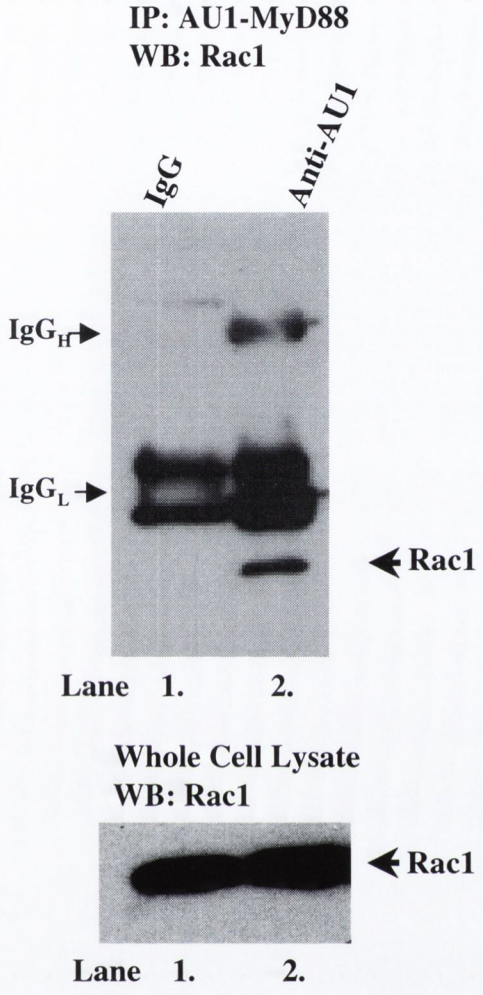


Figure 5.2.12: Rac1 co-immunoprecipitates with over-expressed AU1-MyD88

Lysates of AU1-MyD88-transfectected 293T cells ($1x10^5$/ml) were incubated with 4μg of either control non-specific IgG or anti-AU1 specific monoclonal antibody (2 hours, 4°C) as indicated. Immune complexes were precipitated by the addition of protein G-Sepharose (45 minutes, 4°C) and Rac1 association detected by western blot analysis using an anti-Rac antibody (Upstate Biotechnology). The lower panel shows a western blot of cell lysates to indicate that the total amount of Rac1 present per sample was equivalent.

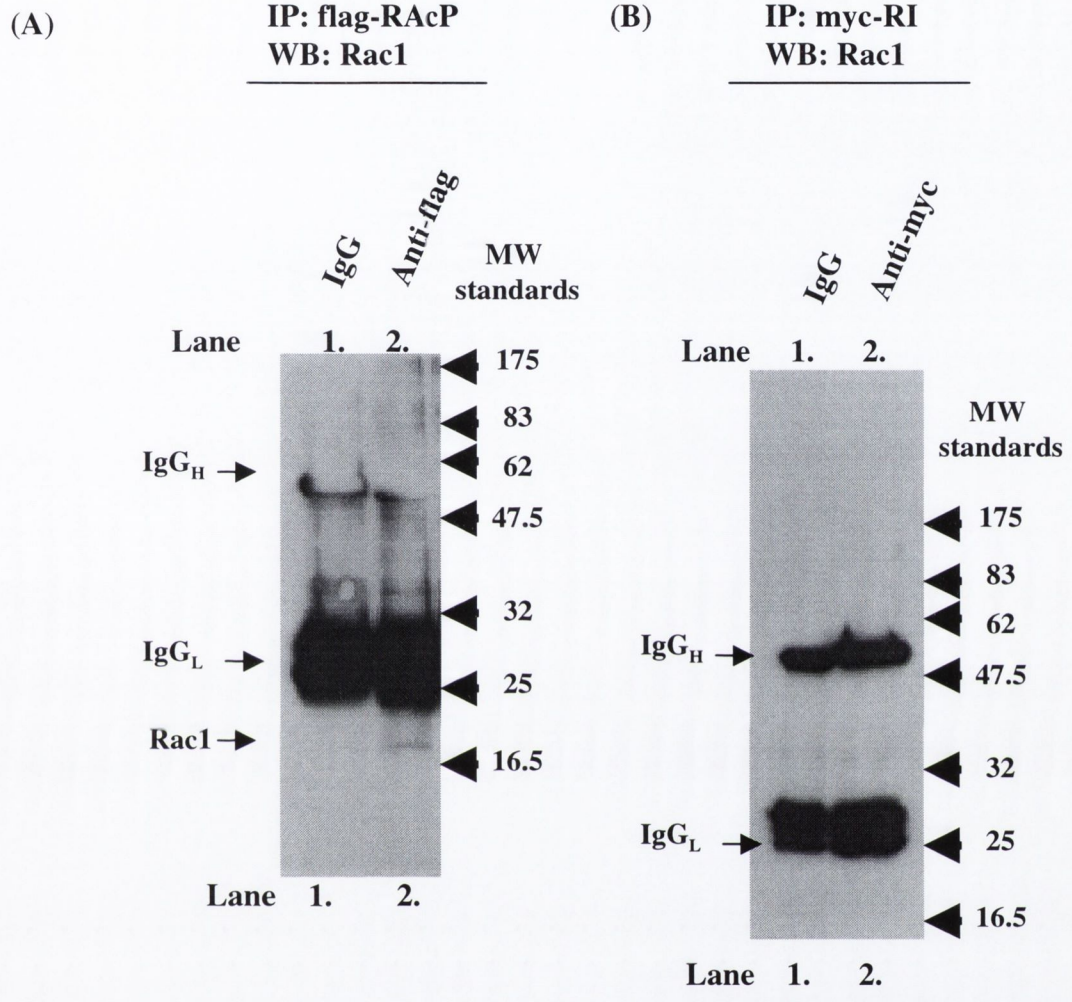


Figure 5.2.13: Rac1 co-immunoprecipitates with IL-1RAcP but not IL-1RI.

Lysates (normalised for protein concentration) of 293 cells ($1x10^5$/ml) stably transfected with flag-tagged IL-1RAcP and myc-tagged IL-1RI were incubated with 4μg of either control non-specific IgG, (A) anti-flag (to IP flag-AcP) or (B) anti-myc (to IP myc-IL-1RI) monoclonal antibody (2 hours, 4°C) as indicated. Immune complexes were precipitated by the addition of protein G-Sepharose (45 minutes, 4°C) and Rac1 association detected by western blot analysis using an anti-Rac antibody.

demonstrate an association between Rac1 and both MyD88 and IL1-RAcP in the receptor complex.

5.3 Discussion

To date the pathway activated by IL-1 resulting in enhanced transactivation by the p65 subunit of NFκB has not been characterised. The key role of MyD88, IRAK-1 and TRAF-6 in IL-1 signal transduction suggested that these proteins may also be important regulators of this pathway. Using plasmids encoding either wild-type or inactive mutants of MyD88, IRAK-1 and TRAF-6, our data clearly indicates the role of these proteins in regulating the signal from the IL-1 receptor complex to p65-mediated transactivation. Transient transfection of EL4.NOB-1 cells with plasmids encoding these proteins demonstrated that in each case the wild type protein activated Gal4-$p65^{1-551}$ activity with IL-1 stimulation eliciting a further stimulation of Gal4-$p65^{1-551}$ activity. Transfection of cells with a plasmid encoding IRAK-2 was unable to drive transactivation of gene expression by p65 but the presence of IRAK-2 potentiated the IL-1 response. Therefore, while IRAK-2 may not have a critical role in this pathway, its presence may enhance the efficiency of IL-1 signalling to p65-mediated transactivation. Inactive MyD88, encoding the TIR domain only, was found to inhibit the IL-1-induced response, indicating that MyD88 is required for the IL-1 response. Transfection of cells with dominant negative IRAK-1 and TRAF-6 completely inhibited IL-1-induced Gal4-$p65^{1-551}$ activity pointing to these proteins as being key regulators of IL-1-induced p65-mediated transactivation.

Previous studies on the involvement of MyD88, IRAK-1 and TRAF-6 in IL-1 signal transduction have demonstrated that MyD88 interacts with the receptor complex via a homotypic interaction via its TIR domain and that IRAK-1 and TRAF-6 are subsequently recruited to the activated complex (reviewed in (141)). MyD88 was originally isolated as an early transcript in IL-6-induced differentiating myeloid cells and was postulated to be a mediator of macrophage differentiation. The similarities between the Toll/IL-1 receptor superfamily and the C terminal domain of MyD88 subsequently suggested a role for MyD88 in signal transduction pathways and further investigation demonstrated its importance in IL-1-mediated signalling to NFκB activation.

In addition, dominant negative versions of IRAK-1 and TRAF-6 were found to inhibit MyD88-induced activation of NFκB demonstrating that IRAK-1 and TRAF-6 lie downstream of MyD88 on the pathway leading to NFκB activation (25). The autophosphorylating activity of IRAK-1 disrupts the interaction of IRAK-1 with the death domain of MyD88 with the result that the interaction of IRAK-1 with the receptor complex is extremely transient. Our data suggests that MyD88, IRAK-1 and TRAF-6 are co-dependent on one another for their ability to activate the pathway regulating p65-mediated transactivation. Cotransfection of cells with wild-type MyD88 and either dominant negative IRAK-1 or TRAF-6 completely inhibited the ability of MyD88 to transactivate gene expression. Similarily, dominant negative MyD88 was able to inhibit both wild-type IRAK-1- and TRAF-6-induced Gal4-p65$^{1-551}$ activity. Together these results suggest that MyD88, IRAK-1 and TRAF-6 form part of a signalling complex which is essential for transducing the signal from the receptor complex resulting in transactivation of gene expression by p65. Similar experiments using wild-type IRAK-1 with dominant negative TRAF-6 and vice versa found that IRAK-1 and TRAF-6 were mutually co-repressive, indicating that IRAK-1 and TRAF-6 are somehow co-dependent on each other in order that they may regulate the pathway leading to p65-mediated transactivation. At this point it must be emphasised that while the use of dominant negative mutants in elucidating signalling pathways is a highly effective tool in cell biology, one must be careful not to over-interpret results obtained using dominant negatives. In the case of pathways signalling to NFκB activation dominant negative MyD88, TRAF-6 and IRAK mutants have all been used effectively in the past to order the pathway from the receptor complex to NFκB activation (25). Therefore their use in this system is highly relevant and the data obtained in this study has been (and will be) used to guide further studies into the precise molecular ordering of this pathway by means of immunoprecipitation of IL-1 receptor or Rac1-containing complexes and determining their composition. The level of expression of transfected constructs in EL4.NOB-1 following transfection was not determined principally due to a lack of

antibodies of sufficient specificity against IRAK-1 and TRAF-6. However, from studies in the laboratory into expression levels of proteins in different expression vectors indicate that the level of expression does not significantly differ (Dr. Andrew Bowie, personal communication).

