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Investigating the role of Small G Proteins in the activation of p38 MAPK by Interleukin-1

Thesis submitted to the University of Dublin

for the degree of Doctor of Philosophy

by

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September 2000

The University of Dublin
Trinity College

Till mamma och pappa

Declaration

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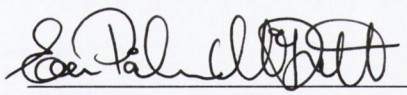

Eva Pålsson McDermott

Table of Contents

This study is an investigation into the role of small G proteins in IL-1 signalling.

Lethal Toxin, from *Clostridium sordellii*, which specifically glucosylates and thereby inactivates the low molecular weight G proteins Ras, Rap, Rac, and Ral, inhibited the activation of p38 and p42/p44 Mitogen Activated Protein Kinase (MAPK) by IL-1 in EL4.NOB-1 cells and primary fibroblasts. *C. difficile* Toxin B, which inhibits Rac, Rho, and Cdc42 had no effect on the activation of p38 and p42/p44 MAPK by IL-1 which indicated that LT was probably targeting a Ras-family G protein on the IL-1 pathway. LT glucosylated proteins of molecular weights 18,19 and 23 kDa to an extent that correlated with the inhibition of the activation of p38 MAPK by IL-1. In addition, LT failed to inhibit p38 MAPK activation by IL-1 in a UDP-glucose deficient cell line, indicating that glucosylation of small G proteins was required for the inhibitory effect. These studies indicate that an LT-sensitive small G protein, possibly belonging to the Ras subfamily, play a critical role in IL-1 signalling.

The target protein involved appeared to be Ras itself, since transient transfections with dominant negative RasN17 inhibited p38 MAPK activation by IL-1. Furthermore, transfections of cells with constitutively active RasVHa activated p38 MAPK. Further evidence for Ras involvement came from the observation that IL-1 caused a rapid activation of Ras in the cells, and from the inhibitory effects of the Ras inhibitors Manumycin A and Damnacanthal. A dominant negative version of Rac (RacN17) (or Rap (Rap1AN17)) did not inhibit the activation of p38 MAPK by IL-1, which is in agreement with the lack of effect of Toxin B.

Intriguingly, transfection of cells with Rap1AN17 activated p38 MAPK. Furthermore, constitutively active Rap1AV12 inhibited p38 MAPK activation by IL-1, consistent with Rap antagonising Ras function. IL-1 also activated Rap in EL4.NOB-1 cells, but with slower kinetics to Ras. In addition, dominant negative RapN17 prolonged the transient nature of the p38 MAPK response to IL-1, suggesting that Rap plays a role in down-regulating this signal. Our studies therefore provide clear evidence using multiple approaches for Ras as a signalling component in the activation of p38 MAPK by IL-1, with Rap having an inhibitory effect.

Transient transfection of cells with constitutively active forms of the known IL-1 signalling components MyD88, IRAK-1, and TRAF-6, or the upstream activators MKK6 and MKK3, activated p38 MAPK. Dominant negative forms of these were found to inhibit activation of p38 MAPK by IL-1. Dominant negative RasN17 blocked the effect of the active forms of all but MKK3 and MKK6, indicating that Ras lies downstream of TRAF-6 but upstream of MKK3 and MKK6 on the pathway. Furthermore, the activation of p38 MAPK caused by overexpressing active RasVHa, could not be inhibited using dominant negative mutants of MyD88, IRAK1 or 2, or TRAF6, but could be inhibited by dominant negative MKK3 or MKK6.

In the same manner, the inhibitory effect of Rap on the activation of p38 by IL-1 occurred at a point downstream of MyD88, IRAK-1, and TRAF6, since the activation of p38 MAPK by these components was inhibited by overexpressing active Rap1AV12, while neither MKK3 nor MKK6 were affected. MyD88, IRAK1, and TRAF6 are therefore involved in the activation of p38 by IL-1, occuring upstream of Ras. Ras may therefore occupy a similar position on the p38 pathway as it does on the classical p42/p44 MAP kinase pathway.

These studies therefore provide evidence for a role for the small G protein Ras on the pathway leading to the activation of p38 MAPK by IL-1, with Rap having an antagonistic effect. Both Ras and Rap occupy a position on the pathway downstream of TRAF6.

First and foremost I would like to thank my supervisor Luke O'Neill for all the help, guidance and support in bringing this work to completion. I wouldn't have managed without him.

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I would also like to acknowledge financial support from Enterprise Ireland, the Health research board, and the European Commission Travel and Mobility Research fund.

aa	Amino acid
A_{260}	Absorbance 260 nm
ABTS	2,2'-Azino-bis(3-Ethylbenzthiazolin-6-sulphonic acid)
ATP	Adenosine triphosphate
AP-1	Activator protein 1
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAS	Crk associated substrate
CAT	chloramphenicol acetyl transferase
CREB	cAMP response element binding factor
DAG	diacylglycerol
DD	Death domain
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbant assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular regulated kinase
FCS	Foetal calf serum
FIL	Family of IL-1

GAP	GTPase-activating protein
G-CSF	granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
hTLR	human Toll like receptor
ICE	IL-1 converting enzyme
IκB	Inhibitor binding protein κB
IIP1	IL-1 receptor interacting protein 1
IKK	IκB kinase
IL	Interleukin
IL1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1RAcPL	Interleukin-1 receptor accessory protein like
IL1RI	Type I IL1 receptor
IL1RII	Type II IL1 receptor
IL-1Rrp1	IL-1R related protein 1
IRAK	IL-1 receptor associated kinase
ITAK	IL-1 TNF activated kinase
JNK	c-jun N-terminal kinase
kDa	kilo dalton

LPS	Lipopolysaccharide
LT	Lethal toxin
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MKK	MAPK kinase
MyD88	Myeloid differentiation marker 88
NEMO	NFκB Essential Modulator
NF-1	neurofibromin 1
NFκB	Nuclear factor kappa B
NIK	NFκB inducing kinase
PAK	p21 activated kinase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
RA	Rheumatoid arthritis
RIPA	Radioimmune precipitation buffer
SIGGIR	Single Ig IL-1R relative
TAK-1	TGFβ activated kinase
TAB-1	TAK-1 binding protein
TLR	Toll like receptor
TIR domain	Toll-IL-1R domain
TNF	Tumour necrosis factor

Tollip	Toll interacting protein
TRAF	TNF-receptor associated factor
UDP	Uridine diphosphate

CHAPTER 1

General Introduction

Interleukin 1 (IL-1) is a central pro-inflammatory mediator. IL-1 was first identified as a factor produced by mononuclear phagocytes that enhanced T cell responses to antigens or polyclonal activators, i.e. as a co-stimulator of T cell activation (reviewed in (Dinarello, 1994; Dinarello, 1995)). Unlike most other factors of the immune system, IL-1 exerts its action on nearly every tissue and cell type of the organism. It is a potent inflammatory cytokine, which when secreted locally provides the co-stimulation necessary for proliferation of $CD4^+$ T cells and the growth and differentiation of B cells. In addition it promotes the synthesis of IL-6, IL-2, members of the IL-8 family, and further synthesis of IL-1. In severe cases of inflammation, IL-1 levels can rise systemically, giving rise to fever, induction of synthesis of acute phase plasma proteins by the liver, and it also initiates metabolic wasting (reviewed in (Dinarello, 1996)). Clinically, IL-1 plays a central role in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. Autoimmune responses develop as a result of multiple interacting factors. One of the principal immunological mechanisms contributing to autoimmunity includes polyclonal lymphocyte stimulation, in which IL-1 plays an important role. The understanding of the way in which IL-1 affects cells and the signalling mechanism involved in the responses to IL-1 is crucial when developing therapeutic strategies for this group of diseases.

1.1 The IL-1 gene superfamily

The IL-1 gene family is made up of at least 8 members: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, and FIL1-δ, –ε, –ζ, and-η. IL-1α and IL-1β are both biologically active molecules, which act by binding to the type I IL-1 receptor (Smith *et al.*, 2000), whereas the IL-1Ra acts as an endogenous regulator of the IL-1 response (Eisenberg *et al.*, 1990). IL-1 is the only cytokine for which a naturally occurring inhibitor has been described. Clinical trials are currently underway using the antagonist in disease states caused by excessive cytokine production. Promising effects have been observed when treating septic shock, and also in relieving the symptoms of rheumatoid arthritis (Bresnihan *et al.*, 1998).

IL-1α and IL-1β are two distinct gene products that share only 25% amino acid homology, however, they bind to the same receptor (Bird and Saklatvala, 1987; Dower *et al.*, 1986). Neither IL-1α nor IL-1β carries a hydrophobic leader sequence that directs the protein to the endoplasmic reticulum. Most of the IL-1 found in the circulation is IL-1β, but it is still unclear how the mature protein is released or secreted. It has been demonstrated that IL-1β release correlates with apoptosis of the producer cell (Hogquist *et al.*, 1991). Human IL-1β is initially synthesised, mainly by mononuclear phagocytes, as a partially active precursor (pro IL-1β) which is cleaved by an IL-1β-converting enzyme (ICE or Caspase-1) to its mature, bioactive form (Dinarello, 1991) (Wingren *et al.*, 1996). The situation is quite different with IL-1α, which is synthesised as a biologically active precursor and, only when the cells die, the pro-IL-1α is released and cleaved extra-cellularly. Unlike human cells, mouse cells produce and release IL-1α and most experimental models have used mouse or rat cells (Dinarello, 1991). The two types of IL-1 seem to provoke distinct effects in the organism, and it has been demonstrated that IL-1β is the form responsible for causing acute phase responses and fever as well as having a key role in collagen induced arthritis (Dinarello, 1991). Pro-IL-1α has been suggested to act as an autocrine growth factor, regulating normal cell differentiation and growth, and it has been suggested to act in a membrane anchored form (Dinarello, 1991).

The IL-1Ra, which is a 22 kDa glucosylated protein, carries a signal sequence and is secreted by the producing cells (Eisenberg *et al.*, 1990).

Recently, another cytokine, Interleukin 18, was recognised as being part of the IL-1 family based on similarity in its amino acid sequence and predicted tertiary structure. IL-18 induces the production of interferon-γ from T cells, and stimulates the killing activity of cytotoxic T lymphocytes and NK cells by upregulating Fas ligand (Dinarello *et al.*, 1998).

Four additional members of the IL-1 family have been described, called FIL1(Family of IL-1)-δ, –ε, –ζ, and-η (Kumar *et al.*, 2000; Smith *et al.*, 2000). These novel genes demonstrate significant sequence similarities

to IL-1α, IL-1β, IL-1ra, and IL-18. The biological activity of these novel IL-1 family members remains to be characterised, although none of the four proteins binds to the type I IL-1R or to the IL-18 receptor IL-1Rrp1.

1.2 The IL-1/TLR receptor superfamily

The IL-1 Receptor family is an expanding family of proteins, which can be divided into two large sub-groups of proteins based on sequence similarities, the IL-1R family and the TLR family. The defining motif of the IL-1R/TLR superfamily is the intracellular Toll-IL-1R (TIR) domain. The minimal TIR domain consists of 128 amino acids, which can be divided into 10 blocks of conserved sequences, with each block containing a discrete secondary structural element (Rock *et al.*, 1998). The division of the family into two sub-groups is based on similarities in the extracellular domains of the receptors (reviewed by O'Neill at *Science's Signal Transduction Knowledge Environment* at http://www.stke.org/cgi/content/full/OC_sigtrans;2000/44/re1).

1.2.1 The IL-1 Receptor family

To date, two separate receptors for IL-1 have been identified: IL-1 receptor type I (IL-1RI)(Sims *et al.*, 1988) of 80 kDa (the founding member of this family) and IL-1 receptor type II (IL-1RII)(McMahan *et al.*, 1991) of 68 kDa (Fig 1.1). The two IL-1 receptors, IL-1RI and IL-1RII, are distinct gene products and have different affinities for IL-1α and IL-1β, although both forms of IL-1 can recognise the two receptors. IL-1α has been shown to bind preferentially to IL-1RI, whereas IL-1β has a higher affinity to IL-1RII (reviewed in (Dinarello, 1991)). Both receptor types belong to the immunoglobulin gene superfamily, sharing a high sequence homology in the three immunoglobulin-like domains in their extracellular regions (reviewed by O'Neill at *Science's Signal Transduction Knowledge Environment* at http://www.stke.org/cgi/content/full/OC_sigtrans;2000/44/re1). Intracellularly, the two receptors differ dramatically, as IL-1RI extends into the cell with a cytoplasmic domain of 215 amino acids, whereas the IL-1RII has a cytoplasmic domain of merely 29 amino acids. Members of this sub-group all contain immunoglobulin (Ig) domains extracellularly, and this group of receptors also includes the IL-1 receptor

accessory protein (IL-1RAcP) which has been shown to associate with the IL-1RI upon IL-1 binding (Greenfeder *et al.*, 1995). This signalling component is thought to be crucial for the ability of IL-1RI to transduce a signal. Another member of this family include the soluble vaccinia virus IL-1RII homologue B15R(Smith and Chan, 1991). This protein has the Ig domains, which define this subfamily of receptors, but lack the intracellular TIR domain. It functions as a soluble IL-1 receptor, and binds only IL-1β, not IL-1α(Alcami and Smith, 1992). The IL-18 Receptor (IL-18R) and its accessory protein AcPL (accessory protein-like) are also members of this receptor family as well as the IL-1Rrp2 and T1/ST2, (as shown in figure 1.2, reviewed by O'Neill at *Science's Signal Transduction Knowledge Environment* at http://www.stke.org/cgi/content/full/OC_sigtrans;2000/44/re1).

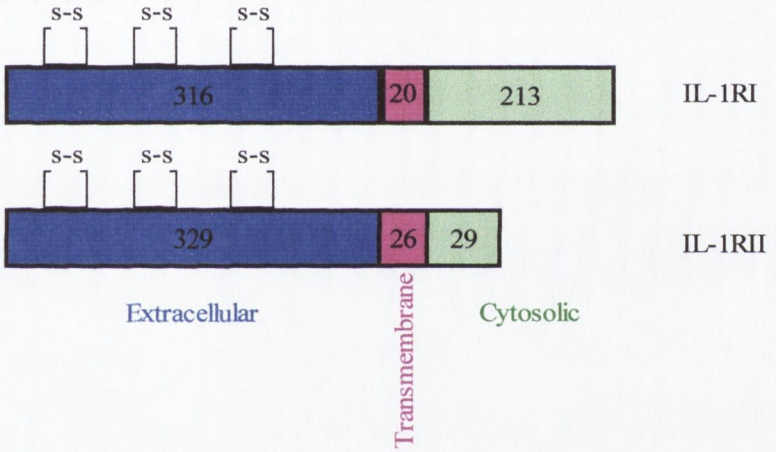


Fig 1.1 **Schematic representation of human IL-1RI and IL-1RII**. Marked with numbers within each domain of the receptors are the number of amino acids that make up the fraction, and each domain is represented as boxes of approximate sizes; Extracellular domain: blue boxes, Trans-membrane domain: pink boxes, Cytosolic domain: green boxes (Dinarello, 1991).

1.2.1i IL-1RI

IL-1RI (Sims *et al.*, 1988) is a heavily glycosylated membrane bound structure found to be present on cell surfaces at typically <500 receptors per cell. It has been shown to transduce a signal at as low as 10 receptors per cell (reviewed in (O'Neill and Greene, 1998)). IL-1RI has an extra-cellular region containing three

immunoglobulin like domains, a single transmembrane domain and a cytoplasmic domain. This receptor has been shown to be the main signalling receptor of IL-1 signalling, and the cytosolic portion of the receptor has been shown to share a 45% amino acid homology with the cytosolic domain of the *Drosophila melanogaster* Toll gene (Gay and Keith, 1991). The product of this gene, the Toll protein, is a membrane protein, which plays an important role in the early developmental stages of *Drosophila melanogaster* (Hashimoto *et al.*, 1988). This apparent sequence conservation has suggested that the well-known signal transduction pathway of Toll leading to the activation of dorsal (the *Drosophila* homologue of NFκB) is homologous to the signalling events occurring in a cell after IL-1 binds to IL-1RI.

1.2.1ii IL-1RII

IL-1RII differs significantly to the IL-1RII due to its short cytoplasmic domain (McMahan *et al.*, 1991). When the intracellular domain of this receptor was identified, experiments using neutralising antibodies to IL-1RI demonstrated that the IL-1RI was responsible for all detectable IL-1 mediated effects (Stylianou *et al.*, 1992). Since then, IL-1RII is known not to be capable of generating a signal, but rather acts as a decoy receptor, i.e. it competes with IL-1RI, binding excess IL-1, and so together with IL-1Ra, it acts to down regulate the IL-1 response.

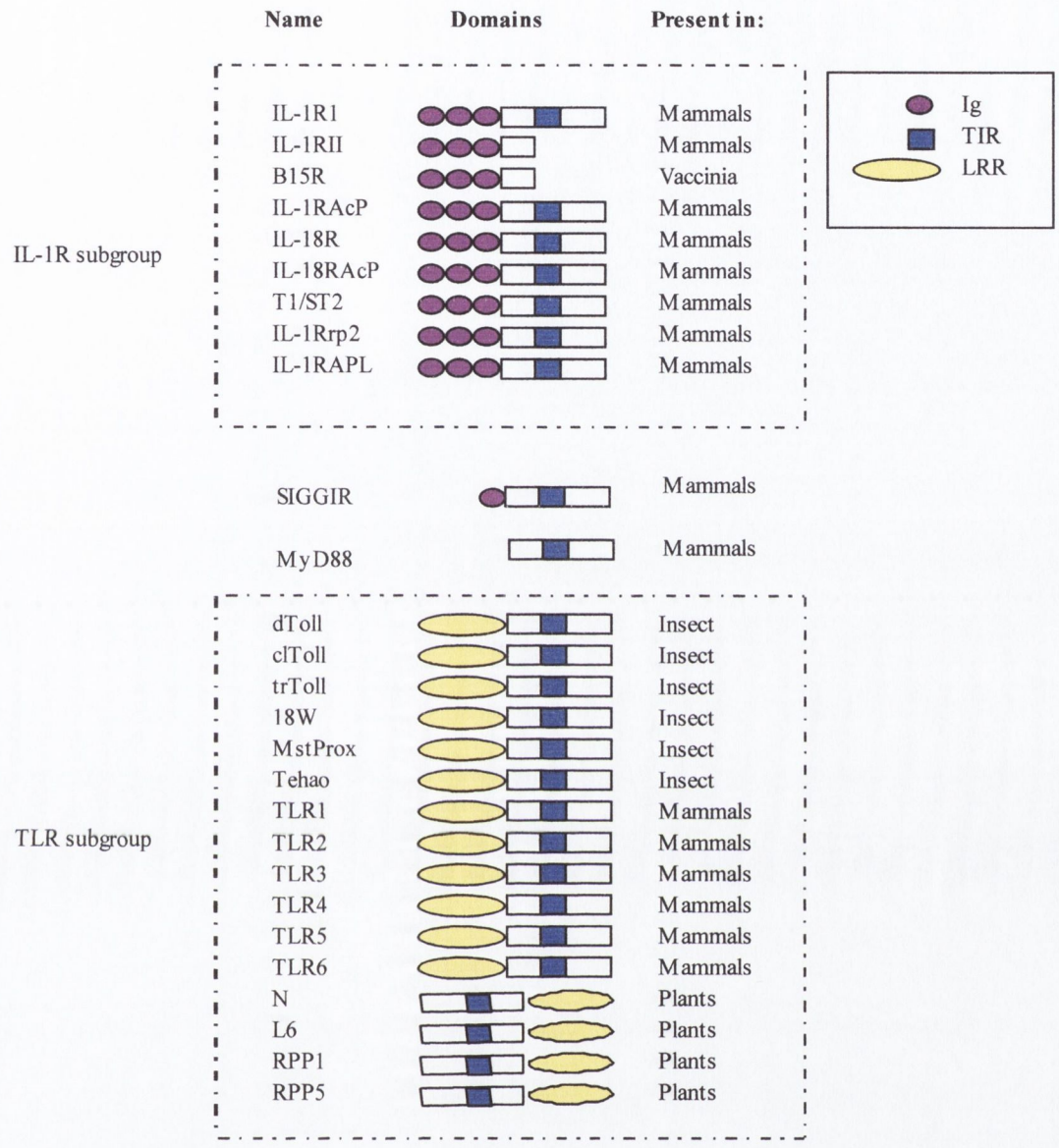


Figure 1.2 **The IL-1R/TLR superfamily.** Details given in text. Taken from L O'Neill, stke August 2000

Ig= Immunoglobulin, TIR= Toll IL-1R Domain, LRR= leucin rich repeat

1.2.2 The TLR family

As mentioned above, the IL-1RI was found to share sequence similarities with the *Drosophila melanogaster* receptor called Toll (Gay and Keith, 1991). Receptors characterised in humans were subsequently found to be homologous with the Toll protein, and these were given the name human Toll Like Receptors (hTLR), and comprise the second subfamily of IL-1 receptors. Toll induces activation of Dorsal, which is a *Drosophila* homologue of NFκB. Dorsal is kept in an inactive state by the IκB homologue Cactus, and following stimulation, Cactus is degraded, freeing Dorsal which can translocate into the nucleus and begin to activate transcription. The protein involved on the signalling pathway culminating in the phosphorylation of Cactus in *Drosophila,* is Pelle, a serine/threonine kinase highly homologous to IRAK (see below). An adapter protein named Tube is involved in regulating Pelle activity through DD interactions, taking on the role of MyD88 in IL-1 signalling (described below, reviewed in (O'Neill and Greene, 1998)). This second sub-group of receptors also has the TIR domain, but instead of extracellular Ig domains, these proteins have a series of leucine-rich repeats (LRRs, figure 1.2). Toll is the founding member of this group(Gay and Keith, 1991; Hashimoto *et al.*, 1988), and there are three other members from the *Drosophila*, namely 18-Wheeler(Eldon *et al.*, 1994), MtsProx(Sims and Dower, 1994), and Tehao(Dower, O'Neill, *The cytokine reference*, Academic Press).

Six human TLR are members of this sub-group (TLR1-6), the best characterised of these being TLR2, which is involved in cellular responses to Gram-positive bacteria (Yoshimura *et al.*, 1999), and TLR4 which is critical for LPS signalling (Poltorak *et al.*, 1998). Some molecules involved in disease resistance in plants are also members of this group.

1.2.3 Other members of the IL-1R superfamily

Apart from the IL-1R and the TLR sub-groups, there are a few molecules which are members of the superfamily, but that do not fit into either of the sub-groups. These include the intracellular adapter protein Myeloid Differentiation protein 88, or MyD88 (Burns *et al.*, 1998; Lord *et al.*, 1990). The C-terminal TIR

domain makes MyD88 a member of the IL-1R family. Structurally, a short linker separates the C-terminal from an N-terminal Death Domain (DD). This DD is related to a motif of approximately 90 amino acids that was initially defined as the region of similarity between the cytoplasmic tails of the FAS and TNF receptors required for their induction of apoptosis. The DD mediates protein-protein interactions through other DD sequences, a mechanism utilised by many signalling complexes to induce responses such as cytotoxicity, activation of mitogen activated protein kinases, and activation of transcription factors such as NFκB. The C-terminal TIR domain of MyD88 is comprised of approximately 130 amino acids, and although this domain is found in an expanding family of proteins, MyD88 is the only non-membrane bound protein carrying this region.

SIGGIR (single Ig IL-1R relative) is a transmembrane protein with only one Ig domain which is also included in the IL-1R superfamily (Thomassen *et al.*, 1999).

1.3 IL-1 signal transduction

The ultimate response of a cell to IL-1 is an increase in the expression of a vast number of genes. The signalling events triggered by the IL-1RI, which lead to such changes have been the subject of much work. Early second messengers implicated include cAMP (Shirakawa *et al.*, 1988), ceramide (Ballou *et al.*, 1992); (Mathias *et al.*, 1993) and diacyl glycerol (reviewed by (Schutze *et al.*, 1994)), although more recent studies have focused on additional signalling events activated by IL-1. A key goal was to unravel the pathways, which culminate in the activation of two transcription factors, NFκB and AP1. AP1 is comprised of two components, c-fos and c-jun and IL-1 has been shown to activate AP-1 in several cell lines. Many genes induced by IL-1 are NFκB and/or AP1 regulated. Some of these genes are listed in table1.1 below.

Table 1.1 IL-1 responsive genes (Baeuerle and Henkel, 1994) (O'Neill, 1995)

	Class of genes	*Target gene*
NFκB regulated genes	Cell adhesion molecules	ELAM-1, VCAM-1, ICAM-1
	Cytokines and hematopoetic growth factors	IFN-β, GM-CSF, G-CSF, M-CSF, IL-2, IL-6, IL-8, TNF-α
	Acute phase proteins	Complement factor B
	Transcription factors	c-myc
AP-1 regulated genes	Cytokines and growth factors	IL-2, IL-8, IL-1α, PDGF
	Cell adhesion molecules	VCAM-1, ICAM-1
	Transcription factors	c-myc
	Others	Collagenase, Methallothionein, E-selectin

1.3.1 Activation of NFκB by IL-1

NFκB is a ubiquitously expressed primary transcription factor which, when activated, is composed of two subunits, most commonly p50 and RelA (p65) (Baeuerle and Henkel, 1994). The inactive form of NFκB is localised to the cytosol and is associated with the inhibitory protein IκB. IκB have multiple functions, including preventing the binding of NFκB to DNA and also preventing nuclear uptake of NFκB.

The activation of NFκB by IL-1 is by far the most thoroughly studied signal induced by IL-1. One important step towards the understanding of IL-1 signalling was the discovery of a second molecule involved in binding and signal generation. This IL-1 Receptor family member IL-1RAcP, mentioned above, becomes associated, by lateral diffusion, with the IL-1RI after IL-1 has become bound (Greenfeder *et al.*, 1995) (Wesche *et al.*, 1997b). Binding of IL-1 to IL-1RI and aggregation with IL-1RAcP leads to the recruitment of the adapter protein MyD88 described above. MyD88 associates with the IL-1RI through its carboxy-terminal TIR domain, and at the same time IL-1 induces recruitment of the IL-1R-associated serine/threonine kinase IRAK1 and/or IRAK2 (Cao *et al.*, 1996a; Croston *et al.*, 1995; Kanakaraj *et al.*, 1998). MyD88

contains an amino terminal death domain (DD) which has been shown to mediate protein-protein interactions with other DD sequences, forming either homo- or heterodimers. With its N terminal DD domain, and its C terminal TIR domain organisation, MyD88 has been suggested to function as an adapter linking TIR and DD modules within the IL-1RI complex (Wesche *et al.*, 1997a). In addition to this, MyD88 has been shown to form homodimers through DD-DD and Toll-Toll interactions and is therefore thought to become recruited to the IL-1 receptor complex as a dimer (Burns *et al.*, 1998). The simplified model for IL-1 signalling can schematically be represented as shown below in Figure 1.3.

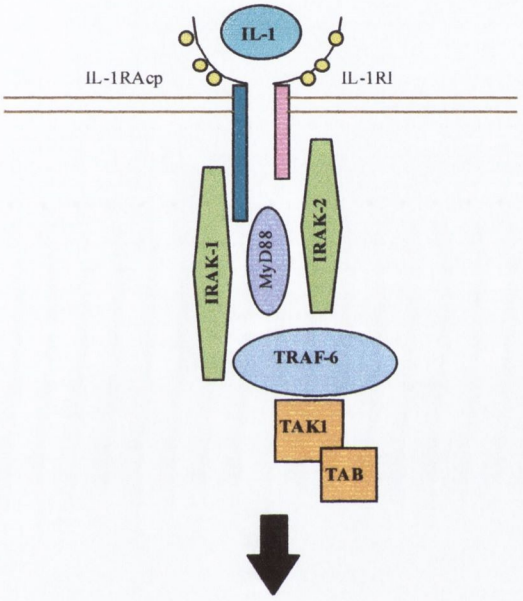


Figure 1.3 **Activation of NFκB by IL-1**. IL-1 binds to IL-1RI, which causes IL-1RAcP to become recruited and MyD88 to interact through its TIR domain with the TIR domain of IL-1RI. The DD of MyD88 can subsequently interact with the DD of IRAK1, causing IRAK1 to become autophosphorylated. Active IRAK1 is released from the signalling complex, and goes on to activate TRAF6, leading to the activation of TAK1(constitutively associated with its adapter protein TAB1), and subsequently the activation of NFκB. Further details are given in the text.

IL-1 causes the activation of IRAK within 30 seconds of IL-1 binding to its receptor, and IRAK becomes auto-phosphorylated and recruited to the IL-1 receptor complex at the cell membrane. Recently, a new

component of the IL-1RI signalling complex has been described called Toll-interacting protein (Tollip) (Burns *et al.*, 2000). A new model for the activation of IRAK by the IL-1RI has been proposed as shown in figure 1.4 below. The suggested model involves the aggregation of IL-1RI and IL-1RAcP following IL-1 binding, and independent recruitment of MyD88 and an IRAK-Tollip complex. Tollip serves to link IRAK to the receptor as well as inhibiting the auto-phosphorylation of IRAK, although the exact molecular mechanism of this has yet to be determined. MyD88 interacts through the TIR domain with the TIR domain of the IL-1RI. Tollip causes IRAK to become recruited and this will allow the DDs of IRAK and MyD88 to interact. Progressive auto-phosphorylation of the N-terminal serine/threonine cluster of IRAK follows, severing the association of IRAK with MyD88 and Tollip, inducing the release of IRAK from the complex.

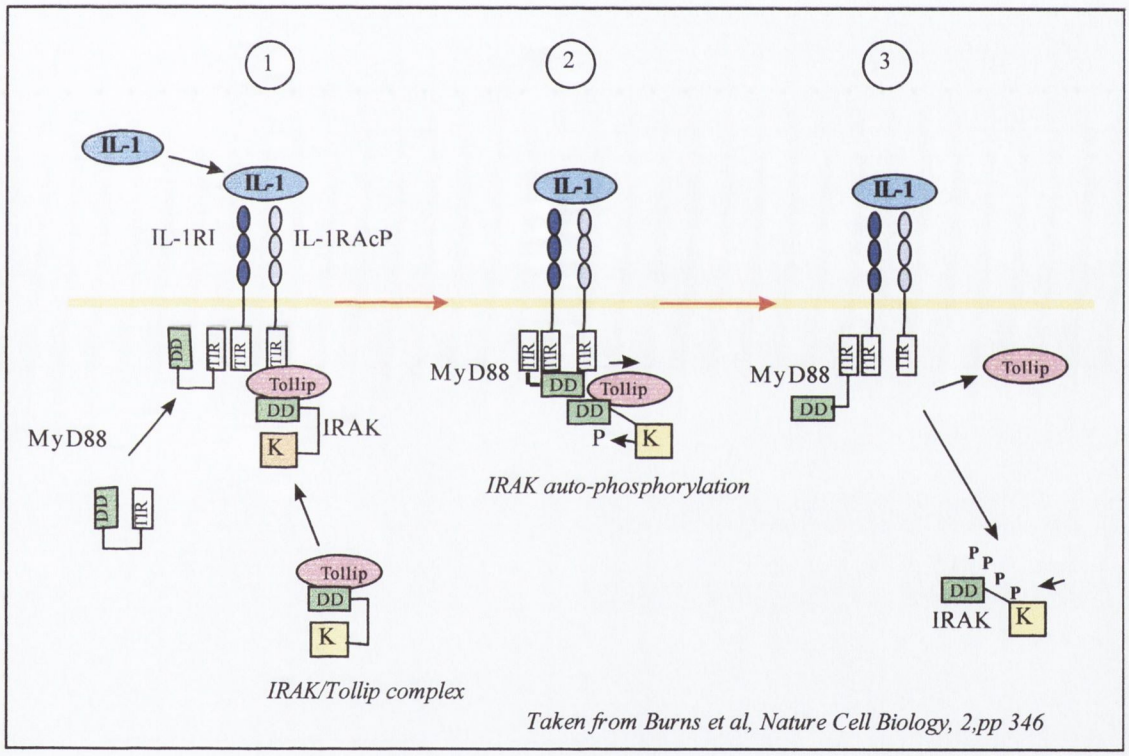


Figure 1.4 **Proposed model for the activation of IRAK by IL-1**. MyD88 and IRAK/Tollip complex become recruited to the IL-1RI complex following IL-1 binding (1) , allowing the DD of MyD88 and IRAK to interact (2) possibly as a result of conformational changes. This causes IRAK to autophosphorylate, affecting residues essential for the IRAK/Tollip and IRAK/MyD88 interactions, inducing the release of IRAK from the receptor complex (3).

TRAF-6 is a member of the TNF receptor-associated factor (TRAF) family of proteins which has been shown to directly interact with IRAK (Cao *et al.*, 1996b). Cao et al demonstrated how a dominant negative mutant of TRAF-6 was able to block IL-1 induced NFκB activation and how overexpression of TRAF-6 gave rise to a permanent activation of NFκB. TRAF-6 then, through the 170 amino acid conserved carboxy-terminal domain, interacts further down stream with NFκB inducing kinase (NIK), which in turn can activate IκB kinase (IKK), giving rise to the activation of NFκB (Beg *et al.*, 1993; Cao *et al.*, 1996b). Evidence, however, now suggests that NIK may not play a direct role in the activation of NFκB by IL-1. Unpublished data (from Goedell, Wu, Yin, and Schreiber, using NIK deficient cells derived from NIK -/- mice, indicates that NIK does not participate in IKK activation in response to either TNF-α or IL-1.

TAK1, an MAPKK kinase (see below) shown to participate in regulation of transcription by transforming growth factor-β, has been proven to directly associate with TRAF6 during IL-1 signalling(Kishimoto *et al.*, 2000; Ninomiya-Tsuji *et al.*, 1999; Shirakabe *et al.*, 1997; Yamaguchi *et al.*, 1995). Endogenous TAK1 is constitutively associated with the TAK1-binding protein TAB1 and is activated by autophosphorylation within its activation loop. This activation gives rise to the activation of NFκB providing another link in the chain of events leading to the activation of NFκB in response to IL-1. Three IκB kinases (IKKα, IKKβ and IKKγ(NEMO)) have been identified (reviewed by (O'Neill and Greene, 1998)). These can, when activated, phosphorylate IκB, which has been shown to be critical for the activation of NFκB. It has been proposed that the IKKs are all part of a 900 000 Da complex, termed a signalsome, which acts to regulate NFκB activity in cells. TAK1 stimulates IKK-α activity, linking TRAF6 to the IKK cascade in IL-1 signalling. NFκB becomes activated when IκB is phosphorylated and degraded, allowing NFκB to become translocated to the nucleus and bind to κB elements of the promoter region of NFκB regulated genes.

Other kinases have also been shown do play a role in the activation of IKK. The atypical PKC isoform PKCζ has been implicated in IKK-2 activation (Lallena *et al.*, 1999). MEKK1, which is critically involved in the

activation of JNK (Xia *et al.*, 2000), was initially implicated in the activation of IKK, and thereby NFκB activation. A novel adapter protein, ECSIT (evolutionary conserved signalling intermediate in Toll pathways), was shown to be a regulator of MEKK1 processing. Kopp et al (1999) demonstrated that a dominant negative fragment of ECSIT blocked MEKK1 processing and therefore activation of NFκB. Although, using mouse embryo fibroblasts derived from MEKK-/- mice, Yujiri et al showed that MEKK1 was indeed required for the activation of JNK but not NFκB in response to selected stress stimuli(Yujiri *et al.*, 2000).

1.3.2 Other signalling pathways activated by IL-1

Another area of IL-1 signalling studied involves activation of Mitogen Activated Protein Kinases (MAPK). To date four MAPK signalling pathways have been described, including the p42/p44 (also called extracellular regulated kinases, ERK1 and ERK2) MAPK pathway, the two stress activated MAPK pathways (p38 MAPK and c-jun N terminal kinase, JNK) and the ERK5 MAPK. These serine threonine kinases are activated by multiple factors and the best described signalling pathways of the four are the p42/p44 MAPK, or classical MAPK pathway, activated by growth factors and phorbol esters, and the two stress activated MAPK pathways: p38 MAPK and JNK MAPK, which are largely activated by environmental stress and cytokines (see section 1.4.2). The importance of these MAPK signalling pathways in IL-1 signalling is an area of much interest. IL-1 is a strong activator of p38 and JNK MAPK but can also activate p42/p44 MAPK although not as strongly (figure 1.5). The IL-1 signalling components IRAK and TRAF6 have from transgenic studies been shown to be critical in the activation of p38 MAPK, giving us some idea of how this signalling pathway may be activated by IL-1(Baud *et al.*, 1999; Kanakaraj *et al.*, 1998; Thomas *et al.*, 1999). The activation of the MAPK cascades by IL-1 give rise to the activation of multiple transcription factors and protein kinases, causing a complex pattern of responses to IL-1.

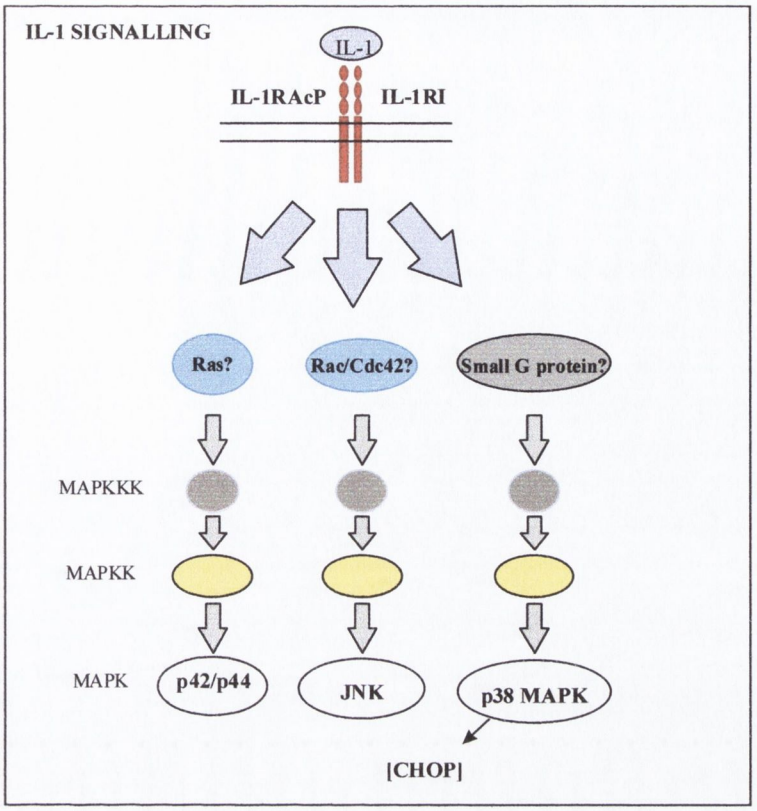


Figure 1.5 **Signalling pathways activated by IL-1**. IL-1 activates the three MAPK cascades p42/p44, JNK and p38 MAPK. The role for a small G protein in the activation of either of these signalling cascades by IL-1 has not been elucidated, although Ras has been suggested to play a role in the activation of p42/p44 and Rac and Cdc42 were proposed to convey the signal leading to the activation of JNK by IL-1. The small G protein involved in the activation of p38 MAPK has not been conclusively identified. Further details are given in the text.

Recently, TLR2 was shown to induce apoptosis, or programmed cell death, in cells stimulated with bacterial lipoproteins (Aliprantis *et al.*, 2000). This induction occurs through the DD of MyD88, the intracellular adapter protein of IL-1RI, which interacts with the DD of FADD, a protein previously shown to be associated with death receptors which convey a signal to cause oligomerization of caspase-8 and induction of apoptosis (Figure 1.6). A role for IL-1 has been implicated in diseases of inappropriate apoptosis such as diabetes mellitus and acute neurodegeneration, with IL-1Ra protecting from diabetogenic processes and ischemic neuronal injury(Rothwell *et al.*, 1997; Sjoholm, 1998). A possible mechanism for this as a direct

comparison to the signalling induced by TLR2, may involve interactions of the DD of MyD88 with FADD in response to IL-1.

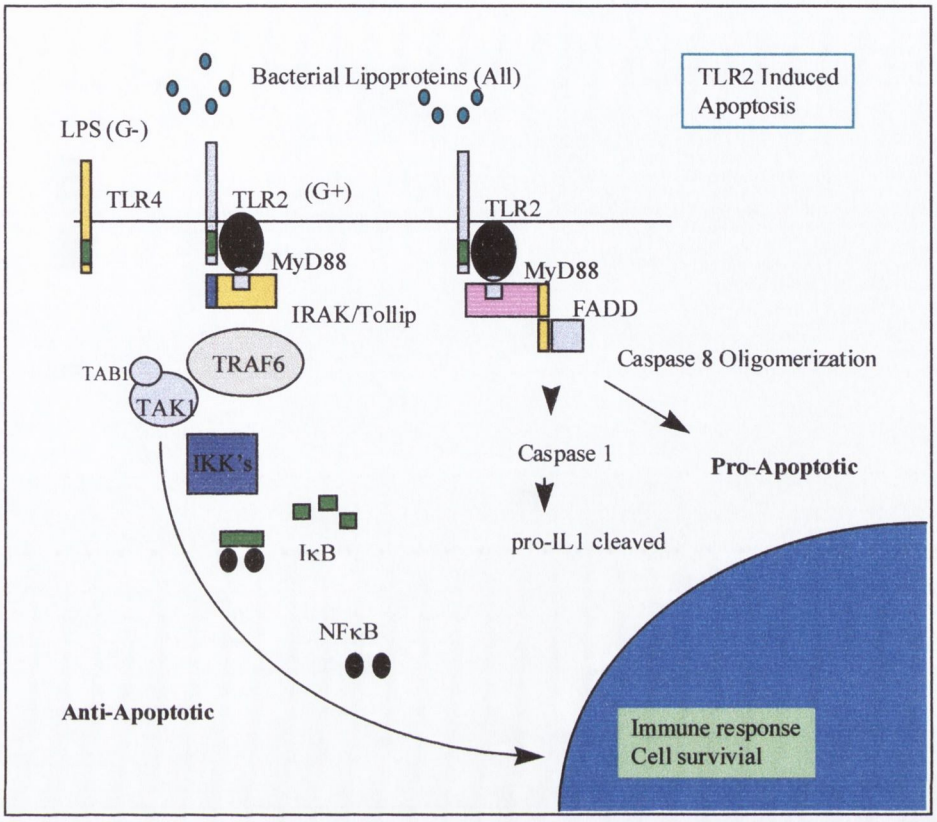


Figure 1.6 **Induction of apoptosis by TLR2**. TLR2 can mediate apoptosis in response to bacterial lipoproteins through recruiting MyD88, which in turn interacts with the death domain of FADD, an early mediator of apoptosis. Further details are given in the text.