We have previously implicated the low molecular weight G protein Rac1 in the IL-1 pathway regulating the transactivation of p65-mediated gene expression (81). Constitutively active RacV12 potently activated the Gal4-$p65^{1-551}$ response while dominant negative RacN17 dose dependently inhibited IL-1-induced Gal4-$p65^{1-551}$ activity thus identifying Rac1 as a key regulator of IL-1-induced p65-mediated transactivation. How Rac1 might fit into this pathway however was unclear. That Rac1 may be involved at the level of the receptor complex and its immediate signalling processes was suggested from previous studies that had shown an interaction between Rac1 and a GST-IL-1RI fusion protein (170). Our data indicates that Rac1 lies downstream of MyD88 on this pathway as dominant negative RacN17 inhibited the ability of wild-type MyD88 to drive Gal4-$p65^{1-551}$ activation in co-transfection experiments. We were unable to show any inhibition of RacV12-induced p65-mediated transactivation by dominant negative MyD88, indicating that Rac1 lies downstream of MyD88 on this pathway. While dominant negative RacN17 totally prevented IRAK-1 and TRAF-6 mediated activation of Gal4-$p65^{1-551}$ activity, the dominant negative mutants of IRAK-1 and TRAF-6 also inhibited RacV12-induced activation of this response. Our data argues for a role for Rac1 in mediating IRAK-1- and TRAF-6-induced Gal4-$p65^{1-551}$ activity and in addition suggests that IRAK-1, TRAF-6 and Rac1 are mutually co-dependent for their activity as inhibition of any of these three signalling components prevents the ability of the other two proteins to drive p65-mediated transactivation. The ability of dominant negative MyD88 to inhibit both IRAK-1- and TRAF-6-, but not Rac1, -induced Gal4-$p65^{1-551}$ activation is initially confusing. However, these disparate effects may be due to a disruption by dominant negative MyD88 of the association between IRAK-1 and TRAF-6 with the receptor complex. The inability of dominant negative MyD88 to inhibit RacV12-induced Gal4-$p65^{1-551}$ activity suggests that, although Rac1 and MyD88 may exist in the same

complex, this association is not fully dependent on MyD88 whereas that of IRAK-1 and TRAF-6 is, as has been previously shown (24, 193). Whereas active MyD88 may be essential for IRAK-1- and TRAF-6-dependent signalling to p65-mediated transactivation our results suggest that Rac1 is not dependent on intact MyD88 (figure 5.3.1). It is possible that the association we observed between Rac1 and MyD88 may be via the TIR domain of MyD88 and therefore dominant negative MyD88 would not be expected to inhibit RacV12-induced transactivation. A further explanation maybe that Rac1 associates with IRAK and TRAF-6 in a multiprotein complex that associates with MyD88 and the activated receptor complex following stimulation of cells with IL-1. Further investigation into the precise make-up of IL-1 receptor-containing complexes and Rac1-containing complexes will address this question.

The involvement of Rac1 in IL-1 signal transduction processes has been previously demonstrated by other groups showing that Rac1 regulates IL-1 signalling to p38 and JNK activation (37, 149). A more direct role for Rac1 in IL-1 signalling has been indicated by the ability of Rac1 to associate with the intracellular domain of IL-1RI fused to GST (170). We used a number of approaches to assess possible interactions of Rac1 with components of the IL1 receptor complex and found that Rac1 associated with both a GST-MyD88 fusion protein in pull-down assays and overexpressed AU1-tagged MyD88 following immunoprecipitation. Treatment of cells with IL-1 increased the amount of Rac1 associating with MyD88. It is likely that MyD88 associates more strongly with active Rac1 as indicated by our results. In addition Rac1 was found to associate with the IL-1RAcP when it was immunoprecipitated from 293 cells stably transfected with flag-tagged IL-1RAcP. However, in contrast to Singh *et al*, following immunoprecipitation of IL-1RI from 293s stably expressing myc-tagged IL-1RI, we were unable to coprecipitate Rac1, indicating that the interaction of Rac1 with the IL-1 receptor complex specifically involves MyD88 and IL-1RAcP rather than IL-1RI. The use of the intracellular domain of IL-1RI fused to GST in a pull-down assay by Singh *et al* to affinity purify the receptor complex rather than immunoprecipitation may partially explain this difference in results. It must be stressed that

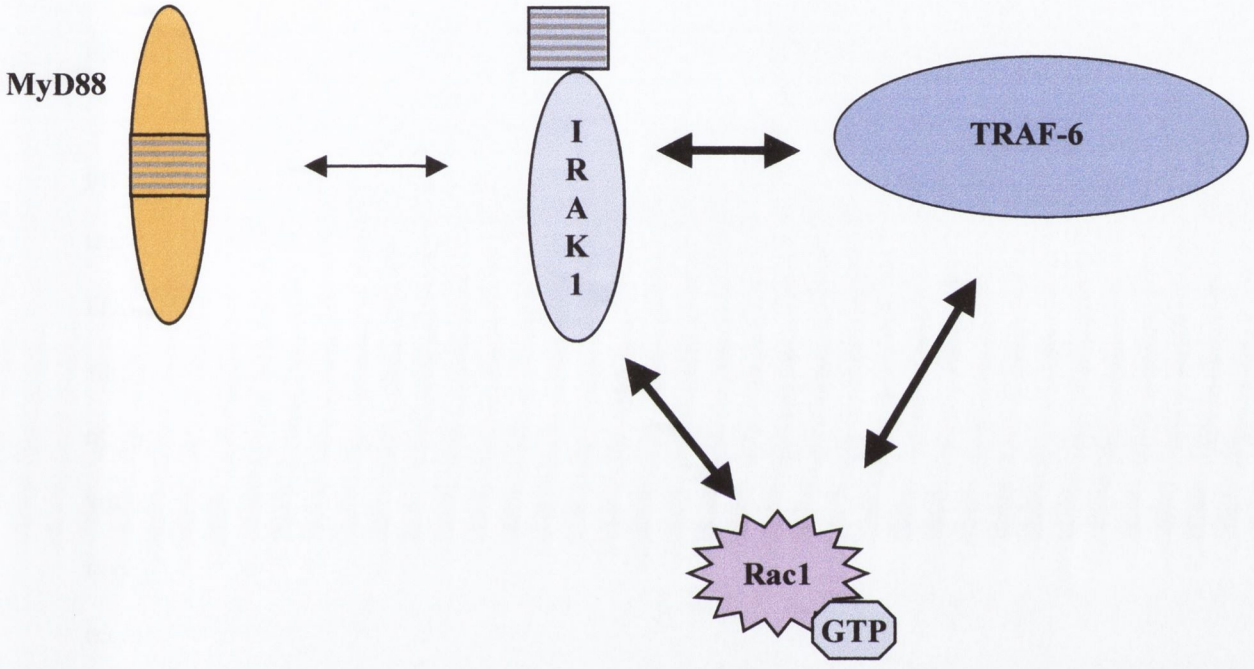


Figure 5.3.1: Schematic organisation of the ordering of Rac1, MyD88, IRAK-1 and TRAF-6 with respect to the pathway regulating p65-mediated transactivation. MyD88 is unable to inhibit RacV12-driven Gal4-p65$^{1-551}$ activity and only partially inhibits IL-1-induced and IRAK and TRAF-6-mediated activity. In contrast, Rac1, IRAK-1 and TRAF-6 all activate the pathway and mutually inhibit one another in their ability to activate Gal4-p65$^{1-551}$. This would suggest a model whereby Rac1, IRAK-1 and TRAF-6 are essential for IL-1-induced activation of this pathway whereas the role of MyD88 is more facilitatory. MyD88 possibly provides the appropriate contacts with the receptor complex for IRAK-1 and TRAF-6 that they require in order to access the transactivation pathway. Rac1, while it requires IRAK-1 and TRAF-6 for its activity on this pathway does not require MyD88.

immunoprecipitation or affinity purification using GST-fusion proteins of complexes does not allow one to discriminate between direct association between proteins and association as part of a multi-protein complex. Use of techniques such as far western blotting or yeast-two-hybrid could be used to determine whether the interaction between Rac1 and MyD88 or IL-1RAcP is direct or as part of a multiprotein complex. How IL-1 activates Rac1, and the significance of this interaction of Rac1 with the receptor complex, is unclear. However, taken together, our data points to a clear role for Rac1 in IL-1 signal transduction pathways.

As to the downsteam signalling events regulated by Rac1 on this pathway, we have previously shown an involvement of p42/p44 and p38 MAPK in both IL-1- and RacV12-induced transactivation of gene expression by p65. A key downstream effector of Rac1, p21 activated kinase (PAK), has been demonstrated to play a role in regulating p42/p44 MAPK activation, suggesting that PAK may be involved in regulation of IL-1-induced p65-mediated transactivation of gene expression downstream of Rac1 (55). We have evidence that stimulation of HeLa cells with IL-1 activates PAK kinase activity, which may suggest a role for PAK in this pathway. An involvement of PAK in regulating NFκB activation in response to multiple signals has been recently suggested (56). The authors show that, while expression of PAK1 in 293 cells does not activate either IKK 1 or 2, PAK1 stimulates nuclear translocation of NFκB. Whether the stimulatory effect of PAK on an NFκB-dependent reporter gene is due to the ability of PAK to enhance the nuclear translocation of p65 or its ability to transactivate gene expression is unclear.

The localization of the IL-1 receptor complexes to focal adhesion complexes in fibroblasts and the recruitment of IRAK-1 to these complexes necessary for the activation of p42/p44 MAPK suggests an involvement of the actin cytoskeleton in regulating signalling pathways emanating from the IL-1 receptor complex (108). The presence of Rac1 in the IL-1 receptor complex may be linked to its role as a critical regulator of the actin cytoskeletal network in cells (reviewed in (70)). Rac1 has been demonstrated to promote the formation of focal

complexes at the cell membrane and to lie downstream of paxillin, a key component of focal complexes, in regulating cytoskeletal reorganisation (107). These complexes, similar to focal adhesions, also contain talin and focal adhesion kinase (FAK), key transducers of signals from cell surface receptors to the cytoskeleton (70). In particular, talin has been demonstrated to become multiply phosphorylated following IL-1 stimulation possibly leading to changes in transmembrane linkage of receptors and the cytoskeleton (147). In addition, the IL-1 receptor complex has been shown to localise to focal adhesion points at the cell membrane, and may thus potentially associate with FAK through its association with these complexes (148). Therefore the association of Rac1 with the receptor complex and the ability of IL-1 to activate Rac1 suggests an involvement of Rac1 downstream of IL-1 in regulating cytoskeletal events in cells. This would explain the effect of transiently transfecting cells with dominant negative RacN17, resulting in the disruption of the signalling complex and inhibition of IL-1-induced transactivation of NFκB-dependent gene expression by p65. T cell activation is accompanied by a dynamic reorganisation of cortical actin, the integrity of which must be maintained in order that the T cells can differentiate and proliferate. This initiates the formation of contacts with antigen presenting cells (APC) and the formation of an immunological synapse (reviewed in (1)). The organisation of receptors within this area (termed SMAC, supramolecular activation cluster) is organised in a concentric fashion into two regions. The T cell receptor and CD4 are confined to the central region which is surrounded by a region containing integrin receptors. The integrin receptors within this region associate with talin and thereby connect through vincullin to actin filaments. The complex formed is highly analogous to focal complexes or adhesions (78). The Rho family of GTPases, including Rac1, play an important role in regulating both T cell activation and actin remodelling in T cells. It is therefore highly likely that Rac1 may be involved in regulating formation of the immunological synapse and activation of signalling pathways that it couples to. Whether other co-stimulatory molecules like the IL-1 receptor localise to or associate with this region remains to be determined. Our results in the murine T cell line, EL4.NOB-1, suggest that

the activated IL-1 receptor complex and Rac1 associate, possibly via changes in the actin cytoskeleton and formation of focal complex-like associations similar to those found in the mature immunological synapse.

In conclusion the results presented clearly indicate that MyD88, IRAK-1 and TRAF-6 are required for p65-dependent transactivation of NFκB in response to IL-1 and suggest that MyD88, IRAK-1 and TRAF-6 form part of a signalling complex that is essential for this pathway. In addition it was demonstrated that Rac1 plays a key role in mediating the signal from MyD88, IRAK-1 and TRAF-6 to p65-mediated transactivation and that the involvement of Rac1 is at the level of the receptor complex with an interaction between Rac1 and both MyD88 and IL-1RAcP being demonstrated. The key role of Rac1 in regulating the formation of focal complexes and the requirement for localization of IRAK to these complexes in order that IL-1 might activate p42/p44 MAPK suggests that Rac1 might provide a link between the activated receptor complex and the actin cytoskeleton, which is essential for engaging with the pathway regulating p65-mediated transactivation of gene expression. Disruption of this link by the introduction of dominant negative Rac1 into cells may inhibit the activated IL-1 receptor complex from engaging with signalling components essential for regulating the pathway mediating transactivation by the p65 subunit of NFκB (figure 5.3.2).

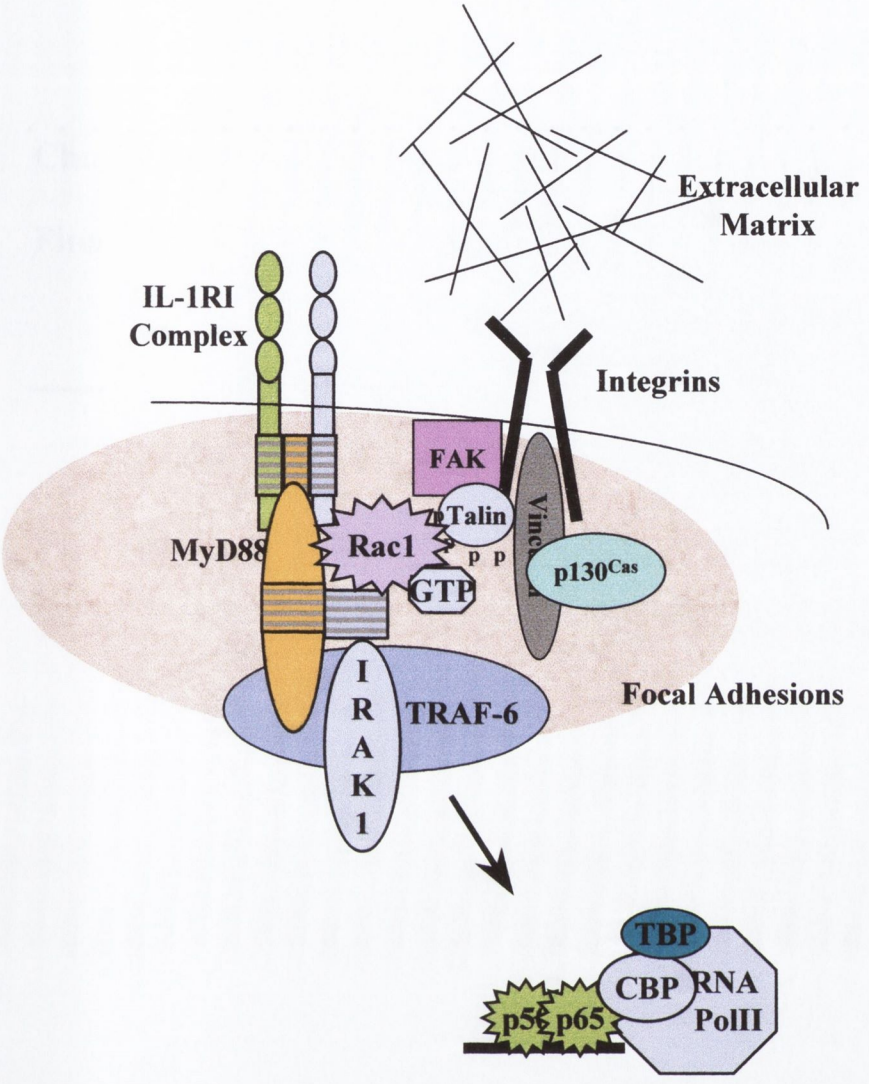


Figure 5.3.2: Model showing the possible relationship between the IL-1 receptor complex and focal complexes in regulating p65-mediated transactivation. The association of Rac1 with focal complexes and the localisation of the IL-1 receptor to such complexes at the cell membrane suggests how the actin cytoskeletal network (and control of it by Rac1) may act to regulate signal transduction processes coming off the IL-1 receptor. The observed association of Rac1 with members of the IL-1 receptor complex (IL-1RAcP and MyD88) suggests that the essential role that Rac1 plays in regulating IL-1-induced Gal-p65$^{1-551}$activity may involve its ability to regulate the actin cytoskeleton. RacV12-induced Gal4-p65$^{1-551}$ activation requires both IRAK-1 and TRAF-6. The requirement for the presence of IRAK-1 at focal adhesions in order that IL-1 might activate p42/p44 MAPK suggests that the IL-1 receptor complex and the multiprotein complex that forms focal complexes and adhesions are inextricably linked.

Chapter Six

Final Conclusion and Comments

6 Chapter six

Final conclusions and future perspectives

This study has focused on the involvement of Rac1 in IL-1-induced activation of the pro-inflammatory transcription factor NFκB. Our initial investigations into the role of Rac1 in IL-1-induced expression of the cytokine IL-2 raised very interesting, and potentially novel questions regarding the role of Rac1 in IL-1 signal transduction.

Our initial finding that the low molecular weight G protein Rac1 is activated by IL-1 prompted further investigation into its possible role in regulating IL-1 signal transduction in EL4.NOB-1 cells. Characterisation of the involvement of Rac1 in IL-1 signalling to IL-2 production has demonstrated a novel role for Rac1 in mediating IL-1-induced activation of NFκB. Rather than regulating IκB degradation, nuclear translocation or DNA-binding of NFκB, Rac1 appears to regulate a separate pathway, independent of these events. We have identified such a pathway, requiring Rac1, which regulates the transactivation of gene expression by the p65 subunit of NFκB in response to IL-1. Initial characterisation of this novel pathway has shown that both IL-1- and Rac1-induced p65-mediated transactivation requires p42/p44 and p38 MAPK pathways, although the full significance of their involvement in this pathway is unclear.

We have characterised the involvement of key regulators of IL-1 signal transduction, MyD88, IRAK-1 and TRAF-6, in regulating transactivation of gene expression in response to IL-1 and have demonstrated the involvement of all three in this pathway with dominant negative versions of each inhibiting the IL-1-induced response. Experiments to position MyD88, IRAK-1 and TRAF-6 with respect to each other on this pathway imply that a multi-protein complex involving all three is required for p65-mediated transactivation as a dominant negative version of any of these proteins will disrupt signalling. Furthermore Rac1 appears to lie downstream of MyD88 in a complex with IRAK-1 and TRAF-6 in regulating p65-mediated transactivation. The observation that Rac1 associates with MyD88 coupled with the inability of dominant negative

MyD88 to inhibit RacV12-induced transactivation by p65 suggests that the role of MyD88 in this pathway may be to recruit and activate Rac1 into the IL-1 receptor complex and thereby facilitate activation of this pathway. The key role of Rac1 in regulating the formation of focal complexes and the localisation of the IL-1 receptor to these complexes suggests that Rac1 might provide a link between the activated receptor complex and the actin cytoskeleton. Interaction of the activated receptor complex with components of focal complexes or the actin cytoskeleton may be essential for engaging with the pathway regulating p65-mediated transactivation of gene expression.

To date the receptor complex is recognised to include the type I IL-1 receptor (IL-1RI), the IL-1 receptor accessory protein (IL-1RAcP) and the adaptor protein MyD88, all of which share a region of homology in their cytosolic domains of approximately 200 amino acids, termed the Toll/IL-1 receptor domain (TIR) (141). While much work has focused on the signals transduced from the IL-1 receptor complex, in contrast comparatively few details of how the components associate and how additional signalling proteins such as Rac1 are assembled at the complex are known.