In conclusion, IL-1 can activate a multitude of signals in the cells. Whether molecules such as IRAK or TRAF are critical for all of these signals has yet to be determined, however, a number of studies indicate that small G proteins may be involved in both NFκB and MAP kinase activation by IL-1.

1.3.3 Role of small GTP-binding proteins in IL-1 signalling

The first indication that a G protein might be involved in IL-1 signalling came from studies demonstrating an increase in GTP binding and hydrolysis in membranes prepared from EL4 cells stimulated with IL-1 (O'Neill *et al.*, 1990). Pertussis toxin, which ADP ribosylates and inhibits heterotrimeric G proteins, inhibits a range

of IL-1 responses. These include induction of IL-2, prostaglandins, IL-2R, and IL-1RI (reviewed in (O'Neill, 1995)).

There have also been several reports suggesting a role for a small monomeric G protein in IL-1 signalling (see also section 1.4). Sims et al (1996) has reported on a protein, which co-precipitated with the IL-1RI. This protein, IL-1 receptor interacting protein (IIP-1), was shown to inhibit IL-1 signalling, if over-expressed, suggesting a possible role for this protein as a negative regulator of IL-1 signalling. When IIP-1 was cloned it was revealed that it contained a GTPase activating protein (GAP) like domain, which plays an important role in the activation/inactivation of Low Molecular Weight G-proteins or small G proteins (see section 1.4). In addition to this, the sequence of IIP1 suggested that this GAP protein could be a specific inactivator of the small G protein, Rap. Apart from this, another protein, IL-1-TNF activated kinase, ITAK, was also shown to associate with IL-1RI and this protein shared sequence homology with a guanine nucleotide exchange factor (GEF). The findings on IIP1 and ITAK have yet to be published however (T Bird personal communications) and are therefore difficult to evaluate.

Finally, several groups have suggested the involvement of the Rho family of small G proteins Rac and Cdc42 (Bagrodia *et al.*, 1995) (Zhang *et al.*, 1995) (Coso *et al.*, 1995) (Just *et al.*, 1996b), in the activation of the stress activated MAP kinases p38 MAPK and c-jun NH_2 terminal MAPK (JNK) by IL-1. Using dominant negative constructs of Cdc42 and Rac, the activation of these MAPK pathways by IL-1 could essentially be blocked, suggesting that these small G proteins are important for IL-1 signalling, however others have failed to show an effect of dominant negative Rac-1 (Davis *et al.*, 1999). This latter group showed how activation of the p38 MAPK and the JNK pathways by IL-1 were not inhibited by dominant negative Rac-1, which suggested that IL-1 did not signal through Rac.

In addition to having a potential role in the activation of p38 and JNK MAPK, Rac has been shown to play an important role in the activation of NFκB. Sulciner et al (Sulciner *et al.*, 1996) demonstrated, using

dominant negative RacN17, how the expression of RacN17 could inhibit the activation of NFκB by IL-1. The study suggested a role for Rac in the regulation of reactive oxygen species (ROS) and that Rac is a component of a redox-dependent signal transduction pathway leading to the activation of NFκB.

Rac and Cdc42, although strong activators of p38 and JNK MAPK, cannot activate the third MAPK pathway; p42 and p44 MAPK. Since IL-1 can, although weakly, activate this MAP kinases signalling pathway, Ras was initially suggested to mediate IL-1 activation of p42 and p44. Bird et al (Bird *et al.*, 1994) however failed to prove this, finding that activation of p42 and p44 MAPK, as well as activation of JNK MAPK by IL-1, are independent of Ras, Raf-1 and MKK1 (see section 1.4.2.2).

Some of these points are somewhat contradictory, but taken together they indicate a role for small G proteins in IL-1 signalling.

1.4 Small Guanine nucleotide binding proteins (G-proteins)

The area of small G proteins is highly complex. A large and growing family of small G proteins has being described, the most prominent of which is the Ras family, which consists of approximately 13 members (reviewed in Bos (1997); Rebollo and Martinez, (1999)). Unlike the heterotrimeric GTP-binding proteins, which consist of three subunits α, β and γ, the Ras like GTP binding proteins are monomeric proteins, which have a typical molecular weight of 21 kDa, but ranging from 19 to 29 kDa. These proteins typically contain 188 or 189 amino acids and because of their size they are therefore also called low molecular weight GTP-binding proteins, or small G proteins. They play key roles in a wide range of cellular functions including growth control, differentiation, cytoskeletal organisation and many other aspects of signal transduction. The Ras superfamily can be divided into several sub-families (Quinn, 1995a). The founding group of proteins make up the Ras sub-family including among others: H-ras, K-ras 4A, K-ras 4B, N-ras and other homologous proteins such as R-Ras, TC21, Rap 1 and 2, and Ral (Bos, 1997; Rebollo and Martinez, 1999).

Ras proteins are well studied proto-oncogene products that are critical components of signalling pathways leading from cell surface receptors to the control of cellular proliferation, differentiation or cell death. Ras proteins are post-translationally modified by prenylation involving the addition of a 15-carbon farnesyl isoprenoid moiety to a conserved cysteine residue in a C-terminal CAAX motif by a farnesyl protein transferase. This process is thought to facilitate membrane targeting and is essential for Ras function. Farnesyl transferase inhibitors such as manumycin A are therefore useful inhibitors of Ras function. Other post-translational modification processes include removal of a C-terminal tripeptide by proteolysis and methylation of the subsequently exposed cysteine residue. Furthermore, Ras proteins can be palmitoylated or phosphorylated.

The complexity of the signalling pathways triggered by Ras becomes evident by the multiple effectors of Ras which are summarised in table 1.2 below.

Table 1.2 Summary of candidate Ras effectors. Amended from (Rebollo and Martinez, 1999)

Stimuli	*Ras*	***Ras effector***	*Downstream signalling events*	*Target*
LPA	**RAS**	**Rin**	Abl	
		Rlf	?	Gene Expression
Insulin		**PKCζ**	?	
		p120GAP	p190/Rac/NFκB&SRF	
Growth factors		**MEKK**	SEK/JNK/ATF2&Jun	
		Raf	MEK/ERK1/2/Elk	
Cytokines		**AF6**	?	
		RalGDS	Ral/PLD Ral/RBP1/Cdc42	Remodelling Actin Cytoskeleton
PMA		**PI3K**	PIP3/Vav/Rac PIP3/Sos/Rac PIP3/Akt/BAD	
		Nore 1	?	Cell survival

DIRECTION OF SIGNALLING →

The Rho sub-family of small G proteins consists of ten proteins including Rac, Rho and Cdc42. Other members of the Ras superfamily are the Rab proteins, which are important in intracellular transport and secretion, the Arf sub-family, which also are implicated in intracellular vesicle transport and the Rad sub-family which are thought to be important for cell proliferation and differentiation (Zerial, Hubert, *Guidebook to the small GTPases,* Sambrook & Tooze publication at Oxford University).

1.4.1 Activation of small G proteins

In addition to post-translational modifications, Ras proteins require binding of GTP to develop functional activity. The activation state of small G proteins is based on the ability of these proteins to cycle between an inactive GDP bound form and an active GTP bound form (Fig 1.3) (Denhardt, 1996; Izquierdo Pastor *et al.*, 1995; Rebollo and Martinez, 1999). Small G proteins have a very low intrinsic GTPase activity and, for the proteins to be able to rapidly cycle between the active and the inactive form, specific regulatory factors are needed. These regulatory proteins include GTPase activating proteins (GAPs) which stimulate hydrolysis of bound GTP to GDP and guanine nucleotide exchange factors (GEFs) which promote the replacement of bound GDP with GTP (as shown in figure 1.7). Upon activation, the specific GEF will interact with the effector domain of a small G protein and stimulate the release of GDP from the inactive small G protein. This allows GTP, which is present at much higher levels, to bind and activate the G-protein. GTPase activating proteins (GAP) are regulatory factors, capable of enhancing the intrinsic GTPase activity of the G-protein, thus shortening the lifetime of the signal response. This is illustrated in figure 1.7.

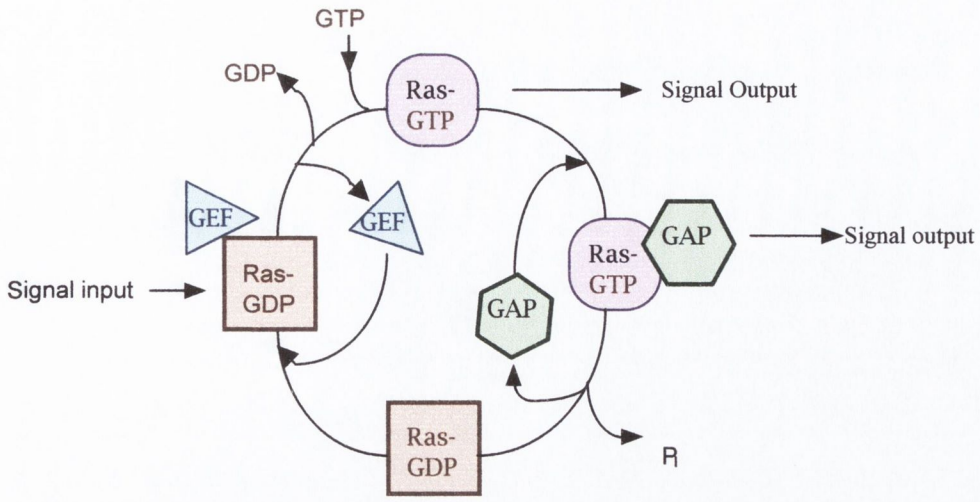


Fig 1.7 **Schematic diagram of the activation/inactivation of the low molecular weight GTP binding (small G) protein Ras**. Once the specific guanine nucleotide exchange factor (GEF) binds to Ras it catalyses the exchange of GDP for GTP. Ras then becomes active and can bind to a downstream effector giving rise to a signal. A specific GTPase activating protein (GAP) which enhances the endogenous GTPase activity of Ras terminates the signal output (Denhardt, 1996).

It has been suggested that the signal output remains for as long as the GTP stays bound to the small G protein. The GAP and GEF proteins have been identified for many of the sub-families of small G proteins. One of the best characterised GEF proteins is son of sevenless-1 (Sos-1) which associates, when activated, with Shc and Grb2 forming a complex capable of activating Ras (Waters *et al.*, 1995). Proline rich regions in the carboxy terminus of Sos-1 bind src homology 3 (SH3) groups present in the adapter protein Grb2. Upon stimulation with growth factors, the Sos-Grb2 complex is recruited to the plasma membrane via the binding of an SH2 domain on Grb2 to tyrosine-phosphorylated sites on activated receptors (Klarlund *et al.*, 1996).

Other Ras GEFs are Sos-2, CDC25 and C3G. C3G has been shown to have activity towards Rap small G proteins also (see table 1.2). Known GEF proteins that target the Rho sub-family of small G proteins are DBL, OST, Vav and smg-GDS(Ridley, 1996).

The GAP proteins for the Ras sub-family include the well-studied p120Ras^{GAP} and neurofibromin (NF1), which can both bind Ras (see table 1.2). p120Ras^{GAP} can, in addition to this, bind Rap and has a higher affinity for Rap-GTP than Ras-GTP but is incapable of catalysing the hydrolysis of Rap-GTP to Rap-GDP. This has caused speculations of the mechanism by which Rap has been shown to reverse the effects of Ras (reviewed in (Bos, 1998)). It has been suggested that Rap does this by competing with Ras for its effector (p120Ras^{GAP}) rendering the effector incapable of transducing a Ras dependent signal in the cell (Cook *et al.*, 1993). A true GAP for Rap has also been purified and cloned, Rap-GAP, which suggests that Rap has additional roles to play apart from acting as an antagonist to Ras signalling (Rubinfeld *et al.*, 1991). Rho sub-family specific GAP proteins include Ral-BP1, p190Rho^{GAP}, and BCR for Cdc42, Rho and Rac respectively (table 1.2) (Denhardt, 1996), although the specificity of these regulatory molecules for their substrates appear less specific and a great deal of cross-reactivity within the sub-family occurs.

A guanyl nucleotide releasing protein for Ras, Ras-GRP, has been described recently, which has a calcium and diacylglycerol binding domain, activates Ras, and causes transformation. Ras-GRP may couple changes in diacylgycerol and possibly calcium concentrations to Ras activation (Downward, 1996), (Ebinu *et al.*, 1998).

Table 1.3 Roles of Small G proteins in mammalian cells and their specific GAPs and GEFs (Denhardt, 1996; Klarlund *et al.*, 1996; McCormick and Wittinghofer, 1996; Ridley, 1996).

G protein	Subfamily	Role in mammalian cells	GAP	GEF
Ras	Ras	Cellular proliferation and differentiation. Involved in immediate early gene responses.	p120RasGAP, neuro-fibromin, GAP1P4BP, GAP1M	Sos, Cdc25
Rap	Ras	Antagonist for Ras. PLC activation. Ca^{2+} fluxes. Involved in immediate early gene responses	Rap-GAP, neurofibromin	C3G, Epac-1, Epac-2, Repac
Ral	Ras	Vesicular transport.	RalGAP	Ral-GDS, Rgl, Rlf
Rho	Rho	Regulation of cytoskeleton. Promotion of assembly of focal adhesions.	Cdc42-GAP, p190RhoGAP	Ost, DBL
Cdc42	Rho	Formation of filopodia and 'focal complexes'.	RalBP-1, BRC, ABR	Cdc24, DBL, Ost
Rac	Rho	Regulation of assembly of actin cytoskeleton, cell proliferation, mast cell secretion and phagocyte NADPH oxidase.	RalBP-1, BRC, ABR	DBL

1.4.2 MAP kinases and their regulation by small G proteins

Many extra-cellular stimuli are converted into specific cellular responses through the activation of mitogen-activated proteins kinase (MAPK) signalling pathways. MAPKs are serine/threonine protein kinases that can phosphorylate both cytoplasmic and nuclear targets. Based on sequence homology and function, the 12 mammalian members of the MAPK family can be divided into 4 sub-families (reviewed in (Garrington and Johnson, 1999)). The two founding members of this group of proteins, p42/p44 MAPK, also called the classical MAPK pathway, or ERK1/2, are activated predominately by mitogenic stimuli, whereas the other two main kinases p38 and JNK become activated in response to non-mitogenic stimuli, such as environmental stresses and pro-inflammatory cytokines such as IL-1 and TNFα. p38 and JNK MAPK play important roles in the regulation of the inflammatory response to these cytokines.

A MAPK pathway generally consists of three levels of kinase activity (Garrington and Johnson, 1999). MAPKs are phosphorylated by MAPK kinases (MAPKK or MAPK2) which are generally dual specificity kinases, phosphorylating MAPK on both tyrosine and threonine residues. MAPKKs are themselves activated by MAPKK serine threonine kinases (MAPKKK or MAPK3). Seven MAPKK and fourteen MAPKKK have been functionally identified in mammalian cells. There has been evidence that small G proteins and certain specific kinases could be considered to act on the level upstream of MAPKKK, i.e. on a MAPKKKK level regulating the activity of MAPKKKs.

General knowledge of the way in which the MAPK pathways are regulated by small G proteins can provide us with useful information on the possible mechanisms behind the activation of MAPK by IL-1.

1.4.2i The regulation of the stress activated MAP kinase pathways by small G proteins

Antimitogenic stimuli such as environmental stress, transforming growth factor-β, and inflammatory cytokines such as IL-1 and TNF activate the two stress activated mitogen activated protein kinases: p38 and c-jun amino-terminal kinases (JNK). p38 MAPK was first identified in lipopolysaccharide (LPS)-stimulated

mouse macrophages and was found to have substantial homology to *Saccharomyces cerevisiae* HOG1 kinase (Han *et al.*, 1994, Brewster *et al.*, 1993). Further studies revealed the importance of p38 MAPK in the regulation of cell growth and apoptosis through the activation of a whole range of transcription factors such as ATF-2, CHOP, and AP-1. In addition p38 MAPK plays a role in the activation of other protein kinases such as MAPKAP-kinase-2, MAPKAP-kinase-3, and Mnk1/2 (Stokoe *et al.*, 1992) (McLaughlin *et al.*, 1996) (Fukunaga and Hunter, 1997), as well as being involved in mRNA stabilisation (Young *et al.*, 1993) (Winzen *et al.*, 1999). p38 MAPK also plays a role in numerous cellular functions, such as cell differentiation, proliferation, and survival (reviewed recently in (Nebreda and Porras, 2000)). Four isoforms make up the p38 MAPK family, p38α, p38β, p38γ and p38δ (Hale *et al.*, 1999), which are widely expressed in many tissues.

The other group of MAPKs which are activated by stress are the JNK MAP kinases. These MAP kinases are activated by cycloheximide, anisomycin, inflammatory cytokines, osmolarity changes, heat shock and ultraviolet light. The JNK MAP kinases were first identified by virtue of their ability to phosphorylate the amino terminal of the transcription factor c-jun and are the products of three distinct genes (human JNK 1,2, and 3), from which a total of 10 different cDNAs have so far been identified. Each JNK may be expressed as either a full length (54kDa) protein or as a C-terminally truncated (46kDa) form arising from differential mRNA splicing (Angel and Karin, 1991).

Rac and Cdc42 are potent activators of the two stress activated protein kinase signalling pathways (Coso *et al.*, 1995; Minden *et al.*, 1995) (Fig 1.5). It has been shown that the first step in the activation of p38 as well as JNK in response to stimuli such as Tumor Necrosis Factor (TNF-α) is the interaction of Rac and Cdc42 with their effectors (Coso *et al.*, 1995). These include the PAK-kinase α,β and γ, SHK1, and Ost, where the PAK-kinases play the most important role (Bagrodia *et al.*, 1995). The subsequent signalling cascade involves a series of kinases (figure 1.8). The MAP kinases become activated through phosphorylation of both threonine and tyrosine within a specific Thr-X-Tyr motif in kinase sub-domain VIII of the protein, where X

corresponds to Pro in JNK, Gly in p38 and Glu in p42 and p44 MAPK (Cobb and Goldsmith, 1995). Once activated, the MAPK exerts its action as a serine/threonine protein kinase, which phosphorylates specific target proteins (figure 1.8). MKK3 and MKK6 were recently identified as specific activators of p38, MKK7 has been shown to activate JNK (Yao *et al.*, 1997) and MKK4 was shown to be able to activate both p38 and JNK giving rise to potential cross talk between the pathways (Derijard *et al.*, 1995). The MAPKKKs responsible for activating the specific MAPKK of each pathway have been determined. MEKK 1 and MEKK 2 are known to phosphorylate MKK4 but not MKK3 or MKK6 (Deacon and Blank, 1997). To date only one MEK kinase activator of both MKK3 and MKK 6 has been identified, transforming growth factor-α activating kinase-1 (TAK-1), but it was recently described how MEKK 3 can phosphorylate MKK3, suggesting that it may be involved in the regulation of the p38 MAPK pathway. Though the targets of p38 MAPK are poorly characterised, biochemical studies indicate that p38 activates the transcription factors AP-1, Max, CREB, ATF1, ATF2, CHOP, and MEF-2C (Tan *et al.*, 1996, Iordanov *et al.*, 1997, Raingeaud *et al.*, 1995, Raingeaud *et al.*, 1996, Wang and Ron, 1996, Han *et al.*, 1997a), as well as other protein kinases such as MAPK activating protein (MAPKAP) kinase 2 and 3 (MAPKAP kinase 2 phosphorylates heat shock protein (hsp)27). Activation of JNK pathway gives rise to the activation of several transcription factors such as c-jun, JunD, ATF-2 and Elk-1 (Wesselborg *et al.*, 1997).

Although a role for Rac in the activation of p38 or JNK has been suggested, other studies have failed to demonstrate this. Davis et al suggested that the synergistic activation of JNK by IL-1 and platelet derived growth factor (PDGF) was independent of Rac and Cdc42(Davis *et al.*, 1999). Further evidence proposes that the activation of p38 MAPK does not always occur through Rac and Cdc42, for example, granulocyte colony-stimulating factor (G-CSF) induced hemopoietic cell proliferation was shown to occur through p38 MAPK with the degree of Ras activation being a critical determinant for the extent of p38 MAPK activation (Rausch and Marshall, 1999). Furthermore, activation of involucrin transcription was shown to involve a pathway that includes protein kinase C, Ras, MEKK1, MEK3 and p38 MAPK, suggesting again that Ras may occur upstream of p38 MAPK (Efimova *et al.*, 1998). Other studies have proposed a role for both Ras

and Rac in p38 MAPK signalling. Turkson (1999) showed by using dominant negative Ras and Rac1 that these constructs could block Stat3-mediated gene regulation induced by Src in a manner consistent with dependence on p38 and JNK. The group proposed that Ras- and Rac- mediated p38 and JNK signals are required for Stat3 transcriptional activity induced by the Src oncoprotein. In addition, a role for Ras in p38 MAPK activation by both hemopoietic cytokines (Matsumoto *et al.*, 1999) and FGF (Tan *et al.*, 1996) has been shown. Taken together, these findings suggest that small G proteins play a critical role in the activation of both p38 MAPK and JNK, although the precise identity of the G protein is uncertain.

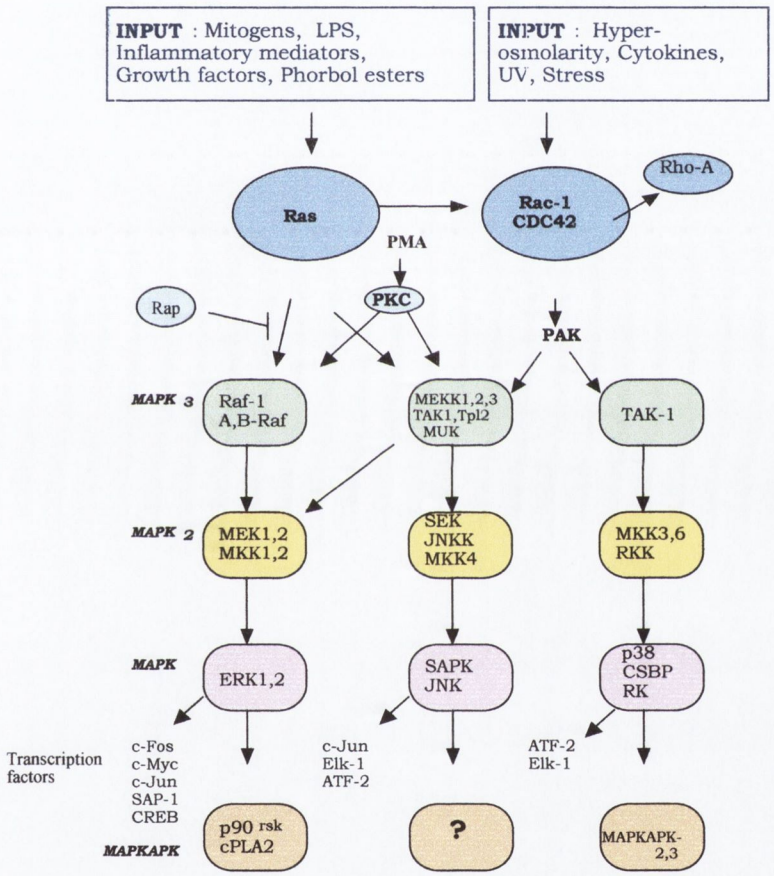


Fig 1.8 **Schematic representation of the three main mitogen activated protein kinase (MAPK) pathways**. The classical MAPK pathway (ERK-1, -2) becomes activated mainly in response to phorbol esters and growth factors, whereas the c-jun N-terminal kinase (JNK) and p38 MAPK pathways become activated in response to external stress and cytokines. When the cell is exposed to an external stimuli, a small G protein becomes activated, which in turn activates a MAPK kinase kinase (MAPK3). The MAPK3 subsequently activates a pathway specific MAPK kinase (MAPK2) which Threonine and Tyrosine phosphorylates the serine/threonine MAPK which can activate a multitude of transcription factors and other kinases causing gene transcription, translation and other cell responses (Denhardt, 1996).

1.4.2ii The regulation of the classical MAP kinase pathway by small G proteins

The third members of the mitogen activated protein kinase family are p42/p44 MAPK. The signalling pathway involving these MAP kinases is sometimes called the classical MAPK pathway and is mostly activated via a receptor tyrosine kinase domain or phorbol esters, but can also become activated, although not as strongly, by cytokines and environmental stresses. Ras is the main activator of this signalling pathway (figure 1.8). When bound to GTP, Ras undergoes conformational changes, allowing it to interact with multiple downstream effectors. Membrane localisation allows Ras to form a dimer, which is essential for activation of downstream effectors (Inouye *et al.*, 2000). The function of Ras on the pathway leading to the activation of p42 and p44 has been intensely studied. The best understood means of activating the p42/p44 pathway through Ras is that used by receptor tyrosine kinases. Ligands cause receptors to auto-phosphorylate and bind SH2 domains of adapters such as Grb2 (growth factor receptor bound protein 2), which can then recruit guanine nucleotide exchange factors for Ras. When activated, the effector domain of Ras interacts with the Ras binding domain of Raf-1 (Zhang *et al.*, 1993), which leads to the binding of the C-terminal cysteine-rich domain of Raf-1 to Ras, recruiting Raf-1 to the membrane. The catalytic domain of Raf-1 can now interact with the down stream kinases MKK 1 and MKK2 (Kyriakis *et al.*, 1992). MKK 1 and MKK 2 activate p42 and p44 MAP kinases through phosphorylation of tyrosine and threonine at the Thr-Gly-Tyr phosphorylation motif (Zhang *et al.*, 1993) as described above. p42 and p44, as with the other MAPKs, activate a range of downstream effectors by phosphorylation of serine and threonine residues. Potential cytoplasmic targets of p42 and p44 MAPK phosphorylation include cytoskeletal elements (microtubule associated proteins and tau), kinases (ribosomal protein S6 kinase, p90rsk), cytoplasmic phospholipase A2, SHP-2, glycogen synthase kinase-3 (GSK-3) and upstream signalling elements such as the EGF receptor, Sos, Raf-1 and MEK. Other effectors include nuclear transcription factors such as c-Fos, c-Myc, CREB, NFAT, and Elk-1, which enable regulation of gene transcription (Genot *et al.*, 1996). Acting through this pathway, Ras plays an important role in T-cell activation and growth (Izquierdo Pastor *et al.*, 1995). Although Ras appears to be the predominant G protein involved in the regulation of the p42 and p44 MAPK pathway, evidence suggest that this pathway can be activated by alternative routes. Bird et al (Bird *et al.*,

1994) demonstrated how the activation of p42 and p44 MAPK by IL-1 was independent of Ras, and additional studies have suggested a role for Rap in the activation of p42 and p44 MAPK signalling, although most probably as a negative regulator of Ras (Cook *et al.*, 1993). Furthermore, arsenite have been shown to activate p42/p44 MAPK through a MKK6 and p38 MAPK dependent pathway, providing evidence for cross-talk between the classical and the stress-activated MAPK cascades (Ludwig *et al.*, 1998).

There is, as yet, no direct evidence for Rac, Cdc42 or any other member of the Rho sub-family of small G proteins activating the p42/p44 MAPK pathway, although the Ras and Rac signalling pathways are thought to be coupled at the level of Sos (Nimnual *et al.*, 1998) enabling growth factors to activate the JNK and p38 MAPK pathways through Rac via Ras.

1.4.3 Other signalling targets for small G proteins

The three Ras proteins, H-Ras, K-Ras and N-Ras are expressed in most, if not all, mammalian cell types and although there are some striking differences in their primary structure, particularly in the C-terminal region, no clear differences in function have yet been described (Bos, 1997).

The first effector described for the Ras proteins in budding yeast was adenylyl cyclase, and from an experiment where it was shown that loss of functional Ras had a more severe phenotype than loss of its effector, the conclusion was drawn that the Ras proteins have multiple effectors (reviewed by (McCormick and Wittinghofer, 1996). To date the best-characterised effector for Ras, as mentioned above, is the c-Raf-1 serine/threonine kinase, and its close relatives A-Raf and B-Raf. Ras is known to interact with a large number of other effectors (as shown in table 1.2 above) such as the catalytic p110 sub-unit of phosphatidyl inositol (PI) 3 kinase, Ral-GEF, protein kinase Cζ, and possibly certain mitogen activated kinase kinases (MEKKs). The Raf-1 protein has an 81-amino acid conserved domain at the amino terminus referred to as the Ras-binding domain (RBD), which has been shown to bind Ras in a GTP-dependent manner (McCormick and Wittinghofer, 1996). The so called Switch One region of Ras proteins has been shown to be

responsible for the main part of the GTP-dependent binding of Ras to the Raf-RBD. This switch domain changes considerably in conformation between the GDP- and the GTP-bound states of the Ras activation cycle.

Another switch domain is present on the Ras proteins, namely the Switch Two domain. This region also differs in conformation between GDP- and GTP-bound Ras but has not been shown to have any part to play in the Raf-RBD interactions. It has however been implicated in the Ras-GAP interactions. Once Ras binds to Raf-1, Raf-1 translocates to the plasma membrane and becomes activated (McCormick and Wittinghofer, 1996). It has been suggested, since Ras is normally found in association with the plasma membrane, that the sole function of Ras is to recruit Raf-1 to the membrane. Raf-1 has been shown to become activated in a Ras independent manner (Leevers and Marshall, 1992) (Stokoe *et al.*, 1994). Other studies have suggested a possible model where Raf becomes recruited to the plasma membrane by Ras and that this enables Raf to become tyrosine phosphorylated which is part of the mechanism whereby Raf-1 is activated (Marais *et al.*, 1995) (Dent *et al.*, 1995). The recruitment model has been argued against and Ras has been shown to activate Raf-1 in a membrane free system (Kuroda *et al.*, 1995) (Yamamori *et al.*, 1995) through the mere binding of the two proteins. As mentioned above, Inouye et al (Inouye *et al.*, 2000) proposed that the membrane localisation allows Ras to form a homodimer, which is essential although not sufficient for Raf-1 activation.

Once Ras is activated this causes distinct downstream effects which are caused by different Ras effectors generating different signals. As well as this, the signalling pathways activated by Ras are not equally activated by any two stimuli due to cross talk with other pathways activated by a different small G-protein in response to the same stimulus.

The Rap proteins, Rap1A, Rap 1B, Rap 2A and Rap 2B, differ from each other only in a few amino acids and they are as much as 50% homologous to Ras (reviewed in (Bos, 1997)). The sequence of Rap proteins share three highly conserved regions with the Ras protein sequence: the nucleotide binding domain, the effector domain, and the C-terminal CAAX motif or membrane attachment site. The identical 32-40 effector

domain of Rap and Ras proteins explains why Rap binds to much the same set of effectors as Ras. Rap signalling is not as clearly mapped as Ras signalling and it appears that Rap can have very different effects in different cell lines studied. Most of the studies of the effectors of Rap in cells have been performed in platelets where Rap makes up close to 0.1% of the total cell protein content. Here, Rap seems to play a role in agonist-induced phospholipase C activation, regulation of plasma membrane Ca^{2+} fluxes and has been shown to associate with the cytoskeleton of activated platelets (Bos, 1997). Rap has been identified in human neutrophils and HL60 cells where Rap 1 appears to be the predominant form. Rap 1 has since been shown to play important roles in leukocyte signal transduction including regulation of the NADPH oxidase system (respiratory burst), modulation of the protein kinase C signal transduction pathway and possibly in granule/vesicle transportation (reviewed by Quinn, 1995).

The signalling roles for Rap are somewhat conflicting. Rap has been shown to activate much the same signalling pathways as Ras including activation of the classical MAPK pathway through B-Raf. It can also cause neurite outgrowth in PC12 cells. On the other hand several studies have demonstrated how Rap V12 (constitutively active mutant of Rap where GTP is constitutively bound to Rap) can antagonise the Ras-dependant activation of p42/p44 MAPK, probably due to competition for downstream effectors (Cook *et al.*, 1993) (Hu *et al.*, 1997). Rap has been shown to maintain human T cell anergy which is also thought to occur through antagonising Ras (Boussiotis *et al.*, 1997). This suggests that Rap can reverse the effects of Ras by sequestering a Ras effector molecule in a non-productive complex (Cook *et al.*, 1993).

The Rho (Ras Homologous protein) sub-family of small G proteins is composed of a number of small G proteins that are 30% homologous with Ras proteins and 50% homologous with each other. The sub-family can be further divided into two sub-groups based on structural similarities, i.e. Rho proteins (Rho A, Rho B, Rho C, and Rho G), and Rac proteins (Rac 1 and 2, TC 10 and Cdc42Hs). The Rho sub-group are involved in distinct cellular functions such as the regulation of the actin cytoskeleton during stress fibre and focal adhesion formation (Nobes and Hall, 1995), regulation of phospholipase D activity, participation in inositol phosphate metabolism and regulation of mast cell secretion (Quinn, 1995b). Effectors of the Rho sub-group

of small G proteins include PKC-1, PKN, and ROK (reviewed by (McCormick and Wittinghofer, 1996)). Activation of Rho can induce a mitogenic response in the cell through its ability to activate the transcription factor, serum response factor (SRF), which binds to the serum response element (SRE) found in the promoters of a number of "early" genes induced by growth factors (Cahill *et al.*, 1996). Rho does not activate any of the known MAP kinase cascades in mammalian cells.

Apart from activating the JNK and p38 MAPK pathways, Rac and Cdc42 can also activate pp70S6K, an important kinase in cell cycle control (Ridley, 1996) and they are required for arachidonic acid release induced by epidermal growth factor (EGF). Other experiments have demonstrated that Rac is essential for cellular transformation by Ras.

1.5 *Clostridia* toxins that target small G proteins

As part of this study we have used bacterial toxins from *Clostridium*, which target small G proteins, in order to explore the involvement of small G proteins in IL-1 signalling. As discussed above, small G proteins are involved in most housekeeping activities of the cell and are vital for cell survival and proliferation. Many of the toxins produced by the bacterial family *Clostridium* act as glucosyl transferases which glucosylate small G proteins in their effector domains preventing them from signalling (Popoff, 1987; Popoff *et al.*, 1996). The consequences of this are obviously dramatic and can be observed in an individual suffering from an infection by the toxin-producing bacteria causing diseases such as gas gangrene and haemorrhagic diarrhoea (Bette *et al.*, 1991), although precisely how the toxins give rise to these symptoms is not fully worked out. Sub-sets of this group of cytotoxins are the *Clostridium difficile* Toxin B (ToxB) and *Clostridium sordellii* lethal toxin (LT). Their effects and targets are summarised in table 1.4.

Table 1.4 **Virulence factors from *Clostridium*** (Popoff *et al.*, 1996) (Chaves Olarte *et al.*, 1997).

Toxin	Effects on mammalian cells	Target small G proteins
C. sordellii LT	Rounding of cell bodies. Cell aggregation. Reorganisation of F-actin into cell surface filopodia. Loss of actin stress fibres	Ras, Rap, Rac, Ral
C. difficile ToxB	Rounding of cell bodies. Destruction of cytoskeleton	Rho, Cdc42, Rac

1.5.1 Clostridium sordellii *LT*

Clostridium sordellii produces two different virulence factors, lethal toxin (LT) and haemorrhagic toxin. LT (Popoff, 1987) is a protein of 270.6 kDa which after prolonged exposure causes morphological and cytoskeletal effects such as rounding of the cell bodies with the reorganisation of F-actin structures into numerous cell surface filopodia and the loss of actin stress fibres (Popoff *et al.*, 1996).

The mechanism of action of LT has been characterised (Popoff *et al.*, 1996). Popoff et al demonstrated that LT from the pathogenic LT strain IP82 specifically targets Rap, Ras, and Rac but not the Cdc42, RhoA, or Rab6 small G proteins. Recently Ral has also been shown to be glucosylated by this strain of LT (Popoff, personal communications). The inhibition of Ras by this toxin was studied in more detail revealing that LT acts as a glucosyl transferase (Just *et al.*, 1996b), targeting threonine 35 in the effector region of Ras and using UDP-Glucose as a co-factor. This glucosylation resulted in the inability of Ras to interact with its down stream effector Raf-1, thereby causing the inhibition. The resulting effects on cells have lead to speculations on the role of Ras in the resting cell. The filopodia induced by LT are very similar to those seen when micro-injecting a cell with the activated form of Cdc42 (Aktories, 1997). This suggests an indirect activation of Cdc42 or a negative regulatory role for Ras, resulting in the filopodia formation caused by Cdc42 to stay masked when Ras is activated in the cell. The same group was also able to determine the preferred conformation of the Ras protein by LT, demonstrating that the GDP bound form of Ras was

glucosylated to a greater extent than the GTP bound form. This is probably due to the way that the hydroxyl group of the threonine 35 becomes exposed in the GDP bound form, rendering it more readily accessible for glucosylation. The substrate targets of the toxins are highly specific and LT isolated from different strains of *C. sordellii* exhibit different substrate specificities (Hofmann *et al.*, 1996). In addition, Hofmann et al showed that it is the N terminal region of amino acids 364 to 516 of LT that primarily defines the substrate specificity (Hofmann *et al.*, 1998).

1.5.2 Clostridium difficile *Toxin B*

Clostridium difficile toxins Toxin A and Toxin B cause rounding of the cell bodies and destruction of the actin cytoskeleton, where Toxin B is 100-1 000 times more potent than Toxin A. The toxins affect the actin cytoskeleton by the inactivation of Rho proteins in much the same way as LT inactivated Ras, Rac and Rap. Toxin B monoglucosylates Rho proteins using UDP-glucose as a co-factor and it targets all tested members of the Rho sub-family of small G proteins, such as Rho, Cdc42, and Rac, but does not target any Ras sub-family member (reviewed by (Aktories, 1997)). Further evidence was provided when it was demonstrated that overexpression of RhoA, -B, and -C could lead to resistance to ToxB but not to LT in HeLa cells (Giry *et al.*, 1995). ToxB, which is also a large protein of 270 kDa, is closely related to LT and they have been shown to cross-react immunologically (Bette *et al.*, 1991).

1.6 Aim of this study

IL-1 is a central mediator in some severe chronic inflammatory diseases. Understanding the mechanism behind the signal transduction of IL-1 is fundamental to preventing its actions. Although recent studies have elucidated in detail some aspects of IL-1 signalling, such as activation of NFκB, several questions still remain, such as the role for small G proteins in IL-1 signalling.

The aim of this study is to identify the small G proteins involved in IL-1 signalling using multiple approaches. The specific aims are as follows:

1 To determine effects of LT from *C. sordellii* on long term responses (including expression of IL-2) to IL-1 and, as a control, PMA. Establish the toxic effects of LT on EL4.NOB-1 cells when using long time frames for these studies.

2 To study the effects of LT and ToxB on short-term responses such as the activation of p42/p44 and p38 MAPKs by IL-1, again using PMA as a control.

3 To study the glucosylating actions of LT in intact cells and *in vitro*, and correlate these actions with the effects seen by LT on short-term responses. By doing this, I could confirm that the glucosylation of a small G protein is the cause of the effects seen on short-term responses to IL-1 and PMA.

4 To verify that the effects seen with LT are caused by glucosylation of the target small G proteins, and not due to non-specific toxic effects. To determine this a UDP-glucose deficient cell line, resistant to LT and ToxB, was used, i.e. Don Cdt^R-Q fibroblasts.

5 To determine the effects of constitutively active and dominant negative constructs of Ras, Rac, and Rap on p38 MAPK activation, to establish the involvement of these in IL-1 signalling.

6 To study the activation of Ras and Rap by IL-1 in order to confirm their roles in the activation of p38 MAPK by IL-1.

7 To determine the roles played by the IL-1 signalling components MyD88, IRAK1, IRAK2, TRAF6 in the activation of p38 MAPK by IL-2. I also wanted to investigate the effects of mutant constructs of the p38 MAPK activators MKK3b and MKK6 on this pathway.

8 To position Ras and Rap on the pathway leading to the activation of p38 MAPK by IL-1 in relation to MyD88, IRAK1/2, TRAF6, MKK3b and MKK6.

These experiments overall therefore aimed to bring us closer to understanding the role for small G proteins in IL-1 signalling, in particular p38 MAPK activation.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Cells

The murine thymoma EL4.NOB-1 cells and Human cervical carcinoma HeLa cells were both obtained from the European Collection of Animal Cell Culture, Salisbury, Wiltshire, UK. The diploid Chinese hamster lung fibroblast cell line, Don and a mutant UDP-glucose deficient cells (described below) were a kind gift from Dr Monica Thelestam, Karolinska Institute, Stockholm, Sweden.

2.1.2 Tissue Culture

RPMI 1640 medium, Bovine Calf Serum, L-Glutamine, penicillin and streptomycin used in culturing the EL4.NOB-1 cells, and Versene and Trypsin-EDTA were all from Gibco BRL, Life Technologies Inc., Gaithersburg, UK or Sigma Co. Ldt. (Dorset, UK). The Minimal Essential Medium with Earle's salts was obtained from Gibco BRL, Life Technologies Inc., Paisley, Scotland and the bovine calf serum, L-Glutamine, penicillin and streptomycin used for culturing the Don fibroblast cell lines were obtained from HyQ Cell Culture Reagents, HyClone® Europe Ltd, Cramlington, UK or Sigma Co. Ldt.(Dorset, UK) from 04-2000. Tissue culture flasks, 24 well plates and cell scrapers were obtained from Greiner Labortechnik, Frickenhausen, Germany. The 96 well plates were obtained from Nunclon TM, Nalge Nunc International, Denmark.

2.1.3 Drugs and Inhibitors

The human recombinant IL-1α was a kind gift from National Cancer Institute Biological Resources Branch, Rockville, MD, USA. The *Clostridium sordellii* Lethal Toxin (LT) and *Clostridium difficile* Toxin B (ToxB) were both a kind gift from Dr M. R. Popoff, INSERM, Nice, France. LT was obtained from culture supernatants of the pathogenic *C. sordellii* IP82 strain and purified as described previously(Popoff, 1987). Toxin B was purified from *C. difficile* as described previously(Shoshan *et al.*, 1990). The Manumycin A and Damnacanthal were purchased from Sigma Co. Ldt.(Dorset, UK) and Calbiochem (Nottingham, U.K.) respectively. The pyridinyl imidazole SB203580 was obtained from Alexis Corporation (Nottingham, U.K.).