Increasingly it is becoming obvious that rearrangement of cytoskeletal networks in cells plays a key role not only in cell motility but also in regulating signal transduction networks in cells. Not only have key adaptor molecules on certain signal transduction pathways, such as proteins in the IκB kinase complex on the Toll/IL-1 pathway regulating phosphorylation of IκB, been identified as scaffold proteins but also compounds which disrupt the microtubule cytoskeleton have been shown to inhibit signalling to NFκB activation (153). In addition the requirement of focal adhesions, and the recruitment of IRAK-1 to these sites, for p42/p44 MAPK activation imply that the cytoskeleton and signal transduction pathways are inextricably linked (106, 108). These findings underline the importance of the cytoskeleton, and proteins that regulate it such as Rac1, in maintaining a molecular environment favorable for signalling. Much attention

has focused on the role of Rac1 in regulating the actin cytokeletal network in cells and the formation of focal complexes at the cell membrane which contain many cytoskeletal and signalling components, some of which (paxillin for example) are also regulated by IL-1. In addition a new target for Rac1 has been described, POSH, which is a scaffold protein involved in regulating both JNK and NFκB activation (179). Other downstream effectors, such as PAK and mixed lineage kinase (MLK), have also been implicated in both JNK and NFκB activation (56, 178). Our results demonstrate an involvement of Rac1 in transactivation of gene expression by the p65 subunit of NFκB in response to IL-1 rather than regulating the pathway leading to IκB degradation or DNA-binding of NFκB (81).

The association of Rac1 with the IL-1 receptor complex via an interaction with MyD88 suggests that the involvement of Rac1 in IL-1-induced transactivation may be as a direct result of this interaction. In addition the association of Rac1 with MyD88 following IL-1 stimulation is enhanced in response to IL-1 stimulation. Whether the association of Rac1 and MyD88 mediates IL-1-induced activation of Rac1 or whether the activation of Rac1 by IL-1 allows interaction of Rac1 with the receptor complex is currently under investigation. The novel observation placing Rac1 at the receptor complex through direct association with MyD88, coupled with the ability of dominant negative RacN17 to inhibit signals from the receptor complex to enhanced transactivation of gene expression by p65, suggests that the interaction of Rac1 and MyD88 provides the necessary link between the receptor complex and this pathway.

It is unknown whether Rac1 activation by an as yet unknown guanine nucleotide exchange factor in the receptor complex is responsible for mediating IL-1-induced transactivation or if Rac1 activation by IL-1 facilitates the association of as yet unidentified proteins which activate downstream pathways responsible for regulating p65-mediated transactivation. No role for key activators of Rac1, Vav or Tiam1, in IL-1 signalling has yet been demonstrated. However, a role for Dbl family members (GEFs specific for the Rho-like GTPases) in NFκB

activation has been demonstrated, although no involvement of any of this family in IL-1 signalling has been reported. We have shown that the MAPK pathways, p42/p44 and p38, lie downstream of IL-1 and Rac1 on this pathway. The means by which p38 MAPK regulates transactivation by p65 has been determined, with p38 MAPK shown to phosphorylate the TATA-binding protein and thereby facilitate gene expression. How p42/p44 MAPK mediates its effects is still unknown. Another important kinase pathway implicated in the regulation of transactivation of gene expression by p65 is protein kinase A (PKA). PKA has been shown to phosphorylate p65 on serine 276 and thus facilitate the interaction between p65 and CBP/p300, a histone acetylase and coactivator of gene expression. Whether IL-1 regulates PKA activity has yet to be determined. Recent work has demonstrated that the Rac1 effector p21 activated kinase (PAK) plays an important role in regulating NFκB activation, although how PAK mediates this effect is unclear. PAK has been reported to lie upstream of p42/p44 MAPK, regulating phosphorylation of, and hence enhanced activation of, its upstream kinase Raf. Whether PAK regulation of the p42/p44 MAPK pathway plays a role in regulating p65-mediated transactivation has yet to be determined. Recently casein kinase II has been demonstrated to be the kinase responsible for phosphorylating serine 529 in the transactivation domain of p65. This phosphorylation event has been previously shown to enhance the transactivation of gene expression by p65. The pathway regulating casein kinase II activity in response to IL-1 has yet to be characterised.

How the various kinase pathways link the IL-1 receptor complex with transactivation of gene expression is under investigation. Given the central role of Rac1 in IL1 signal transduction, future work aims to address the mechanism by which it couples to the receptor. It also seeks to elucidate the molecular architecture of the IL1 receptor complex by means of mass spectrometry, an innovative and highly powerful tool for analysing protein-protein interactions (14, 198). Furthering our knowledge of the various proteins that bind to the activated receptor complex will be invaluable for elucidating how the various signalling pathways activated upon IL1 binding to its receptor are regulated. In addition detailed information on the molecular architecture of the

receptor complex will provide much needed information on the signal transduction pathways activated by IL1 and also on where the various pathways diverge, for example that leading to IκB degradation and that regulating signals enhancing transactivation by NFκB. Mass spectrometric analysis of complexes isolated using methods which would purify Rac1 containing complexes (i.e. anti-Rac immunoprecipitates or GST-PAK pulldowns), will provide additional information regarding the GEFs or GTPase-activating proteins (GAP) which regulate Rac1 activation in response to IL1.

In summary, this work has provided the first characterisation of a novel pathway regulating the transactivation of gene expression by the p65 subunit of NFκB in response to IL-1. Further characterisation of this pathway, coupled with detailed knowledge of the composition of the IL1 receptor complex, will provide valuable insights into possible novel strategies for managing inflammatory diseases.

Chapter Seven

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Chapter Eight

Record of Publications

8 Publication Record

1. Jefferies, C.A., Cooke, E., O'Neill, L.A.J. (2000) Transactivation by the p65 subunit of NFκB in response to interleukin-1 involves MyD88, IRAK-1, TRAF-6 and Rac1. Mol. Cell. Biol. (Submitted August, 2000).

2. Jefferies, C.A., O'Neill, L.A.J. (2000) Rac1 regulates interleukin-1-induced NFκB activation in an IκB-independent manner by enhancing the ability of the p65 subunit to transactivate gene expression. *J. Biol. Chem.* **275,** 3114-3120

3. Jefferies, C.A., O'Neill, L.A.J. (1999) *Cytokine* **11,** A123

4. O'Neill, L.A.J, Matthews, J.S.M., Pålsson, E., Jefferies, C., Brennan, P., Cantrell, D. (1997) IL-1 signaling: Synergy with Rac1 and PI3-kinase. *Biochem Soc Trans,* **25,** S578

Chapter Nine

Appendices

9 APPENDIX I

Addresses of suppliers

Amersham International

Export Sales Division

Lincoln Place, Green End

Aylesbury

Bucks HP20 2TP

United Kingdom.

BDH Limited

P.O. Box 8

Dagenham

Essex RM8 1RY

United Kingdom

Boehringer Mannheim GmbH

Biochemica

P.O. Box 31 01 20

D-6800 Mannheim 31

Germany

Calbiochem

Freepost

Nottingham NG7 2BR

United Kingdom

Clonetics Corporation	9620 Chesapeake Drive Suite 201 San Diego CA 92123 USA
European Collection of Animal Cell Cultures	PHLS Center for Applied Microbiology and Research Porton Down Salisbury SP4 OJG United Kingdom
Gibco BRL ***Life Technologies Ltd.***	P.O. Box 35, Trident House Renfrew Road Paisley PA3 4EF United Kingdom
Genentech	Genentech, Inc. 1 DNA Way South San Francisco, CA 94080-4990 USA
Greiner GmbH	Maybachstrasse

	P.O. Box 1162 D-7443 Frichenhausen Germany
HyClone	1725 South HyClone Road Logan Utah 84321 USA
ICN Biomedicals Inc.	PO Box 26221, Birmingham, AL 35226, USA
Invitrogen	PO Box 2312, 9704 CH Groningen, Holland
National Diagnostics	305 Patton Drive Atlanta Georgia 30336 USA

New England Biolabs	32, Tozer Road, Beverly, MA01915-5599, USA
Nunc A/S	Kamstrupvej 90 DK-4000 Roskilde Denmark
Pharmacia Biosystems Ltd	Pharmacia LKB Biotechnology AB Davy Avenue Knowhill Milton Keynes MK5 8PH United Kingdom
Promega Corporation	2800 Woods Hollow Road Madison WI 53711-5399 USA
QIAGEN Ltd.	Boundary Court Gatwick Road Crawley West Sussex RH10 2AX United Kingdom

R&D Systems Europe Ltd.	4-10 The Quadrant Barton Lane Abingdon OX14 3YS United Kingdom
Santa Cruz Biotechnology, Inc.	2161 Delaware Avenue Santa Cruz California 95060 USA
Sigma Chemical Company Ltd	Fancy Road Poole Dorset BH17 7BR United Kingdom
Southern Biotechnology	Birmingham, Alabama, USA

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Rac1 Regulates Interleukin 1-induced Nuclear Factor κB Activation in an Inhibitory Protein κBα-independent Manner by Enhancing the Ability of the p65 Subunit to Transactivate Gene Expression*

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Caroline A. Jefferies and Luke A. J. O'Neill‡

From the Department of Biochemistry and Biotechnology Institute, Trinity College, Dublin 2, Ireland

We have examined the involvement of Rac1 in nuclear factor κB (NFκB) activation by interleukin 1 (IL1). IL1 induced a rapid and sustained activation of Rac1 in the thymoma cell line EL4.NOB-1. Transient transfection with dominant negative RacN17 inhibited IL1-induced κB-dependent reporter gene expression but not IκBα degradation, whereas constitutively active RacV12 potentiated κB-dependent reporter gene expression in response to IL1 but had no effects on its own. Using porcine aortic endothelial cells stably transfected with RacV12 or RacN17 under the control of an inducible promoter, we confirmed that RacV12 did not affect IκBα degradation, nor did RacN17 inhibit the IL1-induced response. RacV12 was also unable to induce nuclear translocation of NFκB. These effects suggested a role for Rac1 in p65-mediated transactivation of NFκB, independent of IκBα regulation. In support of this we found that IL1 activated a pathway leading to increased p65 transactivation activity and that RacV12 alone could drive this response in both cell systems. Additionally, RacN17 inhibited IL1-driven p65-mediated transactivation. From data using specific inhibitors of p38 and p42/p44 kinases we propose that both p38 and p42/p44 lie downstream of Rac1 on the IL1 pathway leading to enhanced transactivation by p65.

The proinflammatory cytokine interleukin 1 (IL1)[1] is a crucial mediator of both inflammatory and immune responses. The involvement of IL1 in the pathogenesis of inflammatory diseases such as rheumatoid arthritis has led to intensive studies on how IL1 signals are transduced in target cells. Although significant advances have been made in this area recently, particularly with respect to activation of the transcription factor nuclear factor κB (NFκB), several aspects of IL1 signaling remain to be elucidated fully (1). A number of different lines of evidence suggest a role for GTP-binding proteins in IL1 signaling events in cells (2–4). In particular, a role for the small G protein Rac1 (a member of the Rho subfamily of G proteins) in IL1 signaling has been proposed. Although Rac1 was originally described for its effects on the cytoskeleton in cells, more recently it has been shown to play a role in other signaling events (5, 6). Most notably, Rac1 has been suggested to regulate mitogen-activated protein kinase (MAPK) pathways in cells, in particular the stress-activated protein kinase pathways, p38 and c-Jun NH_2-terminal kinase (JNK) (7–9). The proposal that Rac1 may play a role in IL1 signaling came from studies demonstrating that a dominant negative mutant of Rac1 (RacN17) inhibited the activation of both p38 and JNK MAPK pathways by IL1 (7–9), although more recently a role for Rac1 in JNK activation by IL1 has been disputed (10).

In addition, Rac1 has been shown to play a role in activation of NFκB, a ubiquitous transcription factor that regulates the expression of many genes up-regulated by IL1 (11, 12). The best characterized form of NFκB exists in resting cells as a dimer of two proteins, the subunits p50 (which binds the κB motif) and p65 or RelA (which is required for transactivation of gene expression). This heterodimer is complexed to the inhibitory subunit IκBα which, upon stimulation, is phosphorylated and subsequently degraded. NFκB is then free to enter the nucleus and bind to its consensus sequence on target genes (13, 14). Regulation of IκB degradation and the subsequent release of NFκB is a crucial control point in the pathway. However, recent results suggest that an additional IκB-independent pathway is activated, which results in enhanced transactivation potential of NFκB once it is bound to its consensus sequence (15, 16). Activation of these pathways has been shown to result in increased phosphorylation of the p65 (RelA) subunit of NFκB and to promote interaction of p65 with the coactivator protein p300/CBP (17–19). The upstream kinases regulating these events have yet to be identified conclusively, although recent evidence suggests that p38 and p42/44 MAPK pathways may play a role in regulating NFκB transactivation in response to stimulation with both IL1 and TNF-α (15, 16). In addition, several reports indicate that casein kinase II and protein kinase A may be involved in events leading to enhanced phosphorylation of the p65 subunit of NFκB (17, 20). Where Rac1 might participate in either pathway culminating in IκB phosphorylation or p65-mediated transactivation has not been investigated. Evidence for a role for Rac1 in NFκB function is based on the ability of a constitutively active mutant of Rac1 (RacV12) to drive a κB-dependent reporter gene. In addition, dominant negative RacN17 in the same studies inhibited IL1β-stimulated NFκB DNA binding, possibly by inhibiting the generation of reactive oxygen species (ROS) (11, 12).

We have found that the involvement of Rac1 in NFκB activation in response to IL1 appears to be independent of IκBα degradation or nuclear translocation and DNA binding of NFκB. Instead, our data clearly point to a role for Rac1 downstream of these events at the level of enhancing the NFκB transactivating potential of its p65 subunit once bound to its consensus sequence. We therefore propose that IL1 initiates

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‡ To whom correspondence should be addressed. Tel.: 353-1-608-2439; Fax: 353-1-677-2400; E-mail: laoneill@tcd.ie.

[1] The abbreviations used are: IL, interleukin; NFκB, nuclear factor κB; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; CBP, cAMP-responsive element binding protein; IκB, inhibitory protein κB; TNF, tumor necrosis factor; ROS, reactive oxygen species; PAE, porcine aortic endothelial; AP, activated protein; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; PAK, p21-activated kinase.

two pathways in the NFκB system, the well characterized one leading to IκB phosphorylation, and the second, requiring Rac1, which regulates p65-mediated transactivation of gene expression.

MATERIALS AND METHODS

Cell Culture and Reagents—EL4.NOB-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were seeded at 1×10^6 ml^{-1} for experiments and pretreated with inhibitors prior to stimulation with IL1α as indicated in the figure legends. Porcine aortic endothelial (PAE) cells were grown in F-12 nutrient mixture (Ham's F-12; Sigma) containing 15% fetal calf serum, 2 mM L-glutamine, 50 μg/ml gentamycin, and 500 nM puromycin at 37 °C in a humidified atmosphere of 5% CO_2. PAE cells stably transfected with either RacV12 (V12Rac-PAE) or RacN17 (N17Rac-PAE) were grown as above but with the addition of 0.1 mM hygromycin B. 24 h prior to the induction of Rac1 mutants, cell lines were cultured in serum-free medium containing 0.2% fetal calf serum, 0.1% fatty acid-free bovine serum albumin, 2 mM L-glutamine, 50 μg/ml gentamycin, 500 nM puromycin, and 0.1 mM hygromycin B. Expression of RacV12 and RacN17 was induced by the addition of 15 mM filter-sterile isopropyl-β-D-thiogalactopyranoside to the starvation medium for the time periods indicated in the figure legends. Human recombinant IL1α was a kind gift from Prof. J. Saklatvala (Kennedy Institute of Rheumatology, U. K.). The pyridinyl imidazole SB203580 was kindly provided by Peter Young, Smithkline Beecham Pharmaceuticals, King of Prussia, PA. PD98059 (2′-amino-3′-methoxyflavone) was a kind gift from Alan Saltiel, Parke-Davis Research Division, Warner Lambert Company, Ann Arbor, MI. All inhibitors were prepared in dimethyl sulfoxide.

Plasmid Constructs—The pEF expression vector encoding myc-tagged constitutively active RacV12 and dominant negative RacN17 and the AP1 reporter plasmid (AP1 chloramphenicol acetyltransferase, AP1-CAT) were all kind gifts from Dr. D. Cantrell (ICRF, London) and have been described elsewhere (21). GST-PAK (residues 1–252) was also a kind gift from Dr. Cantrell. The κB-luciferase reporter gene (pGL3–5xκB-luc) was a kind gift from Dr. R. Hofmeister (Universität Regensgurg, Regensburg, Germany). Gal4-$p65^{1-551}$ plasmid encoding the full p65 subunit (amino acids 1–551) fused to the DNA binding domain of Gal4 was obtained from Dr. Lienhard Schmitz (German Cancer Research Center, Heidelberg, Germany) and has been described previously (22, 23). The Gal-luciferase reporter gene was purchased from Stratagene. IκBctag was constructed by cloning IκBα into the pcDNA3 expression vector, which contained a sequence encoding the SV5 Pk tag, and was a kind gift from Prof. R. T. Hay (University of St. Andrews, Scotland). All plasmids were purified using an endotoxin-free protocol (Wizard® PureFection DNA Purification, Promega, Madison, WI).

Affinity Precipitation of Active Rac1 using GST-PAK—EL4.NOB-1 cells (1×10^7) were stimulated for various time points with IL1. Activation was terminated by washing cells with ice-cold phosphate-buffered saline followed by lysis in 1 ml of lysis buffer (25 mM HEPES, pH 7.5, 1% Nonidet P-40, 0.25% deoxycholate, 10% glycerol, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na_3VO_4, 2 μg/ml aprotonin) containing 10 μg of GST-PAK and incubated for 1 h at 4 °C. Cell lysates were cleared by centrifugation and supernatants incubated with 30 μl of glutathione agarose beads for 60 min at 4 °C. The bead pellet was washed three times with lysis buffer and finally resuspended in 30 μl of Laemmli sample buffer. Proteins were separated by 15% SDS-polyacrylamide gel electrophoresis, and associated active Rac1 was detected by Western blot analysis using an anti-Rac-specific antibody (Upstate Biotechnology, Lake Placid, NY).

Transient Transfection and Reporter Gene Assays—EL4.NOB-1 cells (1.4×10^7) were transfected with plasmids as described in the figure legends in a final volume of 1.2 ml using DEAE- dextran (24). PAE cell lines were transfected using Fugene (Roche Diagnostics Ltd., East Sussex, U. K.) according to the manufacturer's recommendations. After a period of recovery (16–18 h) cells were treated as indicated in the figure legends. To assay luciferase activity, cells were lysed using passive lysis buffer (Promega) and luciferase activity determined using standard procedures. All experiments were done in triplicate, and luciferase activity was normalized to protein concentration as determined by the method of Bradford (25). Cell lysates for assessing the activity of CAT were prepared by repeated freeze-thaw cycles and enzyme activity determined as described previously (26).

Immunoprecipitation and Western Blot Analysis—EL4.NOB-1 cells were treated as described in the figure legends, and treatment was terminated by the addition of 5 ml of ice-cold phosphate-buffered saline. Cells were lysed on ice (30 min) in buffer containing 25 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_3VO_4, and 0.5% Nonidet P-40. Lysates were cleared by centrifugation, and after preclearing for 30 min with protein A-insoluble (Sigma), Rac1 was immunoprecipitated with 4 μg of mouse monoclonal anti-Rac (Upstate Biotechnology) for 60 min at 4 °C. The immune complexes were precipitated by incubation with protein A-Sepharose for 60 min at 4 °C, and PAK association was detected using a polyclonal anti-PAK antibody (Santa Cruz). For Western blot analysis of IκBα degradation and expression of myc-tagged constructs, total cell lysates were prepared using radioimmune precipitation buffer (27). Equivalent amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose or polyvinylidene difluoride membranes; after incubation with primary antibodies as indicated (1 h at room temperature), blots were incubated with the appropriate peroxidase-conjugated secondary antibody (45 min at room temperature). Visualization was by enhanced chemiluminescence according to manufacturer's recommendations (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as described by Osborn *et al.* (28) from cells (5×10^6) treated as described in figure legends. Nuclear extracts (4–8 μg of protein) were incubated (30 min at room temperature) with 10,000 cpm of double-stranded [γ-^{32}P]ATP NFκB oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). Incubations were performed in the presence of 2 μg of poly(dI·dC) as nonspecific competitor and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 4% glycerol, and 100 μg/ml nuclease-free bovine serum albumin. DNA-protein complexes were resolved on native (5%) polyacrylamide gels that were subsequently dried and autoradiographed.