2.1.4 Antibodies

The purified rat anti-mouse IL-2 antibody, recombinant IL-2 and biotinylated rat anti-mouse IL-2 antibody were all obtained from Pharmingen, Cambridge Bioscience, Cambridge, UK. PhosphoPlus® p38 MAPK (Thr180/Tyr182) Antibody Kit, PhosphoPlus® MAPK Antibody Kit, HRP linked goat anti-rabbit antibody and the ECL reagents were obtained from New England BioLabs (U.K.) Ltd, Hitchin, Hertfordshire, England. The mouse monoclonal antibody to human IκBα recognises an epitope between amino acids 21 and 48 and was a kind gift from Dr R. T. Hay (University of St. Andrews, Fife, U.K.)(Jaffray *et al.*, 1995). The antibody used for detecting Apopain/CPP32/pro-caspase 3, p12 subunit was supplied by Upstate Biotechnology (Lake Placid, NY). The pan-Ras antibody is a product of Oncogene Research Products (Cambridge, MA), and the polyclonal anti-Rap1A antibody was a kind gift from Dr Jean de Gunzburg (Institute Curie, Paris, France).

2.1.4 Plasmid preparation

Bacto® Tryptone, Bacto® Yeast extract, Bacto® Agar and Bacto® Luria broth were all obtained from Difco Laboratories, Detroit, MI, USA. Agarose was a product of Boeringer Mannheim (Diagnostics & Biochemicals) Ltd, East Sussex, UK. The QIAGEN® Plasmid Midi Kit came from QIAGEN® GmbH, Hilden, Germany, alternatively, plasmids were prepared using the Wizard ® PureFection Plasmid Kit (Promega, Madison, MI).

2.1.5 Miscellaneous

AcetylCoA and [^{14}C]-chloramphenicol were both supplied by Amersham LifeScience, Amersham International, Amersham place, Buckinghamshire, UK.

Thin liquid chromatography (TLC) plates were supplied by MERCK KGaA, Darmstadt, Germany, and the Instant Imager® system was bought from Packard Instruments Company, Meriden, CT, USA.

The 22 base pair oligonucleotide probe 5'-AGT TGA GGG GAC TTT CCC AGG C-3' along with [γ-^{32}P]ATP and T4 polynucleotide kinase were all obtained from Promega Corporation, Madison, WI, USA. Accugel® and Protogel® were supplied by National Diagnostics, Atlanta, Georgia, USA.

Non-fat dry milk was of the brand name Marvel®, and was provided by Premier Beverages, Adbaston, Stafford, U.K.

UDP-^{14}C-Glucose in ethanol (300 mCi/mMol) was obtained from DuPont NEN, Les Ulis, France.

The Fugisep Mini® filters were purchased from Intersep filtration systems, Wokingham, Berkshire, UK. Ampholines (pH 3-10) were obtained from Pharmacia Biotech, Uppsala, Sweden. Whatman® 3MM Chr chromatography filterpaper was supplied by Whatman International Ltd, Maldstone, UK.

Avidin-peroxidase, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), trypan blue dye, dithiothreitol (DTT), DMSO, ampicillin, DEAE-Dextran, bovin serum albumine, IPEGAL CA-630 (alias NP40), heparin, Kodak diagnostic film (X-OMAT RP), Rnase and nitrocellulose membrane were purchased from Sigma Co. Ldt., Dorset, UK. UDP-[^{14}C]-Glucose in ethanol (300 mCi/mMol) was purchased from DuPont NEN (Les Ulis, France).

When required for cell culture, preparation of nuclear extracts, electrophoretic mobility shift assay and preparation of plasmid DNA, all reagents were of molecular biology grade and all solutions were autoclaved. Laboratory chemicals were of analytical grade and supplied by BDH Laboratory Supplies, Poole, UK or by MERCK, Darmstadt, Germany, unless otherwise stated.

2.2 Expression vectors

The NFκB-CAT construct containing three κB elements upstream of a chloramphenicol acetyl-transferase (CAT) gene was a kind gift from Dr. T. Bird, Immunex Corp., Seattle, WA. The components for the PathDetect® CHOP *trans*-Reporting System (pFA-CHOP, pFC2-dbd, pFR-Luc and pFC-MEK3) were purchased from Stratagene (La Jolla, CA). Rap 1A wild type, constitutively active Rap 1A V12, dominant negative Rap 1A N17, wild type Rap 2A, constitutively active Rap 2A V12, dominant negative Rap 2A N17 and the corresponding empty vectors pRK5 or pCDNA3 were all a kind gift from Dr J. de Gunzburg,

(Institute Curie, Paris, France) along with the primary rabbit-polyclonal anti-peptide Rap 1 and Rap 2 antibody.

The expression vectors encoding constitutively active RasVHa, dominant negative Ras N17 (described previously (Izquierdo and Cantrell, 1993)), constitutively active Rac V12 and dominant negative Rac N17 (described in (Genot *et al.*, 1996)), together with the expression vectors encoding amino acids 1-149 of human c-Raf1 in pGex-KG, i.e. GST-RBD (de Rooij and Bos, 1997) , and pGex-4T3-GST-RalGDS-RBD (Franke *et al.*, 1997) were all kind gifts from Dr Doreen Cantrell (ICRF, London, U.K.). Full length MyD88, the mutant truncated ΔMyD88 (amino acids 152-296) lacking the death domain, full length IRAK2, C-terminal K-IRAK2 [C-terminal kinase like domain in pCDNA3], and N-terminal death domain ddIRAK2 [cloned into pCDNA3] were all kind gifts from Marta Muzio (Mario Negri Institute, Milan, Italy, (Muzio *et al.*, 1997). Dominant negative IRAK1 (amino acids 1-211) was a kind gift from Emma-Louise Cooke (Glaxo Wellcome, Stevenage, U.K.). Mutant ΔTRAF6 [mutation in N-terminal zinc finger binding region acting as dominant negative mutant], wild type TRAF6, and wild type IRAK1 were gifts from Tularik (South San Francisco) (Greene and O'Neill, 1999). The general structure of the MyD88, IRAK1, IRAK2, and TRAF6 deletion mutants are shown in figure 2.1 below.

The pRK5 expression vectors encoding constitutively active Rap1AV12 and dominant negative Rap1AN17 were a kind gift from Dr Jean de Gunzburg (Institute Curie, Paris, France). The vector encoding FLAG-tagged p38 MAPK, was a kind gift from Dr Jeremy Saklatvala (Kennedy Institute of Rheumatology, London, UK). The constructs encoding for MKK3b, mutant MKK3b(A), MKK6, and mutant MKK6(A), were all kind gifts from Dr Jiahuai Han (The Scripps Research Institute, La Jolla, CA, USA). MKK mutants have Alanine as a replacement for the conserved Lysine in the ATP binding site. The expression vectors encoding for full-length pFlag-TAK1 and pcDNA-HA-TAB1, together with the mutant version of TAK1, pFlag-δTAK1K63W were all kind gifts from Dr. Hiroaki Sakurai, Tanabe Seiyaku Co., Ltd., Osaka, Japan.

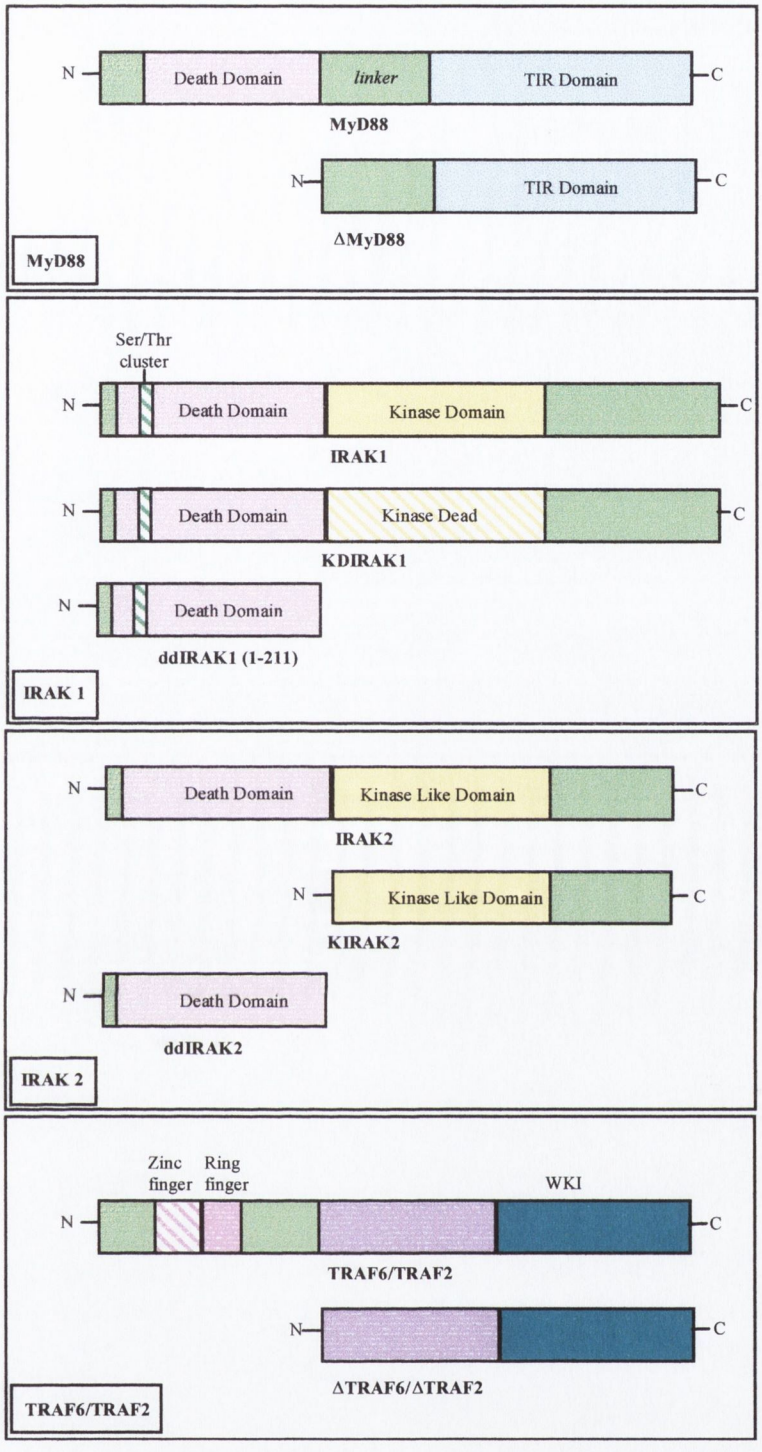


Figure 2.1 **Schematic representation of MyD88, IRAK1, IRAK2, and TRAF6 deletion mutants**. ΔMyD88 is deleted in the N terminal Death domain. KDIRAK1 is full-length kinase-dead IRAK1. ddIRAK1 contain amino acids 1-211 of full length IRAK1, consisting of the death domain region only. K IRAK2 is the kinase domain like region of IRAK2. DDIRAK2 is the N-terminal death domain of IRAK2 only. ΔTRAF6 and ΔTRAF2 are lacking the N-terminal zing and ring fingers. Abbreviations used: KD: Kinase Dead, dd: Death Domain.

2.3 Cell lines

2.3.1 EL4.NOB-1 cells

The murine ascites thymoma cell line, EL4.NOB-1 is derived from EL4.BU.OU6, selected for a high level of IL-1 receptors. The EL4.NOB-1 cells were cultured in complete medium consisting of RPMI 1640 medium supplemented with 10% Foetal Calf Serum, 2 mM L-Glutamine and 100 IU/ml of penicillin and streptomycin respectively and were always kept at 1-10x10^5 cells per ml at 37°C, 5% CO_2. Cell cultures were passaged by centrifuging at 170 x g for 5 minutes and the cells were resuspended in 1 ml of complete medium for every 25 ml cell culture. Viable cells were counted by haemocytometry of a mixture of 10 µl cell suspension, 10 µl 0.4% trypan blue, and 40 µl complete medium. Viable cells will exclude the trypan blue dye whereas dead and/or membrane damaged cells become stained and appear blue under bright field microscopy therefore differing from the translucent viable cells. 2x10^5 cells/ml were seeded and left for 36 hours before passaging again.

Stocks of EL4.NOB-1 were kept in liquid nitrogen and were frozen down when the cells were in log growing phases for optimal viability. The cells were seeded at 2.5x10^5 cells per ml in complete RPMI 1640 medium and grown over night. The following day the cell culture was centrifuged and counted as described above. 5x10^6 cells were resuspended per ml of FCS:DMSO (at a ratio of 9:1) and aliquoted in 1 ml per cryotube. The cryotubes were then slowly frozen at -80°C in a polystyrene box for 2.5 hours after which the cells could be transferred to liquid nitrogen.

The cells could subsequently be thawed by defrosting the cryotube at 37°C, and adding 1 ml of complete medium. This was mixed gently and centrifuged at 170g for 5 minutes. The pellet was resuspended in 8 ml of complete medium, centrifuged, counted as above and seeded at usual density in RPMI 1640 supplemented with 20% FCS, 2 mM L-Glutamine and 100 IU/ml of penicillin and streptomycin respectively. The medium was changed 24 hours later. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2

2.3.2 Don fibroblasts

The LT and ToxB resistant Diploid Chinese hamster lung fibroblast cell line denoted Don-Cdt^{R}-Q, was first isolated after mutagenization of the parental Don cell line with ethyl methanesulphonate and a two step selection with *Clostridium difficile* ToxB (Florin, 1991). The mechanism behind the resistance to LT and Toxin B lies in a lowered intracellular level of UDP-glucose, due to a single point mutation in the UDP-Glucose pyrophosphorylase gene as shown in figure 2.1 below. The synthesis of UDP-Glucose depends on the amount of glucose-6-phosphate (Glc-6-P), and UTP available in the cell. One of the enzymes needed for UDP-glucose synthesis is UDP-glucose pyrophosphorylase (the gene mutated in Don Cdt^{R}-Q) which catalyses the formation of UDP-Glc from UTP and Glc-1-P.

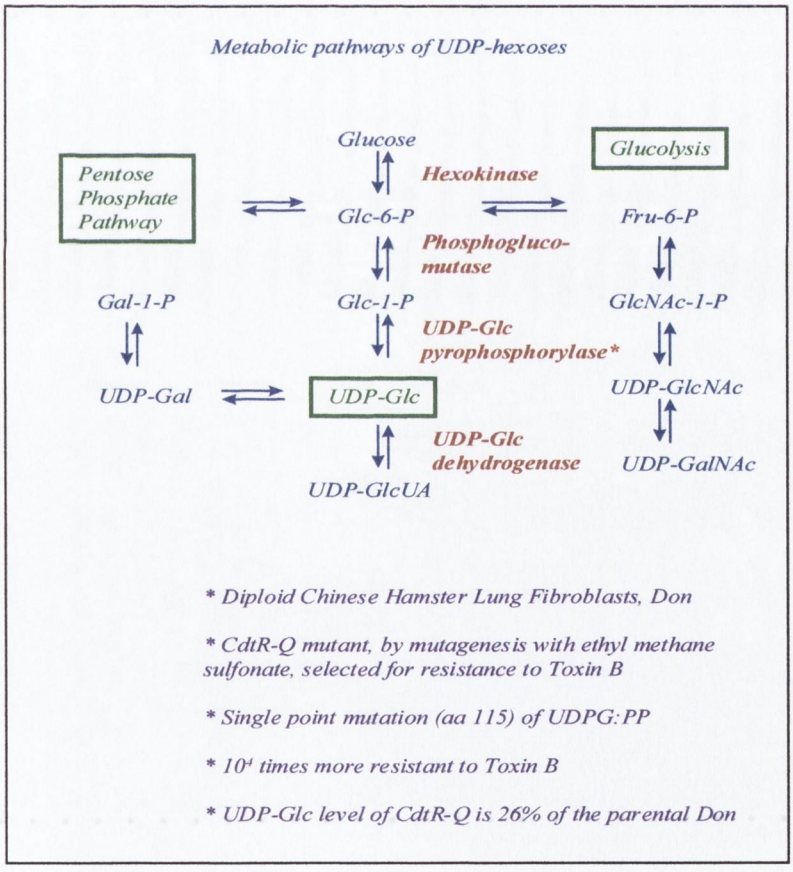


Figure 2.1 **Metabolic pathways of UDP-hexoses**. Abbreviations used: *Glc, glucose; Fru-6P, fructose-6-phosphate; GlcNAc-1-P, N-acetylglucoseamine-1-phosphate; UDP-GlcNAc, UDP-N-acetylglucoseamine; UDP-GalNAc, UDP-N-acetylgalactosamine* (adapted from (Flores-Diaz *et al.*, 1997)).

The parental Don and the UDP-Glucose deficient mutant Don-Cdt^R-Q cells were cultured in Minimal Essential Medium supplemented with 10% Foetal Calf Serum, 2 mM L- Glutamine and 100 IU/ml of penicillin and streptomycin (total volume of 7 ml, 15 ml or 35 ml for a 25, 80 or 175 cm^2 culture flask respectively). The cells were kept sub-confluent at all times. The 80-90% confluent monolayer cells of an 80 cm^2 flask was passaged by washing with 1-2 ml Versene for 1 minute. The cells were then incubated with 0.25% (w/v) Trypsin-EDTA in Versene at 37°C until the cells detached from the flask and became resuspended. The flask was tapped gently to increase the single cell suspension and to break up any aggregated cells. The trypsinization was terminated by adding 2 ml of complete Minimal Essential Medium (containing 10% FCS) after which the cells were centrifuged (170g, 5 minutes), the pellet was resuspended in complete medium and the cells were seeded at $1x10^5$ in a fresh 80 cm^2 culture flask in a total volume of 15 ml. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2

The Don cells were frozen and thawed in a similar way to the EL4.NOB-1 cells described above.

2.3.3 HeLa cells

Human cervix carcinoma epithelial HeLa cells were kept at 0.5-5 x 10^5 cells per ml DMEM medium supplemented with 2 mM Glutamine, 100 IU/ml gentamicin and 10 % FCS. 24 hours prior to transfection, cells were set up at 0.5-1x 105 cells per ml, in a total of 3 ml, in 6 well plates.

HeLa cells were frozen and thawed as described above. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2

2.4 IL-2 ELISA

2.4.1 Sample preparation

2.4.1i IL-1 dose response

$1x10^6$ EL4.NOB-1 cells were seeded in 0.5 ml complete RPMI 1640 medium in a 24 well plate. The cells were then stimulated with indicated doses of IL-1α for 24 hours after which the plate was centrifuged at 170g for 10 minutes and the supernatant removed for determination of IL-2 production by IL-2 ELISA.

2.4.1ii Clostridium sordellii *LT pre-treatment*

Prior to use, the LT toxin was first diluted 10 times in 5 mM dithiothreitol (DTT) in PBS and left at room temperature for 3-5 minutes to minimize oxidation of the toxin when added to cell culture. The toxin was then diluted to working concentrations in PBS and added to the cells, which were seeded as described above for the preparation of samples treated with IL-1α. The cells were pre-treated with LT for 3 hours and subsequently washed and incubated with IL-1α for an additional 24 hours. The plate was centrifuged and the supernatant collected for IL-2 analysis.

2.4.2 ELISA procedure

The wells of a 96 well plate were coated with primary anti-IL-2 antibodies by adding 50 µl of 4 µg/ml purified rat anti-mouse IL-2 antibody in 0.1 M $NaHCO_3$, pH 8.2 and incubating the plate at 4°C overnight. The plate was washed twice in PBS/0.5% Tween-20 (PBS: 0.145M NaCl, 3.9 mM NaH_2PO_4, 18 mM Na_2HPO_4, pH 7.0) and non-specific activity was blocked using 200 µl PBS/10% FCS per well, at room temperature for 2 hours. After subsequently washing the plate twice in PBS/Tween, the samples were added at 100 µl per well. A set of dilutions of recombinant IL-2 ranging from 0.1 to 10 ng/ml IL-2 were also added to give a curve of standard IL-2 values. The plate was covered and incubated at 4°C overnight. The plate was once again washed in PBS/Tween-20 four times and 100 µl of 2 µg/ml Biotinylated rat anti-mouse IL-2 antibody was added and left to bind at room temperature for 45 minutes. The plate was then washed in

PBS/Tween six times and incubated at room temperature for 30 minutes with 100 µl of 2.5µg/ml avidin-peroxidase in PBS/10% FCS. After washing the plate eight times in PBS/Tween the wells were incubated with 100 µl 0.1 M Citric acid buffer, pH 4.3 with 0.3 mg/ml 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 0.03% H_2O_2 and was allowed to develop at room temperature in the dark for 30-60 minutes. The colour developed was measured at OD 405 nm, and the IL-2 concentration of the unknown samples could be calculated from the graph created when plotting the OD against the concentration of the known recombinant IL-2 standard samples.

2.5 Preparation plasmids for transfection

2.5.1 Preparation of competent E. coli *cells*

Competent *E. coli* were obtained by treating an *E. coli* culture with $CaCl_2$ which creates pores in the cell walls and facilitates the uptake of plasmids when exposed to heat at a later stage. The competent cells were prepared by diluting 1 ml of an overnight culture of *E. coli* into 500 ml Luria Broth (10mg/ml tryptone, 5 mg/ml yeast extract, 5 mg/ml NaCl). This was grown at 37°C in an orbital shaker (200 rpm) for approximately 2 hours until A_{500}nm reached 0.6. The cells were subsequently centrifuged (10 000g, 10 minutes, 4°C) and resuspended in 10 ml of ice-cold 100 mM $CaCl_2$ and left on ice for 20 minutes. After centrifuging again (5 000g, 10 minutes, 4°C) the now competent cells were resuspended in 500 µl (i.e. 1/100th the culture volume) of 100 mM $CaCl_2$ and stored at -70°C until needed.

2.5.2 Transformation of competent E. coli

All the work was done aseptically. 200 µl competent *E. coli* cells were incubated on ice for 15 minutes with 0.2 µg of the plasmid DNA to be amplified, to allow the cells and the DNA to associate. Following this the cell solution was heat shocked at 43°C for 2 minutes to allow DNA uptake into the cells and then cooled on ice for 1-2 minutes to allow the cells to close again. The solution was transferred to 1 ml Luria broth without drug selection and was left at 37°C in an orbital shaker at 200 rpm for 1 hour. Following this 100 µl of the cell suspension was plated on Luria agar (Luria broth with 15 mg/ml Agar added) supplemented with 0.1

mg/ml Ampicillin or Kanamycin and was incubated for 16-18 hours at 37°C. Following this, the colonies of transformed cells were plated out as single colonies onto fresh plates with ampicillin or kanamycin selection and were grown for an additional 16 hours at 37°C. The single colony purified transformed cells could then be used for plasmid purification.

2.5.3 Plasmid purification procedure according to QIAGEN® (from 1996 till 1998)

Plasmid preparations were made using QIAGEN® Plasmid Midi Kit according to manufacturers instructions.

Buffers used:

P1: 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 mg/ml RNase A

P2: 200 mM NaOH, 1% SDS

P3: 3.0 M potassium acetate, pH 5.5

QBT: 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100

QC: 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol

QF: 1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% ethanol

The procedure in brief: 25 ml Luria Broth with 0.1 mg ampicillin/ml was inoculated with a single CFU and was incubated at 37°C for 16-18 hours. The cells were centrifuged (10 000g, 10 minutes, 4°C) and resuspended in 4 ml buffer P1, mixed with 4 ml buffer P2 and incubated at room temperature for 5 minutes. 4 ml of chilled Buffer P3 was then added and the solution was incubated on ice for 15 minutes. During this time a QIAGEN-tip 100 was equilibrated with Buffer QBT and the solution with lysed cells could then be centrifuged (20 000g, 45 minutes, 4°C) and the supernatant applied to the tip. The tip was washed twice with 10 ml of Buffer QC as soon as the supernatant had been allowed to pass through the tip, and the DNA could then be eluted with 5 ml Buffer QF. The eluted DNA was precipitated with 3.5 ml isopropanol and centrifuged (15 000g, 30 minutes, 4°C). After finally washing the pellet twice with 70% ethanol, the DNA could be re-dissolved in 50 µl water. This resuspension was further aided by freezing the sample at -20°C,

and the DNA concentration could then be estimated by running a small amount on a 1% agarose gel, comparing it to a sample of known concentration run on the same gel.

2.5.4 Plasmid purification according to Wizard ® PureFection Plasmid Kit (from 1998 till 2000)

Plasmid purification was performed according to manufacturer's recommendations, using buffers provided in the kit.

In brief, 100 ml of confluent transformed *E. coli* cultures were pelleted, and resuspended in 6.25 ml Cell Resuspension Buffer. The cells were lysed using 6.25 ml of Cell Lysis Solution, and were incubated for 5 minutes at room temperature. After 8.75 ml of Neutralization Solution had been added, the lysates were centrifuged at 10,000xg for 20 minutes at room temperature. Endotoxins were removed from the supernatant by adding 1.25 ml of Endotoxin Removal Resin and incubating the samples for 10 minutes at room temperature, following which the magnetic beads were removed using the Promega magnetic tube stand.

After 5 ml of 5M GTC was added, the plasmid DNA of each supernatant could be captured by adding 3.75 ml of MagneSil™ Paramagnetic Particles, which were incubated with the sample for 2-3 minutes at room temperature. The particles were washed once in 5 ml of 4/40 Wash Solution, and then a further three times in 80% ethanol. After the final wash the beads were drained of all ethanol by leaving the tubes on the magnets for 10 minutes, allowing residual ethanol to collect at the bottom of the tube. The DNA was eluted from the beads using 6 ml high quality water, and then precipitated using 3 ml of 7.5 M ammonium acetate and 15 ml of 95% ethanol, following which the DNA was recovered by centrifugation at 14,000 x g for 15 minutes. The DNA pellet was washed once in 70% ethanol, and after a short period of air-drying, the DNA was resuspended in up to 1 ml of water, and the plasmid concentration was determined by running 3 µl of each sample on a 1% agarose gel, together with a DNA sample of known concentration.

2.6 Chloramphenicol acetyl-transferase assay

2.6.1 Transfection and preparation of cytosolic extracts

EL4.NOB-1 cells were seeded in complete medium at $2.5x10^5$ cells per ml and grown overnight to bring the cells into exponential growth phase. The following day the cells were washed in 10 ml of PBS and counted as described above. $1.4x10^7$ cells were resuspended in 10 ml PBS and centrifuged at 170g for 5 minutes. Two solutions (I and II) were prepared, the first containing 250 µg DEAE-Dextran per ml, made up to a total volume of 600 µl with TBS (25 mM Tris, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM NaH_2PO_4). This permeabilises the membrane and enables the cells to take up the plasmids. Solution II contained the NFκB-CAT construct containing three κB elements upstream of a chloramphenicol acetyl-transferase (CAT) gene, 10 µg DNA in total, and 40 µg/ml chloroquine to inhibit any DNAse and so protect the vector. Solution I and 600µl of solution II were mixed just prior to use and the $1.4x10^7$ washed cells from above were resuspended in the mixture(Moynagh *et al.*, 1993). The cells were incubated at room temperature for 30 minutes, and were during this time agitated frequently to prevent sedimentation of the cells. The cells were subsequently centrifuged and washed with 10 ml complete RPMI 1640 medium containing 5 IU/ml heparin. The heparin gives a better recovery of the cells, possibly by binding excess plasmid, but the precise mechanism is unknown. The cells were washed twice more in complete medium, seeded in 40 ml complete medium supplemented with 20% FCS and left to recover for at least 16 hours. Where indicated in figure legends in chapter 5, half the amounts of cells, solutions, and DNA was used, in order to enable a higher through-put of samples.

The samples were prepared by seeding the transfected EL4.NOB-1 cells at $1x10^6$ cells in 1 ml complete medium per sample in a 24 well plate. Each sample was then stimulated with IL-1α for 24 hours, or pre-treated with LT (pre-treated with DTT as described above) for 3 hours before being washed in complete medium and stimulated with IL-1α for 24 hours.

The supernatant of each sample was recovered for IL-2 determination by ELISA as described above, and the cells of each sample were resuspended in 1 ml PBS, centrifuged (10 000g, 5 minutes) and the pellets were resuspended in 80 µl of 0.25 M Tris-HCl, pH 8. The cells were subsequently lysed by alternatively freezing the samples in liquid nitrogen and thawing them in a 37°C water bath. The samples were centrifuged at 13 000g for 10 minutes so the cytosolic fraction (supernatant) could be recovered and assayed for chloramphenicol acetyl transferase (CAT) activity.

2.6.2 Bradford assay

The protein content of each sample was determined by the method of Bradford (Bradford, 1970). The Bradford reagent (0.1 mg/ml Coomassie Brilliant Blue G250, 4.75% ethanol, 8.5% phosphoric acid) was stored at 4°C, stirred vigorously and used at room temperature. The Bradford assay was performed in a 96 well plate, and for each cytosolic extract, 2 µl sample, 18 µl 0.25 M Tris and 200 µl Bradford reagent was added and incubated at room temperature for 5 minutes to allow the colour to develop. The result was measured at Abs 570 nm. A standard curve could be created from the results of a series of bovine serum albumin (BSA) samples of known protein concentrations.

2.6.3 CAT Assay procedure

The CAT activity of each cell extract could be assayed by incubating a volume of each sample corresponding to 40 µg protein with 0.98 mM AcetylCoA and 3 µl (0.3 µCi) [^{14}C]-chloramphenicol made up to a total volume of 91.5 µl, at 37°C for 24 hours. 350 µl ethyl acetate was then added and after vortexing each sample for 30 seconds, and centrifuging at 13 000 g for 1 minute, the organic phase containing the chloramphenicol could be recovered. The ethyl acetate could then be evaporated using a vacuum dryer (Integrated SpeedVac® System, Savant) and the dried chloramphenicol could be re-dissolved in 12 µl ethyl acetate. This could subsequently be spotted on to a thin liquid chromatography (TLC) plate and separated by standing the plate in a TLC chamber containing a total volume of 150 ml chloroform: methanol, 19:1(v/v) which had been left to equilibrate for 30 minutes prior to the chromatography. The plate was run for 30 minutes after which it was removed and left to dry for 5 minutes. The resulting radioactivity could then be detected by

autoradiography and subsequent counting of the β-radiation of each spot on an Instant Imager® system. The results could then be expressed as percentage (%) acetylation obtained by dividing the cpm from the acetylated chloramphenicol (upper two spots, see figure 3.1.4) with the sum of the cpm from the non-acetylated chloramphenicol (bottom spot) and the cpm from the acetylated chloramphenicol (upper two spots).

2.7 Electrophoretic mobility shift assay

2.7.1 Buffers

Buffer A: 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 100 mM PMSF, 0.5 mM DTT

Buffer C: 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% Glycerol (v/v), 100 mM PMSF

Buffer D: 10 mM Hepes, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 10% Glycerol, 100 mM PMSF, 0.5 mM DTT

10x Binding reaction buffer: 40% glycerol, 10 mM EDTA, 50 mM DTT, 100 mM Tris, pH 7.5, 1 M NaCl, 1 mg/ml nuclease free BSA

10xTBE running buffer: (0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA),

5% polyacrylamide gel: 2.5 ml 10X TBE 3.125 ml 40% acrylamide:bis-acylamide mix (29:1 ratio), 5 µl 1 M DTT, 15 µl N,N,N',N'-tetra-methylethylenediamine (TEMED) and 0.05g ammonium persulphate, made up to 25 ml with water

Loading buffer: 33% glycerol, 0.25 mg/ml Brom Phenol Blue, 0.25 Xylene Cyanol FF, made up to volume with TBE.

2.7.2 Preparation of nuclear extracts

$5x10^6$ EL4.NOB-1 cells were seeded in 1 ml per well in a 24 well plate. The cells were then pre-treated with varying doses of LT as indicated and then either with IL-1α (10ng/ml) for 1 hour or with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) for 5 hours. The reaction was terminated by transferring each sample

to a sterilin containing 5 ml ice-cold PBS after which the cells were centrifuged (170g, 5 minutes) and washed with an additional 1 ml of PBS before resuspending them in 1 ml of Buffer A. After centrifuging the samples at 13 000g for 10 minutes, the pellet was resuspended in 20 µl Buffer A with 0.1% NP40 and incubated on ice for 10 minutes. The samples were again centrifuged at 13 000g for 10 minutes, and the pellet was resuspended in 15µl Buffer C and incubated on ice for 15 minutes. After again centrifuging the samples as above, the supernatant was recovered and 75 µl of Buffer D was added to this before the protein contents were determined according to Bradford. The samples could then be stored at -80°C for up to 6 months without loss of activity.

2.7.3 Labelling of oligonucleotides for electrophoretic mobility shift assay

5' end-labelling of the 22 base pair oligonucleotide 5'-AGT TGA GGG GAC TTT CCC AGG C-3', containing the NFκB consensus sequence (underlined) was based on the method of Sambrook *et al* (1989).

The reaction mixture containing 10 pmol of the oligonucleotide probe, 50 pmol (150 µCi) [γ-^{32}P]ATP, 20 U T4 polynucleotide kinase and 5 µl of 10x kinase buffer (100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine, 500 mM Tris-HCl, pH 7.5) was made up to 50 µl with H_2O and incubated at 37°C for 10 min. The reaction was terminated with 2 µl of 0.5M EDTA. 50 µl of a phenol:chloroform solution (1 part TE-saturated phenol plus 1 part chloroform:isoamyl alcohol (24:1 ratio)) was added to extract the DNA. After vortexing for 1 min, the solution was centrifuged at 13,000g for 2 min.

The resulting top aqueous layer was transferred to a fresh tube, 2 µl of 5 M NaCl added, the tube vortexed and 100 µl of ethanol added. This was incubated at -70°C for 30 min to allow ethanol precipitation of the DNA. After centrifugation (13,000 g, 5 min), the supernatant was carefully removed and discarded. The pellet was dried in an incubator (30°C, 45 min) and resuspended in 50 µl TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8). 1 µl of this solution was counted in5 ml scintillant fluid, and the stock solution subsequently diluted to 10,000 cpm/µl immediately before use in the electrophoretic mobility shift assay (EMSA).

2.7.4 Electrophoretic mobility shift assay procedure

Nuclear extracts were analysed for NFκB by electrophoresis, as described by Sen and Baltimore (1986). The principle of this assay is that any activated NFκB present in nuclear extracts will bind to a labelled probe containing the NFκB binding motif, thus retarding the electrophoretic mobility of this DNA in a polyacrylamide gel.

The NFκB-probe binding reaction was set up by incubating at room temperature for 30 minutes a mixture of 1/10th of the total volume of 10x binding reaction buffer, a volume of nuclear extract corresponding to 4 µg protein, 2 µg non-specific competitor DNA (poly d(I-C), Pharmacia Biosystems), and [^{32}P] labelled NFκB consensus oligonucleotide corresponding to a total activity of 10 000 cpm per reaction. Following this incubation, the binding reaction was terminated by the addition of 1/10th of a volume of loading buffer (final concentration 0.025% (w/v) bromophenol blue, 0.025% xylene cyanole FF, 33% glycerol in TBE).
The samples were then run on a 5% native polyacrylamide gel (20x20 cm) for 2-3 hours at 120-180V with 0.5xTBE as running buffer. The gel was dried and the radioactivity detected by autoradiography using Kodak diagnostic film in autoradiography cassettes with intensifying screens for 16-48 hours at -70°C.

2.8 MAP Kinase phosphorylation state specific western blotting assay

2.8.1 Preparation of whole cell lysates for p42/p44 MAPK assay

EL4.NOB-1 cells were seeded at 3x10^6 cells per ml in RPMI 1640 medium supplemented with 0.5 % FCS as well as L-Glutamine, penicillin and streptomycin as described above. After 24 and 48 hours the medium was changed and following this the cells were given fresh medium 2 hour prior to stimulation, reducing background activation of p42/p44 caused by FCS and factors secreted by the cells. The cells were then seeded in fresh medium at 2x10^6 cells per ml in a 24 well plate with each well containing 4-5 ml of cells. In a similar manner the Don fibroblasts were seeded on day 1 at 2x10^6 cells per well in a 24 well plate in 4 ml of

MEM medium supplemented with 0.5 % FCS as well as L-Glutamine, penicillin and streptomycin as described above. The medium was changed as above at 24, 48 and 50 hours after the seeding.

Following the 2 hour culturing in the new medium, the cells were if indicated below, pre-treated in fresh medium with LT or Toxin B, for indicated length of time. The cells were then incubated with IL-1α at 10 ng/ml, or PMA at 1 ng/ml for varying times. The reaction was terminated by adding 5 ml of ice-cold PBS.

The EL4.NOB-1 cells were subsequently centrifuged (170g, 10 minutes) and the medium was aspirated. The EL4.NOB-1 cells were resuspended in 1 ml of PBS and transferred to a microcentrifuge tube which was centrifuged (10 000g, 5 minutes). Following this the EL4.NOB-1 cells were lysed in 100 µl SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromphenol blue) and the viscosity of the sample was reduced by sonication for 30 seconds.

The Don fibroblasts were simply washed with PBS in the 24 well plate. After aspirating the PBS the cells were lysed straight in the well by adding 100 µl SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50mM DTT, and 0.1% w/v bromphenol blue). Each well was then scraped using a cell scraper and the sample was transferred to a microfuge tube in which it was sonicated as described above. The cell lysates were subsequently heated to 95°C for 5 minutes and were then ready to be assayed by western blotting.

2.8.2 Preparation of whole cell lysates for p38 MAPK assay

EL4.NOB-1 cells were seeded at $2x10^6$ cells per ml in a 24 well plate with each well containing 4-5 ml of cells in complete RPMI 1640 medium (10% FCS). In a similar manner the Don fibroblasts were seeded at $2x10^6$ cells per well in a 24 well plate in 4 ml of complete MEM medium (10% FCS, 2mM L-Glutamine, 100 IU/ml penicillin and streptomycin). The cells were pre-treated with LT or ToxB as indicated in figure legends. Following this the samples were incubated with IL-1α (10 ng/ml), or PMA (1 ng/ml) for indicated times until the reactions were terminated by the addition of 5 ml of ice-cold PBS.

The EL4.NOB-1 cells were subsequently centrifuged (170g, 10 minutes) and the medium was aspirated. The cells were washed in 1 ml of PBS and transferred to a microcentrifuge tube which was centrifuged (10 000g,

5 minutes). Following this the samples were lysed in 100 µl SDS sample buffer (as described above) and the viscosity of each sample was reduced by sonication for 30 seconds.

The Don fibroblasts were washed with PBS in the 24 well plate, and following the aspiration of the PBS they were lysed straight in the wells by adding 100 µl SDS sample buffer. Each well was then scraped using a cell scraper and the samples were transferred to microfuge tubes, which were sonicated as described above. The cell lysates were subsequently heated to 95°C for 5 minutes before they were assayed by western blotting.

2.8.3 Western blotting procedure

The phosphorylation status of p38 MAPK and p42/44 MAPK were analysed using PhosphoPlus® p38 MAPK (Thr180/Tyr182) Antibody Kit and PhosphoPlus® MAPK Antibody Kit respectively. 20 µl of each whole cell lysate was loaded on to a 10% SDS-PAGE gel (for 12 ml: 5.93 ml H_2O, 5.03 ml Protogel®, 3.82 ml 1.5 M Tris, pH 8.8, 150 µl 10% SDS, 150 µl 10% ammonium persulphate, 6µl TEMED; for 6 ml 5% Stacking gel: 4.1 ml H_2O, 1.0 ml Protogel®, 750 µl 1.0 M Tris, pH 6.8, 60 µl 10% SDS, 60 µl 10% ammonium persulphate, 6µl TEMED) which was run at 25 mA per gel for 1 hour at room temperature (essentially according to the method of Laemmli(Laemmli, 1970)). Also loaded as controls were one of the following: for detection of phosphorylated p38: total cell extract from C-6 glioma cells prepared with Anisomycin; for detection of total p38 content: total cell extract from C-6 glioma cells prepared without Anisomycin; for the detection of phosphorylated p42/p44: fully phosphorylated Erk2 protein; and for total cell content of p42/p44: bacterially expressed, kinase inactive, Erk2 protein, 10 µl of each. The gel was subsequently equilibrated in transfer buffer (25 mM Tris-HCl, 0.2 M glycine, 20% methanol) for 15 minutes before the protein on the gel was transferred onto a nitrocellulose membrane using a wet transfer system, transferring at 150 mA for 1 hour while cooling with ice blocks. The membrane was after the transfer washed once in TBS (0.2 M Tris-HCl, 1.4 M NaCl, pH 7.6) and thereafter the non-specific binding was blocked by incubating the membrane in 25 ml of blocking buffer (TBS with 0.1% Tween-20 with 5% w/v nonfat dry milk) at 4°C overnight. The blocking buffer was subsequently removed by washing the membrane with 25 ml of TBS-0.1%Tween-20 three times for 5 minutes each. To detect the phosphorylated p38 MAPK or p42/44 MAPK the membrane was incubated with the primary antibody which is phosphorylation state

specific, detecting only phosphorylated MAP kinases. As a control, a second membrane containing the same samples was incubated with a primary antibody which was phosphorylation state independent, enabling a comparison of the total to the phosphorylated p38 or p42/44 MAPK content of the samples, and therefore acting as a control of the loading. All the primary antibodies were diluted 1:1000 in a total volume of 5 ml. After incubating the membranes with the primary antibody over night at 4°C with gentle agitation, the membranes were again washed three times in TBS-Tween, after which they were incubated with the secondary antibody, HRP-conjugated anti-rabbit antibody, diluted 1:2000, for 1 hour at room temperature with gentle agitation. Following this the membranes were again washed tree times with TBS-Tween and a final wash was performed in TBS alone. The HRP could then be detected by ECL according to manufacturers instructions and could be recorded using autoradiography. The reaction involves the conversion of luminol to 3-aminophtalate, N_2 and light by horse radish peroxidase (HRP) in the presence of H_2O_2.