RESULTS

Rac1 Is Activated after IL1 Stimulation—Although previous evidence points to Rac1 playing an important role in IL1-induced signaling it has not been shown conclusively that stimulation of cells with IL1 activates the small G protein. To address this question we used a murine thymoma cell line, EL4.NOB-1, which is strongly responsive to IL1 (27) and employed a technique that relies on the fact that only in its active state will Rac1 bind its downstream effector PAK1 (29). We therefore used a GST-PAK fusion protein (residues 1–252), which contained the crucial domain essential for Rac1 binding, in GST pull-down experiments followed by anti-Rac immunoblot analysis to assess the level of Rac1 activation in our cells. As shown in Fig. 1*A*, virtually no PAK-associated Rac1 was detected in unstimulated cells (*lane 1*). After stimulation with IL1, however, the amount of associated Rac1 increased, with Rac1 being activated as early as 5 min (*lane 2*) and activation increasing up to 60 min (*lane 4*). We confirmed this result by immunoprecipitating endogenous Rac1 in our cells and tested for PAK1 association by immunoblot analysis. Fig. 1*B* shows that IL1 stimulation of cells for 15 min results in increased association of endogenous PAK1 with Rac1, indicating increased activation of the G protein. This method also confirms that the effector with which Rac1 associates after IL1 activation is PAK1, which has been shown previously to regulate p42/p44 MAPK activation (30).

Dominant Negative RacN17 Inhibits IL1-induced κB-dependent Reporter Gene Expression—To investigate the role of Rac1 in IL1-induced activation of NFκB we used two mutants of Rac1, constitutively active RacV12 and dominant negative RacN17 (5). These mutants, which have been characterized extensively, have point mutations in the GDP/GTP binding site which prevent GTP hydrolysis or GDP exchange, respectively. Fig. 2 demonstrates the effect of IL1 in cells transiently co-transfected with a NFκB-dependent reporter gene, κB-luciferase, and a plasmid encoding dominant negative RacN17. Treatment of cells for 3 h with 10 ng/ml IL1 increased κB-luciferase activity 10-fold (Fig. 2*A*). This effect was inhibited in cells

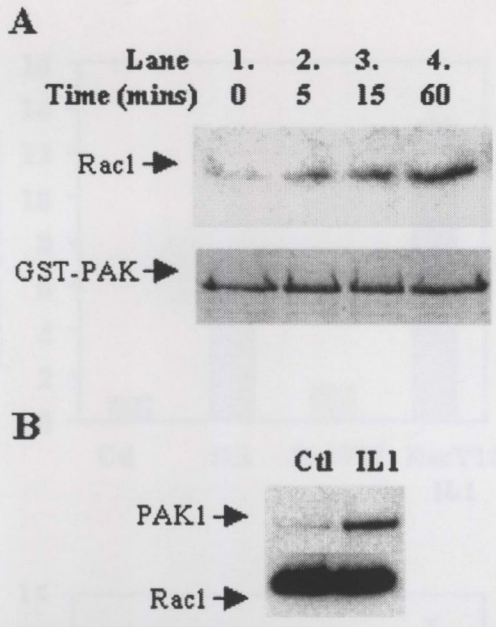

FIG. 1. **IL1 stimulation of EL4.NOB-1 cells activates Rac1.** *A*, activated Rac1 was affinity purified from EL4.NOB-1 (2×10^7) cell lysates (stimulated with IL1 (10 ng/ml) for various time points as indicated) using GST-PAK and detected by Western blot analysis using an anti-Rac antibody (Upstate Biotechnology). After transfer, gels were stained to show that equivalent amounts of GST-PAK were added to each sample (*lower panel*). Identical results were observed in a further experiment. *B*, total Rac was immunoprecipitated from EL4.NOB-1 cells (1×10^7) stimulated with IL1 (10 ng/ml) as indicated and PAK association determined by Western blotting using an anti-PAK antibody. Blots were stripped using 50 mM glycine buffer (pH 2) and reprobed using the anti-Rac antibody to demonstrate that equal levels of Rac were immuoprecipitated in both samples (*lower panel*).

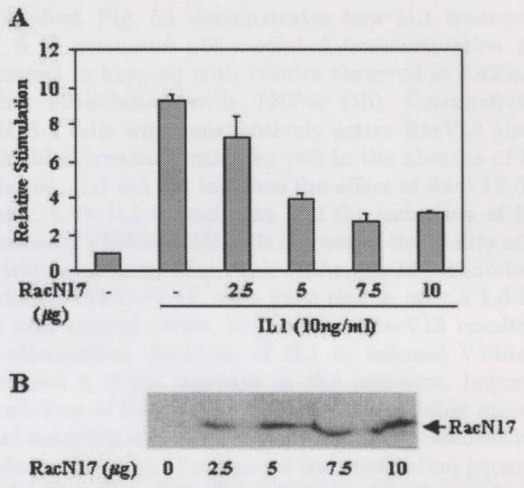

FIG. 2. **Effect of RacN17 on NFκB-dependent transcription.** *A*, EL4.NOB-1 cells (1.4×10^7) were transiently transfected with the NFκB-dependent reporter gene κB-luciferase (2.5 μg) and increasing amounts of plasmid encoding RacN17 (0–10 μg). The total amount of plasmid transfected in each case was kept constant by adding the appropriate amounts of relevant empty vector plasmid. After 18-h recovery, cells (1×10^6/ml) were stimulated as indicated with IL1 (10 ng/ml, 3 h) and extracts assayed for luciferase activity. Results are expressed as fold increase compared with unstimulated control samples (mean ± S.D. of triplicate determinations) and are representative of at least three separate experiments. *B*, myc-tagged RacN17 expression was detected by Western blot analysis of whole cell lysates following transfection of cells with increasing amounts of plasmid encoding myc-tagged RacN17 (0–10 μg) using a monoclonal antibody that recognized the myc epitope. The band detected was at a molecular mass of 22 kDa as would be expected for myc-tagged RacN17.

cotransfected with increasing amounts of RacN17 with 10 μg of plasmid reducing the effect of IL1 by 70%. This effect correlated with the level of expression of RacN17 in the cells as judged by Western blot analysis using an anti-myc antibody, which recognized epitope-tagged RacN17 (Fig. 2*B*).

Constitutively active RacV12 has been shown previously to activate NFκB in rabbit synovial fibroblasts and HeLa cells (11, 12). In our system cotransfection with RacV12 had no effect on κB-luciferase activity but did potentiate the IL1-driven response by 2-fold (Fig. 3*A*). In contrast, transfection of cells with RacV12 activated an AP1-driven reporter gene, *AP1-CAT*, without the need for additional stimuli. IL1 alone had only a marginal effect on this response but potentiated the effect of RacV12 (Fig. 3*B*). Expression of myc-tagged RacV12 in transfected cells was detected by Western blot analysis using an anti-myc antibody (Fig. 3*C*).

Rac1 Does Not Participate in the Pathway to IκBα Degradation Induced by IL1—A crucial regulatory control point on the pathway to NFκB activation is the phosphorylation, ubiquitination, and subsequent degradation of IκB. We therefore examined the effect of constitutively active RacV12 and dominant negative RacN17 on IL1-induced IκB degradation. For this we used a tagged version of IκBα (IκBctag) which, when expressed in cells, can be distinguished from endogenous IκBα in Western blot analysis because of its higher molecular weight. Cells were cotransfected with expression plasmids encoding IκBctag and either RacV12 or RacN17 to ensure that the effects on IκBα degradation could be analyzed on transfected populations of cells. After stimulation with IL1 (10 ng/ml, 30 min) the ability of either RacV12 or RacN17 to induce or inhibit IκBctag degradation, respectively, was analyzed by Western blot analysis using an antibody that recognized IκBα. As shown in Fig. 4*A*, stimulation with IL1 resulted in degradation of both endogenous and tagged IκBα (compare *lane 2* with *lane 1*). The expression of RacV12 had no effect on IκBctag degradation (*lane 3*), nor did it enhance the effect of IL1 (*lane 4*) as had been seen on κB-luciferase (Fig. 3*A*). Furthermore, and most importantly, RacN17 did not inhibit IL1-induced degradation of IκBctag (*lane 6*) as would be expected if Rac1 was involved in regulating this crucial regulatory step on the pathway to NFκB activation.

We next analyzed the effects of constitutively active RacV12 on nuclear translocation and DNA binding of NFκB as determined by electrophoretic mobility shift assay on RacV12-transfected cells. As shown in Fig. 4*B*, IL1 induced strong activation of NFκB (compare *lane 2* with *lane 1*). However, transfection of cells with RacV12 (*lanes 3* and *4*) or RacN17 (*lanes 5* and *6*) had no effect on NFκB DNA binding as judged by electrophoretic mobility shift assay, either on their own or in IL1-treated cells.

These results were confirmed using PAE cells that were stably transfected with epitope-tagged constitutively active RacV12 (V12Rac-PAE) or dominant negative RacN17 (N17Rac-PAE) under the control of an isopropyl-β-D-thiogalactopyranoside-responsive promoter. Expression of these constructs was detected readily using an anti-Rac antibody because of their higher molecular weight compared with endogenous Rac1, and expression of either was found to occur from 6 h (not shown) and was maximal at 24 h postinduction (Fig. 4*C*). Comparison of *lanes 1* and *3* in Fig. 4*D* shows that induction of RacV12 expression was unable to induce IκBα degradation, nor was it able to enhance the DNA binding activity of NFκB (Fig. 4*E*). RacN17 induction similarly did not affect IL1-induced IκBα degradation (Fig. 4*D*, compare *lanes 7* and *8*) or DNA binding activity of NFκB (Fig. 4*E*, *lanes 7* and *8*). The lack of effect of RacV12 or RacN17 was also evident at earlier induction times (data not shown).

Rac1 Is Required for Increased Transactivation Potential of p65 in Response to IL1—Because RacN17 inhibited IL1-in-

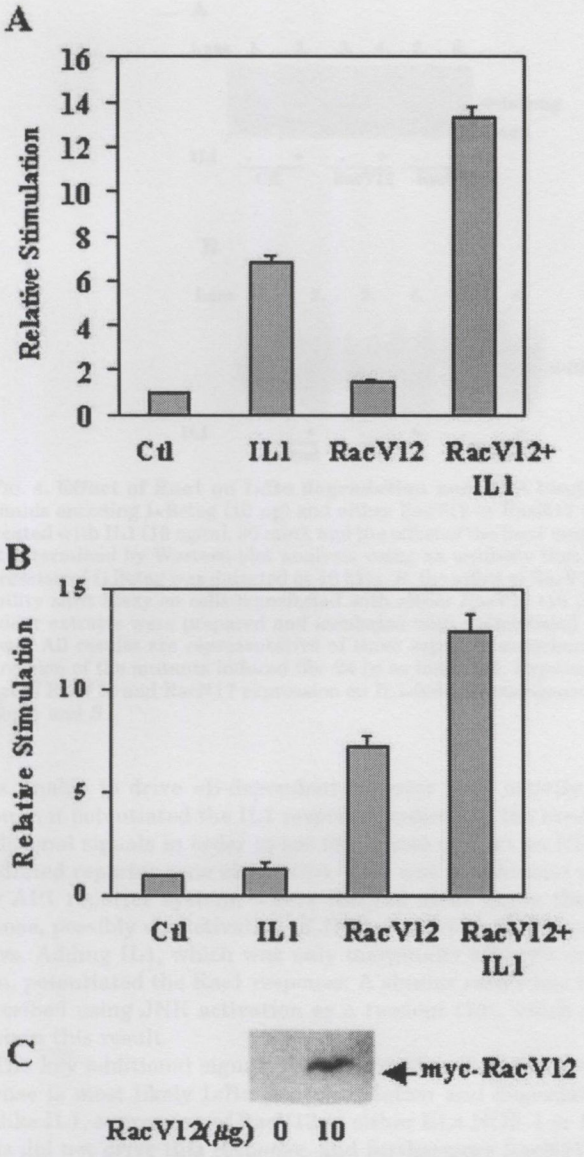

FIG. 3. **Effect of RacV12 on κB-dependent gene expression.** *A*, EL4.NOB-1 cells (1.4×10^7) were transiently transfected with κB-luciferase (2.5 μg) and RacV12 (10 μg). After 18-h recovery, cells (1×10^6/ml) were stimulated as indicated with IL1 (10 ng/ml, 3 h) and extracts assayed for luciferase activity. Results are expressed as fold increase compared with unstimulated control samples (mean ± S.D. of triplicate determinations) and are representative of at least three separate experiments. *B*, cells were transiently transfected with AP1-CAT reporter gene (10 μg) and RacV12 (10 μg). After recovery, cells (1×10^6/ml) were stimulated with IL1 (10 ng/ml, 24 h) and extracts assayed for CAT activity. Results are expressed as fold increase over unstimulated control for three separate experiments (mean ± S.E. of four individual experiments). *C*, myc-tagged RacV12 expression was detected by Western blot analysis as described in the legend to Fig. 1. The band detected was at a molecular mass of 22 kDa as would be expected for myc-tagged RacV12.

duced κB-dependent reporter gene expression but not IκBα degradation and because RacV12 potentiated κB-dependent reporter gene expression in response to IL1 but had no effects on its own, our results indicated a role for Rac1 in transactivation by NFκB. To investigate this possibility we cotransfected EL4.NOB-1 cells with the p65 subunit of NFκB fused to the DNA binding domain of Gal4 (Gal4-$p65^{1-551}$) and a Gal4-responsive reporter gene, Gal-luciferase (22, 23). The advantage of this assay is that Gal4-$p65^{1-551}$ is exclusively nuclear and is regulated independently of IκB thus allowing the effects of various stimuli or genes of interest on transactivation by p65 to be studied. Fig. 5*A* demonstrates how IL1 treatment (10 ng/ml, 5 h) increased p65-mediated transactivation 2.5-fold over control in keeping with results observed in L929sA cells following stimulation with TNF-α (15). Cotransfection of EL4.NOB-1 cells with constitutively active RacV12 also more than doubled transactivation by p65 in the absence of further stimulation. IL1 did not increase the effect of RacV12 further. Similarly, both IL1 stimulation and the induction of RacV12 expression in V12Rac-PAE cells increased the ability of p65 to drive transactivation (Fig. 5*A*). Although IL1 stimulation of noninduced V12Rac-PAE cells gave rise to only a 1.5-fold increase over control levels, induction of RacV12 resulted in a 2-fold stimulation. Addition of IL1 to induced V12Rac-PAE cells caused a slight increase in the response. Importantly, cotransfection of EL4.NOB-1 cells with increasing amounts of plasmid encoding dominant negative RacN17 resulted in dose-dependent inhibition of enhanced transactivation potential induced by IL1 (Fig. 5*B*). Transfection with 5 μg of plasmid reduced the transactivation potential of p65 to basal levels. Furthermore, plasmid amounts greater than 5 μg lowered the response below basal, indicating a role for Rac1 in the basal signal (Fig. 5*B*). We were unable to test the N17Rac-PAE cells in this assay because IL1 was unable to drive transactivation in noninduced cells (not shown). Prolonged exposure of blots from N17Rac-PAE cells revealed a low level of constitutive expression of RacN17 in noninduced cells (Fig. 5C, *lane 1*), which provided a possible explanation for the lack of effect of IL1 on noninduced cells. In comparison, the V12Rac-PAE cells, under the same conditions, did not show this basal level of expression of RacV12 (Fig. 5*C*, *lane 2*).

p42/p44 and p38 MAPK Are Involved in Enhanced p65-mediated Transactivating Activity in Response to IL1 and RacV12—Recent reports have demonstrated an involvement of both p42/p44 and p38 MAPK pathways in NFκB transactivation in response to TNF stimulation (15). We therefore tested the involvement of these kinases in regulating transactivation using specific inhibitors of each of these pathways, the MEK1 inhibitor PD98059 (33) and the p38-specific inhibitor SB203580 (34, 35). Fig. 5*D* demonstrates how treatment of cells with both PD98059 and SB203580 inhibited transactivation by p65 in response to both IL1 and RacV12. With respect to both IL1- and RacV12-induced increase in transactivation activity, PD98059 reduced the effect to basal levels indicating that p42/p44 MAPK lies downstream of both IL1 and Rac1 in events leading to enhanced transactivation activity of p65. In addition SB203580 inhibited both responses by at least 50%, suggesting that p38 MAPK is also involved in regulating these pathways. Our result indicates that p42/p44 and, to a lesser extent, p38 MAPK mediate the effects of IL1 and Rac1 on enhanced p65 transactivation activity.

DISCUSSION

In this study we provide evidence that Rac1 does not lie on the IL1-induced signaling pathway leading to IκBα phosphorylation and degradation, but instead participates in a second process required for NFκB function, namely the ability of the p65 subunit of NFκB to transactivate gene expression. Our data indicate that there are two separate signals activated by IL1 in the NFκB system. First, NFκB becomes activated by phosphorylation and degradation of IκBα by the signalsome resulting in the subsequent release of NFκB. The second signal enhances the transactivating potential of NFκB, acting on the complex once it is bound to its consensus sequence. Using the two well characterized mutants of Rac1, constitutively active RacV12 and dominant negative RacN17, our results clearly show a role for Rac1 in the latter of these two pathways activated by IL1. Transfection of EL4.NOB-1 cells with RacV12

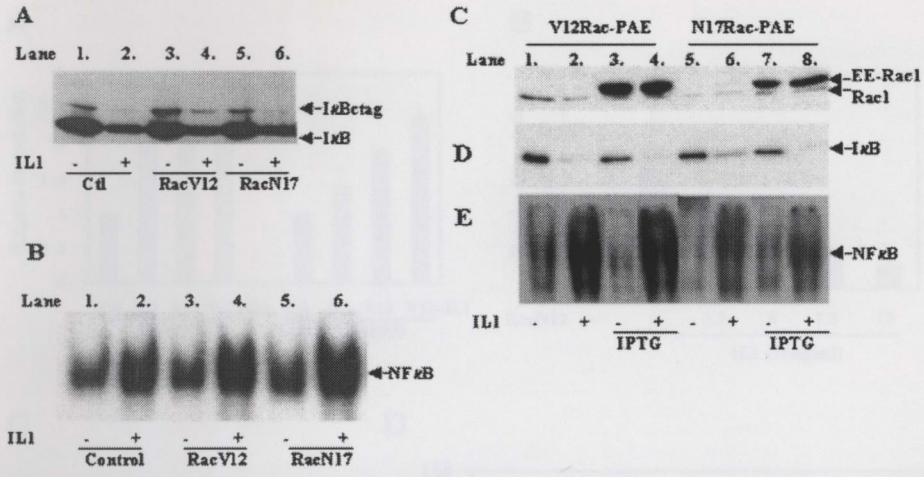

FIG. 4. **Effect of Rac1 on IκBα degradation and DNA binding activity of NFκB.** *A*, EL4.NOB-1 cells (1.4×10^7) were transfected with plasmids encoding IκBctag (10 μg) and either RacV12 or RacN17 (10 μg) as indicated. After 18-h recovery, cells (1×10^6/ml) were stimulated as indicated with IL1 (10 ng/ml, 30 min), and the effect of the Rac1 mutants on IL1-induced degradation of both endogenous and tagged IκBα (IκBctag) was determined by Western blot analysis using an antibody that recognized IκBα. As expected, endogenous IκBα was detected at 38 kDa, and expression of IκBctag was detected at 40 kDa. *B*, the effect of RacV12 and RacN17 on DNA binding ability of NFκB was assessed by electrophoretic mobility shift assay on cells transfected with either RacV12 (10 μg) or RacN17 (10 μg) after stimulation with IL1 (10 ng/ml, 1 h) as indicated. Nuclear extracts were prepared and incubated with radiolabeled κB-dependent probe (30 min, room temperature). DNA-protein complexes are shown. All results are representative of three separate experiments. *C*, V12Rac-PAE and N17RacPAE cells were serum starved for 24 h and expression of the mutants induced (for 24 h) as indicated. Expression of the EE-tagged constructs was detected using an anti-Rac antibody. The effect of RacV12 and RacN17 expression on IL1-induced endogenous IκBα degradation (*D*), and DNA binding ability of NFκB (*E*) was determined as for *A* and *B*.

was unable to drive κB-dependent reporter gene activity, although it potentiated the IL1 response, indicating the need for additional signals in order to see the effects of Rac1 on NFκB-mediated reporter gene expression. This was not the case with the AP1 reporter system, where RacV12 alone drove the response, possibly via activation of JNK or p42/p44 MAPK pathways. Adding IL1, which was only marginally effective on its own, potentiated the Rac1 response. A similar result has been described using JNK activation as a readout (10), which may explain this result.

The key additional signal required for the κB-luciferase response is most likely IκBα phosphorylation and degradation. Unlike IL1, expression of RacV12 in either EL4.NOB-1 or PAE cells did not drive this response, and furthermore RacN17 did not block the effect of IL1. In addition, RacV12 alone was unable to induce nuclear translocation and DNA binding of NFκB. In EL4.NOB-1 and PAE cells, however, RacV12 enhanced the transactivating potential of p65 in the absence of IL1, and importantly RacN17 inhibited IL1-induced transactivation by p65. Taken together these results strongly indicate a role for Rac1 in the pathway leading to enhanced transactivation by p65 but not IκBα phosphorylation and degradation.