2.9 Western blotting procedure for detecting IκB degradation and caspase-3 cleavage

2.9.1 Preparation of whole cell lysates for western blotting

For use in western blotting, 8 x 10^6 EL4.NOB-1 cells in 4 ml complete medium were incubated with medium alone or with indicated doses of inhibitors for 1-4 hours. Following this, the cells were stimulated with 10 ng/ml IL-1α for indicated times. The cells were subsequently washed with 10 ml PBS and centrifuged at 170 g for 5 minutes. The pellet was subsequently resuspended in 1 ml ice cold RIPA buffer (1% (v/v) Nonidet P40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS all made up in PBS; within 5 minutes of use the following was added: 100 µg/ml PMSF, 30 µl/ml aprotonin, 10 µl/ml sodium orthovanadate) and incubated on ice for 30 minutes. The cells were further disrupted and homogenised by passage through a 21 gauge needle 10 times after which a further 10 µl of 10 mg/ml PMSF was added (final concentration of 200 µg/ml) and the cells were incubated on ice for 30 minutes. The sample was then centrifuged at 15 000g for 20 minutes at 4°C and the resulting supernatant was collected and used as whole cell lysate for western blotting.

2.9.2 Western blotting procedure

The protein content of each whole cell lysate was analyses according to the method of Bradford as described in section 2.5.2. RIPA buffer strongly interferes with the Bradford reagent and was therefore used as a blank sample, the value of which could be subtracted from the values of the samples. 30-50 µg of protein of each sample was loaded on to a 15 % SDS-PAGE gel (for 10 ml: 5 ml Protogel®, 2.3 ml H_2O, 2.55 ml 1.5 M Tris, 0.1 ml 10% (w/v) SDS, 0.1 ml ammonium persulphate, 4 µl TEMED, with stacking gel: for 6 ml: 4.1 ml H_2O, 1.0 ml Protogel®, 750 µl 1.0 M Tris, pH 6.8, 60 µl 10% SDS, 60 µl 10% ammonium persulphate, 6µl TEMED) and run for 1 hour at 25 mA per gel. The gel was subsequently equilibrated in transfer buffer (25 mM Tris-HCl, 0.2 M glycine, 20% methanol) for 15 minutes before the protein on the gel was transferred onto a nitrocellulose membrane using a wet transfer system, transferring at 150 mA for 1 hour while cooling with ice blocks. The membrane was after the transfer washed once in TBS (0.2 M Tris-HCl, 1.4 M NaCl, pH 7.6) and blocked in 5% nonfat dry milk (Marvel), made up with TBS, over night at 4°C. The following day the membrane was washed in TBS-Tween (0.05%), three times by five minutes each wash, after which they were incubated for 1 hour at room temperature with the primary anti-IκB primary antibody used at a dilution of 1:200 in 1% fat free dry milk in TBS/0.1% Tween, or anti-CPP32, used at a dilution of 1:1000 in 1% fat free dry milk in TBS/0.1% Tween. After being washed again three times by five minutes in TBS-Tween (0.05%), the membranes were incubated with the secondary HRP linked goat anti-rabbit antibody diluted 1:1000 in TBS-Tween (0.05%) at room temperature for one hour. The secondary antibody was subsequently detected using a kit for enhanced chemiluminescense substrate development (New England BioLabs (U.K.) Ltd).

2.10 Glucosylation assay

2.10.1 Preparation of whole cell lysates.

$5x10^7$ EL4.NOB-1 or Don fibroblasts were used for each sample when analysing the glucosylation substrate targets of these cells (performed essentially as described in (Popoff *et al.*, 1996)). If pre-treatment was

performed with either of the toxins (LT or ToxB), the cells were seeded in a 6 well plate prior to the addition of the toxin of interest. The pre-treatment time as well as the doses used is indicated below in the results section. Toxicity was monitored by trypan blue staining, as described in section 2.2.1, of 10 µl of the treated cells. Following the pre-treatment, the cells were washed twice in 10 ml of PBS after which the cells were resuspended in 400 µl glucosylation buffer (50 mM triethanolamine, pH 7.5, 100µM dithiothreitol, 1µg/ml leupeptin) and lysed by freezing the cells in liquid nitrogen alternated by thawing in a 37°C water bath three times.

2.10.2 Glucosylation assay procedure

After determining the protein contents of the cell lysates, a sample volume corresponding to 50-150 µg of protein was incubated for 1 hour at 37°C with 2 µg/ml LT or Toxin B, and 20 µl UDP- [14C]-Glucose (DuPont) in ethanol from which the ethanol had been totally evaporated using a vacuum centrifuge dryer (Integrated SpeedVac® System, Savant). The reaction was stopped by adding SDS sample buffer and loading the total volume of 20 µl sample on to a 15% SDS PAGE gel and running this at room temperature for 1 hour at 25 mA per gel. The gel was washed briefly in PBS, and after transferring the protein of the gel on to a nitrocellulose membrane as described previously, the membrane was dried and dipped in 20% PPO (w/v) in toluene, dried again and the radio-labelled proteins were detected by autoradiography.

2.11 Two dimensional gel electrophoresis of glucosylated cell extracts from EL4.NOB-1 cell

2.11.1 Preparation of glucosylated whole cells lysates for two dimensional gel electrophoresis

The method used for separating proteins in two dimensions; by isoelectric focusing, and by molecular weight, was modified from O'Farrell by Celis et al (1991) involved in the Human 2D PAGE Database (http://biosun.biobase.dk/~pdi/jecelis/mouse_data_select.html). Whole cell lysates from $5x10^7$ EL4.NOB-1 cells were prepared as described previously, and the glucosylation assay was performed essentially as described above, except that twice the volume of the glucosylation reaction mixture was prepared. To desalt

the sample, Fugisep Mini® with a cut-off of 4 000 Da was used according to manufacturers instructions. In brief, the sample was added to the Fugisep, which had been centrifuged with distilled water prior to use. The fugisep was then centrifuged at 5 000g until the nearly all the sample had passed through. The protein of the sample was then washed with 1 ml sample buffer (9.8 M Urea, 100 mM DTT, 2% NP40) and reduced to about 50 µl using the Fugisep®.

2.11.2 Two dimensional gel electrophoresis procedure

2.11.2i Isoelectric focusing (First dimension)

The first dimension gel was prepared between two glass plates (10x12 cm, 1 mm thick) overlaid with water-saturated butanol and left to set at 37°C. For one gel: 1.5 ml 40% Accugel, 1.62 ml 40% sucrose, 325 µl Ampholines (pH 3-10), 4 ml 4M Urea, 250 µl 10% NP40, 25 µl 10% Ammonium persulphate, 7 µl TEMED. The IEF electrophoresis was run on a system with a connected water cooling system, using 1 M NaOH, and 40 mM DL-Aspartic acid as running buffers for the cathode and anode respectively. The two glass plates were separated, and the IEF gel was left lying on the ridge-free plate. A large tip for a micropipette was used to pierce holes in the gel for where the samples were to be applied, slightly to the anode side of the middle. Generally, 2-3 samples were run per gel. Once the plate had been placed on the IEF running system the samples were added to the prepared holes. Two pieces of Whatman® chromatography filterpaper were cut to approximately 10x12 cm in size, and one was dipped into each tank and left in connection with the running buffer with one of the short ends, and overlaying the gel with about 1 cm with the other end. The gel was subsequently run at 100 V for 30 minutes after which it was increased with 100 V every 30 minutes, always making sure the current stayed below 6-7 mA, until it reached 450 V at which the gel was run for 4 hours or until the current had reached 1-2 mA.

2.11.2ii SDS-Polyacrylamide gel electrophoresis of IEF slab gel (second dimension)

After the IEF gel was taken off, 1 cm wide strips of the gel were cut along where the sample had run and soaked in 10 ml of sample buffer (2 ml glycerol, 2 ml 10% SDS, 0.5 ml methanol, 1.25 ml 1 M Tris, 0.02 g brom phenol blue, 4.25 ml H_2O), for 20 minutes, in order to visualise the protein migration and aid the

denaturing of the proteins. One 1 cm wide strip free of sample was kept and cut into 1 cm long pieces and left in 2 ml H_2O each for determining pH the following day. A 20x20 cm, 2 mm thick, 15% denaturing gel was cast (for 50 ml: 11.5 ml H_2O, 25 ml 30% Protogel, 12.75 ml 1.5 M Tris pH 8.8, 0.5 ml 10% SDS, 0.5 ml 10% Ammonium persulphate, 20 µl TEMED) with a 5% stacking gel (for 24 ml: 16.4 ml H_2O, 4 ml 30% Protogel, 3 ml 1.0 M Tris pH 6.8, 240 µl 10% SDS, 240 µl 10% Ammonium persulphate, 24 µl TEMED). One "sample strip" was applied to the top of the stacking gel and moulded into place with 1% agarose (1g Agarose, 12.5 ml 1 M Tris, pH 6.8, 90 ml H_2O). The gel was run for 16 hours at 50 V after which the protein of the gel was transferred to a nitrocellulose membrane, dried, dipped in 20% PPO in toluene as described previously and the radioactivity was detected by autoradiography at -80°C for 2-3 weeks.

2.12 GAL4-CHOP$^{(1-101)}$ assay

2.12.1 Principle of GAL4-CHOP$^{(1-101)}$ assay

This assay is based on the specific ability of p38 MAPK to phosphorylate and activate the transcription factor CHOP. The system consists of a fusion protein, where the activation domain of CHOP is fused to the DNA binding domain of GAL4. When transfected into cells, the fusion protein becomes expressed and the GAL4 portion of the protein binds to five GAL4 binding sites present on the second component of this system, the luciferase reporter gene (as illustrated in figure 2.2 below). If p38 MAPK is active in these cells, the CHOP domain of the fusion protein can be phosphorylated by p38 MAPK and this will cause transactivation of the luciferase gene, and expression of luciferase. By measuring luciferase activity in cell extracts from these cells a measurement of p38 MAPK activity can be obtained. The great advantage of this system is the ability to use cotransfections of other signalling components, and study their effect on p38 MAPK activity. The assay was set up essentially according to the manufacturer's recommendations (Stratagene).

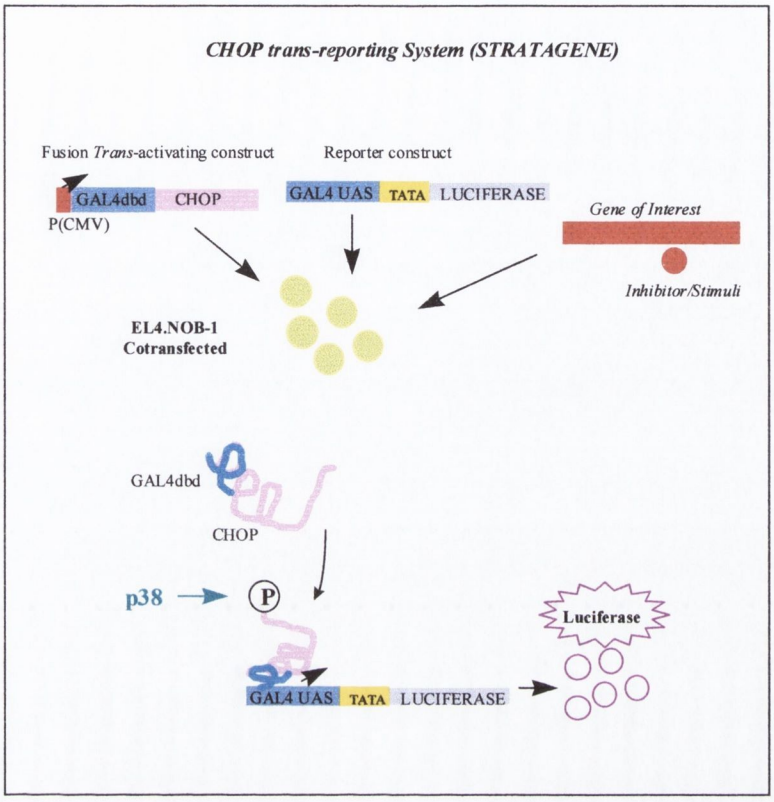


Figure 2.2 **The GAL4-CHOP$^{(1-101)}$ reporter assay**

2.12.2 Transfection of EL4.NOB-1 cells

Cells (1.4 x 10^7) were harvested [in exponential growth phase] and resuspended in a final volume of 0.6 ml of Tris-buffered saline (25 mM Tris (pH 7.4), 137 mM NaCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM NaH_2PO_4) containing 5-15 μg DNA (1.25μg pFA-CHOP, 5 μg pFR-Luc, 1 ug pEF-lacZ and 2.5-5 μg signalling component expression vector), 250 μg/ml DEAE-Dextran and 40 μg/ml chloroquine as described above in detail. Following incubation for 30 minutes at 37°C, the cells were washed twice in complete RPMI 1640 medium and resuspended in 7 ml of RPMI/20% (v/v) FCS medium.

2.12.3 Protocol for the GAL4-CHOP$^{(1-101)}$ assay

After a recovery period of 16-24 hours, the cells were harvested, seeded at 1 x 10^6 cells/ml (samples assayed in triplicates) and, when required, stimulated with IL-1α (10 ng/ml) for 6 hours. Luciferase activity was measured using standard procedures in cell lysates which were prepared using 30 µl Passive lysis buffer (Promega Corporation, Madison, WI) diluted 1:5. The firefly luciferase enzyme catalyzes a reaction using D-luciferin and ATP in the presence of oxygen and Mg2+ resulting in light emission. The luciferase reaction is quantitated using a luminometer which measures light output. The total amount of light measured during a given time interval is proportional to the amount of luciferase reporter activity in the sample. Coenzyme A improves the assay, providing a longer sustained light reaction with greater sensitivity. The sensitivity of the luciferase assay is in the sub-attomole range, approximately 30-1,000 times greater compared to the sensitivity of the CAT assay. Luciferase activity was measured by adding 40 µl of luciferase assay mix (470 µM Luciferin, 270 µM Coenzyme A, 530 µM ATP, 33.3 mM DTT, 0.1 mM EDTA, pH 8.0, 2.67 mM $MgSO_4{*}7H_2O$, 1.07 mM $(MgCO_3)4Mg(OH)_2{*}5H_2O$, 20 mM Tricine, 570 µl of 2M NaOH), and the activity was measured in a luminometer. Samples were normalised for transfection efficiency, by incubating equal amounts of cell lysate with 120 ul PM2 buffer, and 40 ul ONPG (4 mg/ml), incubating at 37°C, and measuring β-gal activity at Abs 410nm. The lacZ gene from E.coli encodes the β-galactosidase enzyme. This enzyme is a tetrameric enzyme with subunits 116 kDa in size. It catalyzes the hydrolysis of β-galactoside sugars such as lactose.

2.13 Activation assay for Ras/Rap

2.13.1 Basis for Ras and Rap activation assay

Ras and Rap1A activation assays using c-Raf-RBD and RalGDS-RBD respectively have been described elsewhere (de Rooij and Bos, 1997; Franke *et al.*, 1997; Herrmann *et al.*, 1995). The assay is based on the specific ability of active GTP-bound Ras or Rap to bind to their respective binding sites on their downstream effectors Raf1 and RalGDS. This binding domain can be purified from bacteria, coupled via a GST linker to glutathione agarose beads, and incubated with cell lysates, whereby active Ras or Rap will be pulled down.

When denatured, the proteins will detach from the agarose beads, and the quantity of pulled down Ras/Rap can be determined by western blotting using antibodies to Ras or Rap respectively. The principle of this assay is shown in figure 2.3 below.

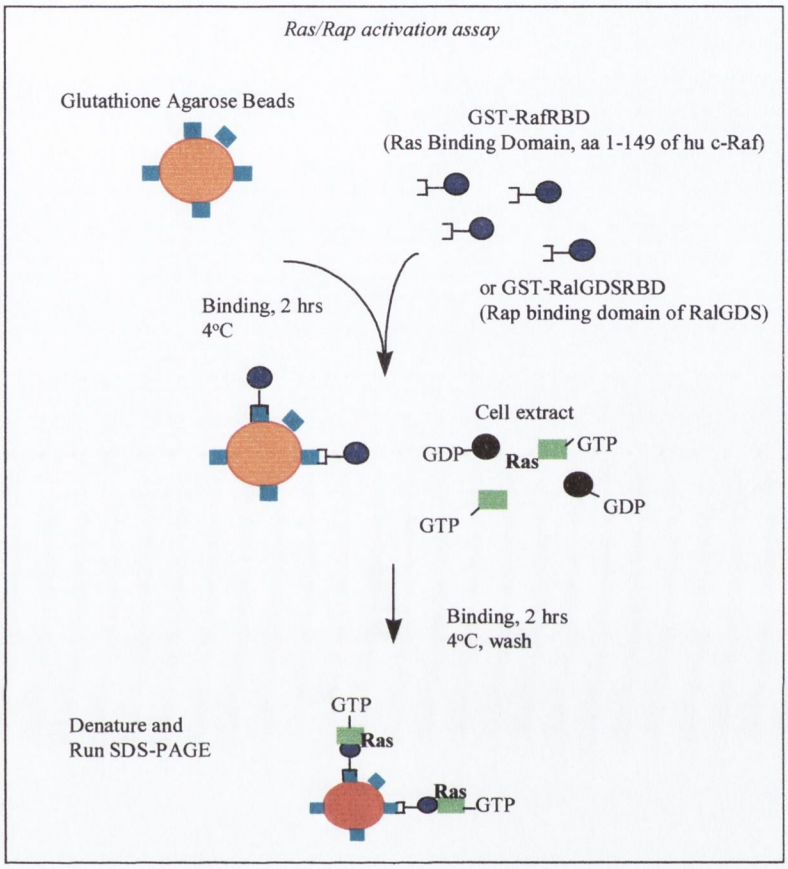


Figure 2.3 **Ras and Rap activation assay using RafRBD and RalGDS RBD**.

2.13.2 Preparation of GST-linked proteins and beads for Ras and Rap activation assay

E.coli, transformed with pGEX-KG-Raf-RBD-GST or pGEX-4T3-Ral-GDS-GST were grown to OD(600)=0.6-0.8, and induced by adding 0.4 mM IPTG after which the culture was grown for an additional 4 hours. The bacteria were lysed in 10 ml lysis buffer (1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 1 μg/ml SP1, PBS, 0.1μM aprotinin) and sonicated for 5 minutes (pulses of 5 sec). The glutathione S-transferase (GST) fusion proteins were loaded onto a glutathione agarose bead column, and eluted using 10 mM reduced glutathione (pH 8.00) in 50 mM Tris-HCl. Protein concentration was determined by reading

OD(280), and by SDS-PAGE. The protein could subsequently be coupled to glutathione agarose beads for use in the Ras and Rap pulldown assays. This was done by incubating 1 mg of fusion protein with 0.1 ml of a 50% glutathione agarose bead solution for 2 hours at 4°C on a roller. The beads were washed three times in PBS, and once in storage buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 50 % glycerol), and stored at -20°C, ready for use.

2.13.3 Ras and Rap pulldown in EL4.NOB-1 cells

$2x10^6$ EL4.NOB-1 cells were incubated with IL-1α (10ng/ml) as indicated in figure legends. Whole cell extracts were prepared using a lysis buffer (50 mM HEPES, pH 7.4, 10mM NaF, 10 mM iodacetamide, 75 mM NaCl, 1% NP40, 10mM $MgCl_2$, 1mM PMSF, 1mM Na_2VO_3) and equal amounts of protein per sample, as determined by the method of Bradford (Bradford, 1970), were incubated with 10 µl of a 50% solution containing GST-RalGDS-RBD or GST-RafRBD, pre-coupled to glutathione agarose beads (coupled by incubating 10 mg fusion protein per ml 50% slurry glutathione agarose beads, for 2 hours at 4°C). The beads were subsequently washed in lysis buffer and coupled protein was released by heating the samples to 95°C, while resuspended in sample buffer. The samples were subjected to SDS/15%PAGE (Laemmli, 1970) and detected by immunoblotting using monoclonal anti-pan-Ras antibody, or polyclonal anti-Rap1 antibody as described above. The antibodies recognizing Rap1A , was used at a dilution of 1:1000 in 1% fat free dry milk in TBS/0.1% Tween . The pan-Ras antibody was used at a dilution of 1:20 in 1% fat free dry milk in TBS/0.1% Tween. The antibody-antigen complexes were detected using a horse radish peroxidase(HRP) coupled anti-rabbit or a HRP linked anti-mouse antibody, each used at a dilution of 1:2000 in 5% fat free dry milk in TBS/0.1%Tween. The secondary antibody was subsequently detected using a kit for enhanced chemiluminescense substrate development (New England BioLabs (U.K.) Ltd).

2.14 ATF-2 kinase assay and FLAG-p38 MAPK western blotting

2.14.1 Transfection of HeLa cells using FuGene™ transfection reagent and p38 MAPK western blotting procedure

HeLa cells were seeded at 1.5x105 cells in 3 ml of complete DMEM medium 24 hours prior to transfection. 7.5 ul of Fugene transfection reagent per transfection was then added dropwise to 242.5 ul of serum free DMEM medium, and left to incubate at room temperature for 5 minutes. The FuGene/DMEM mix was then added dropwise to 2 ug of vector encoding for FLAG-tagged p38 MAPK, together with 2.5-5 ug of vector encoding for various components of the IL-1 signalling pathway, as indicated in figure legends. Equal total amounts of DNA were added to samples, which were to be directly compared, ensuring equal transfection efficiencies. The FuGene/DNA mixture was left to incubate for 15 minutes, before being added dropwise to the HeLa cells. The cells were subsequently harvested, 48 hours after the transfection. This was done by adding 150 µl SDS sample buffer to each sample, after which the cells were scraped off the plate using a cell scraper. Samples were transferred into microfuge tubes, sonicated, and western blotting was performed as described above.

2.14.2 ATF-2 kinase assay procedure

HeLa cells were seeded at $1x10^5$ cells per ml in a total volume of 10 ml in a 100mm petridish 24 hours prior to the experiment. The cells were subsequently transfected with FLAG tagged p38 MAPK using FuGene transfection reagent as described above. Following pre-treatment with LT and stimulation with IL-1 (10 ng/ml) as indicated in figure legends, the cells were washed in ice-cold PBS, and transferred into microfuge tubes in a total of 300 µl of lysis buffer (120 mM NaCl, 50 mM Tris, pH 8.0, 20 mM NaF, 1 mM benzanidine, 1 mM EDTA, 6 mM EGTA, 7.5 mM Disodiumpyrophosphate, 1% NP40, 40 mM β-galactosidase, 0.1 mM PMSF, 0.1 mM Na_3VO_4). The cells were lysed on ice for 20 minutes, and the cell debris was removed by centrifugation (10 minutes, 13,000xg, 4°C). 30 µl of a 10% solution (V/V) Protein A sepharose was added per sample, which were subsequently incubated at 4°C for 20 minutes as a pre-clearing step. When the protein concentration had been determined according to the method of Bradford (described

above), a volume of sample corresponding to equal amounts of protein was incubated with 2-5 µg anti-Flag antibody in a total volume of 300 µl (made up with lysis buffer), and incubated on a roller at 4°C for 2 hours. 30 µl of a 50% (v/v) Protein A sepharose solution was added per sample which were incubated for a further 45 minutes. The beads, with bound p38 MAPK, were washed three times in 1 ml of lysis buffer, and once in 1 ml kinase assay buffer (25 mM Hepes, pH 7.4, 20 mM β-glycerophosphate, pH 7.0, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM Na_3VO_4). 15 µl of kinase reaction mixture was added per sample (13.3 µl kinase assay buffer, 5 µg recombinant ATF-2, 20 µM ATP) which were incubated at 37°C for 20 minutes. Reaction was stopped by adding 10 µl of 5x SDS Sample buffer, whereafter the samples were boiled for 8 minutes, centrifuged for 5 minutes, 13,000xg, and run on a 10% SDS-PAGE gel. Western blotting was performed as described above, using a primary anti-phosphorylated ATF-2 antibody at a dilution of 1:1000.

CHAPTER 3

Lethal Toxin as a tool for investigating the role of Small G proteins in IL-1

INTRODUCTION

The aim of the first part of this study was to determine if a small G protein was involved in IL-1 induced activation of NFκB, expression of IL-2 and activation of the two MAPK cascades leading to the activation of p38 and p42/p44 MAPK. To do this I utilised a highly specific inhibitor from *Clostridium sordellii* called Lethal Toxin (LT). This glucosyltransferase glucosylates and thereby inhibits the small G proteins Ras, Rap, Rac and Ral (Just *et al.*, 1996a), allowing me to determine if these play a role in IL-1 signalling.

IL-1 activates NFκB by binding to the IL-RI and IL-1AcP, and this signalling cascade also involves the IL-1 signalling components MyD88, IRAK1, Tollip, IRAK2, TRAF6, IKK and IκB. Although much effort has gone into elucidating this signalling pathway, the role for a small G protein in the activation of NFκB by IL-1 is not clear. I therefore set out to investigate this using a NFκB-linked reporter gene assay, which allowed me to study the activation of NFκB using the expression of CAT as a read-out. In order to investigate the roles of Ras, Rap, Rac or Ral on this pathway, I utilised the inhibitor LT.

Another strong signal induced by IL-1 is the expression of IL-2. IL-2 is a glycoprotein of 15.5 kDa which is important for antigen-specific proliferation and differentiation of activated B cells into antibody-secreting cells. IL-2 also plays a role in the activation of the cytotoxic properties of T cell clones. The IL-2 promoter contains binding sites for a range of transcription factors, including NF-AT, AP-1, NFκB and Oct-1/OAP40, and all these binding sites must be occupied for optimal transcription. IL-1 strongly induces the expression of IL-2 and this could therefore be used as a model when investigating the role of small G proteins in IL-1 signalling.

Both the reporter gene assay and the IL-2 assays involved prolonged incubation times, stretching up to 24 hours. LT is known to cause morphological changes after prolonged exposure and, consequently, a concern regarding secondary non-specific effects was raised. I therefore decided to study, in addition, the effect of LT

on short-term responses to IL-1. p38 MAPK is strongly phosphorylated and activated by IL-1, and another short term response that is responsive to IL-1 is p42/p44 MAPK. The activation of these kinases was used as a third IL-1 responsive signal when investigating the role of small G proteins in IL-1 signalling.

A role for a small G protein in the activation of p38 MAPK has already been studied to some extent. Using dominant negative versions of the small G proteins Cdc42 and Rac, these were implicated in IL-1 activation of the p38 MAPK and JNK (Bagrodia *et al.*, 1995) (Coso *et al.*, 1995) (Just *et al.*, 1995). However, in a different system, (Davis *et al.*, 1999) these constructs were incapable of blocking IL-1 mediated JNK activation, suggesting that IL-1 did not signal this response through Rac. Ras has also been implicated in p38 MAPK activation by hemopoietic cytokines, PDGF, as well as FGF (Efimova *et al.*, 1998; Matsumoto *et al.*, 1999; Rausch and Marshall, 1999; Tan *et al.*, 1996). These studies strongly implicate that a small G protein plays a role in the activation of p38 MAPK by IL-1, although the specific G protein involved has yet to be identified.

RESULTS

3.1 Effect of LT on IL-1 induced expression of IL-2 and NFκB-CAT reporter gene

3.1i LT inhibits IL-1α and PMA induced IL-2 production in EL4.NOB-1 cells.

IL-1α is known to be a potent inducer of IL-2 expression in EL4.NOB-1 cells, although it is not clear if a small G protein is involved in conveying this response. Figure 3.1.1A show the results from stimulating EL4.NOB-1 cells with increasing doses of IL-1α for 22 hours. IL-1 can induce IL-2 expression at doses starting from 0.1 ng/ml, peaking at 10 ng/ml giving rise to a 10-20 fold increase in IL-2 production. Phorbol 12-myristate 13-acetate (PMA) is also a potent inducer of IL-2 expression in EL4.NOB-1 cells (fig 3.1.1B), inducing close to 5 times as much IL-2 as that induced by IL-1α. PMA induced IL-2 peaks at 100 ng/ml PMA, inducing levels of close to 100 ng/ml IL-2, with 1 ng/ml PMA inducing similar amounts of IL-2 as 10 ng/ml IL-1α.

To test the effect of LT on IL-1 and PMA induced IL-2 expression, EL4.NOB-1 cells were pre-treated with increasing doses of LT for 30 minutes prior to stimulation with IL-1 and PMA for 22 hours. Figure 3.1.2 shows how LT potently inhibits IL-1 (A) and PMA (B) induced IL-2 expression, causing an 80-90% inhibition of IL-1 induced IL-2 production at a dose of 150 ng/ml LT. The effect on PMA is less pronounced because of the strength of the signal, although 150 ng/ml LT is clearly inhibitory (figure 3.1.2A).

3.1ii LT inhibits IL-1α induced expression of NFκB-induced CAT

I next examined NFκB activation as another IL-1 signal.

The activation of NFκB by IL-1 can be measured by transiently transfecting EL4.NOB-1 cells with a reporter construct encoding a chloramphenicol acetyl transferase (CAT) reporter gene downstream of five NFκB binding elements. IL-1 activates NFκB and induces transcription and transactivation of the CAT reporter gene via the NFκB binding sites. The CAT activity gives a measure of the NFκB activity of the cells. Figure 3.1.3 shows the increased CAT activity of cell lysates from EL4.NOB-1 cells transfected with

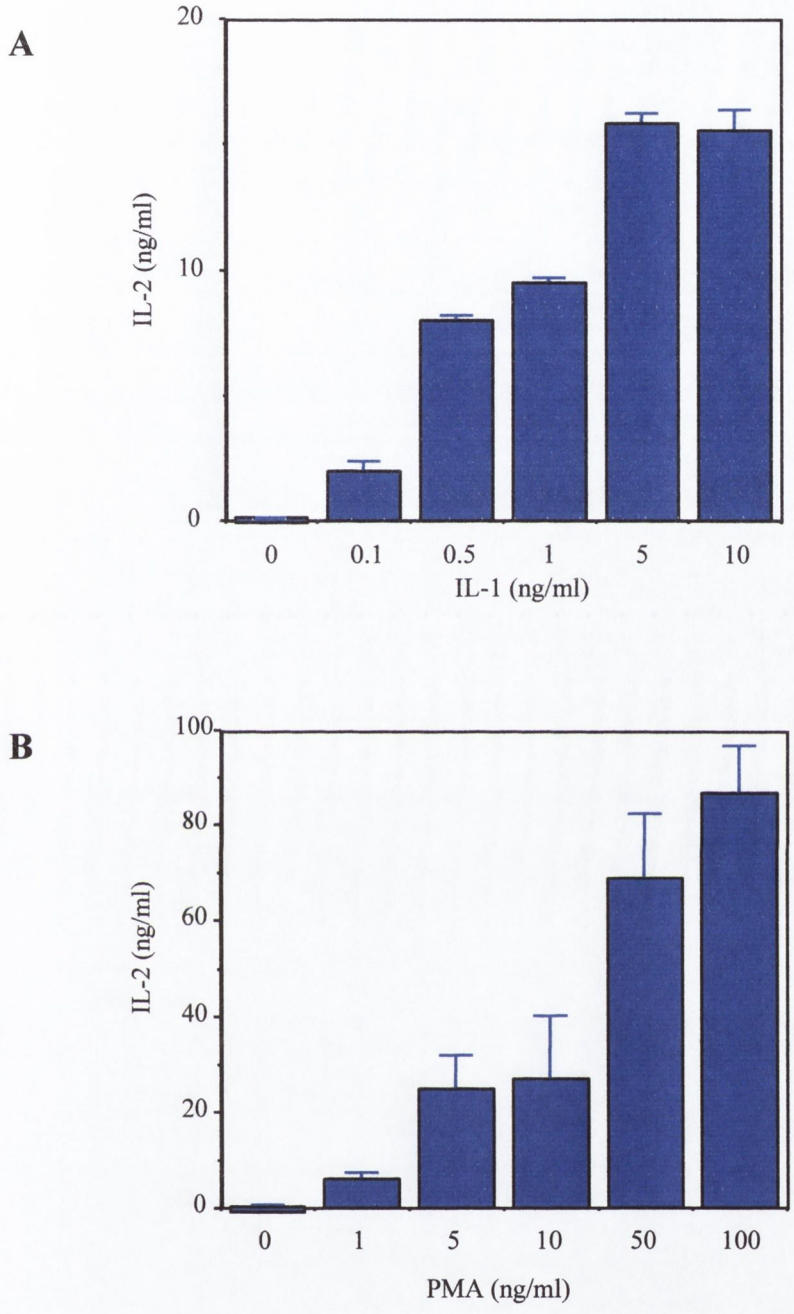


FIGURE 3.1.1

IL-1 α and PMA induced IL-2 expression in EL4.NOB-1 cells. $1x10^6$ Murine Thymoma EL4.NOB-1 cells in 1 ml medium, were stimulated with indicated doses of IL-1 α or PMA for 22 hours at 37°C. The supernatants were subsequently recovered and the IL-2 concentrations of these were measured by ELISA as described in the methods section. The results are shown as one single representative experiment out of three performed, expressed as mean ± SD from triplicate determinations.

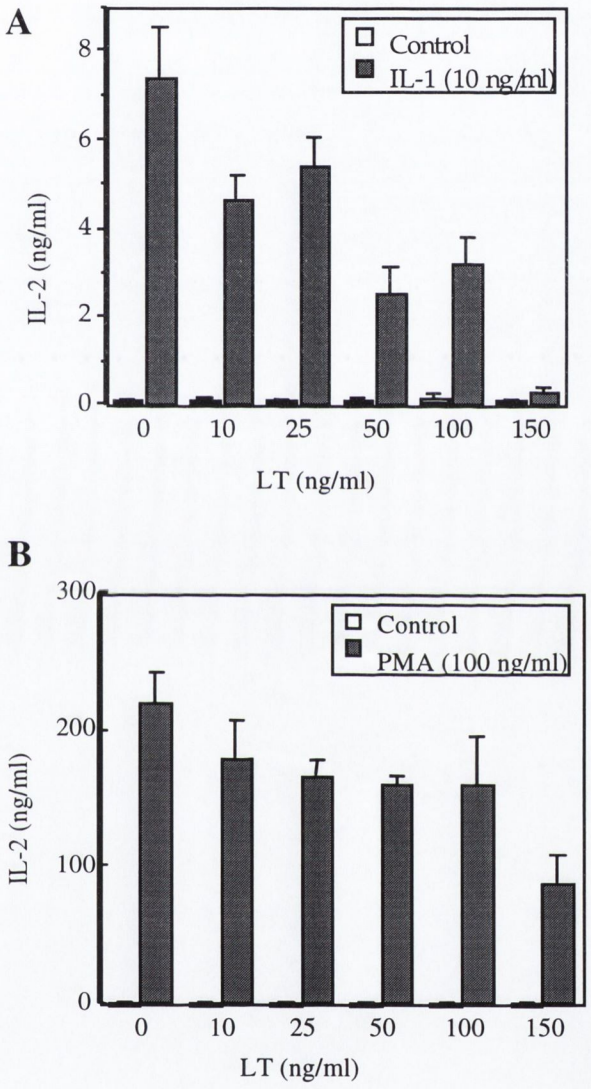


FIGURE 3.1.2

Effect of LT on IL-1α and PMA induced IL-2 expression. $1x10^6$ EL4.NOB-1 cells were incubated with indicated doses of LT for 30 minutes. The cells were then stimulated with 10 ng/ml IL-1α (A) or 100 ng/ml phorbol 12-myristate 13-acetate (PMA, B) for 22 hours after which the cell were centrifuged (1000xg, 10 minutes) and the supernatants were removed and assayed for IL-2. Results are shown as one single representative experiment out of three performed, expressed as mean ± SD from triplicate determinations.

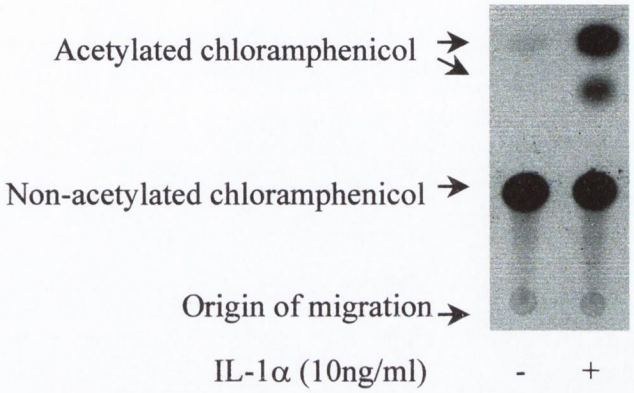


FIGURE 3.1.3

Representative autoradiographic film showing typical results from the chloramphenicol acetyl transferase assay (CAT assay) as described in the methods section. In brief, $2.5x10^5$ EL4.NOB-1 cells were transiently transfected with a plasmid construct encoding the chloramphenicol acetyl transferase (CAT) gene downstream of five kB binding elements. After a 20 hour recovery time the cells were stimulated with 10 ng/ml IL-1α for 16 hours after which whole cell lysates were prepared and assayed for CAT activity. The two upper spots represents the acetylated forms of chloramphenicol, and the bottom spot represents non-acetylated chloramphenicol.

the NFκB-CAT construct, stimulated with IL-1α at 10 ng/ml. The two uppermost spots in figure 3.1.3 represents chloramphenicol that have been acetylated at one or the other of two potential sites. Sometimes a third faster migrating spot can be detected, corresponding to diacetylated chloramphenicol. The lowest, intense spot represents non-acetylated chloramphenicol. Figure 3.1.4 show how IL-1α (A) and PMA (B) both are potent activators of NFκB, causing the transcription of the CAT reporter gene in EL4.NOB-1 cells. The radioactivity of the acetylated chloramphenicol in relation to the non-acetylated chloramphenicol gives a measure of the CAT activity of each sample. This measure (% acetylation) is achieved when dividing the cpm of the acetylated chloramphenicol with the sum of the cpm of acetylated chloramphenicol and non acetylated chloramphenicol. The effect of IL-1 was optimal at the lowest point tested (0.1 ng/ml), while an optimal effect of PMA was observed at 50 ng/ml. Figure 3.1.5 shows the results when assaying the supernatants of EL4.NOB-1 cells transfected with the NFκB-CAT construct, for IL-2 after stimulation with IL-1α (A) or PMA (B). The cells maintain their ability to produce IL-2 in response to IL-1 after being transiently transfected with the construct, although not to the same extent as untransfected EL4.NOB-1 cells (fig 3.1.1). IL-1α induced CAT expression and IL-2 production in EL4.NOB-1 cells transiently transfected with the NFκB-CAT expression vector was inhibited by LT (fig 3.1.6). 150 ng/ml LT causes a nearly total inhibition of IL-1 induced NFκB-CAT expression, and IL-2 production in these cells.

3.1iii Prolonged exposure of EL4.NOB-1 cells to LT gives rise to toxicity.

Reports in the literature have suggested that, although LT at the doses used here is not toxic to cells, prolonged exposure can cause morphological changes and toxicity(Bette *et al.*, 1991; Popoff, 1987). To test whether the results obtained using the above assays were specific, and not caused by general toxicity to the cells, I performed a comparative assay, looking at toxicity in parallel with inhibitory effect of LT on IL-1 induced IL-2 expression (fig 3.1.7). The experiment described in figure 3.1.2 was repeated and EL4.NOB-1 cells were pre-treated with increasing doses of LT prior to stimulation with IL-1α or PMA for 22 hours (fig 3.1.8). At this time point, cell viability was measured by analysing cell uptake of trypan blue, which indicates membrane leakage, i.e. non-viable cells. When IL-1 induced IL-2 production was inhibited by 50

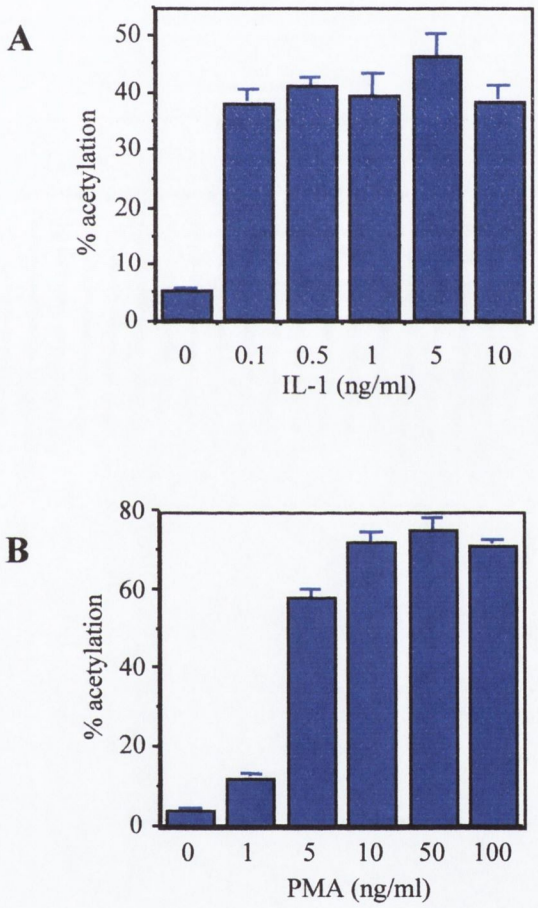


FIGURE 3.1.4

IL-1 and PMA induced activation of NFκB. $2.5x10^5$ Murine thymoma EL4.NOB-1 cells were transiently transfected with a plasmid construct encoding chloramphenicol acetyl transferase down stream of five NFκB binding elements. After a 16 hour recovery period the cells were stimulated with IL-1α (A) or phorbol 12-myristate 13-acetate (PMA, B) for 22 hours, lysed and assayed for CAT activity. Result show one representative out of two identical experiments performed, expressed as mean ± SD for triplicate determinations.