Previous studies in HeLa cells and rabbit synovial fibroblasts have proposed that Rac1 mediates NFκB activation via a redox-dependent pathway involving ROS (11, 12). Although Rac1 has been shown to regulate ROS production in a number of different systems, this ability has been demonstrated to be highly cell type-specific (36, 37). Indeed, recent studies in lymphocytes have clearly shown no role for Rac1 in ROS-dependent activation of NFκB, supporting our view that in our system Rac1 lies on an alternate pathway regulating NFκB activation (38). We have been unable to find a role for ROS in NFκB activation by IL1 in EL4-NOB-1 (39).

A key question concerns how Rac1 might enhance p65 transactivating activity. Recent studies have pointed to the involvement of both p42/p44 and p38 MAPK pathways in p65 function in response to TNF-α stimulation (15). Using specific inhibitors of p42/p44 and p38 MAPK pathways (PD98059 and SB203580, respectively), our results also indicate a role for these MAPK pathways downstream of IL1. We have shown previously that IL1 activates both p42/p44 and p38 MAPK in these cells and that this response is blocked by their respective inhibitors (24). Recently it has been shown that SB203580 inhibits phosphorylation of TATA-binding protein, preventing interaction with p65 and thereby blocking transactivation. This provides a possible mechanism for the effect of SB203580 in our studies, implying that activation of p38, via a pathway involving Rac1, leads to TATA-binding protein phosphorylation, promoting transactivation by p65.

Previous work in our laboratory using the T cell distal element of the IL2 promoter found that neither p42/p44 nor p38 MAPK pathways were involved in IL1-induced activation of this element as determined using the CAT reporter gene linked to the T cell distal element (24). This site, although capable of binding NFκB, is not a canonical κB site. It binds additional (but as yet unidentified) factors, and studies have clearly shown differences in how the T cell distal element and the NFκB element are regulated in response to IL1 (40). Our results here clearly indicate that both p42/p44 and p38 MAPK are involved in regulating NFκB transactivation by p65, and hence κB-linked gene expression. In addition it appears that both p42/p44 and p38 MAPK lie downstream of Rac1 on the pathway leading to p65-mediated transactivation of NFκB as PD98059 and SB203580 inhibited RacV12-driven transactivation by p65. Although a role for Rac1 in IL1-mediated activation of p38 MAPK has been demonstrated previously (41), the involvement of Rac1 in p42/p44 activation in response to IL1 has yet to be shown. Our results indicate that Rac1 lies upstream of p42/p44 MAPK and is in keeping with a report that has shown that the downstream effector of Rac1, PAK, can regulate p42/p44 activation via a Raf-independent pathway (42). As well as demonstrating Rac1 activation by IL1, our results demonstrate that the downstream effector with which Rac1 associates after IL1 stimulation is PAK1. Our results therefore support a role for IL1 and Rac1, possibly via PAK1 activation, in regulating p42/p44 and p38 activation and indicate that Rac1, via the p42/p44 and p38 MAPK pathway, is critically involved in regulating the transactivation potential of

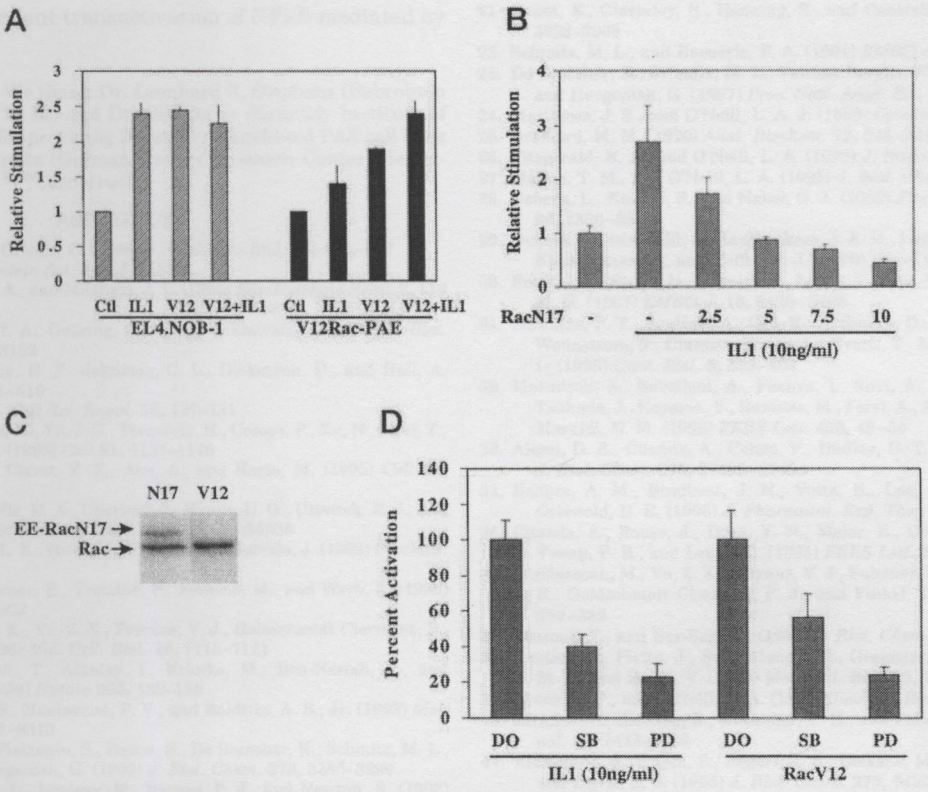

FIG. 5. **Effect of Rac1 on p65-mediated transactivation of NFκB.** *A*, EL4.NOB-1 cells (1.4×10^7) and V12Rac-PAE (4×10^4) were transfected with Gal-luciferase reporter plasmid (5 μg and 350 ng, respectively) and Gal4-$p65^{1-551}$ (2.5 μg and 350 ng, respectively). In addition, EL4.NOB-1 cells were cotransfected with RacV12 (10 μg) as indicated. RacV12 expression was induced 6 h post-transfection in V12Rac-PAE cells, and both cell types were stimulated with IL1 (10 ng/ml, 6 h) 18 h later. *B*, EL4.NOB-1 cells (1.4×10^7) were transfected with Gal-luciferase reporter plasmid (5 μg) and expression plasmids encoding Gal4-$p65^{1-551}$ (2.5 μg) and increasing amounts RacN17 (0–10 μg) as indicated. Cells were allowed to recover for 16–18 h after which they were stimulated with IL1 (10 ng/ml, 6 h) and cell extracts prepared and assayed for luciferase activity. *C*, the expression of EE-RacN17 and EE-RacV12 in noninduced N17Rac-PAE and V12Rac-PAE cell lines, respectively, was detected by Western blotting using an anti-Rac antibody. *D*, EL4.NOB-1 cells were transfected as in *A*, and after recovery they were incubated in complete medium containing 0.5% fetal calf serum. Cells were pretreated with inhibitors or vehicle control (dimethyl sulfoxide) as indicated (1 h, 37 °C): *DO*, dimethyl sulfoxide; *PD*, PD98059 (30 μM); SB203580, 30 μM SB203580. Subsequent to this they were stimulated with IL1 (10 ng/ml, 6 h) as indicated and lysates assayed for luciferase activity. Results (mean ± S.D. for triplicate determinations) in *A* and *B* are represented as fold increase compared with unstimulated controls; in *C* they are shown relative to response to IL1 or RacV12. In all cases results are representative of at least three separate experiments.

NFκB in response to IL1.

Several reports have demonstrated that upon stimulation with either TNF-α or IL1 the p65 subunit of NFκB becomes phosphorylated on multiple serines thus potentially acting to enhance p65 transactivating potential (17, 18). Although p38 and p42/p44 may be involved, these are unlikely to phosphorylate p65 directly because of the lack of consensus sites for phosphorylation. The kinase(s) directly responsible for phosphorylating p65 have yet to be identified, although a role for casein kinase II has been proposed as it has been shown to phosphorylate the transactivation region found in the COOH-terminal domain of p65 (20). Recently, an as yet unidentified kinase has been shown to regulate phosphorylation of the transactivation domain of p65 on serine 529, regulating the transactivational activity of NFκB (18). In addition, recent work demonstrated that protein kinase A is involved in events leading to the phosphorylation of serine 276 in the Rel homology domain of NFκB (17). The NH_2-terminal Rel homology domain is crucial for regulating the binding of p65 to its consensus sequence. This domain has also been shown recently to play an important role in regulating transactivation signals in response to TNF-α stimulation, with protein kinase Cζ (activated by Ras) shown to play a role in regulating phosphorylation of this domain on a site other than serine 276 (43). In addition to p65 subunit phosphorylation, interaction with the coactivator p300/CBP has been shown to enhance NFκB transcriptional activity. p300/CBP is constitutively associated with RNA polymerase II, and interaction with p65 via its COOH-terminal transactivation domains results in increased transcriptional activity of NFκB which is enhanced after phosphorylation of the Rel homology domain by protein kinase A (17, 19). We would speculate that Rac1 is required for some or all of these events in our system via the activation of both p38 and p42/p44 MAPK pathways. As mentioned above, TATA-binding protein would be another possible target for p38 here.

How IL1 may mediate Rac1 activation is as yet unclear, although a recent report indicates that Rac1 may associate with the IL1 receptor complex (44). Our data clearly support this observation in that we provide direct evidence for Rac1 activation by IL1 and the subsequent interaction between Rac1 and PAK1. Furthermore, the intracellular domain of the IL1 receptor has been shown to associate with the p85 regulatory domain of phosphatidylinositol 3-kinase (which has previously been shown to activate Rac1 (31, 45)) via a potential phosphotyrosine motif on the receptor (32). Whether phosphatidylinositol 3-kinase interaction with IL1 type I receptor results in Rac1 activation in response to IL1 stimulation remains to be elucidated.

In conclusion, our results indicate that IL1 mediates the activation of two separate signaling pathways that, combined, regulate the activity of the transcription factor NFκB. We have demonstrated a role for Rac1 in IL1-induced enhancement of NFκB transactivation potential independent of both IκB degradation and nuclear translocation and DNA binding of NFκB. Furthermore, both p38 and p42/44 MAPK pathways are re-

quired for p65-dependent transactivation of NFκB mediated by both IL1 and Rac1.

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