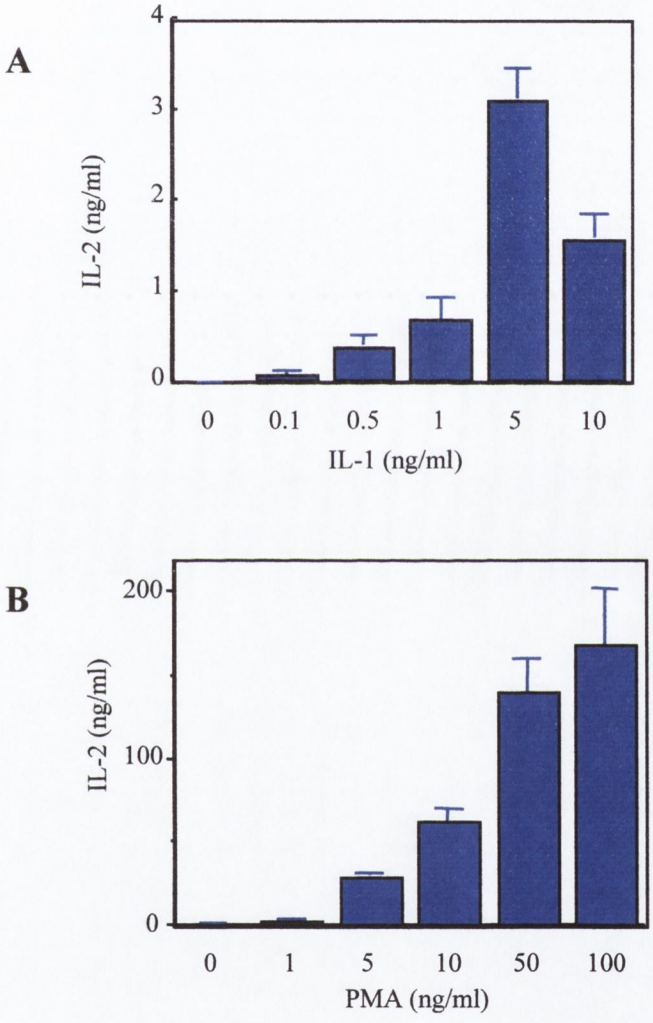


FIGURE 3.1.5

IL-1 and PMA induced expression of IL-2 in EL4.NOB-1 cells transfected with NFκB-CAT. $2.5x10^5$ Murine Thymoma EL4.NOB-1 cells were transiently transfected with a construct encoding chloramphenicol acetyl transferase down stream of five consensus NFκB binding sites. 16 hours after the transfection the cells were stimulated with IL-1α (A) or phorbol 12-myristate 13-acetate (PMA, B) for 22 hours after which the supernatants were removed and assayed for IL-2. Results show one representative out of two identical experiments performed, expressed as mean ± SD for samples assayed in triplicates.

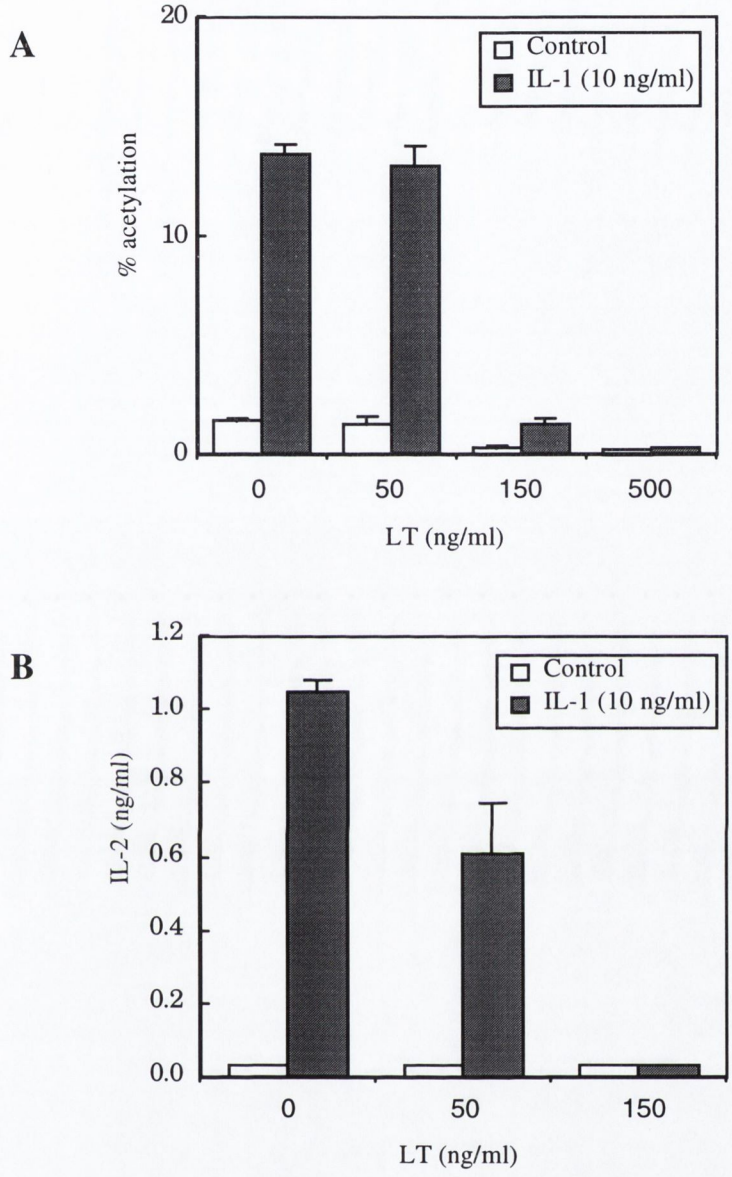


FIGURE 3.1.6

Effect of LT on IL-1 induced activation of NFkB, and expression of IL-2 in EL4.NOB-1 cells. $2.5x10^5$ Murine thymoma cells, EL4.NOB-1, were transiently transfected with a construct coding for chloramphenicol acetyl transferase downstream of five NFκB sites. After a 16 hour recovery period, the cells were pretreated with LT for 30 minutes and then stimulated with IL-1α for 22 hours. Cell lysates were analysed for CAT activity (A) as described in the methods section and the IL-2 concentration of the supernatants was measured by ELISA (B). Results show one representative out of three identical experiments performed, expressed as mean ± SD for triplicate determinations.

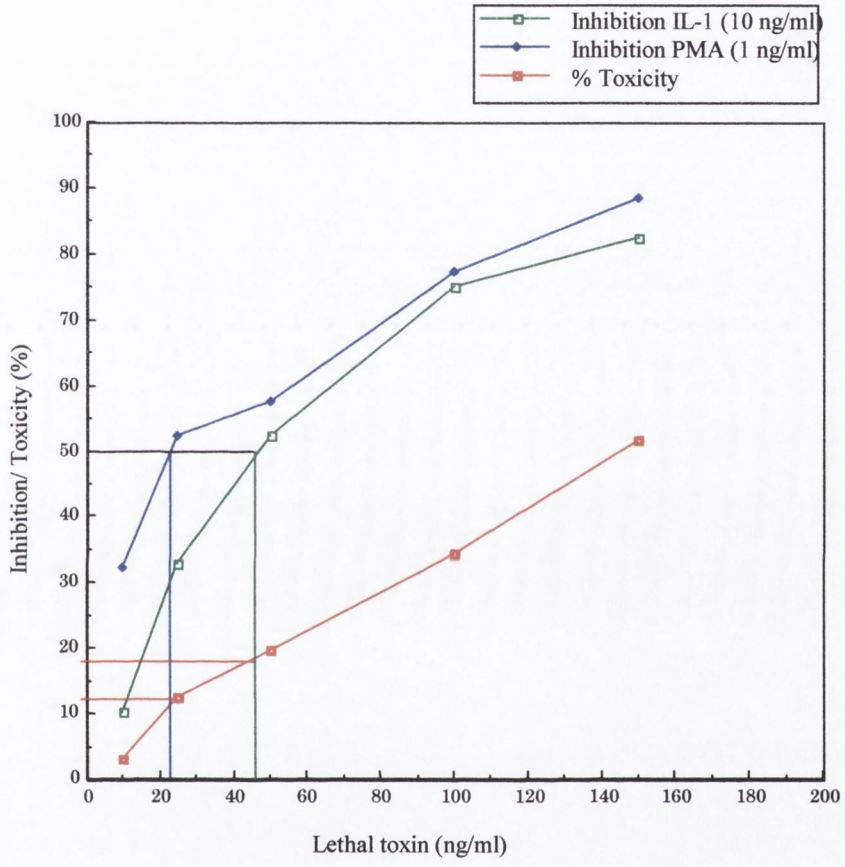


FIGURE 3.1.7

Comparison of toxicity and inhibitory effect of LT in EL4.NOB-1 cells. $1x10^6$ EL4.NOB-1 cells were incubated with indicated doses of LT for 30 minutes. The cells were then stimulated with 10 ng/ml IL-1α (A) or 1 ng/ml phorbol 12-myristate 13-acetate (PMA, B) for 22 hours after which the cells were centrifuged (1000xg, 10 minutes) and the supernatants were removed and assayed for IL-2. At this time point, cell samples were taken and toxicity was measured by staining with trypane blue as described in the methods section. Results show one representative of two identical experiments performed. An inhibitory effect of 50% of IL-1 induced IL-2 corresponded to a toxicity of 18%, whereas a 50% inhibition of PMA induced IL-2 corresponded to a mere 12% toxicity.

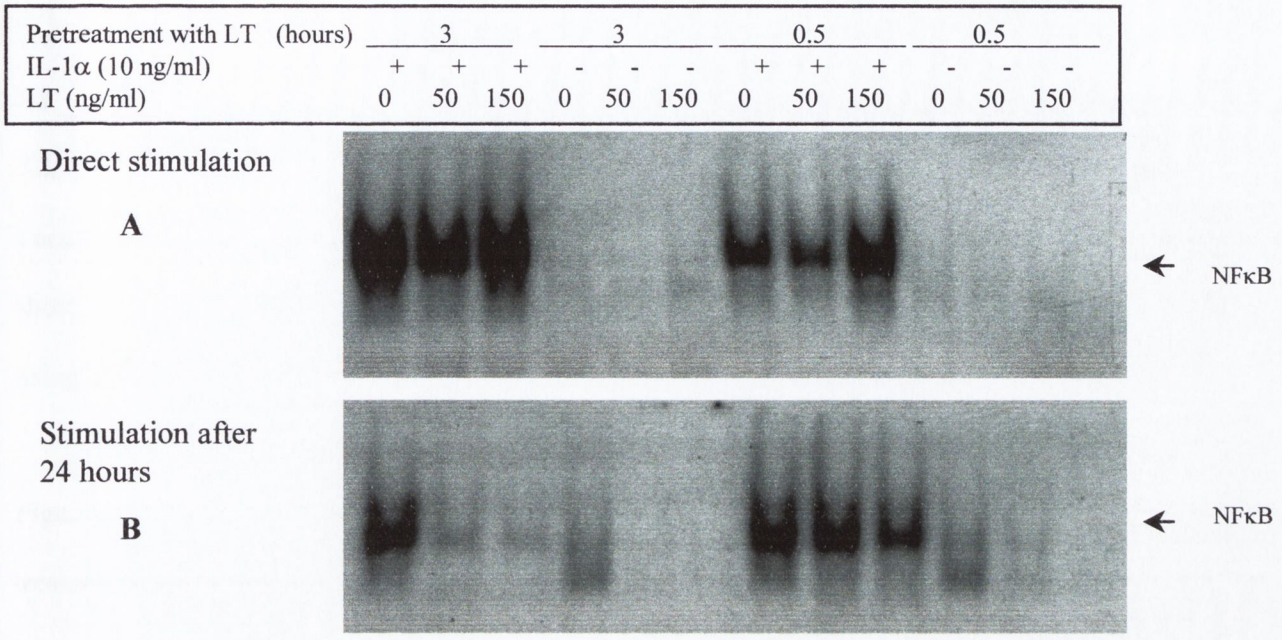


FIGURE 3.1.8

Effect of LT on IL-1 induced NFκB binding to DNA. $5x10^6$ Murine thymoma EL4.NOB-1 cells were pretreated for 30 minutes or 3 hours with indicated doses of LT. The cells were then directly stimulated with 10 ng/ml IL-1α for 1 hour (A) or left for a further 24 hour (B) before stimulation with IL-1α. Nuclear extracts were prepared as described in the methods section and an electrophoretic mobility shift assay was performed to measure activated NFκB in the cells. NFκB-DNA complexes are shown.

%, 18% toxicity was evident. When PMA was used as a stimulus, 12% toxicity was evident at 50 % inhibition. The results indicated that although the level of toxicity did not correlate directly with the inhibition, it was of a sufficient level to question the specificity of the effect.

3.2 Effect of LT on earlier IL-1 responses

3.2i Lack of effect of LT on the activation of NFκB by IL-1.

I next decided to measure a more rapid response to IL-1, since the cells would be exposed to the toxin for a short time, whereby toxicity would be minimal. The first signal choice was NFκB activation, as measured using an electrophoretic mobility shift assay.

Figure 3.1.8A shows how LT does not inhibit NFκB activation by IL-1 in EL4.NOB-1 cells which had been treated with increasing doses of LT for 30 minutes or 3 hours prior to stimulation with 10 ng/ml IL-1 for 1 hour. This of course is in contrast to the NFκB-CAT assay as seen in figure 3.1.6. Another experiment was therefore performed (3.1.8B), where the cells were stimulated with IL-1 24 hours after being pre-treated with LT, as to re-create the conditions of the CAT assay of figure 3.1.6. During these circumstances, an inhibitory effect of LT on the activation of NFκB by IL-1 became evident. Since there was evidence of toxicity in the more prolonged protocol it was possible that these effects were due to toxicity.

In support of the lack of effect of LT on NFκB activation as examined by EMSA, figure 3.1.9 demonstrates that LT has no effect on IL-1 induced degradation of IκB, as treatment with up to 500 ng/ml LT for 4 hours, followed by stimulation with 10 ng/ml IL-1 for 1 hour, caused full degradation of IκB in response to IL-1.

3.2ii LT inhibits IL-1α activated p38 MAPK in EL4.NOB-1 cells.

I next examined the effect of LT on the activation of p38 MAPK by IL-1. Figure 3.2.1A illustrates how IL-1α causes phosphorylation and therefore activation of p38 MAPK within a few minutes, peaking after about 10 minutes stimulation and reaching basal levels again after 60 minutes. Extract from Anisomycin treated C6

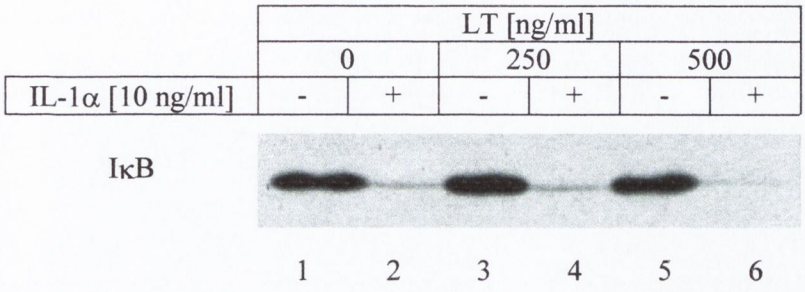


FIGURE 3.1.9

Effect of LT on IL-1α induced degradation of IκBα. EL4.NOB-1 cells (8 x 10^6 in 4 ml) were incubated with indicated concentrations of LT (0-500 ng/ml) for 4 hours at 37°C. Extracts were prepared as described in Experimental Procedures, after which the levels of IκBα could be measured by western blotting. Identical results were obtained in a further experiment.

cells was used as a positive control. To ensure equal loading of total cellular p38 MAPK, a phosphorylation status independent anti-p38 MAPK antibody was used, and the results from this blotting are shown in 3.2.1B. Whole cell extract from C6 cells was used as a positive control (data not shown).

Most importantly, I found this response to be sensitive to LT. As shown in figure 3.2.2, EL4.NOB-1 cells were treated with increasing doses of LT for 3 hours after which they were stimulated with IL-1α for 10 minutes and analysed for active p38 MAPK by western blotting using an antibody specific for phosphorylated p38 MAPK (A). 500 ng/ml LT gives rise to a near total inhibition of the activation of p38 MAPK by IL-1. Figure 3.2.2B illustrates loading of total p38 MAPK for each sample of this experiment with no differences being observed. LT also inhibits the activation of p38 MAPK by IL-1α in a time dependent manner (figure 3.2.3A). EL4.NOB-1 cells were pre-incubated with 500 ng/ml LT for 1, 2 or 4 hours, prior to stimulation with IL-1α for 10 minutes and assaying the cells for p38 MAPK activity by western blotting. Again a strong inhibition of the activation of p38 MAPK by IL-1 occurs at four hours pre-treatment with 500 ng/ml LT. Equal loading of total p38 MAPK was ensured by using a phosphorylation state independent anti p38 MAPK antibody. Toxicity was monitored by staining cells with trypan blue, and closely monitoring procaspase-3 processing (as a marker for apoptosis(Polverino and Patterson, 1997)). No difference in cell viability could be established by trypane blue staining (data not shown) or by caspase-3 analysis (figure 3.2.4).

Phorbol esters are known not to activate the p38 MAPK signalling pathway (Sanghera *et al.*, 1996), and this was confirmed when EL4.NOB-1 cells were preincubated with PMA for 10 minutes (fig 3.2.5A), as PMA could not activate p38 MAPK.

3.2iii LT inhibits the activation of p42 and p44 MAPK by IL-1α and PMA in EL4.NOB-1 cells.

I next examined another signal, p42/p44 MAPK activation. In my system, IL-1α induces phosphorylation, and activation, of p42 MAPK and to a lesser extent p44 MAPK in EL4.NOB-1 cells (fig 3.2.6 A). Maximum

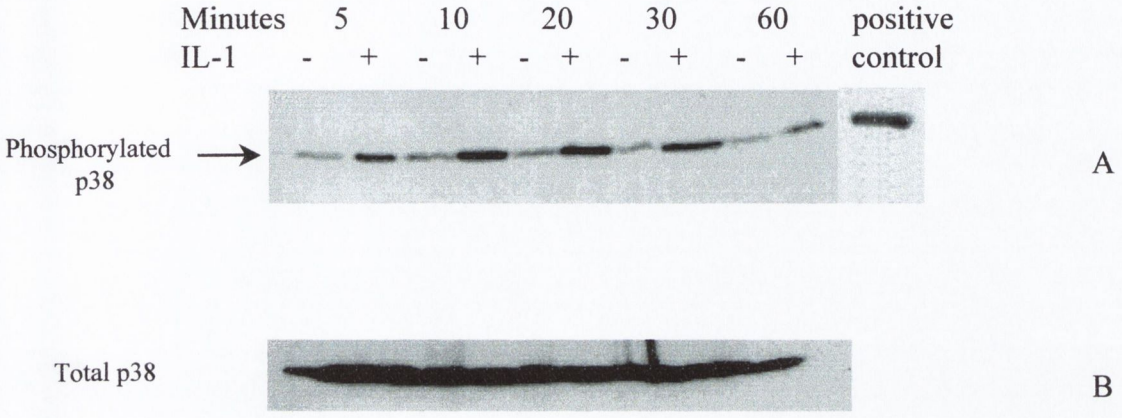


FIGURE 3.2.1.

IL-1 activates p38 MAPK in EL4.NOB-1 cells. $8x10^6$ murine thymoma EL4.NOB-1 cells in 4 ml of medium were treated with 10 ng/ml IL-1α for times indicated above. Cell lysates were prepared and the phosphorylation status of p38 MAPK was measured by western blotting, described in the method section. Results show one representative experiment out of two performed. (A) represents autoradiographic film from western blotting using an antibody recognising phosphorylated p38 MAPK and (B) represents autoradiographic film from western blotting using phosphorylation state independent anti-p38 MAPK antibody, which measures total p38 MAPK in each extract and acts as a loading control. (A) shows the result from the positive control of Anisomycin treated C6 cell extracts, showing the migration of phosphorylated p38.

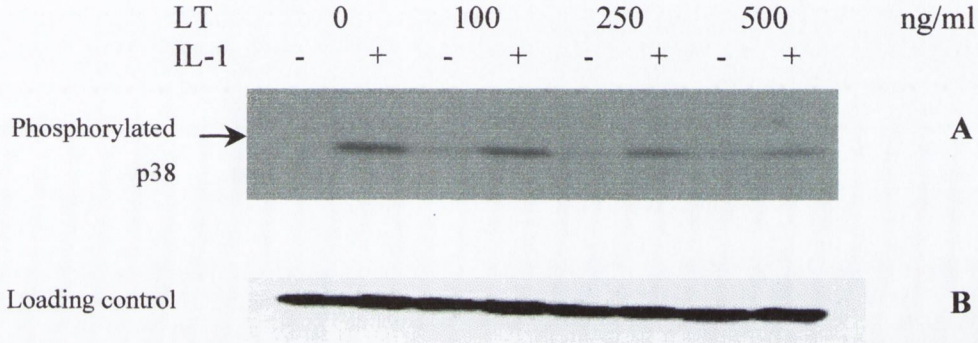


FIGURE 3.2.2

Effect of LT on the activation of p38 MAPK by IL-1. $8x10^6$ murine thymoma EL4.NOB-1 cells in 4 ml of medium were treated with indicated doses of LT for 3 hours. The cells were subsequently stimulated with 10 ng/ml IL-1α for 10 minutes after which cell lysates were prepared as described in the methods section. The samples were analysed for phosphorylated p38 MAP kinase by western blotting as described above in the methods section. (A) represents autoradiographic film from western blots using an antibody for phosphorylated anti-p38 MAPK, and (B) represents autoradiographic film from western blotting using a phosphorylation state independent p38 MAP kinase antibody, measuring total p38 MAPK of each extract acting as a loading control. Results show one representative of four identical experiments performed.

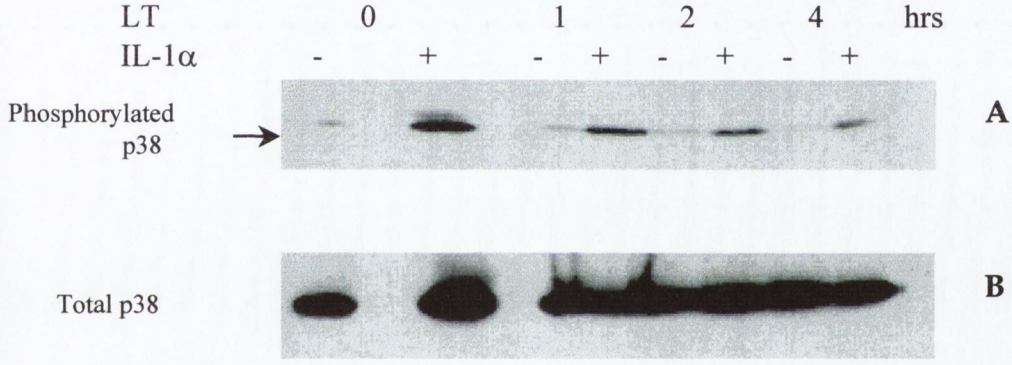


FIGURE 3.2.3

Effect of LT on the activation of p38 MAPK by IL-1. $8x10^6$ murine thymoma EL4.NOB-1 cells in 4 ml of medium were pretreated with 500 ng/ml of LT for times indicated above. The cells were subsequently stimulated with 10 ng/ml IL-1α for 10 minutes after which cell lysates were prepared as described in the method section. The samples were analysed for phosphorylated p38 MAP kinase by western blotting as described above in the method section. (A) represents autoradiographic film from western blotting with an antibody that recognises phosphorylated p38 MAP kinase, and (B) represents autoradiographic film from blots treated with phosphorylation state independent anti-p38 MAP kinase antibody to ensure that equal amounts of p38 MAPK were being analysed.

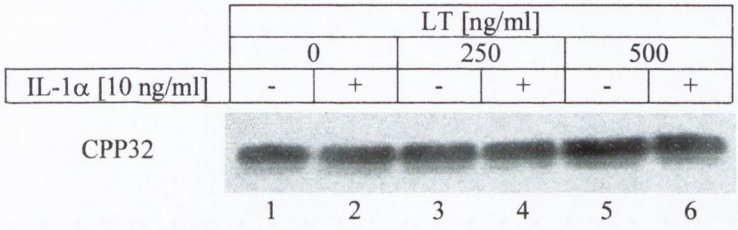


FIGURE 3.2.4

Effect of LT on IL-1α induced degradation of pro-caspase 3 (CPP32), as a marker for apoptosis. EL4.NOB-1 cells (8 x 106 in 4 ml) were incubated with indicated concentrations of LT (250-500 ng/ml) for 4 hours at 37°C. Extracts were prepared as described in Experimental Procedures, after which the levels of IκBα, and pro-caspase 3 could be measured by western blotting. Identical results were obtained in a further experiment.

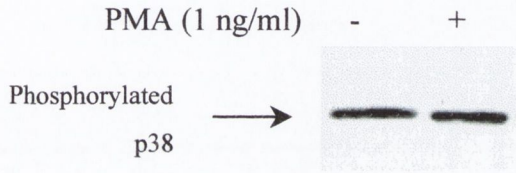


FIGURE 3.2.5

PMA does not activate p38 MAPK in EL4.NOB-1 cells. $8x10^6$ murine thymoma EL4.NOB-1 cells in 4 ml of medium were treated with 1 ng/ml phorbol 12-myristate 13-acetate (PMA) for 10 minutes after which cell lysates were prepared as described in methods above. The samples were analysed for phosphorylated p38 MAPK by western blotting. Figure represents autoradiographic film of samples treated with an antibody that recognises phosphorylated p38 MAPK from one of four identical experiments performed. Equal loading for each sample was ensured by blotting with an antibody that recognises total p38 MAPK.

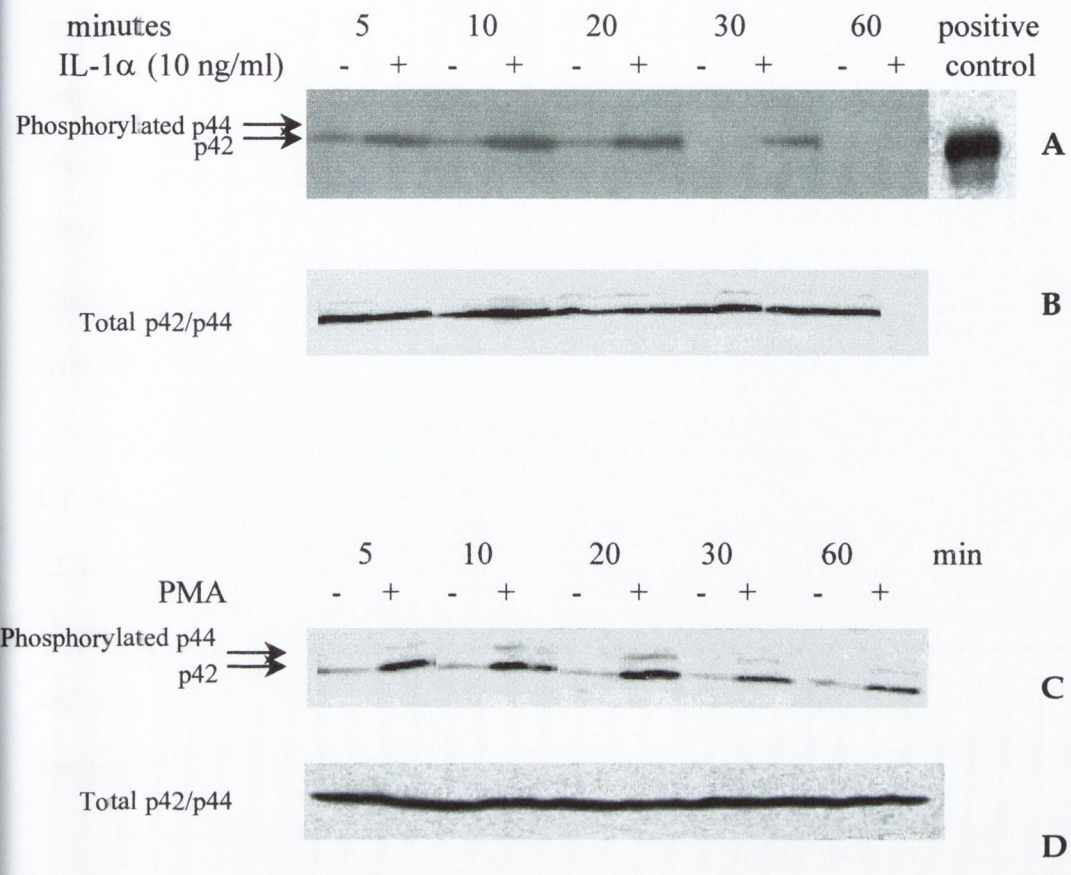


FIGURE 3.2.6

IL-1 and PMA activate p42 and p44 MAPK in EL4.NOB-1 cells. Murine thymoma EL4.NOB-1 cells were seeded at high density in RPMI 1640 with 0.5% FCS (serum starve) 50 hours prior to the experiment. $8x10^6$ EL4.NOB-1 cells in 4 ml medium were stimulated with IL-1α (10 ng/ml, A and B) or phorbol 12-myristate 13-acetate (PMA, 1 ng/ml, C and D) for times indicated above. Cell extracts were prepared as described in the methods section and the activation of p42/p44 MAPK was measured by western blotting. (A) and (C) represents autoradiographic films of western blotting using an antibody that recognises phosphorylated p42/p44 MAPK. (B) and (D) represents western blotting using a p42/p44 MAPK phosphorylation state independent antibody, to ensure equal loading for each sample. Positive control of recombinant p42 MAPK run for all western blots is shown in A. Results show one representative out of two identical expriments performed.

activation occurs after 10 minutes, reaching basal levels again within 60 minutes stimulation with IL-1. Fully phosphorylated recombinant p42 MAPK was loaded as a positive control. Figure 3.2.6 B shows equal loading of the MAPK proteins using western blotting with a primary antibody detecting p42 and p44 MAPK independent of phosphorylation status. Recombinant p42 MAPK was used as a positive control (data not shown). Figure 3.2.6 C illustrates how PMA can activate p42 and p44 MAPK in EL4.NOB-1 cells using the same western detection technique as above. The activation of p42/p44 MAPK by PMA peaks after about 10 minutes stimulation, reaching basal levels within 60 minutes. Figure 3.2.6D shows results from detection with the p42/p44 MAPK antibody detecting total p42 and p44 MAPK in the samples.

LT inhibited the activation of p42 and p44 MAPK by PMA in a dose dependent manner (fig 3.2.7A). EL4.NOB-1 cells were pre-treated with increasing doses of LT for 4 hours after which the cells were stimulated with PMA (1 ng/ml) for 10 minutes. 500 ng/ml LT almost totally inhibited the activation of p42 and p44 MAPK by PMA. Total p42 and p44 MAPK content of each sample was analysed to ensure equal loading (fig 3.2.7 B).

LT also inhibits the activation of p42 and p44 MAPK by PMA in a time dependent manner as shown in figure 3.2.8A. EL4.NOB-1 cells were incubated with 100 ng/ml LT for 1, 2, or 4 hours. The cells were then stimulated with PMA, 1 ng/ml, for 10 minutes, and p42 and p44 MAPK activity was analysed by western blotting. 4 hours pre-treatment with 100 ng/ml LT nearly totally blocked the activation of p42 MAPK by PMA, and inhibited all detectable phosphorylated p44 MAPK in these cells. 3.2.8B illustrates total p42 and p44 MAPK contents of each sample.

I next attempted to demonstrate inhibition of p42/p44 MAPK activated by IL-1. Basal activity however proved to be elevated over the 4 hour treatment time and no activation could be detected (data not shown). This was not evident in the PMA experiment as PMA was a much stronger stimulus and a shorter exposure time to the autoradiographic film in the assay did not reveal the basal activity. I therefore shortened the pre-treatment time with LT in order to limit basal activity, as such activity would not be expected from the time

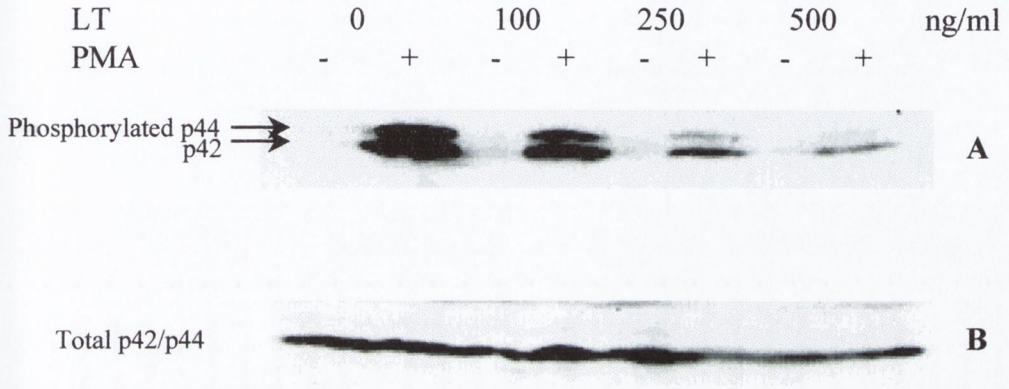


FIGURE 3.2.7 **Effect of LT on PMA activated p42/p44 MAPK in EL4.NOB-1 cells.** Murine thymoma EL4.NOB-1 cells were seeded at high density in RPMI 1640 with 0.5% FCS (serum starving) 50 hours prior to the experiment. $8x10^6$ EL4.NOB-1 cells in 4 ml medium were treated with LT at doses indicated above. After 4 hours the cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 1 ng/ml) for 10 minutes. After cell extracts were prepared as described in the methods secion, the phosphorylation status of the p42/p44 MAPK was determined using western blotting as described. (A) represents autoradiographic film of western blotting using an antibody that recognises phosphorylated p42/p44 MAPK. (B) represents western blot treated with a phosphorylation state-independent p42/p44 antibody, that recognises total p42/p44 and acts as a loading control. Results show one representative of three identical experiments performed.

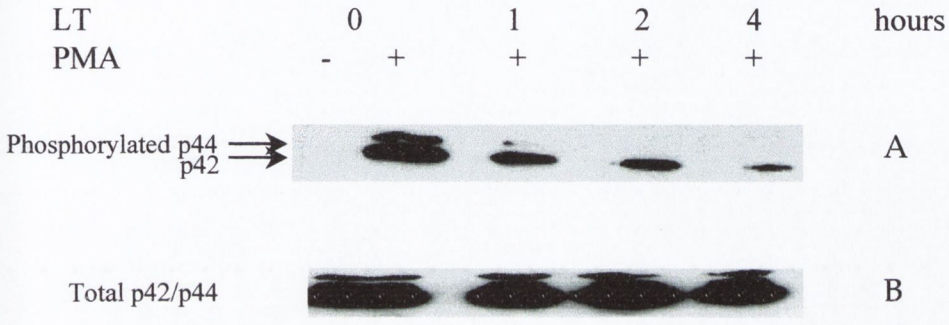


FIGURE 3.2.8 **Effect of LT on PMA activated p42/p44 MAPK in EL4.NOB-1 cells.** Murine thymoma EL4.NOB-1 cells were seeded at high density ($3x10^{6}$ ml^{-1}) in RPMI 1640 with 0.5% FCS (serum starving) 50 hours prior to the experiment. $8x10^{6}$ EL4.NOB-1 cells in 4 ml medium were treated with LT (100 ng/ml) for times indicated above. The cells were then stimulated with phorbol 12-myristate 13-acetate (PMA) for 10 minutes, cell extracts were prepared and the phosphorylation status of p42/p44 MAP kinase of the cells was analysed using western blotting as described. (A) represents autoradiographic film of western blotting using an antibody that recognises phosphorylated p42/p44 MAPK. (B) represents western blot treated with an antibody that recognises total p42/p44 MAPK which acts as a loading control. Results show one representative out of three identical experiments performed.

course study (figure 3.2.6A). As can be seen in figure 3.2.9A, basal activity of p42/p44 MAPK after a preincubation time of 1 hour was much lower compared to a preincubation time of 4 hours (simulating the pretreatment with LT) and a clear activation by IL-1 was observed after the shorter incubation time. This activation of p42/p44 MAPK was potently inhibited by pretreating the cells for 1 hour with LT (fig 3.2.9C) with 250 ng/ml LT having the optimal effect. Figures 3.2.9B and 3.2.9D show the results from western blotting using a phosphorylation state independent anti p42 and p44 MAPK antibody. Throughout the p42/p44 western blotting experiments using LT, toxicity was monitored by trypan blue staining, and was never shown to be significantly increased compared to non-treated cells.

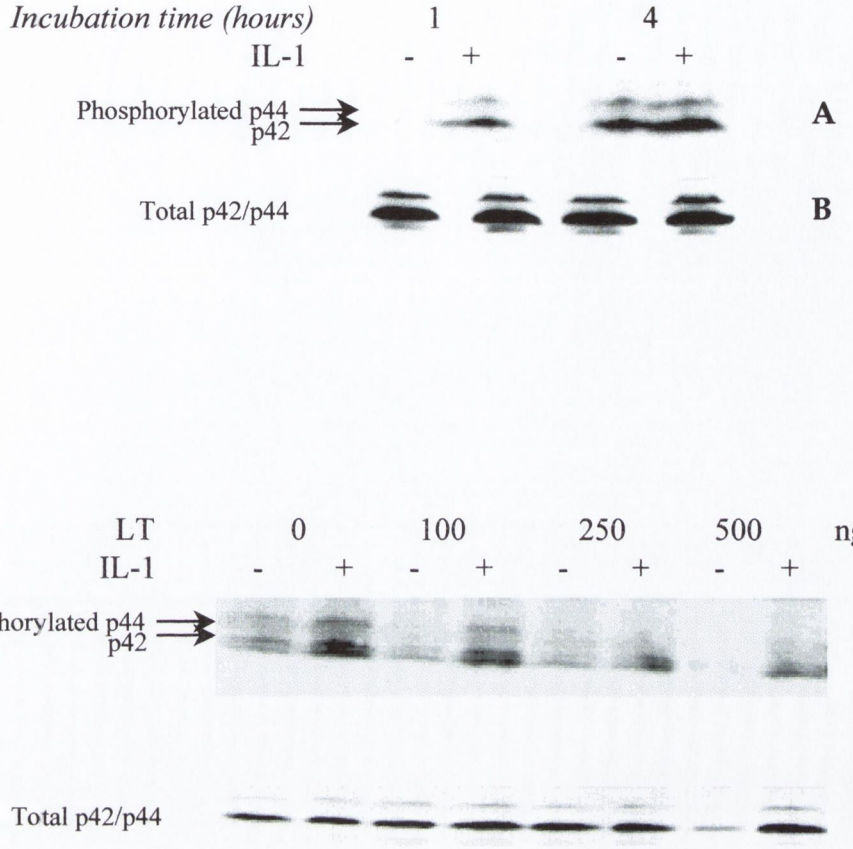


FIGURE 3.2.9 **Effect of LT on the activation of p42/p44 MAPK by IL-1 in EL4.NOB-1 cells.** Murine thymoma EL4.NOB-1 cells were seeded at high density ($2x10^6$ ml^{-1}) in RPMI 1640 with 0.5% FCS (serum starving) 50 hours prior to the experiment. $8x10^6$ EL4.NOB-1 in 4 ml medium were left in medium for 1 or 4 hours (A and B) to mimic treatment with LT. In (C) and (D) EL4.NOB-1 cells were pretreated with LT for 1 hour at doses indicated above. The cells were then stimulated with IL-1α (10 ng/ml) for 10 minutes, cell extracts were prepared, and the phosphorylation status of p42/p44 MAPK was analysed using western blotting as described in the methods section. (A) and (C) represent autoradiographic film of western blots using an antibody that recognises phosphorylated p42/p44 MAPK. (B) and (D) represents corresponding samples treated with a phosphorylation state independent p42/p44 MAPK antibody which acts as a loading control. The results depicted in C and D show one representative out of two identical experiments performed.

DISCUSSION

IL-1 exerts its effects by activating multiple signalling pathways in the cells. In order to investigate the role of small G proteins in IL-1 signalling I first studied the effect of LT on two of these signals: IL-1 induced IL-2 expression and NFκB activation. The cell line chosen for these studies was a murine thymoma cell line EL4.NOB-1, which was isolated from EL4 cells since they displayed an increased number of IL-1R on the cell surface. This elevated level of IL-1R causes the cells to be highly responsive to IL-1, and has since been a well studied system for IL-1 signal transduction. IL-1 causes a strong increase in the expression of IL-2, as does PMA, which was used throughout these experiments as a positive control.

IL-1 still causes an increased expression of IL-2 in cells transfected with NFκB –CAT, although not to the same extent as in untransfected cells. Furthermore, stimulating these cells with an increased amount of IL-1 does not improve the expression level of IL-2, but sometimes causes a slight reduction compared to the lower doses of IL-1 (figure 3.1.5A). The reason for this is thought to be competition for transcription machinery by the NFκB–CAT construct and the IL-2 gene in cells transfected with NFκB –CAT. As a result, the levels of IL-2 induced by IL-1 in these cells are generally 5-10 times lower than in untransfected cells. PMA is a very strong inducer of IL-2 in these cells, in fact it is so strong that the PMA induced IL-2 expression is unaffected in cells transfected with NFκB–CAT.

Pretreating EL4.NOB-1 cells with LT inhibits IL-1- as well as PMA-induced expression of IL-2. Furthermore, pretreating cells transfected with NFκB–CAT with LT causes an inhibition of IL-1 induced activation of NFκB and consequently a reduction in CAT activity. These results initially suggested to us that a small G protein targeted by LT was involved in IL-1 induced activation of NFκB and the expression of IL-2 in these cells.

LT has been reported to cause morphological changes to cells exposed for a prolonged period of time to the toxin(Bette *et al.*, 1991). Furthermore, although at doses much higher than what was used in this study, LT

can cause other toxic effects. While no morphological changes were evident 24 hours after first contact with LT, I nevertheless wanted to further investigate the toxic effects of LT in our cell system. Since EL4.NOB-1 cells were stimulated with IL-1 for 22-24 hours in order to express sufficiently increased levels of IL-2 and CAT, the cells consequently were left for up to 24 hours after the first contact with the toxin. While the cells were washed with medium before stimulation with IL-1 for 22 hours, some LT may have already been taken up by the cells and could consequently not be washed off. As a measure of inhibitory effect versus toxicity, the cells that were used for the IL-2 assay were treated with trypan blue to measure viable cells in relation to non-viable cells. This was then directly compared to the inhibitory effect of LT on IL-1 induced IL-2 expression. Even though LT caused close to 100% inhibition of the IL-1 induced IL-2 expression and only a 45% toxicity at the higher doses used, at 50 ng/ml LT the inhibitory effect was 50% and the toxicity was close to 20%. Since trypan blue is a crude method of assaying toxicity, as only dead cells will take up the dye, cells that are affected by the toxicity of LT may still exclude the stain and will consequently be counted as viable. Lack of DNA laddering effect in response to LT proved that LT was not causing apoptosis in these cells.

I decided that the toxic effects evident in the IL-2 and NFκB–CAT assays, while not directly comparable to the inhibitory effect of LT, indicated that non-specific effects could not be ruled out. To avoid the problem of non-specific inhibitory effects of LT on later signalling events, I turned my investigation to studying the role of small G proteins in early signalling events of IL-1, which would involve short time exposures to LT, thus avoiding later toxic effects. As controls for the toxic effects of LT at these treatment times, rapid phosphorylation dependent events were studied. The first involved NFκB activation, as measured by EMSA and the degradation of the inhibitory subunit IκB in response to IL-1. The EMSA showed that LT did not inhibit NFκB activation. Similarly, the degradation of IκB was not affected by pre-treatment with LT.

Next I examined the activation of the 2 early response MAPK signalling cascades leading to the activation of p38 MAPK and p42/p44 MAPK. Most importantly, LT potently inhibited the activation of p38 MAPK by

IL-1. This suggested that at least one of the small G proteins Ras, Rap, Ral or Rac, (or a novel small G protein not yet identified as a target for LT), may be involved in the signal leading from IL-1 to the activation of p38 MAPK. PMA, although unable to activate p38 MAPK, is a potent activator of p42/p44 MAPK. This activation is thought to occur through the small G protein Ras, and PMA was therefore used as a control for the effect of LT. Accordingly LT strongly inhibited the activation of both p42 and p44 by PMA. Over the time course examined in these studies no toxicity was observed, either by trypan blue exclusion or by procaspase-3 processing (Polverino and Patterson, 1997).

IL-1 activates p42/p44 MAPK in EL4.NOB-1 cells, although not to the same extent as PMA does. When studying the activation of p42/p44 MAPK, the cells mush be starved from serum for at least 24 hours, preferably 48 hours, prior to the experiment, to reduce basal levels of active p42/p44 caused by factors in the serum. 2 hours prior to the experiment the medium must be changed to further reduce the basal level of activity. As IL-1 is not a very strong activator of this pathway, reduced basal activity of p42/p44 proved to be critical in order to study the effects of IL-1. When treating the cells with LT for 4 hours I noticed that the basal activity of p42/p44 had increased compared to when stimulating with IL-1 alone. In order to determine if this was a true effect of the toxin, I simulated the treatment-time with LT by leaving the cells in serum free medium for the 4 hour duration. This extended time in low serum medium was sufficient to cause an increase in basal activity of p42 and p44 MAPK, making it difficult to observe an additional activity caused by IL-1. When this pre-treatment time, on the other hand, was reduced to 1 hour, the activation of p42/p44 by IL-1 was readily detectable, as was the inhibition of this signal caused by pretreating the cells for 1 hour with up to 500 ng/ml LT. This suggested that a small G protein targeted by LT mediates the activation of p42/p44 MAPK by IL-1.

In conclusion, LT can be used as a tool for investigating the role of small G proteins in early event signal transduction. By using LT I found that none of the small G proteins, targeted by LT, were likely to be involved in the activation of NFκB by IL-1 up to the point of binding of NFκB to its consensus sequence

DNA. Furthermore, I found that a small G protein targeted by LT is involved in the activation of p38 MAPK and p42/p44 MAPK by IL-1 and also in the activation of p42/p44 MAPK by PMA. I then proceeded to attempt to identify the G protein involved.

CHAPTER 4

Exploring the target for Lethal Toxin

INTRODUCTION

Four isoforms make up the p38 MAPK family, p38α, p38β, p38γ and p38δ (Hale *et al.*, 1999). The signalling pathway leading to the activation of p38 by IL-1 is not as well elucidated as the pathway leading to the activation of NFκB (O'Neill and Greene, 1998), although early events in the two pathways share similarities, as both TRAF6 and IRAK1 have been shown to play a role in the activation of p38 MAPK by IL-1 (Thomas *et al.*, 1999) (Baud *et al.*, 1999; Kanakaraj *et al.*, 1998). p38 MAPK is activated by the MAPK kinases MKK3b (the predominant isoform of MKK3)(Han *et al.*, 1997b), and MKK6(Han *et al.*, 1996). A variety of MAPKKK can activate MKK3 and MKK6, including PAK, TAK1 and ASK1(Bagrodia *et al.*, 1995) (Moriguchi *et al.*, 1996) (Ichijo *et al.*, 1997).

As I have shown in Chapter 3, LT inhibits the activation of p38 MAPK by IL-1, suggesting that one of the small G proteins Ras, Rap, Rac or Ral (or a novel G protein not yet identified) must be involved in the activation of this pathway.

Studies using dominant negative RacN17 and Cdc42N17 have implicated these G proteins in p38 MAPK activation as well as in the activation of JNK (Coso *et al.*, 1995; Minden *et al.*, 1995). However, more recently, Ras was shown to convey the signal leading to the activation of p38 MAPK by hemopoietic cytokines (Efimova *et al.*, 1998; Rausch and Marshall, 1999). In addition, a role for Ras in p38 MAPK activation by both PDGF and FGF has been shown (Matsumoto *et al.*, 1999; Tan *et al.*, 1996).

Ras has previously been proposed to play a part in IL-1 signalling. Induction of the collagenase promoter by IL-1 in chondrocytes has been shown to require Ras (Grumbles *et al.*, 1997), as has induction of brain natriuretic peptide in myocytes (He and LaPointe, 1999). Others however have failed to demonstrate activation of Ras by IL-1 (Bird *et al.*, 1994).

The role for Rap in cell signalling is less clear. A number of extracellular signals including PDGF, EGF, endothelin and 1-oleoyl-lyso-phosphatidic acid (LPA), have recently been shown to activate Rap (Bos, 1998; Zwartkruis *et al.*, 1998), although a role for Rap in downstream events was not investigated. Recent reports have pointed to Rap as a positive regulator in cell signalling, although the major role for Rap appears to be as an antagonist towards Ras(Boussiotis *et al.*, 1997; Cook *et al.*, 1993; Hata *et al.*, 1990; Kitayama *et al.*, 1990; Kitayama *et al.*, 1989). The mechanism of this antagonism is likely to be due to competition for downstream effectors.

Ral, a small G protein expressed in human neutrophils, was also shown to be glucosylated by LT. The role for Ral in signal transduction however has not been studied in great detail (reviewed in (Bos, 1998)). Ral is activated by fMet-Leu-Phe, ionomycin and platelet-activating factor in primary human neutrophils, and the activation of Ral by 12-O-tetradecanoylphorbol-13-acetate occurs through protein kinase C(M'Rabet *et al.*, 1999; Voss *et al.*, 1999; Wolthuis *et al.*, 1998a; Wolthuis *et al.*, 1998b). As Ral-GEFs were found to bind directly to the active, GTP-bound form of Ras, Rap-1, R-Ras and TC21 (Herrmann *et al.*, 1996), Ral has been proposed to act as a downstream target for these small G proteins. Another report shows a role for Ral in receptor tyrosine kinase-induced phospholipase D stimulation in HEK-293 cells, mediated by a protein kinase C and a Ras/Ral signalling cascade(Voss *et al.*, 1999).

Attempts to identify small G proteins involved in IL-1 actions have, to date, relied on the use of dominant negative constructs of small G proteins, which can have non-specific effects and can give results difficult to interpret. While transient transfections are a very important technique, I have found that additional approaches must be taken in combination with these studies to avoid artefactual results. Using a naturally occurring virulence factor, such as LT when targeting a particular protein in the cell has, over the years, proven to be a highly specific approach which is less prone to non-specific effects.

In this part of the study I have sought to investigate further the role of small G proteins in the activation of p38 MAPK by IL-1. I have used 3 distinct approaches: (i) treatment of cells with the glucosyltransferase

Clostridium difficile Toxin B (LT related protein that specifically glucosylates and inhibits the small G proteins Cdc42, Rac, and Rho (Aktories, 1997)); (ii) treatment of cells with 2 Ras inhibitors, Manumycin A and Damnacanthal; and, finally (iii) transient transfection of cells with plasmids encoding mutant versions of Ras, Rac and Rap. My data strongly indicates a role for Ras in the activation of p38 MAPK by IL-1, with Rap having an antagonistic effect.

RESULTS

4.1 Effect of ToxB on the activation of p38 MAPK by IL-1α or PMA in EL4.NOB-1 cells.

4.1i ToxB does not inhibit IL-1α activated p38 MAPK or PMA activated p42 and p44 MAPK.

To further investigate the involvement of a small G protein in IL-1 signalling, *Clostridium difficile* Toxin B was used. This is a toxin that glucosylates and inhibits the small G proteins Rho, Rac and Cdc42 from the Rho sub-family of small G-proteins. In other words, its substrate specificity overlaps with that of LT [Rac] but differs in that it does not affect the Ras sub-family. Figure 4.1.1A shows how ToxB was unable to inhibit the activation of p38 MAPK by IL-1α in EL4.NOB-1 cells. The cells were pre-treated with increasing doses of ToxB for 1 hour, and subsequently stimulated with 10 ng/ml IL-1α for 10 minutes. The phosphorylation status of p38 MAPK was then analysed by western blotting. Figure 4.1.1B illustrate the results from western blotting of the same samples using a phosphorylation state independent p38 antibody to measure total p38 MAPK.

ToxB was also unable to inhibit the activation of p42 and p44 MAPK by PMA in EL4.NOB-1 cells. Figure 4.1.2A illustrates this with the results from a western blotting with samples generated from EL4.NOB-1 cells that had been treated with increasing doses of ToxB prior to stimulation with PMA for 10 minutes. Figure 4.1.2B B show the results from corresponding samples analysed by western blotting using a phosphorylation state independent anti-p42 and p44 MAPK antibody, which helps to ensure equal loading. Morphological changes such as clumping of EL4.NOB-1, characteristic of the effects of ToxB, confirmed that the toxin was taken up by the cells (data not shown).

4.2 Exploring the target proteins for LT and Toxin B in EL4.NOB-1 cells

4.2i LT glucosylates at least 3 proteins of approximately 18,19 and 23 kDa in EL4.NOB-1 cells.

As both LT and Toxin B are glucosyltransferases, this enzymatic ability can be utilised when studying the target proteins in the cells. A glucosylation assay can be performed by exposing whole cell lysates to the

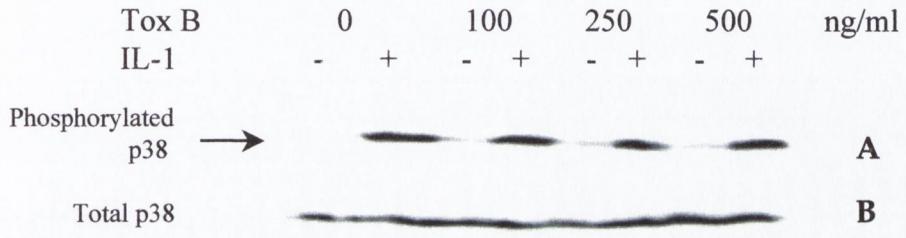


FIGURE 4.1.1

Effect of Toxin B on the activation of p38 MAPK by IL-1 in EL4.NOB-1 cells. $8x10^6$ murine thymoma EL4.NOB-1 cells were pretreated with indicated doses of ToxB for 1 hour. The cells were subsequently stimulated with 10 ng/ml IL-1α for 10 minutes after which cell lysates were prepared as described in the methods section. The samples were analysed for phosphorylated p38 MAPK by western blotting as described in the methods section. (A) represents autoradiographic film from western blotting using an antibody for phosphorylated p38 MAPK, and (B) represents autoradiographic film from western blot treated with phosphorylation state independent anti-p38 MAPK antibody which measures the total p38 MAPK contents of the extracts and acts as a loading control. Results show one representative of three identical experiments performed.

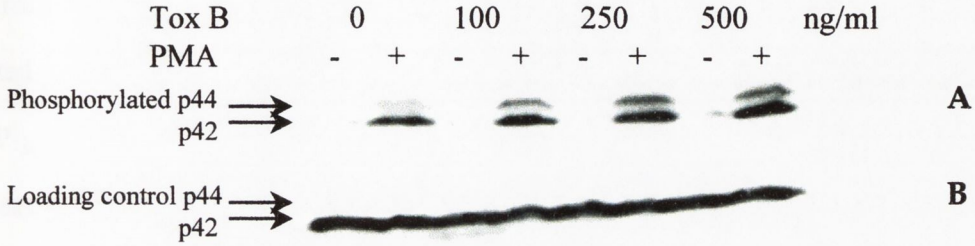


FIGURE 4.1.2

Effect of Toxin B on the activation of p42/p44 MAPK by PMA. Murine thymoma EL4.NOB-1 cells were seeded at high density in RPMI 1640 with 0.5% FCS, 50 hours prior to the experiment (serum starve). $8x10^6$ EL4.NOB-1 cells in 4 ml medium were treated with ToxB at doses indicated above. After 4 hours the cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 1 ng/ml) for 10 minutes. Cell extracts were than prepared and the phosphorylation status of p42/p44 MAP kinase of the cells were detected using western blotting as described in the methods section. (A) represent autoradiographic film of western blotting using an antibody that recognises phosphorylated p42/p44 MAPK. (B) represent western blots treated with an antibody that recognises p42/p44 MAPK independent of phosphorylation status, which measures total p42/p44 MAPK in each extract acting as a loading control.

toxin in the presence of radio-labelled UDP-glucose. The glucosylated and radiolabelled target proteins can subsequently be visualised carrying out an SDS-PAGE and autoradiography. Figure 4.2.1 illustrates that the major target for glucosylation by LT is a protein of 20 kDa (compare lane 2 to lane 1). In non-LT-treated cells endogenous glucosylation of a number of proteins can be seen (lane 1).

In order to verify that LT can enter the cell and exert its action in intact cells, EL4.NOB-1 cells were pre-treated with LT, and subsequently lysed and treated *in vitro* with the toxin in the presence of [^{14}C]-labelled UDP-glucose as shown in figure 4.2.2. The results show how the substrate for the toxin becomes increasingly glucosylated with increasing treatment times with 500 ng/ml LT (4.2.2A) and therefore becomes unavailable for the toxin to glucosylate *in vitro* in the presence of the radiolabelled cofactor. At least 50% of the substrate small G protein becomes glucosylated in intact cells at 500 ng/ml for 4 hours. Figure 4.2.2B illustrate in a similar manner how pretreatment of EL4.NOB-1 cells with increasing doses of LT for 4 hours lessens the subsequent *in vitro* glucosylation of the small G protein substrates of the toxin. Figure 4.2.2A and B illustrate how in some experiments glucosylated proteins can be seen. A band at 23 kDa can be seen and the predominant 20 kDa band appears to be composed of two bands, which can be seen particularly clearly in figure 4.2.2B.

As the extent of glucosylation *in vivo* appeared to correlate with the extent of inhibition by LT of p38 MAPK, I attempted to quantify the data (figure 4.2.3). The diagrams demonstrate how the inhibition of the activation of p38 MAPK by IL-1 and the degree of glucosylated small G proteins by LT in intact cells correlate. The level of inhibition or glucosylation was quantified by subjecting autoradiographic film to densitometric analysis. 4.2.3A shows the inhibitory effect of LT on the activation of p38 MAPK by IL-1 in a time dependent manner in relation to the time dependent glucosylation of target proteins in intact cells. A correlation can clearly be seen. Figure 4.2.3B shows a similar correlation of the inhibitory effect of LT in relation to the dose dependent glucosylation of target proteins, although slightly more inhibition of p38 MAPK can be seen relative to glucosylation at 250 ng/ml LT.

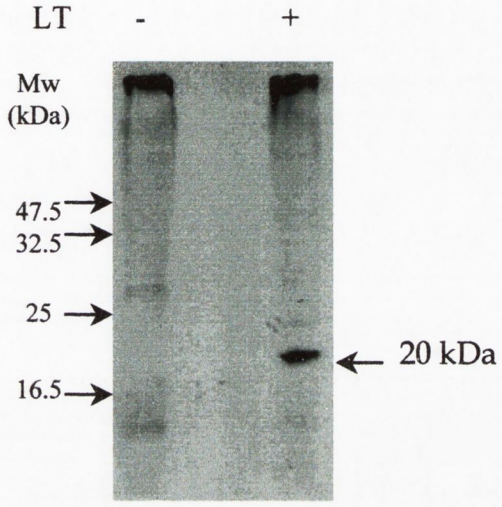


FIGURE 4.2.1

LT glucosylates a protein of 20 kDa in extracts prepared from EL4.NOB-1 cells. Cell extracts using $5x10^7$ murine thymoma EL4.NOB-1 cells were prepared as described in the methods section. After the protein levels of each sample was established, 20 µl (33 µg protein) of the cell extract was incubated with 20 µl [^{14}C]-UDP glucose and 2 µg/ml LT for 1 hour at 37° C. The proteins of the samples were separated by electrophoresis using a 15% SDS-PAGE gel which was transferred to a nitrocellulose membrane, dried, dipped in 20% PPO and exposed to autogradiographic film. Results show one representative out of three identical experiments performed.

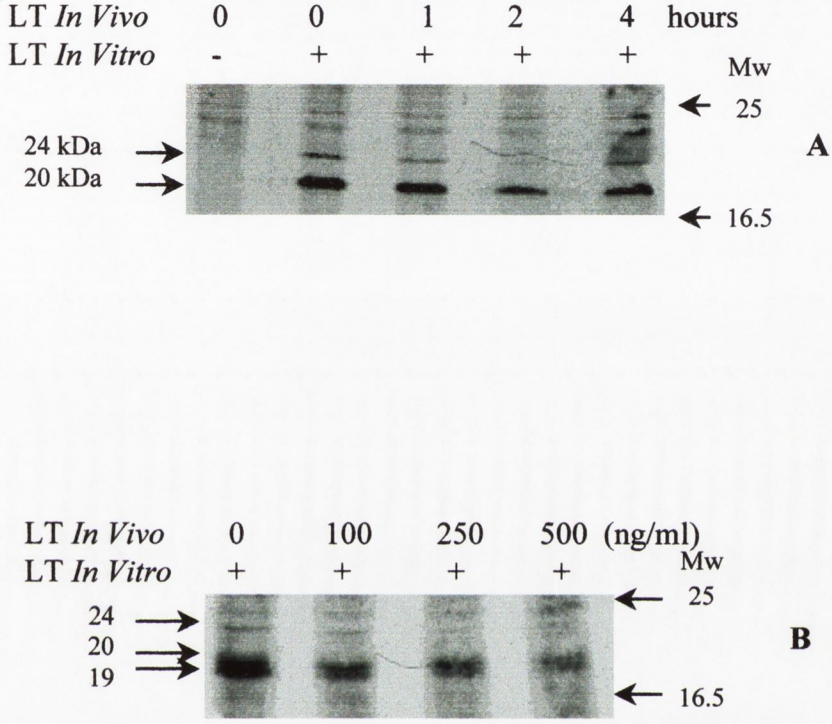
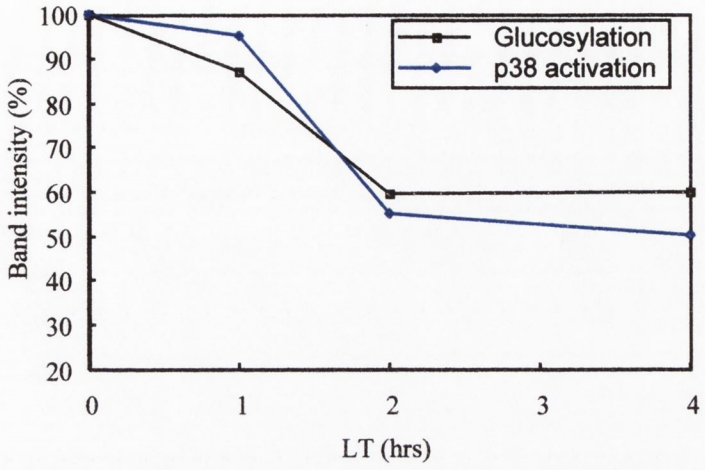


FIGURE 4.2.2

Effect of pretreatment with LT on the subsequent glucosylation of target proteins *in vitro*. $5x10^7$ murine thymoma EL4.NOB-1 cells were pretreated with LT for indicated length of time at 500 ng/ml(A) or with increasing doses for 4 hours (B). Cell lysates were prepared as described in the methods section. After the protein levels were measured, 27μg (A) or 46 μg (B) of the cell extracts were incubated with 20 μl [^{14}C]-UDP glucose and 2 μg/ml LT at 37°C for 1 hour. The samples were subsequently separated on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane which was dried, dipped in 20% PPO and exposed to autoradiographic film. Results are shown as one representative experiment out of two performed.

A



B

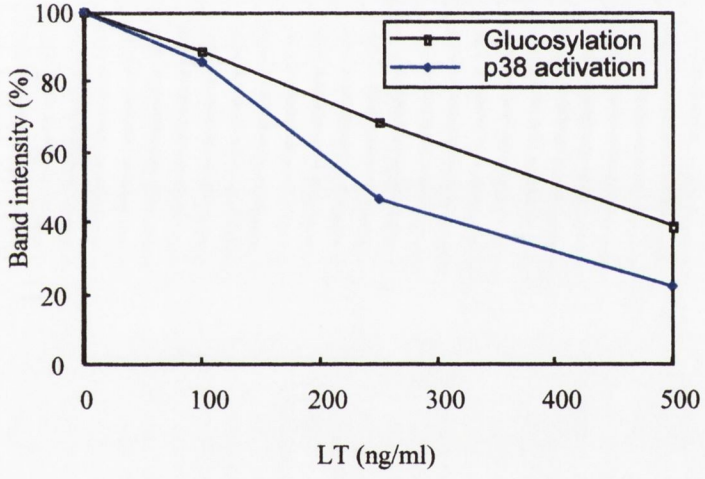


FIGURE 4.2.3

Comparison of the effect of LT on glucosylation of target proteins and inhibition of the activation of p38 MAPK by IL-1. The results obtained in experiments shown in 4.2.2 are here shown together with the results obtained from the experiments shown in figures 3.2.2 and 3.2.3. The intensities of the bands of the autoradiographic films were quantitated by densitometry, and compared within each experiment. The density of the bands from the samples were compared to that of the samples that had not been treated with LT, and are expressed as % band intensity. Figure A shows the results from the glucosylation experiment of figure 4.2.2A and the p38 assay of figure 3.2.3. Figure B represents the results from figure 4.2.2B and figure 3.2.2.

One of the G proteins targeted by LT is required for p38 MAPK activation by IL-1. Four target proteins for the toxin have already been reported, Ras, Rap, Ral and Rac. To further resolve the proteins that are shown to be glucosylated by the toxin (figure 4.2.2), an attempt to separate them in two dimensions was made (figure 4.2.4). The proteins were first separated according to their pI, using isoelectric focussing, after which the gel from this was analysed by SDS-PAGE, to separate the proteins according to molecular weight. Detection is low since the specific activity of the [^{14}C] labelled UDP-glucose is low, but spots can be seen at pI of about 9-10 and 7-6, and molecular masses of approximately 20 kDa. Some unknown high molecular weight proteins were also glucosylated by LT, or by other glucosyltransferases present in the cell lysates, as seen in this figure. Figure 4.2.4 also demonstrates the results from staining the gel with Coomassie stain, showing how the proteins have migrated both according to pI and molecular mass. Although further attempts were made at these experiments, the results presented here were difficult to reproduce, due to the detection difficulties.

4.2ii Toxin B glucosylates a protein of 23 kDa in EL4.NOB-1 cells

To determine if the substrates for Toxin B were present in EL4.NOB-1 cells, whole cell lysates from these cells were treated with Toxin B in the presence of [^{14}C]-labelled UDP glucose. The radiolabelled proteins were detected by SDS-PAGE and autoradiography. The results are presented in figure 4.2.5 and show the presence of a 23 kDa substrate for ToxB in EL4.NOB-1 cells.

4.3 Glucosylation is required for the inhibitory effect of LT

4.3i LT, but not ToxB, inhibited the activation of p38 MAPK by IL-1α in Chinese hamster lung fibroblast (Don) cells, but not in the UDP-glucose deficient mutant Don strain, CdtR-Q.

To confirm that glucosylation was required for inhibition of IL-1 actions by LT, a cell line, resistant to LT and ToxB due to a low intracellular level of UDP-glucose, occurring because of a single point mutation in the UDP-glucose pyrophosphorylase gene (Flores-Diaz *et al.*, 1997) was used. Figure 4.3.1 illustrates how

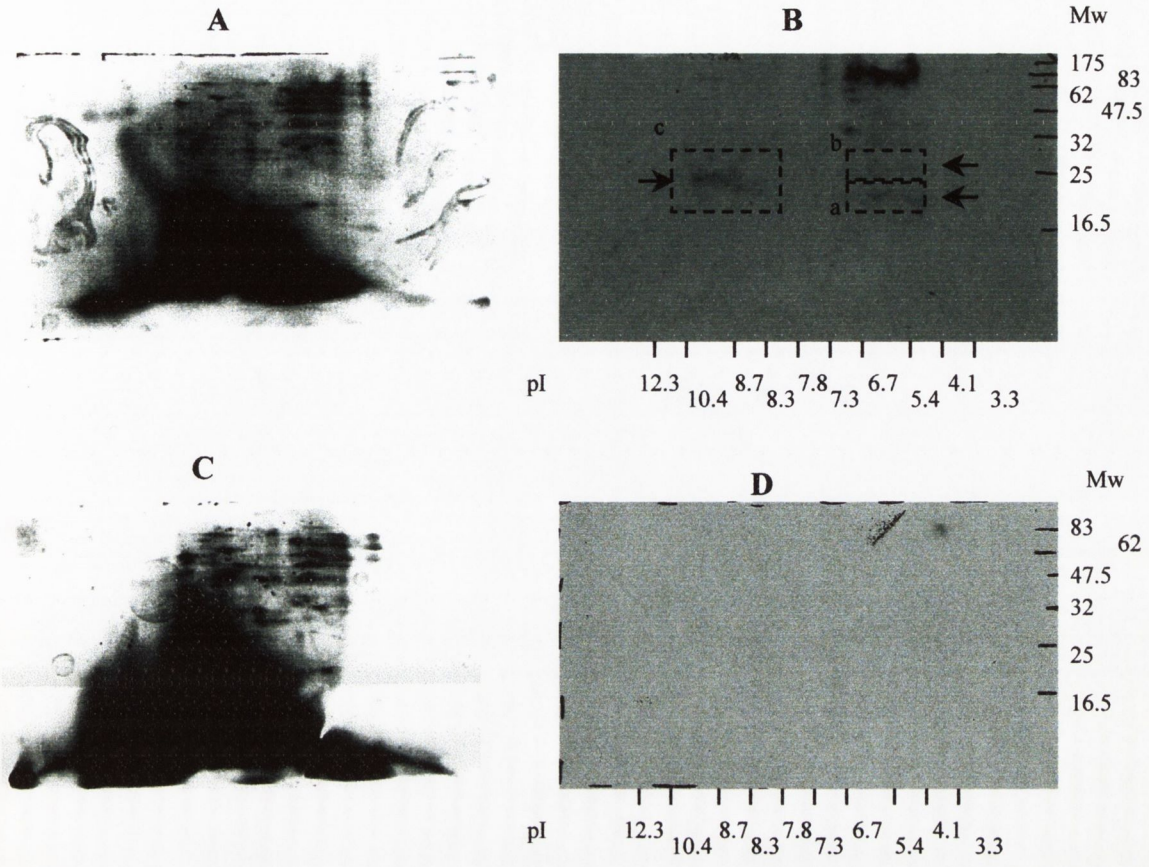


FIGURE 4.2.4

2-dimension gel electrophoresis of EL4.NOB-1 cell extracts glucosylated by LT. Whole cell lysates were prepared using $5x10^7$ murine thymoma EL4.NOB-1 cells as described in the methods section. 73 µg of protein from these extracts were incubated with 50 µl [^{14}C] UDP-glucose and with (A and B) or without (C and D) 2 µg/ml LT and for 1 hour at 37°C. The proteins of the samples were then separated in two dimensions, according to isoelectric point (pI) and molecular weight (Mw) as described in the methods section. Figures A and C represents photographs of the gels with LT-treated and non-treated samples respectively stained with coomassie stain, and figures B and D show the corresponding autoradiographic films after the proteins had been transferred to a nitrocellulose membrane which had been dipped in 20% PPO in toluene to enhance detection sensitivity. Arrows indicate glucosylated proteins.

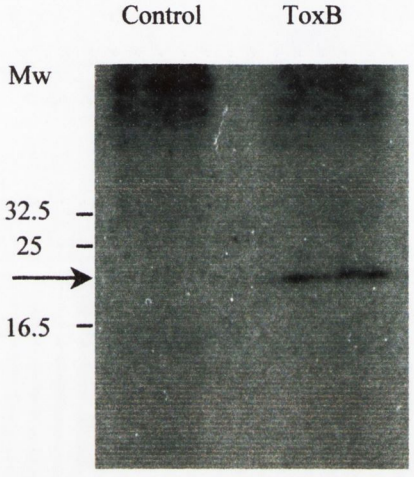


FIGURE 4.2.5

Glucosylation of target proteins by Toxin B in extracts prepared from EL4.NOB-1 cells. Whole cell lysates were prepared from $5x10^7$ Murine thymoma EL4.NOB-1 cells. 176 μg of protein was treated with or without 2 μg/ml ToxB for 1 hour at 37°C in the presence of [^{14}C]-labelled UDP-Glc. The samples were run on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane which was dried and dipped in 20% PPO in toluene before detection using autoradiographic film.

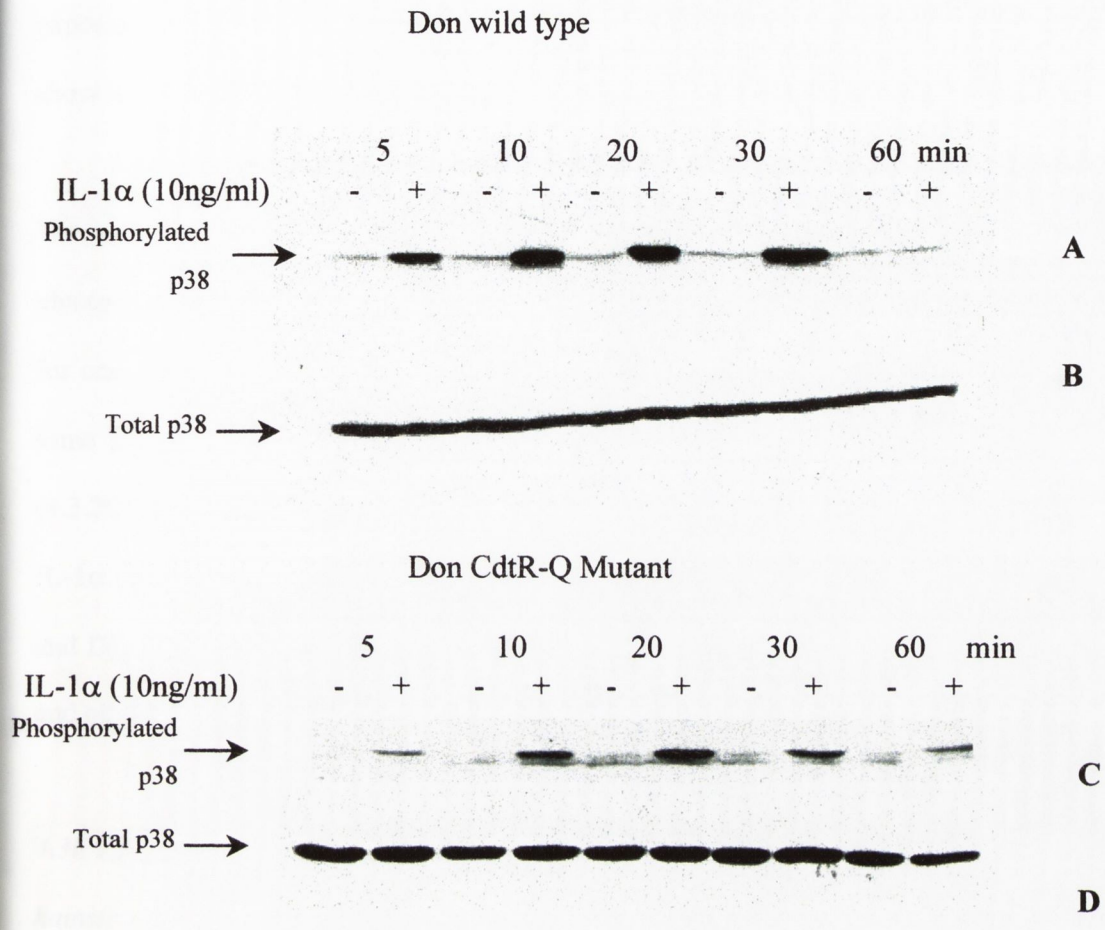


FIGURE 4.3.1

Activation of p38 MAPK by IL-1 in Don wild type and UDP-glucose deficient mutant fibroblasts. $2x10^6$ Diploid Chinese hamster Don lung fibroblast cells were treated with 10 ng/ml IL-1α for times indicated. Cell lysates were prepared and the phosphorylation status of p38 MAPK was analysed using western blotting according to the protocol described in the methods section. Results show one representative of two identical experiments performed. (A) and (C) show results from blotting using a specific anti-phosphorylated p38 MAPK antibody for Don wild type and UDP-glucose deficient Don CdtR-Q mutant cells repectively. (B) and (D) represents the results from the same samples using a phosphorylation state independant p38 MAPK antibody, to ensure that equal amounts of p38 MAP kinase was loaded for each sample.

p38 MAPK can become transiently activated by IL-1α in both the parental Don cell line (A and B) as well as in the UDP-glucose deficient Don CdtR-Q strain (C and D). p38 MAPK became activated after 5 minutes exposure to IL-1α reaching a maximum activation after 20 minutes in the two cell lines. Figure 4.3.1B and D show how an equal amount of total p38 MAPK was loaded for each sample.

The activation of p38 MAPK by IL-1α was inhibited by LT in Don parental cells, but not in the UDP-glucose deficient mutant strain Don CdtR-Q (fig 4.3.2). Don wild type cells pre-treated with 200 ng/ml LT for one hour showed almost complete inhibition of IL-1α activated p38 MAPK (fig 4.3.2A), whereas the same pre-treatment with LT in the mutant CdtR-Q Don cell line did not cause any detectable inhibition (4.3.2C). Figure 4.3.3 shows, as expected, that Toxin B was unable to inhibit the activation of p38 MAPK by IL-1α in both Don parental cells (A and B) and in the UDP-glucose deficient CdtR-Q mutant Don cells (C and D), although the toxin was taken up by the cells, as evident from rounding of the parental cell bodies, and other morphological changes characteristic of Toxin B.

4.3ii LT, but not ToxB, inhibited the activation of p42 and p44 MAPK by IL-1α more potently in Chinese hamster lung fibroblast (Don) cells, compared to in the UDP-glucose deficient mutant Don strain, CdtR-Q.

The activation of p42/p44 MAPK by IL-1 could be inhibited by LT in the Don parental cells to a greater extent than in the mutant CdtR-Q strain as seen in figure 4.3.4. Pre-treatment of the cells with 500 ng/ml LT for four hours showed a total inhibition of the activation of p42/p44 MAPK by IL-1 in the parental cells (figure 4.3.4A) compared to the mutant strain where no inhibition could be noticed at 500 ng/ml LT. Again the total MAPK in each sample was monitored as seen in figure 4.3.4 B and D.

ToxB did not inhibit IL-1α activated p42 and p44 MAPK (figure 4.3.5) in neither the parental Don cell line (A and B) nor in the UDP-glucose deficient Don CdtR-Q strain (C and D). Pre-treatment of the two cell lines with 500 ng/ml ToxB for 1 hour had no effect on IL-1 activated p42 and p44 MAPK.

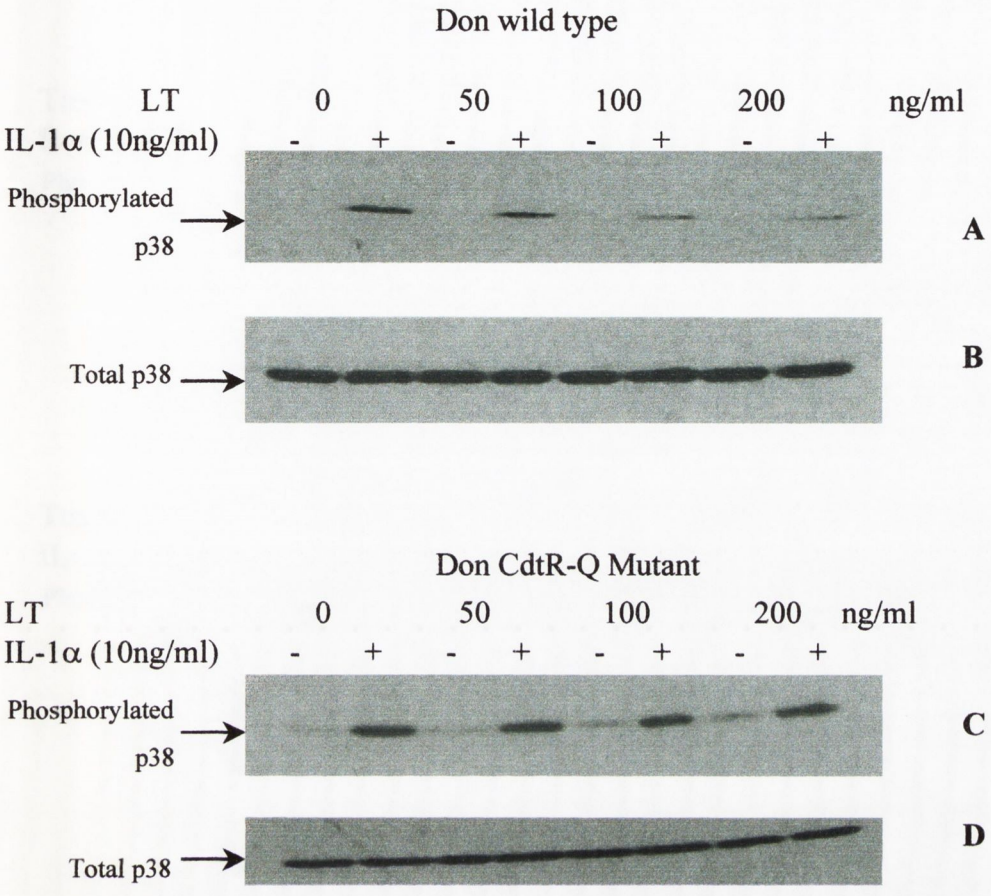


FIGURE 4.3.2

Effect of LT on the activation of p38 MAPK by IL-1 in Don wild type and UDP-glucose deficient fibroblasts. $2x10^6$ Diploid Chinese hamster Don lung fibroblast cells were pretreated with indicated doses of LT for one hour at 37°C. Following this the cells were stimulated with 10 ng/ml IL-1α for 20 minutes after which cell lysates were prepared and the phosphorylation status of the p38 MAPK of each sample was detected by western blotting as described in the methods section. Results show one representative out of four identical experiments performed. (A) and (C) show results from western blotting using an antibody recognising phosphorylated p38 MAPK for Don wild type and the UDP-glucose deficient strain Don CdtR-Q mutant cells repectively. (B) and (D) represents the results from the same samples as in (A) and (C) detected in western blotting using a phosphorylation state independent p38 MAPK antibody, to ensure that equal amounts of p38 MAP kinase was loaded for each sample.

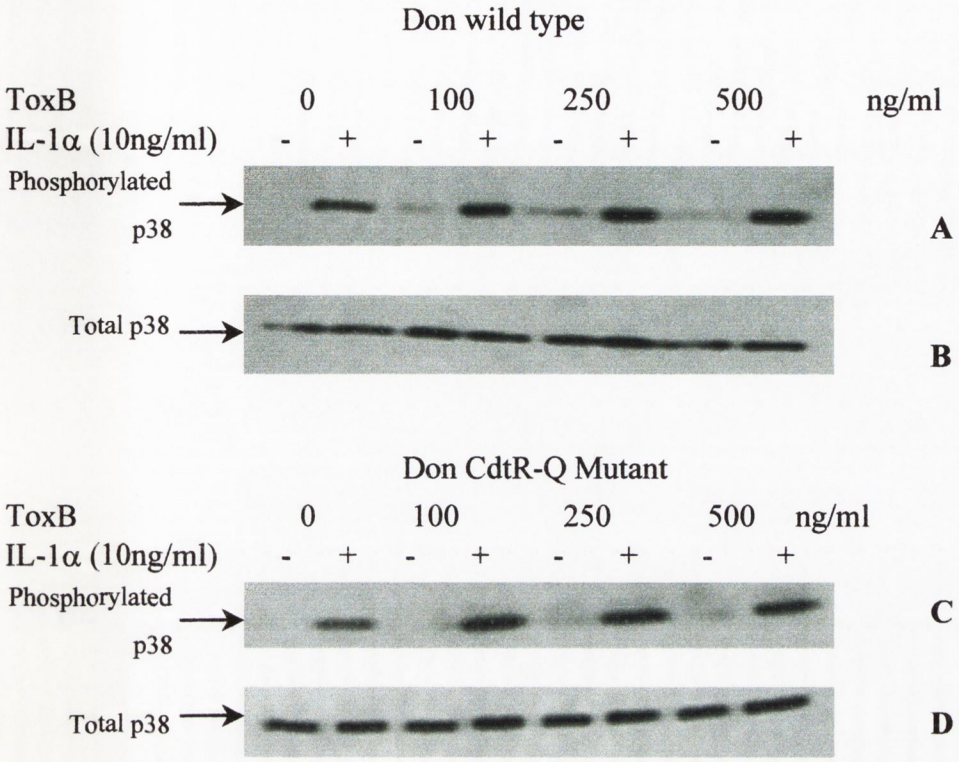


FIGURE 4.3.3

Effect of Toxin B on the activation of p38 MAPK by IL-1 in Don wild type and UDP-glucose deficient mutant fibroblasts. $2x10^6$ Chinese hamster Don lung fibroblast cells were pretreated with indicated doses of ToxB for one hour at 37°C. Following this the cells were stimulated with 10 ng/ml IL-1α for 20 minutes after which cell lysates were prepared and the phosphorylation status of the p38 MAPK of each sample was analysed by western blotting as described in the methods section. Results show one representative out of two identical experiments performed. (A) and (C) show results from western blotting using a phosphorylation status specific anti-phosphorylated p38 MAPK antibody for Don wild type and UDP-glucose deficient Don CdtR-Q mutant cells repectively. (B) and (D) represents the results from the same samples as in (A) and (C) detected in western blotting using a phosphorylation state independant p38 MAPK antibody, to ensure that the total amount of p38 MAP kinase analysed was equal.

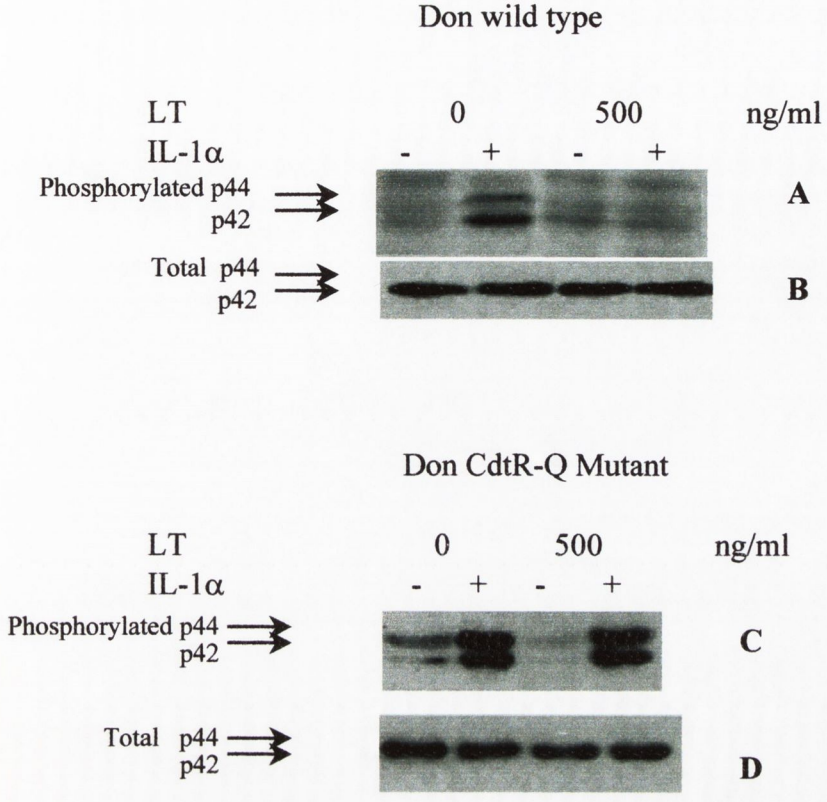


FIGURE 4.3.4

Effect of LT on the activation of p42/p44 MAPK by IL-1 in Don wild type and UDP-glucose deficient mutant fibroblasts. $2x10^6$ Chinese hamster Don lung fibroblast cells were treated with indicated doses of LT for four hours at 37°C. Following this the cells were stimulated with 10 ng/ml IL-1α for 20 minutes after which cell lysates were prepared and the phosphorylation status of the p42 and p44 MAPK of each sample were detected by western blotting as described above. Results show one representative of five identical experiments performed. (A) and (C) show results from western blotting using an antibody that recognises phosphorylated p42/p44 MAPK for Don wild type and UDP-glucose deficient Don CdtR-Q mutant cells repectively. (B) and (D) represents the results from the same samples but detected in western blotting using a phosphorylation state independent p42/p44 MAPK antibody, to ensure that equal total amounts of p42 or p44 MAP kinases were analysed.

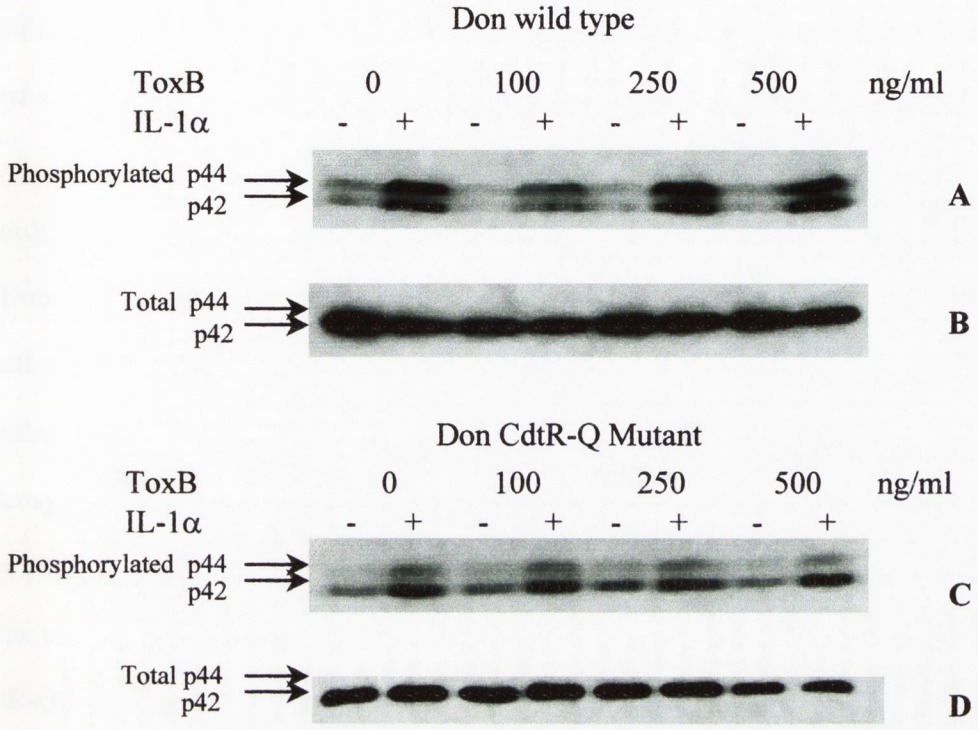


FIGURE 4.3.5

Effect of Toxin B on the activation of p42/p44 MAPK by IL-1 in Don wild type and UDP-glucose deficient mutant fibroblasts. $2x10^6$ Chinese hamster Don lung fibroblast cells were pretreated with indicated doses of ToxB for one hour at 37°C. Following this the cells were stimulated with 10 ng/ml IL-1α for 20 minutes after which cell lysates were prepared and the phosphorylation status of the p42 and p44 MAPK of each sample were analysed by western blotting as described in the methods section. Results show one representative of two identical experiments performed. (A) and (C) show results from western blotting using an antibody recognising phosphorylated p42/p44 MAPK antibody for Don wild type and Don CdtR-Q mutant cells repectively. (B) and (D) represents the results from the same samples detected by western blotting using a phosphorylation state independent p42/p44 MAPK antibody, to ensure that the total amount of p42/p44 MAP kinases analysed was equal.

4.3iii LT and ToxB glucosylate 20 kDa proteins in Don wild type and Don CdtR-Q mutant cells in vitro *and in intact wild type cells.*

In order to establish that the substrates for LT and Toxin B are present in parental Don fibroblast as well as in Don CdtR-Q, cell lysates from both cell lines were incubated with each toxin in the presence of [^{14}C]-labelled UDP-glucose (fig 4.3.6). The proteins were subsequently separated by SDS-PAGE and the radio-labelled bands were detected by autoradiography. The results show at least one band that has been glucosylated by LT, and also a band, although fainter, which is glucosylated by ToxB for both the wild type Don cells and the CdtR-Q mutant strain. This indicates that the varying sensitivity of the wild type compared to the mutant Don cell line to LT is not due to the lack of target proteins in the UDP-glucose deficient Don CdtR-Q.

To verify that LT can enter the cells and exert its action on intact cells as well as *in vitro*, Don wild type and CdtR-Q mutant cells were pre-treated with increasing doses of LT (Fig. 4.3.7). The cells were then lysed and exposed to the toxin *in vitro* in the presence of radiolabelled UDP-glucose. Don wild type cells pre-treated with 500 ng/ml LT show a clear inhibition of substrate glucosylation *in vitro* (fig. 4.3.7A), suggesting that the substrate has been glucosylated during the pre-treatment with LT. The graph corresponding to the Don Wild type experiment (fig 4.3.7A) clearly shows that, measured by densitometry, an 80% decrease in band intensity occurs when the cells were treated with 500 ng/ml LT. No such decrease in available substrate can be detected for the CdtR-Q mutant (fig 4.3.7B), treated with the same doses of LT, but instead the LT-treated samples appears to be equally well glucosylated by the toxin during the subsequent *in vitro* glucosylation assay. This demonstrates that no glucosylation of target protein can occur in intact Don CdtR-Q cells, although the target proteins are there, and can be glucosylated *in vitro* following addition of the missing

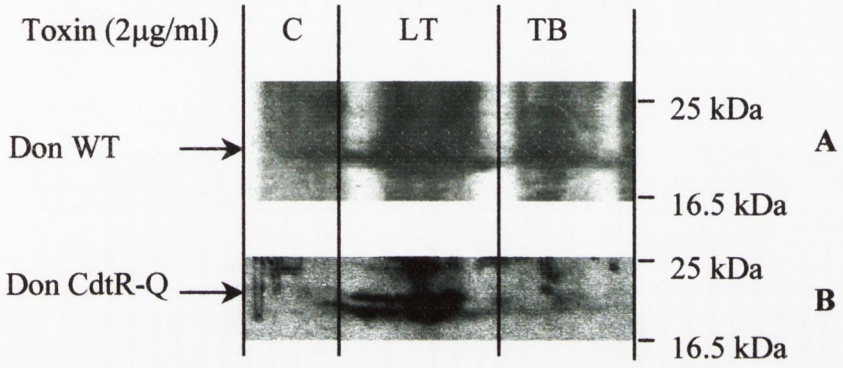


FIGURE 4.3.6

Glucosylation of target proteins by LT and Toxin B in extracts prepared from Don wild type, and UDP-glucose deficient mutant fibroblasts. $1x10^7$ Diploid Chinese hamster Don lung fibroblast cells were prepared for glucosylation *in vitro* as decribed in the methods section. 100 μg of protein was incubated at 37°C for 1 hour with 20 μl of ^{14}C labelled UDP-Glc and 2 μg/ml of LT, or Toxin B (TB). An untreated control sample (C) was included. The samples were run on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane which was dipped in 20% PPO in toluene before the glucosylated proteins were detected by autoradiography. (A) show the results from samples derived from the parental Don cell line, and (B) represents the samples derived from the glucose deficient Don CdtR-Q mutant strain.

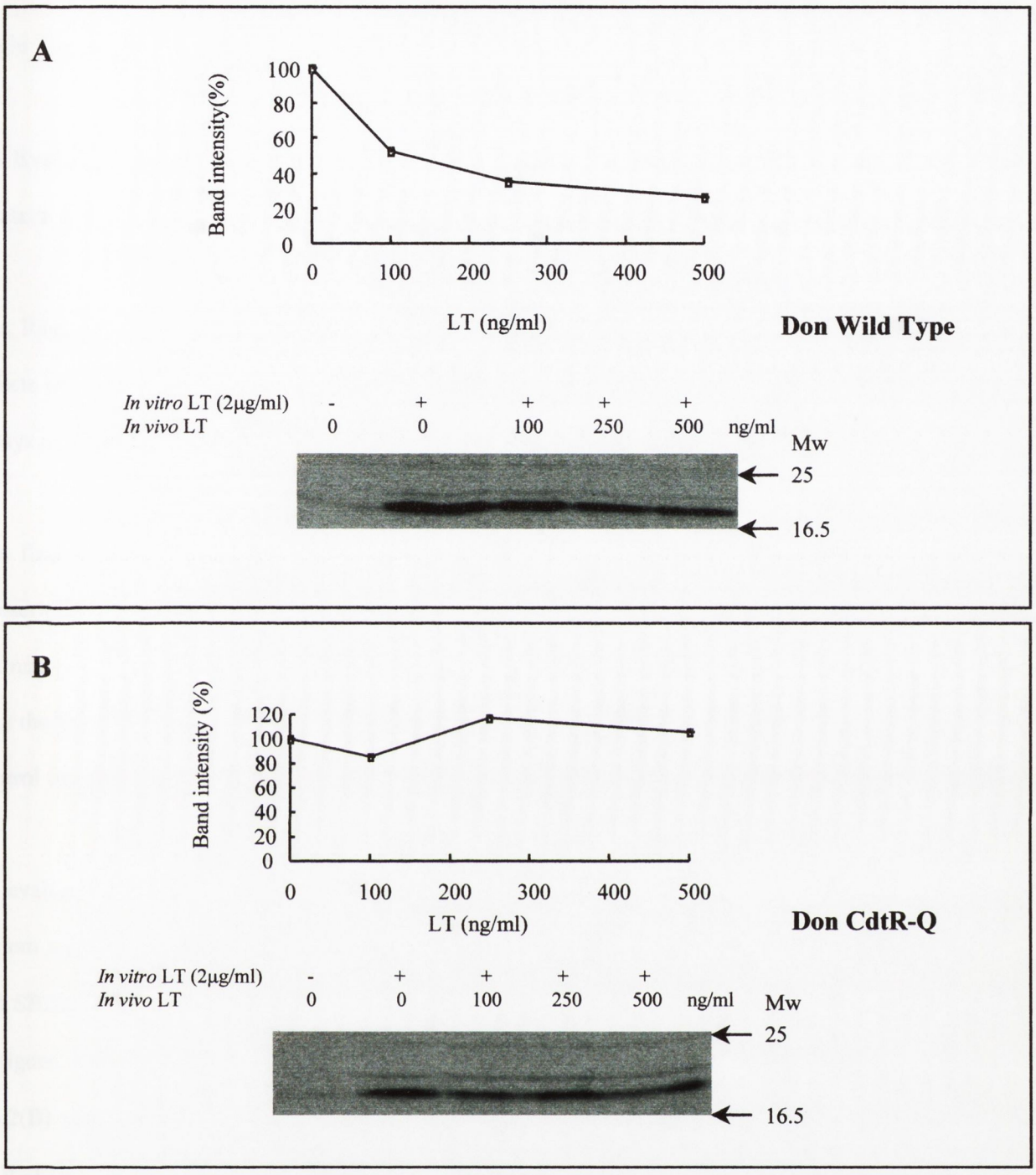


FIGURE 4.3.7

Effect of pretreatment with LT on the glucosylation of target proteins *in vitro* in cell extracts prepared from Don wild type or UDP-glucose deficient mutant fibroblasts. $1x10^7$ Diploid Chinese hamster Don lung fibroblast cells were pretreated with indicated doses of LT for 3.5 hours at 37°C. The sample preparation was performed as described in the methods section. 38 μg of protein was incubated at 37°C for 1 hour with 20 μl of [^{14}C]-labelled UDP-Glc and 2 μg/ml of LT (not included in control sample). After this incubation the samples were run on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane which was dipped in 20% PPO in toluene before the glucosylated proteins were detected by autoradiography. The intensity of the resulting bands were measured by densitometry, and the intensity of the band corresponding to the sample from cells which had not been pre-treated with LT (second band in A and B lower panels) was set to 100%, after subtracting basal glucosylation activity (first lanes). (A) show the results from samples derived from the parental Don cell line, and (B) represents results from samples derived from the glucose deficient Don CdtR-Q mutant strain.

cofactor UDP-glucose. Taken together, these results strongly indicate that the inhibitory effect of LT is due to glucosylation of a Ras family G protein.

4.4 Evaluation of the GAL4-CHOP$^{(1-101)}$ trans-reporter system, and setting up a transfection based western blotting assay for p38 MAPK.

Ras, Rap, Rac, and Ral are four known targets for LT. The next approach I therefore took was to examine the effects of dominant negative forms of Ras, Rac and Rap on p38 MAPK activation using transfection-based assays as described in the methods section.

The first of these involved the GAL4-CHOP$^{(1-101)}$ p38 MAPK assay. In this system, IL-1 induces the transcription of the GAL4Luciferase reporter gene within 1.5 hours of stimulation with IL-1, reaching optimal expression after about 6 hours (fig 4.4.1). When using the GAL4 DNA binding domain alone, rather than the GAL4-CHOP fusion construct, the system is unresponsive to the effects of IL-1, acting as a negative control (data not shown).

To evaluate the specificity of this assay, cells transfected with the components of the GAL4-CHOP$^{(1-101)}$ system were treated with the specific p38 MAPK inhibitor SB203580. Pretreating EL4.NOB-1 cells with 1 μM SB203580 for 1 hour totally inhibited IL-1 induced transcription of the luciferase reporter gene as seen in figure 4.4.2A. This verifies that the CHOP assay is specific for p38 MAPK. In addition to this, figure 4.4.2(B) also shows how in this independent assay, LT is a potent inhibitor of the activation of p38 MAPK by IL-1, providing further evidence of a role for one, or more, of the small G proteins Ras, Rap, or Rac in IL-1 signalling. Treating EL4.NOB-1 cells with 500 ng/ml of LT caused a close to total inhibition of the activation of p38 MAPK by IL-1 in the GAL4-CHOP$^{(1-101)}$ system.

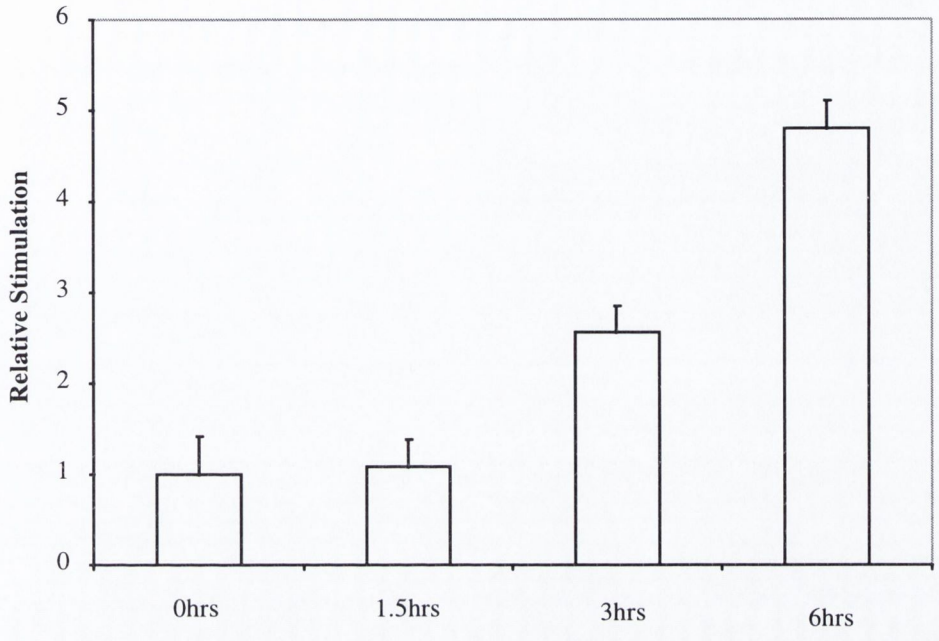


FIGURE 4.4.1

IL-1 induces the phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were transfected with the components of the GAL4-CHOP$^{(1-101)}$ system as described in Experimental Procedures. Transfected cells (5 x 10^5 per sample) were stimulated with 10 ng/ml IL-1α for indicated times. Cells were subsequently lysed, the luciferase activity of each sample was measured, and readings were corrected for protein measured according to the method of Bradford . Results show one representative experiment of three identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

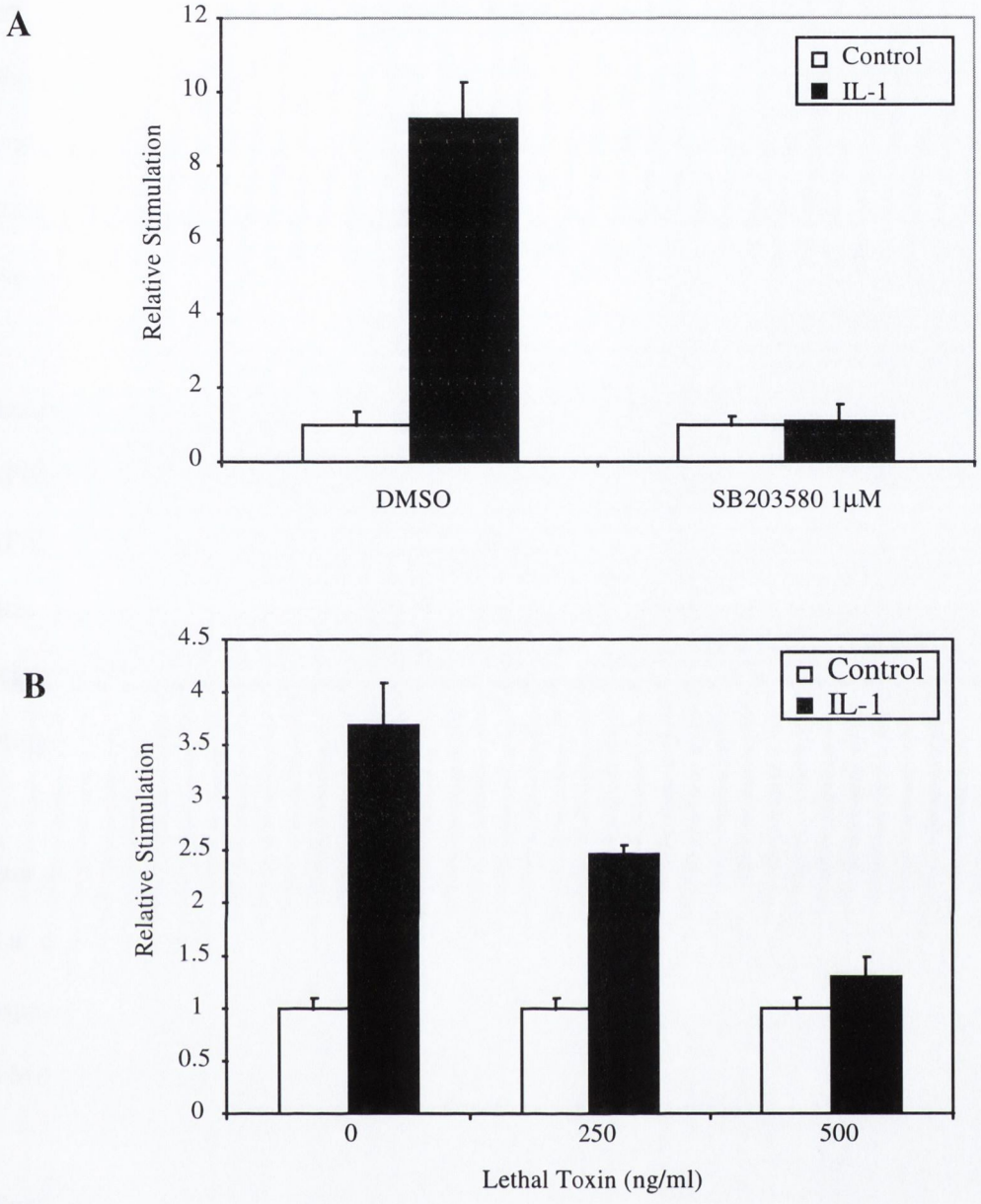


FIGURE 4.4.2

Effect of SB203580 and LT on IL-1 induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were transfected with the components of the GAL4-CHOP$^{(1-101)}$ system as described in Experimental Procedures. Transfected cells (5 x 10^5 per sample) were pre-treated with SB203580 (A) for 1 hour or LT (B) for 4 hours at indicated doses, following which cells were stimulated with IL-1α for a further 4 hours (filled bars) or left unstimulated (white bars). Cells were subsequently lysed, the luciferase activity of each sample was measured, and readings were corrected for protein measured according to the method of Bradford . Results show one representative experiment of three identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

Furthermore, to ensure that the inhibitory effect of LT in this system was not due to a non-specific inhibitory effect on the GAL4-CHOP$^{(1-101)}$ transfection assay, I co transfected EL4.NOB-1 cells with the components of the GAL4-CHOP$^{(1-101)}$ system, together with a constitutively active mutant of MKK3, an upstream activator of p38 MAPK. This activator of p38 MAPK was not expected to be inhibited by LT, and as can be seen in figure 4.4.3, LT (500 ng/ml, 4 hours) does not inhibit the activation of p38 MAPK by constitutively active MKK3.

A second transfection-based assay was also established. HeLa cells were transfected with FLAG tagged p38 MAPK, which could be detected in the western blot assay using an antibody specific for phosphorylated p38 MAPK. Figure 4.4.4 shows a western blot (using a primary antibody specific for FLAG) of whole cell lysates from HeLa cells transfected with FLAG tagged p38 MAPK, and demonstrate the high transfection efficiency of FuGene when used on HeLa cells. This provided the basis for the FLAG p38 MAPK western blotting in HeLa cells.

Figure 4.4.5 shows that IL-1 activates p38 MAPK in this assay (compare lane 2 to lane 1). Pretreating the HeLa cells with up to 5 μM of the p38 MAPK specific inhibitor SB203580, totally inhibited the phosphorylation of both endogenous and transfected FLAG tagged-(migrating slower, i.e. upper band on gel) p38 MAPK by IL-1.

A final means of examining p38 MAPK is by an *in vitro* kinase assay, using ATF-2 as a substrate (as described in the methods section). Figure 4.4.6 show how LT can inhibit the activation of p38 MAPK by IL-1 in this assay. 500 ng/ml LT for 4 hours inhibited the IL-1 induced phosphorylation of ATF-2 by p38 MAPK by 49.5% (measured by densitometry). This provides further evidence for a role for the small G proteins Ras, Rap or Rac in the activation of p38 MAPK by IL-1.

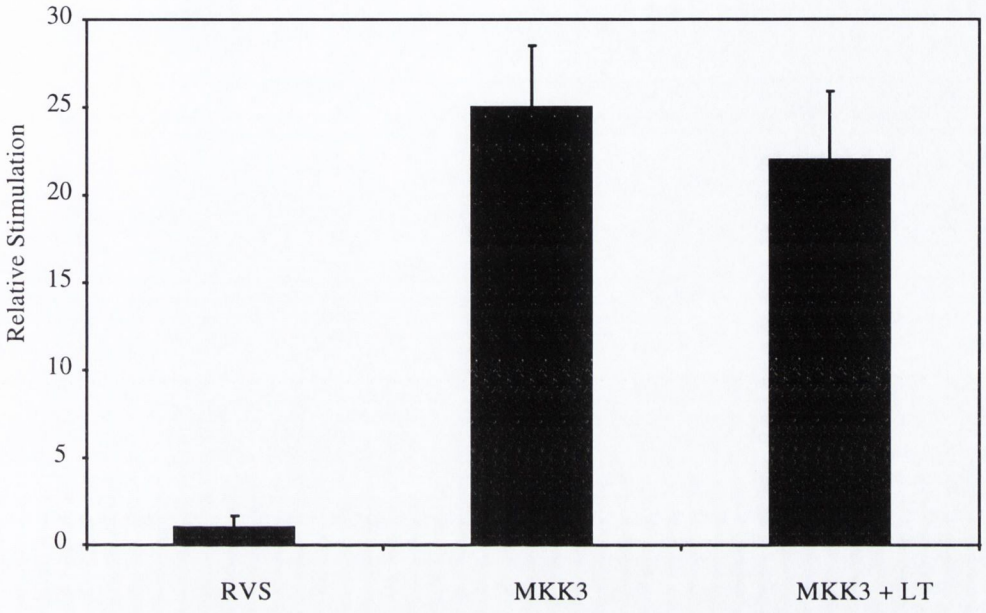


FIGURE 4.4.3

Effect of LT on MKK3-induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system and constitutively active MKK3 as described in Experimental Procedures. Transfected cells (5 x 10^5 per sample) were then treated with LT for 4 hours at 500 ng/ml. The cells were subsequently lysed, the luciferase activity of each sample was measured, and readings were corrected for protein measured according to the method of Bradford . Results show one representative experiment of three identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

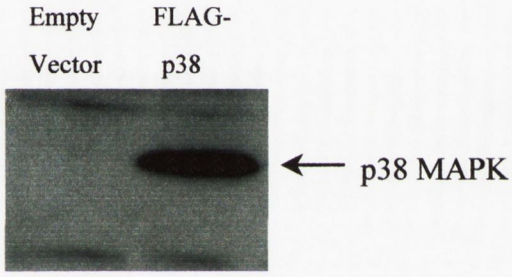


FIGURE 4.4.4

Expression of FLAG tagged p38 MAPK in HeLa cells. HeLa cells were seeded at $1.5x10^5$ cells in 3 ml of complete DMEM medium 24 hours prior to transfection. The cells were subsequently transfected with FLAG-tagged p38 MAPK according to the method of FuGene™ as described in the methods section. 48 hours after transfection the cells were harvested and western blotting was performed on the cell lysates, detecting transfected p38 MAPK using an anti-FLAG antibody.

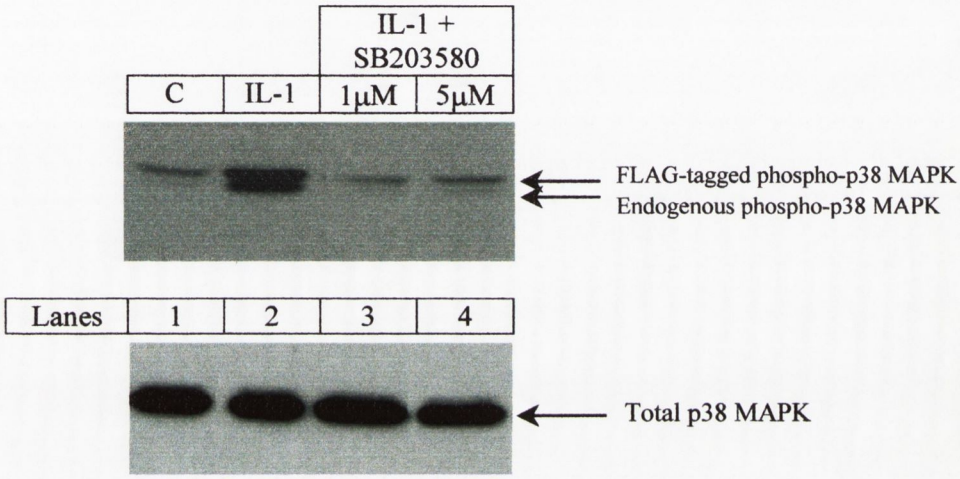


FIGURE 4.4.5

Effect of SB203580 on IL-1 induced phosphorylation of p38 MAPK in HeLa cells transfected with FLAG-tagged p38 MAPK. HeLa cells were seeded at $1.5x10^5$ cells in 3 ml of complete DMEM medium 24 hours prior to transfection. The cells were subsequently transfected with FLAG-tagged p38 MAPK according to the method of FuGene™ as described in the methods section. 48 hours after transfection the cells were treated with SB203580 at indicated doses for 1 hour, and subsequently stimulated with IL-1 for 10 minutes. The cells were then harvested and western blotting was performed on the cell lysates, detecting transfected and endogenous p38 MAPK using an anti-phosphorylated p38 MAPK antibody, as described in the methods section.

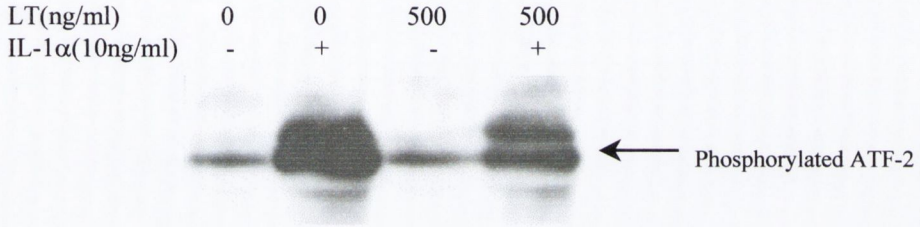


FIGURE 4.4.6

LT inhibits IL-1 induced phosphorylation of ATF-2 by p38 MAPK in HeLa cells transfected with FLAG-tagged p38 MAPK. HeLa cells were seeded at $1.5x10^5$ cells in 3 ml of complete DMEM medium 24 hours prior to transfection. The cells were subsequently transfected with FLAG-tagged p38 MAPK using FuGene™ as described in the methods section. 48 hours after transfection the cells were treated with LT at indicated doses for 4 hours, and subsequently stimulated with IL-1 for 10 minutes. Cell extracts were prepared, FLAG-tagged p38 MAPK was immunoprecipitated as described in the methods section, and an ATF-2 phosphorylation assay using [^{32}P]-ATP was performed as described in the methods section.

4.5 Effect of dominant negative and constitutively active Rac on the activation of p38 MAPK.

Having established the assays, I went on to examine the effects of mutant Rac, Ras and Rap. I first examined Rac. EL4.NOB-1 cells were cotransfected with the components of the GAL4-CHOP$^{(1-101)}$ system, together with dominant negative RacN17. As can be seen in figure 4.5.1A, dominant negative Rac N17 did not interfere with the ability of IL-1 to induce phosphorylation of CHOP, and transcription of the luciferase gene. On the other hand, co-transfecting cells in this system with constitutively active RacV12 (fig 4.5.1B) caused an increased expression of the luciferase reporter gene, proving that Rac can indeed activate this pathway, although it may not be critical when IL-1 is used as a stimulus.

4.6 A role for the small G protein Ras in the activation of p38 MAPK by IL-1

4.6i Effect of dominant negative and constitutively active Ras on the activation of p38 MAPK in EL4.NOB-1 cells transfected with the components of the GAL4-CHOP$^{(1-101)}$ system

I next turned to the small G protein Ras, a prominent target for LT, but a protein which is unaffected by Toxin B. Ras therefore was a prime candidate for the role of the small G protein in the activation of p38 MAPK by IL-1. As depicted in Figure 4.6.1A, transfection of cells with 2.5 μg of plasmid encoding dominant negative Ras N17 totally inhibited IL-1 induced luciferase expression. Transfection of cells with constitutively active RasVHa, on the other hand, activated p38 MAPK (figure 4.6.1B). Figure 4.6.1B also shows how treatment of RasVHa transfected cells with IL-1 caused a further increase in the response.

Further evidence for the involvement of Ras was provided when using two Ras inhibitors, Manumycin A and Damnacanthal (fig 4.6.2). Treatment of cells with Manumycin A, which inhibits Ras by acting as a farnesyl transferase inhibitor (Hiwasa *et al.*, 1997), dose-dependently inhibited the activation of p38 MAPK by IL-1, optimum inhibition occurring at 5 μM Manumycin A (fig 4.6.2A). Damnacanthal, a Ras function inhibitor

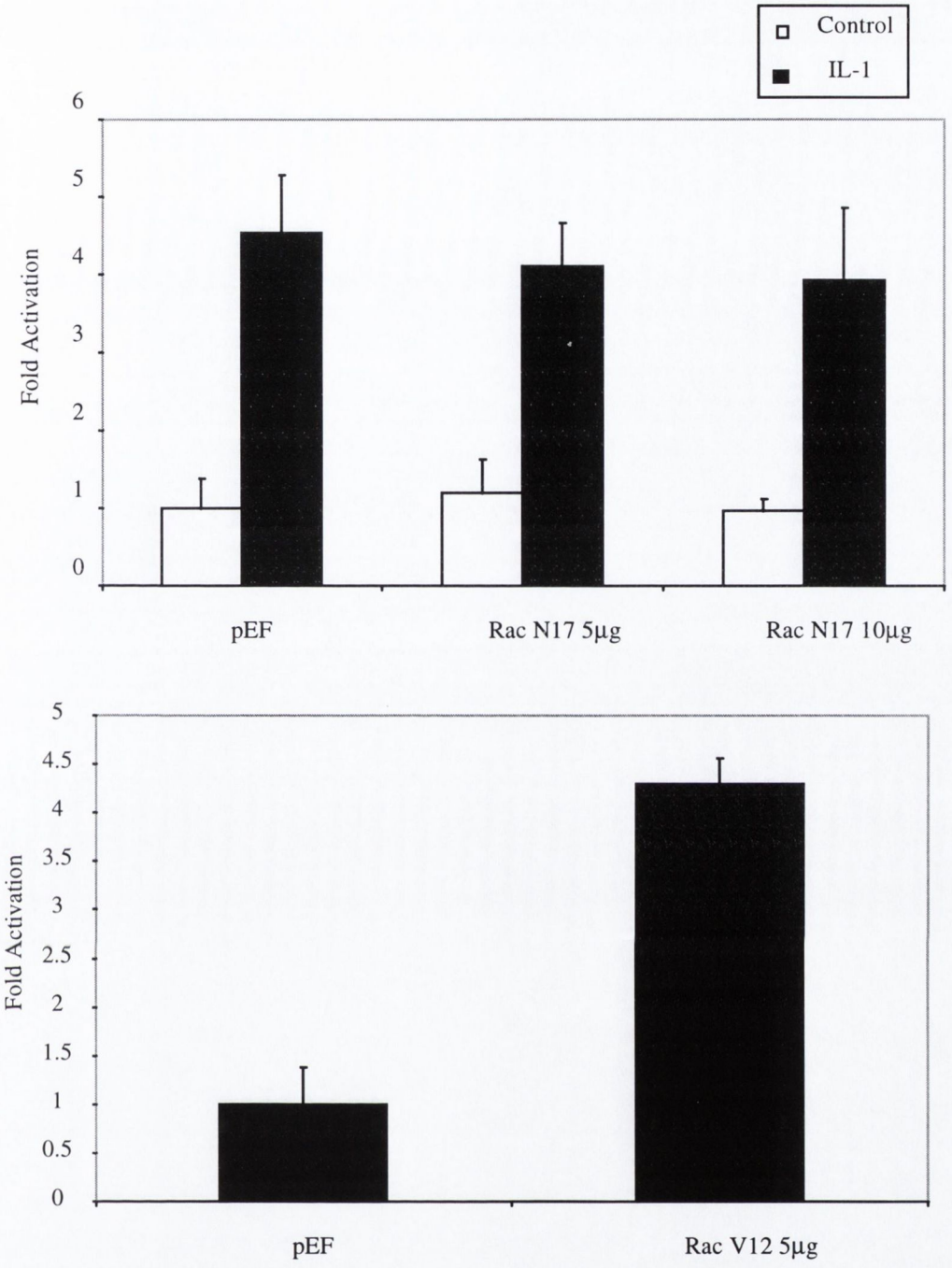


FIGURE 4.5.1

Effect of dominant negative RacN17 and constitutively active RacV12 on IL-1 induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with indicated amounts of RacN17 (A) or RacV12 (B) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α (A) for 6 hours at 37°C (filled bars) or left unstimulated (white bars), following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for protein as measured according to the method of Bradford. Results show one representative experiment of three identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

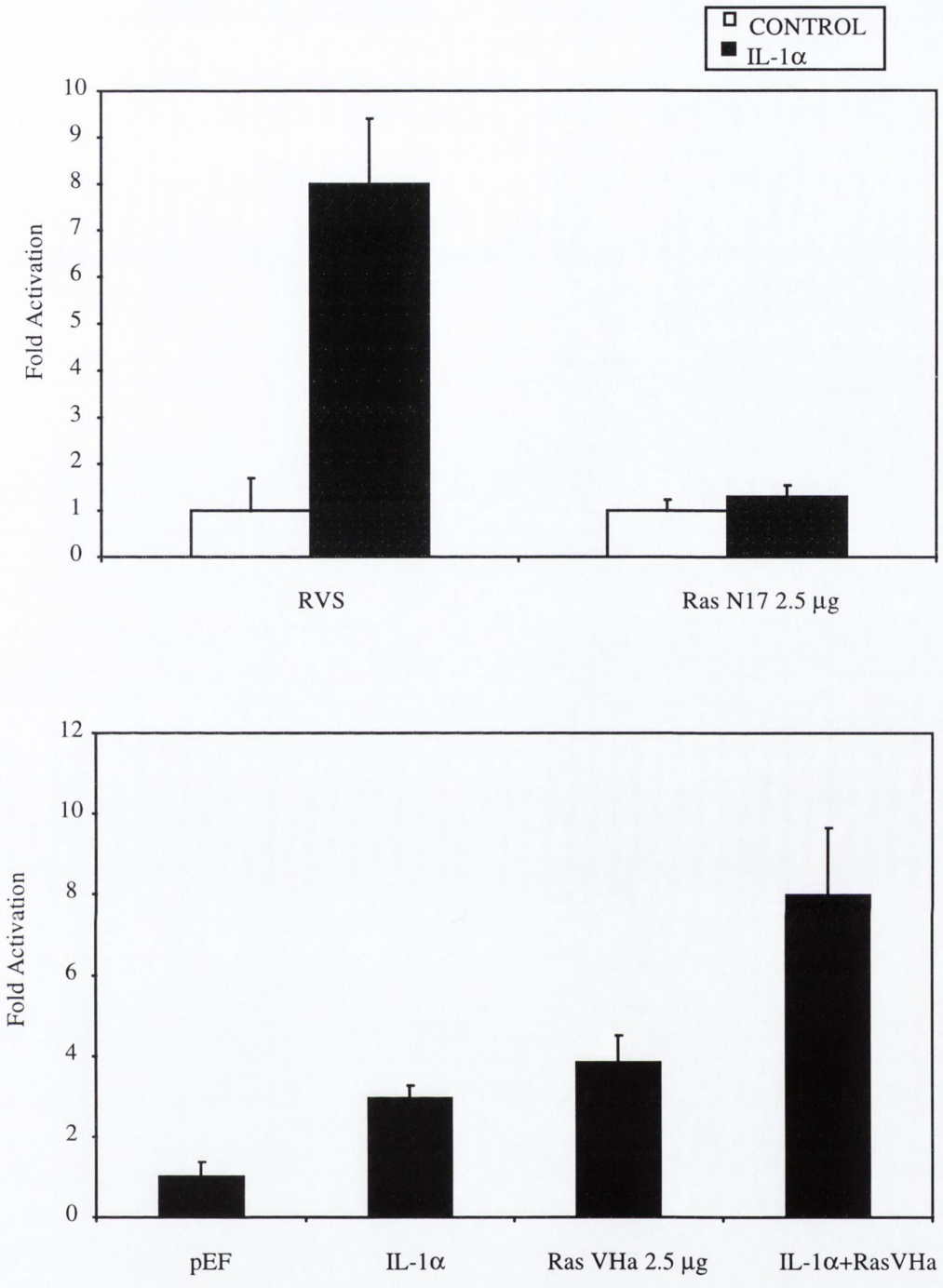


FIGURE 4.6.1

Effects of RasN17 and RasVHA on IL-1α induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system and indicated amounts of RasN17 (A) or RasVHa (B) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml medium per sample) were stimulated with IL-1α for 6 hours at 37°C (filled bars, A) or left unstimulated (white bars), following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for protein as measured according to the method of Bradford. Results shown are from a single experiment (mean of fold stimulation ±SEM, n=4). Identical results were obtained in 3 further experiments.

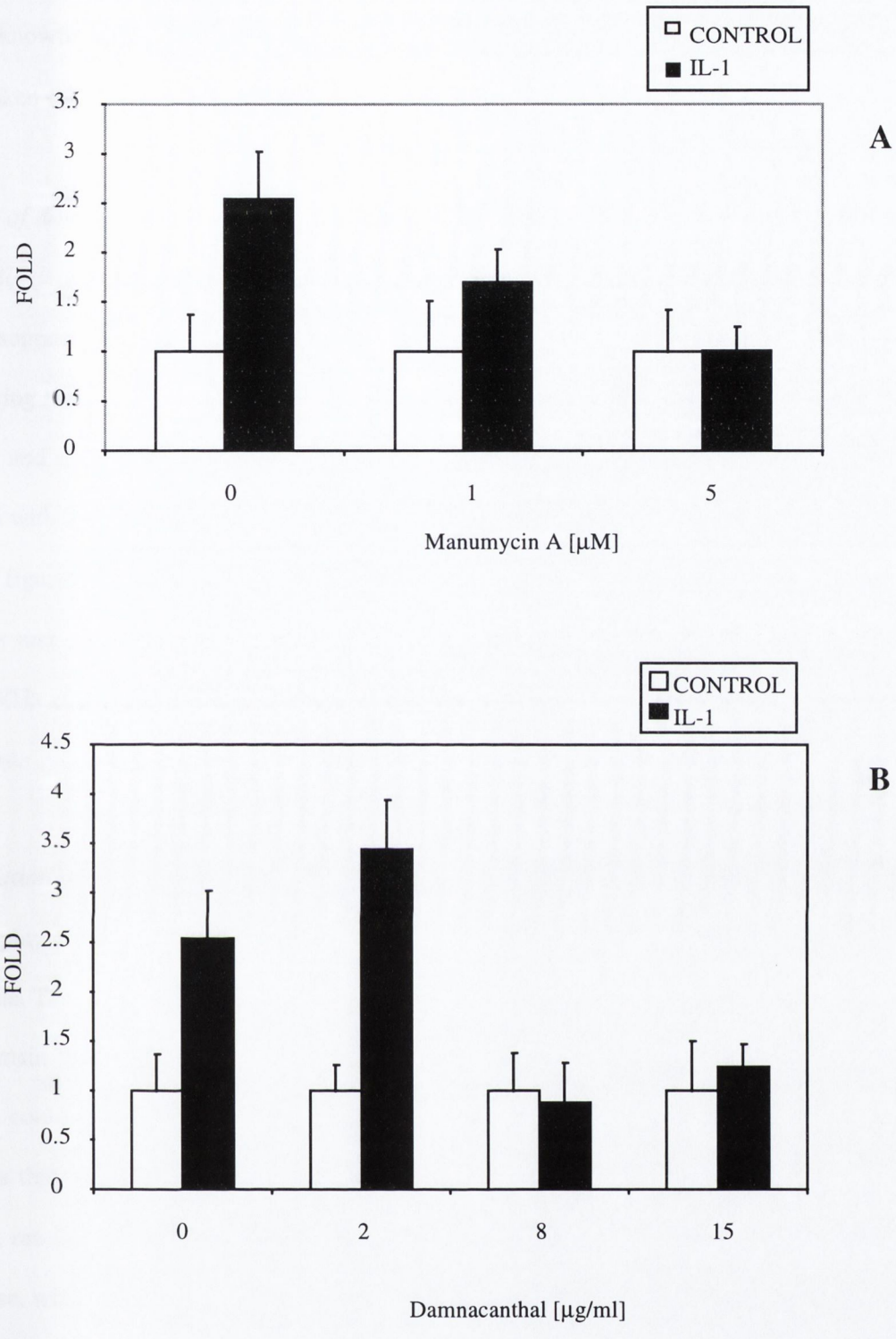


FIGURE 4.6.2

Effects of Manumycin A and Damnacanthal on IL-1 induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were transfected with the components of the GAL4-CHOP$^{(1-101)}$ system as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml of medium per sample) were pre-treated with Manumycin A (A) or Damnacanthal (B) at indicated concentrations for 1 hour at 37°C, following which cells were stimulated with IL-1α for a further 6 hours (filled bars) or left unstimulated (white bars). Cell extracts were subsequently prepared after which the luciferase activity of each sample was measured, and readings were normalised for protein as measured according to the method of Bradford. Results show one representative experiment of three identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

with an unknown mechanism also inhibited the response, an optimum effect being evident at 8 μg/ml (fig 4.6.2B). Taken together, these data strongly implicate Ras in p38 MAPK activation by IL-1.

4.6ii Effect of dominant negative and constitutively active Ras on the phosphorylation of FLAG-p38 MAPK in HeLa cells

To further support the data obtained from using the GAL4-CHOP$^{(1-101)}$ system, I used the independent assay of transfecting HeLa cells with FLAG-tagged p38 MAPK together with dominant negative or constitutively active Ras, and assayed cell extracts by western blotting. The phosphorylation status of FLAG-p38 MAPK provides us with a measure of the activation status of p38 MAPK in the transfected population of cells. As is shown in figure 4.6.3A, dominant negative RasN17 inhibits IL-1 induced phosphorylation of both endogenous and transfected FLAG-p38 MAPK in this system. Furthermore, overexpressing constitutively active RasVHa activated FLAG-p38 MAPK in these cells, showing that Ras indeed is a strong activator of this pathway.

4.6iii Activation of endogenous Ras by IL-1

For Ras to play a role in the activation of p38 MAPK by IL-1, IL-1 must be able to activate endogenous Ras in these cells. To investigate this, I relied on the ability of active GTP-bound form of Ras to bind to the Ras-binding domain of the Ras effector Raf-1. This specific binding provided us with a means of isolating active Ras, which could then be detected by Western Blotting (described in detail in the methods section). Figure 4.6.4 shows that the incubation of EL4.NOB-1 cells with IL-1 gave rise to a rapid increase in active GTP bound Ras, reaching a maximum activation after 5 minutes (lane 2). Also evident is the transient nature of this response, which returned to basal levels after 15 minutes (lane 5).

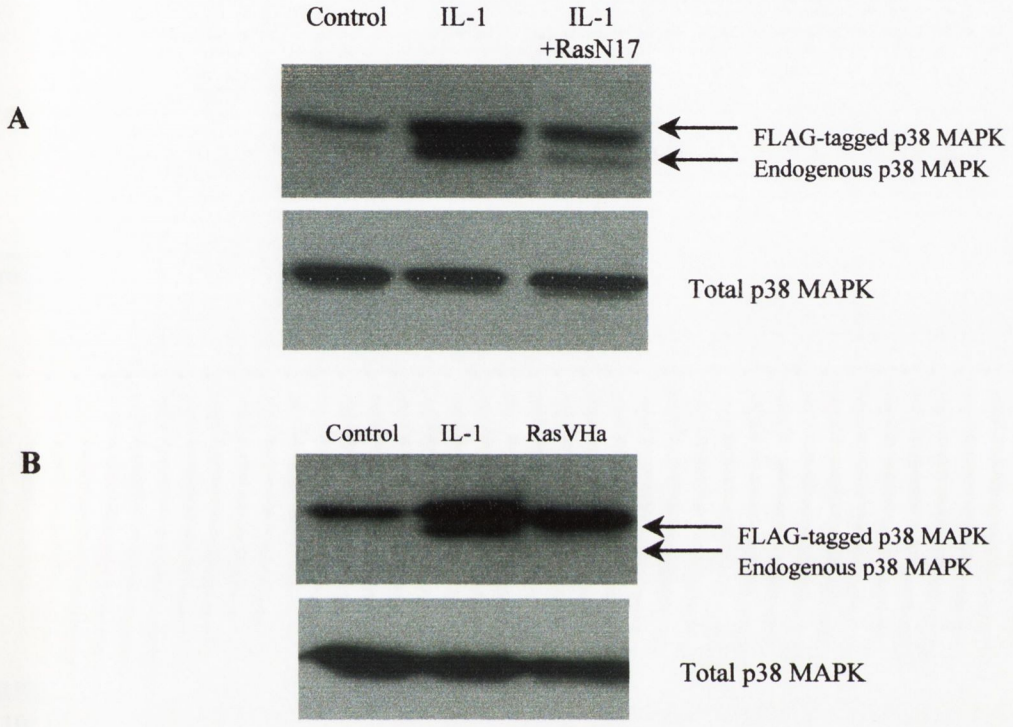


FIGURE 4.6.3

Effect of RasN17 and RasVHa on IL-1 induced phosphorylation of p38 MAPK in HeLa cells transfected with FLAG-tagged p38 MAPK. HeLa cells were seeded at $1.5x10^5$ cells in 3 ml of complete DMEM medium 24 hours prior to transfection. The cells were subsequently co-transfected with FLAG-tagged p38 MAPK and 2.5 μg of RasN17 (A) or RasVHa (B) using FuGene™ transfection reagent as described in the methods section. 48 hours after transfection the cells were stimulated with IL-1 for 10 minutes, harvested and western blotting was performed on the cell extracts, detecting transfected and endogenous p38 MAPK using an anti-phosphorylated p38 MAPK antibody, or total p38 MAPK, as described in the methods section.

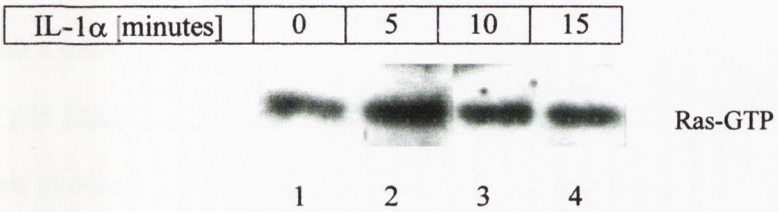


FIGURE 4.6.4 **Activation of Ras by IL-1 in EL4.NOB-1 cells** Cell extracts from 2 x 10^6 EL4.NOB-1 cells pre-treated with IL-1α for indicated times (5-15 minutes), were incubated with GST-RafRBD pre-coupled to glutathione agarose beads for 2 hours at 4°C as described in Experimental Procedures. Following subsequent washing of the beads, bound Ras-GTP was detected by western blotting, using an anti-pan Ras antibody. Identical results were obtained in a further experiment.

4.7 Exploring the role of the small G protein Rap in the activation of p38 MAPK by IL-1

4.7i Effect of dominant negative and constitutively active Rap on the activation of p38 MAPK by IL-1 in EL4.NOB-1 cells transfected with components of the GAL4-CHOP$^{(1-101)}$ system.

Finally, I investigated the role of Rap on p38 MAPK activation by IL-1. Intriguingly, I found that transfections using dominant negative Rap1AN17 activated the GAL4-CHOP$^{(1-101)}$ reporter system (Figure 4.7.1A). Transfection of cells with 2.5μg of expression plasmid encoding Rap1AN17 induced a response similar to stimulation with IL-1. Adding IL-1 to transfected cells further enhanced this response. This result implied that Rap was having a negative effect on p38 MAPK activation by IL-1, possibly by relieving a tonic inhibition caused by Rap antagonising Ras function. In agreement with this, the same experiment performed with a constitutively active version, Rap1AV12 (Figure 4.7.1B), showed a clear inhibition of the activation of p38 MAPK by IL-1. This suggested that Rap has an antagonistic effect on p38 MAPK activation by IL-1. This phenomena has been seen in previous studies, and may reflect the antagonistic role of Rap towards Ras function, which is thought to occur through the competition for down stream effectors (discussed further below).

4.7ii Effect of constitutively active RapV12 on the activation of p38 MAPK by IL-1 in HeLa cells transfected with FLAG-p38 MAPK.

Further evidence of the antagonistic effects of Rap was provided when co-transfecting HeLa cells with constitutively active RapV12, as seen in figure 4.7.2. Here, HeLa cells transfected with constitutively active RapV12, together with FLAG-tagged p38 MAPK were stimulated with IL-1. As evident from the western blotting using a phospho-p38 MAPK primary antibody, active RapV12 inhibited IL-1 induced phosphorylation of endogenous, as well as transfected FLAG-p38 MAPK. This supports the theory of Rap playing an antagonistic role towards Ras in the activation of p38 MAPK by IL-1. RapV12 also reduced the basal activity of p38 MAPK.

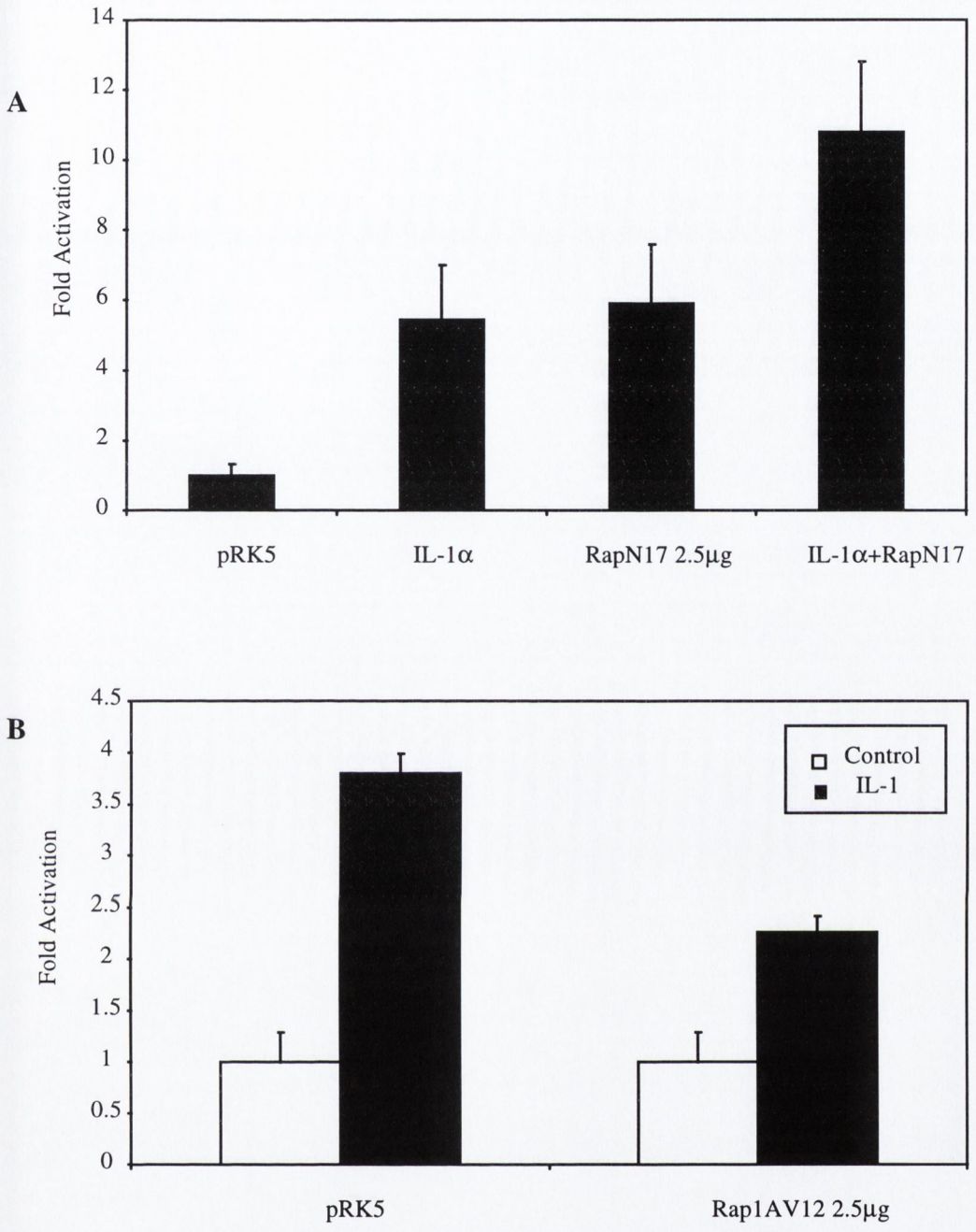


FIGURE 4.7.1

Effects of RapN17 and RapV12 on IL-1α induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with RapN17 (A) or RapV12 (B) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α for 6 hours at 37°C (B, filled bars) or left unstimulated (B, white bars) following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for protein as measured according to the method of Bradford . Results show one representative experiment of four identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

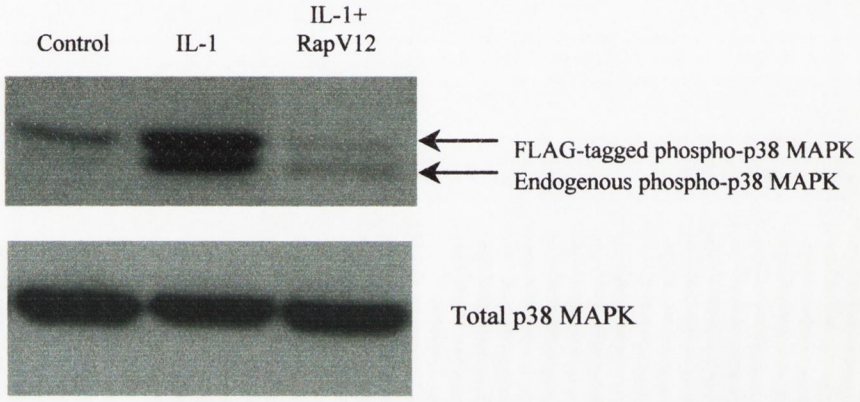


FIGURE 4.7.2

Effect of constitutively active RapV12 on IL-1 induced phosphorylation of p38 MAPK in HeLa cells transfected with FLAG-tagged p38 MAPK. HeLa cells were seeded at $1.5x10^5$ cells in 3 ml of complete DMEM medium 24 hours prior to transfection. The cells were subsequently co-transfected with FLAG-tagged p38 MAPK and 2.5 μg of RapV12 using FuGene™ transfection reagent as described in the methods section. 48 hours after transfection the cells were stimulated with IL-1 for 10 minutes, harvested and western blotting was performed on the cell extracts, detecting transfected and endogenous p38 MAPK using an anti-phosphorylated p38 MAPK antibody (upper panel), or total p38 MAPK (lower panel), as described in the methods section.

I then asked what impact this antagonistic effect would have on IL-1 induced p38 MAPK activity. Does Rap play a role in down-regulating the p38 MAPK response, thereby explaining the transient nature of p38 MAPK activation by IL-1?

To answer this question, I transfected HeLa cells with FLAG-p38 MAPK and either control vector, or dominant negative RapN17. These cells were subsequently exposed to IL-1 for increasing lengths of time, and the phosphorylation status of p38 MAPK was determined by western blotting. As in EL4.NOB-1 cells, IL-1 activates p38 MAPK within a few minutes in HeLa cells transfected with control vector (fig 4.7.3, lanes 1-5), peaking at 10 minutes (lane 2), and rapidly returning to basal levels within 40-60 minutes (lanes 4,5). However, in cells transfected with RapN17 (lanes 6-10), the activation of p38 MAPK by IL-1 became prolonged as well as intensified. As seen in figure 4.7.3 (compare lane 2 to lane 7, and lane 3 to lane 8), RapN17 causes the activation of p38 MAPK by IL-1 at the 10 and 20 minutes time points to be upregulated compared to cells transfected with control vector alone. More importantly, the transient nature of the p38 MAPK signal appears to be delayed, as p38 MAPK, after 40 minutes stimulation in cells transfected with RapN17, is still activated compared to in control cells, which display only a slight activation over basal levels (compare lane 4 to lane 9). This may provide a possible function for the antagonistic effect of Rap, suggesting that Rap may be responsible for the transient nature of the p38 MAPK response to IL-1.

4.7iii Activation of endogenous Rap by IL-1

For Rap to play a role in IL-1 signalling, IL-1 must activate endogenous Rap. Using a similar approach as the activation assay for Ras, the activation of Rap can be measured by using the Ral-GDS-Rap Binding Domain (Ral-GDS-RBD) domain coupled by GST to glutathione agarose beads. Only activated Rap-GTP will bind to the Ral-GDS-RBD, and Rap-GTP can be precipitated and detected by Western blotting using a Rap specific antibody. An increase in Rap-GTP was evident after 6 minutes (fig 4.7.4, lane 2), reaching maximum activation after 13 minutes (lane 3), and was shown to be transient reaching nearly basal levels after 18 minutes (lane 4). This slower kinetics to Ras activation implies that the later activation of Rap may be responsible for the transient nature of p38 MAPK activation in IL-1 treated cells.

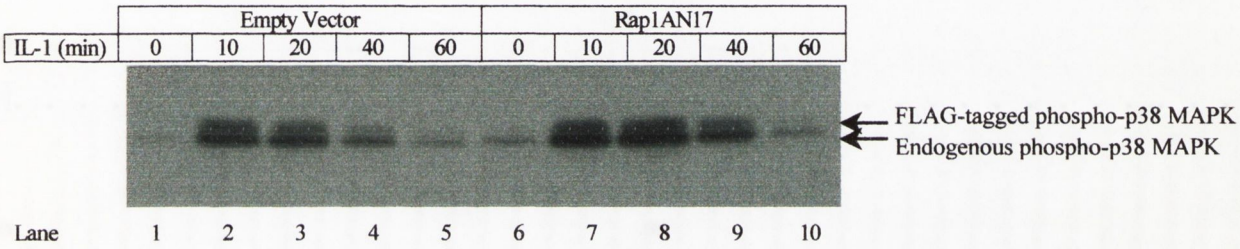


FIGURE 4.7.3

Effect of dominant negative RapN17 on the transient nature of the activation of p38 MAPK by IL-1 in HeLa cells transfected with FLAG-tagged p38 MAPK. HeLa cells were seeded at $1.5x10^5$ cells in 3 ml of complete DMEM medium 24 hours prior to transfection. The cells were subsequently co-transfected with FLAG-tagged p38 MAPK and 2.5 μg of RapN17 using FuGene™ transfection reagent as described in the methods section. 48 hours after transfection the cells were stimulated with IL-1 for indicated times, harvested and western blotting was performed on the cell extracts, detecting transfected and endogenous p38 MAPK using an anti-phosphorylated p38 MAPK antibody, as described in the methods section.

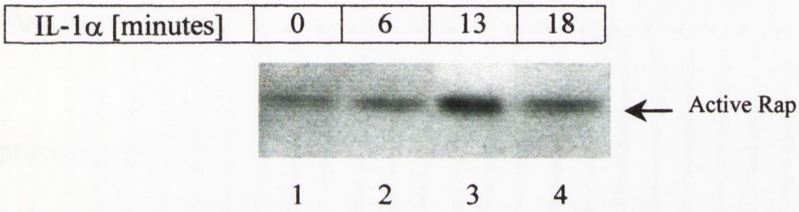


FIGURE 4.7.4

Activation of Rap by IL-1 in EL4.NOB-1 cells. Cell extracts from 2 x 10^6 EL4.NOB-1 cells treated with IL-1α for indicated times (up to18 minutes), were incubated with GST-RalGDS-RBD pre-coupled to glutathione agarose beads for 2 hours at 4°C as described in Experimental Procedures. Following subsequent washing of the beads, the bound active Rap-GTP was detected by western blotting, using a polyclonal anti-Rap antibody. Identical results were obtained in a further experiment.

DISCUSSION

I have shown that a small G protein targeted by LT is involved in the activation of p38 MAPK and p42/p44 MAPK by IL-1. As IL-1 potently activates p38 MAPK, I decided to use the p38 MAPK signal as my model for further studies into identifying the small G proteins involved in this signal. In order to do so I used multiple approaches. The first approach involved the Clostridial factor Toxin B. This LT-related glucosyltransferase inhibits a distinct subset of small G proteins, namely Rac, Rho and Cdc42, enabling me to further narrow down the potential candidates involved in IL-1 signalling. While Toxin B was taken up by the cells, evident from morphological changes which occurred after prolonged treatment time, Toxin B was nevertheless unable to inhibit the activation of p38 MAPK by IL-1, even after treatment times which caused the cells to round up and clump. This suggested that Rac, which is targeted by both Toxin B and LT, was not involved in conveying this signal. In addition, PMA induced activation of p42/p44 MAPK was unaffected by Toxin B, acting as a negative control, since Ras is thought to be the G protein involved in PMA signalling.

I proceeded then to study the proteins targeted by LT and Toxin B. To do this, I performed glucosylation assays, utilising the glucosyltransferase activity of the two virulence factors. LT glucosylated at least 3 proteins in EL4.NOB-1 cells, all within the right molecular weight range of small G proteins with approximate weights of 18, 19 and 23 kDa. Toxin B glucosylated a protein of about 23 kDa in these cells, suggesting that it was not a lack of target proteins that caused the lack of effect on the activation of p38 MAPK. As mentioned in the General Introduction section, the molecular masses of small G proteins vary slightly. The predicted molecular weights of proteins may differ significantly from those observed experimentally. Ha-Ras has an estimated molecular weight of 20.7 kDa determined by the amino acid sequence of the protein and migrates as a 21.3 kDa protein on a 2-D gel. Ral, on the other hand, has a predicted molecular mass of 26.7 kDa, but appears as a 23.6 kDa band on an SDS-PAGE gel. Rap 1 is estimated to have a molecular weight of 18.5 kDa, but migrates as a 21 kDa band on an SDS-PAGE gel, and Rap 2 appears as a 20.6 kDa band, although the predicted weight is only 19 kDa. Rac 1, on the other hand, migrates close to the predicted molecular weight of 21.4 kDa (all data from SWISS PROT Data base at: http://www.expasy.ch/).

The glucosylation of target proteins by LT correlated with the degree of inhibition of p38 MAPK activated by IL-1. The degree of glucosylation in cell extracts using [^{14}C]UDP-glucose and LT will be inversely proportional to that in intact cells treated with LT. Since proteins glucosylated in intact cells will be unavailable to the toxin during a subsequent glucosylation assay in cell extracts, this gives a measure of the degree of glucosylation in intact cells. A direct comparison shows that the extent of glucosylation of target proteins in intact cells and the inhibitory effect of LT on the activation of p38 MAPK by IL-1 correlate, providing evidence that inhibition occurs by glucosylation.

To further determine the number and identity of the small G proteins targeted by LT, separation of target proteins by two-dimension gel elecrophoresis was attempted. Due to the low specific activity of the radiolabelled [^{14}C]-UDP-glucose, glucosylated proteins were difficult to detect by this method and no further attempts were made. However, preliminary results show several interesting areas of the gel, for example, one large area with increased radioactivity with co-ordinates 23 kDa and a pI of 8-10 and another two bands of 24 and 20 kDa both with a pI of around 5-7. These represent proteins targeted by LT in EL4.NOB-1 cells. As was mentioned above, the members of the Ras super-family of small G proteins vary in their molecular mass and in addition to this, small G proteins have pI values that vary from 4.5 to 8.5. For example, Ras has a pI of 5.2, Rap 1A: 6.84. Rap 1B: 5.88, Rap 2A and B: 4.55, and Rac: 7.56 (Zerial et al). The two dimensional gel therefore provides another method for identifying target G proteins. Many of the proteins in the cell have been mapped in the mouse 2D PAGE database project and can be localised by searching for a protein with particular pI and Mw co-ordinates, in the databases at:
(http://biosun.biobase.dk/~pdi/jecelis/mouse_data_select.html).

Another approach to verifying the specificity of LT involved a mutant Chinese Hamster Lung fibroblast cell line, denoted Cdt^{R}-Q. This cell line was derived by mutagenesis and isolated by selecting for resistance to Toxin B (Florin, 1991). Further studies revealed that the cells had a single point mutation in the UDP-glucose pyrophosphorylase gene, resulting in cells with an intracellular level of UDP-glucose of only 26% of

that of the parental cell line (Chaves Olarte *et al.*, 1996; Flores-Diaz *et al.*, 1997). Using this UDP-glucose deficient cell line, I was able to demonstrate that UDP-glucose is critical for the inhibitory effect of LT on the activation of p38 MAPK by IL-1. The lack of UDP-glucose proved to be sufficient to cause a total lack of effect of LT on the activation of p38 MAPK by IL-1 in Cdt^R-Q cells. This verified that the inhibitory effect of LT was due to glucosylation of target small G proteins in the cells. Furthermore, the Don cell line provided another system in which Toxin B had no effect on IL-1 signalling, although it caused rounding of the cell bodies when taken up by the cells. Furthermore, the activation of p42 and p44 MAPK by IL-1 in these cells was inhibited by LT in the parental Don cells but it was unaffected by LT in the UDP-glucose deficient Don strain, again proving that glucosylation of target proteins is crucial for the inhibitory effect. Toxin B was again without effect on the activation of p42/p44 MAPK by IL-1 in both cell types, suggesting that Rac, Cdc42, and Rho do not play a role in the activation of this pathway.

The lack of effect in the Don Cdt^R-Q cells could have been due to a deficiency in target proteins present in the mutant cell line. This, however, was shown not to be the case, as both wild type Don and Cdt^R-Q displayed comparable amounts of target proteins for both LT and Toxin B when a glucosylation assay was performed.

The Cdt^R-Q mutant cells show a different pattern of glucosylation during the glucosylation assay on intact cells. While the cells may take up LT, no glucosylation can occur in intact cells due to the lack of UDP-glucose. When *in vitro*, [^{14}C]-UDP glucose is added, all the target proteins for LT are available for radiolabelling, therefore, no differences are observed between pre-treated compared to untreated samples. This further reflects the lack of glucosylation taking place in Cdt^R-Q mutant cells, and provides yet another control for the specificity of the effects of LT.

My data with LT indicated that Ras, Rac, Ral or Rap was critical for p38 MAPK activation by IL-1. The effect of Ral has not been investigated in this study, and the reasons for this are twofold. Firstly, when LT from the Clostridial strain IP82 was first characterised, no glucosylation effect on Ral by LT was detected,

and the target proteins were determined to be Ras, Rac and Rap. Subsequently, when this study was well under way, further characterisations showed that Ral was indeed, to a small extent, targeted by LT. Secondly, there was a lack of reagents specific for Ral. I therefore focussed on Ras, Rap and Rac using an assay which would enable the use of transient transfections with mutant constructs of each of these G proteins. The technique is based on a *trans*-acting one-hybrid system, involving phosphorylation of the p38 MAPK specific substrate CHOP (described in detail in materials and methods).

The second approach for studying the effect of individual G proteins involved overexpressing FLAG-tagged p38 MAPK in HeLa cells, and by western blotting detecting the phosphorylation status of the transfected p38 MAPK after cotransfecting the cells with constructs for dominant negative or constitutively active small G proteins. This approach again allowed us to study the effects of these components in a transfected population of cells. The cell line chosen for this experiment were human cervical carcinoma HeLa cells, because these cells have a high transfection efficiency when transfecting using FuGene transfection reagent. HeLa cells also provide a second IL-1 responsive cell line in which results from the CHOP reporter assay can be confirmed.

As the results from using Toxin B suggested that Rac might not be involved in the activation of p38 MAPK by IL-1, I first tested the effect of dominant negative Rac on the activation of p38 MAPK. These results indicated that Rac was indeed not involved in this response. As described above, dominant negative Rac has been shown by others to inhibit NFκB and p38 MAPK activation by IL-1 however. The basis for the discrepancy with my results is not clear, but given that both ToxB and transfection of RacN17 failed to have any effect, I conclude that Rac is not important in my system. A lack of effect of Rac in JNK activation by IL-1 has also been shown (Davis *et al.*, 1999), and I have therefore concluded that Rac may not be an important regulator of p38 MAPK in the IL-1 system, although as RacV12 activates p38 MAPK Rac may be important in the activation of p38 MAPK in response to other stimuli.

A role for Ras in p38 MAPK activation by PDGF, FGF, and hemopoietic cytokines has been demonstrated (Efimova *et al.*, 1998; Matsumoto *et al.*, 1999; Rausch and Marshall, 1999; Tan *et al.*, 1996). My data here clearly adds IL-1 to the list of p38 MAPK activators which require Ras. Constitutively active RasVHa activated p38 MAPK in both the GAL4-CHOP$^{(1-101)}$ as well as in the FLAG-p38 MAPK assays. IL-1 in combination with RasVHa causes an additional increase in CHOP activity, probably reflecting activation of endogenous Ras by IL-1 adding to the effect. In addition, dominant negative RasN17 inhibited the activation of p38 MAPK by IL-1 in both assays. A role for Ras in IL-1 signalling has also been indicated in other studies. Induction of the collagenase promoter by IL-1 in chondrocytes has been shown to require Ras(Grumbles *et al.*, 1997), as has induction of the brain natriuretic peptide in myocytes(He and LaPointe, 1999). Others, however, have failed to demonstrate activation of Ras by IL-1(Bird *et al.*, 1994). My data, using the GST-RBD assay, clearly shows rapid and transient activation of Ras in EL4.NOB-1 cells however. This is a very specific activation assay for Ras, which relies on the specific ability of activated Ras to bind the Ras binding domain of c-Raf (described in detail in materials and methods section). Only active Ras will interact with c-Raf, and the assay therefore reflects a real interaction of Ras with a known downstream effector. How IL-1 activates Ras, and the means by which Ras activates the p38 MAPK cascade is unclear. Generally, Ras becomes activated by specific Ras-GEFs, which catalyse the exchange of GDP for GTP, causing Ras to become activated. Ras then forms a homodimer and alters its conformation to interact with multiple downstream effectors(Inouye *et al.*, 2000). The best-characterised effector, as described in chapter 1, is Raf-1, a kinase responsible for the activation of the classical p42/p44 MAPK pathway, in response to stimuli such as growth factors. The GEF involved in activating Ras in response to IL-1 has not yet been described.

A number of recent studies have suggested that the different Ras homologues could preferentially mediate distinctive cellular processes (reviewed in (Rebollo and Martinez, 1999)). K-Ras, but not H- or N-Ras, for example, plays an important role in mouse development. K-Ras, is induced during differentiation of pluripotent embryonal stem cells and has been shown to specifically interact with microtubules and disrupt basolateral polarity in colon epithelial cells. Furthermore, selective activation of K-Ras expression attenuates

the ability of the EGF receptor to activate MAPK pathway by interfering with receptor autophosphorylation. A novel subclass of Ras proteins has been identified. The Ras proteins of this subgroup differ from other Ras proteins in amino acid positions 12 and 61, as the amino acids of this region are similar to those present in oncogenic forms of Ras. These novel Ras proteins have been given the names κb-Ras1 and κB-Ras2 as they regulate the degradation of IκB (Fenwick *et al.*, 2000).

Unfortunately, the RasN17 construct used in this study, does not allow for distinguishing between the function of different Ras isoforms and, therefore, no conclusion as to which type of Ras protein mediate the effect seen here can be drawn. As reviewed in (Feig, 1999), RasN17 mutants inhibit as they fail to bind to downstream target proteins even when bound to GTP, and instead, the GTPRasN17 stay bound to Ras-GEF, causing this activator of Ras to become 'mopped up'. Consequently, endogenous Ras cannot become activated. The specificity lies in which RasGEF becomes occupied by RasN17, and this is where the technique fails to distinguish between Ras isoforms which are inhibited. As the different GEFs are, to a certain extent, specific for the different Ras isoforms, and since all RasGEFs become bound non-specifically to RasN17, all Ras isoforms will compete with RasN17 for GEFs. RasVHa also mimics Ras in an isoform unspecific manner, again providing no information as to which Ras isoform is utilised by this pathway.

Apart from the activation of Ras, I also found IL-1 to be an activator of Rap. A number of other extracellular signals including PDGF, EGF, endothelin and 1-oleoyl-lyso-phosphatidic acid (LPA), have recently been shown to activate Rap (Bos, 1998; Zwartkruis *et al.*, 1998), although a role for Rap in downstream events was not investigated. The mechanism whereby Rap becomes activated is not as well studied as the activation of Ras, although several Rap specific GEFs and GAPs have been identified (see Introduction). Furthermore, two independent groups have published data, which provide a link between heterotrimeric G proteins and Rap1. In the first report, co-immunoprecipitation data proposed a direct interaction between the α-subunit of a inactive heterotrimeric G_o protein with Rap1GAP (Jordan *et al.*, 1999). This provides evidence for a novel function of $G\alpha_o$, which in its resting state can sequester Rap1GAP, regulating Rap1 activity. The second

report (Mochizuki *et al.*, 1999) shows that the Rap GAP: rap1GAPII can bind specifically to the α-subunit of a G_i heterotrimeric G protein. Activation of the heterotrimeric G protein causes the RapGAP to translocate to the membrane, decreasing the amount of available GTP-bound Rap1. As Rap has been shown to anatagonise Ras function, removing active Rap causes a reduction of inhibition of Ras function which, in this study, resulted in activation of p42/p44 MAPK.

Recent reports have pointed to Rap as a positive regulator in cell signalling, although the major role of Rap in cell signalling appears to be as an antagonist towards Ras. The mechanism of this antagonism is likely to be due to competition for effectors. Evidence has been presented for Rap interacting with Raf-1, blocking its activation by Ras. Furthermore, RapV12 has been shown to inhibit p42/p44 MAPK activation by LPA and EGF (Cook *et al.*, 1993). Since Rap activation occurred at a later time to Ras activation it is possible that the transient nature of p38 MAPK activation by IL-1 may be due to Rap inhibiting the response.

The activation of Rap may also be important for the induction of anergy in T cells. Boussiotis et al (Boussiotis *et al.*, 1997) showed that Rap-GTP was present in anergic T cells and proposed a negative regulatory role for Rap in T-cell Receptor mediated IL-2 gene transcription, suggesting that Rap may be responsible for the specific defect in IL-2 production in T cell anergy. This may be relevant for IL-1 signalling, since IL-1 induces IL-2 in EL4. It is therefore possible that the activation of Rap by IL-1 would lead to an inhibition of signalling initiated as a result of Ras activation, thereby limiting the effects of IL-1 on IL-2 production (and as stated above on p38 MAPK activation), acting as a negative feedback loop. The activation of the GAL4-$CHOP^{(1-101)}$ system by overexpressing mutant RapN17 may be the result of removing the tonic inhibitory effect of Rap in resting cells, suggesting that the pathway leading to p38 MAPK is regulated by Rap in the absence of IL-1. This basal activity of the pathway, however, is not sufficiently strong to phosphorylate FLAG p38 MAPK in HeLa cells to a level which is detectable in the western blotting assay. This activity, however, can be seen in the accumulative CHOP reporter system, demonstrating this effect of RapN17.

As the activation of p38 MAPK by IL-1 is transient, I wanted to study the effect of dominant negative Rap on the nature of this signal. By removing the down-regulating element of the pathway (Rap) by transfecting cells with dominant negative RapN17, I was able to show a prolonged activation profile of p38 MAPK in response to IL-1. This suggests that Rap may be needed to maintain the transient nature of the activation of p38 MAPK by IL-1 and it points to an important negative regulatory role for Rap.

In conclusion, my study thus far identifies Ras as a key signal in the activation of p38 MAPK by IL-1 (fig 4.8). Furthermore, this is the first demonstration of Rap activation by IL-1, which, given its inhibiting effect, provides further evidence for a role for Ras in the effect of IL-1 and suggests a mechanism for the transient nature of IL-1 signalling.

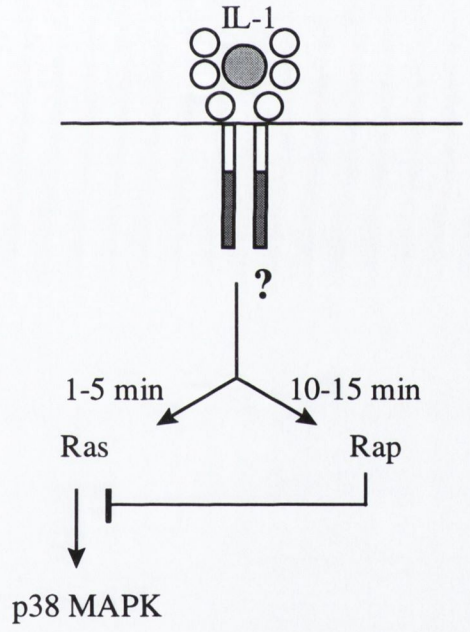


Figure 4.8 Proposed model of the role for Ras and Rap in the activation of p38 MAPK by IL-1. Further details are given in the text.

CHAPTER 5

Investigating how known IL-1 signalling components couple to Ras and Rap in the activation of p38 MAPK

INTRODUCTION

Having demonstrated a role for Ras and Rap in p38 MAPK activation by IL-1, I next addressed how my observation related to other components in the IL-1 signalling system. The signalling pathway leading to the activation of the transcription factor NFκB by IL-1 has been studied in great detail (reviewed in (O'Neill and Greene, 1998)). As described in the General Introduction, IL-1 binds to the Type I IL-1 Receptor (IL-1RI), after which the receptor forms a complex with the IL-1 Receptor Accessory Protein (IL-1RAcP)(Wesche *et al.*, 1997b). This triggers the recruitment of the adapter protein MyD88, which associates with IL-1RI through its carboxy-terminal Toll/IL-1 Receptor (TIR) domain (Burns *et al.*, 1998). Binding of IL-1 to its receptor also causes the recruitment of the serine/threonine kinase IL-1R-associated kinases (IRAK)-1 and –2(Cao *et al.*, 1996a; Wesche *et al.*, 1997a), which is pre-associated with the regulatory protein Toll-interacting protein (Tollip, see introduction) (Burns *et al.*, 2000). Once recruited, MyD88 can, through its death domain, interact with the death domain of IRAK, which induces auto-phosphorylation of the serine/threonine rich N-terminus of IRAK. Once phosphorylated, IRAK dissociates from MyD88 and Tollip and becomes associated with TNF Receptor Associated Factor 6 (TRAF6)(Cao *et al.*, 1996b). The MAPKKK TGF-β-activated Kinase (TAK1), constitutively associated with TAB1 (Ninomiya-Tsuji *et al.*, 1999), then associates with the TRAF6/IRAK complex, triggering the autophosphorylation of TAK1. TAK1 can then activate two kinase cascades: one that leads to the activation of jun N-terminal kinase (JNK) and the other cascade leads to the activation of the IκB kinase complex which ultimately leads to the activation of NFκB (Baud *et al.*, 1999; Song *et al.*, 1997).

The signalling pathway leading to the activation of p38 by IL-1 is not as well elucidated as the pathway leading to the activation of NFκB, although early events in the two pathways share similarities, since both TRAF6 and IRAK1 have been shown to play a role in the activation of p38 MAPK by IL-1 (Thomas *et al.*, 1999) (Kanakaraj *et al.*, 1998) (Baud *et al.*, 1999). p38 MAPK is activated by the MAPK kinases MKK3b (the predominant isoform of MKK3), and MKK6. A variety of MAPKKK can activate MKK3 and MKK6, including PAK, TAK1, and ASK1 (Bagrodia *et al.*, 1995; Ichijo *et al.*, 1997; Moriguchi *et al.*, 1996).

In chapters 3 and 4 I demonstrated that the Small G Protein Ras plays a critical role in the activation of p38 MAPK by IL-1. In the last part of this study, I have investigated the role of MyD88, IRAK1/2, and TRAF6, in the activation of p38 MAPK, and I have found that they all play a role in mediating this activation. Furthermore, I have sought to identify the role of Ras on this pathway, and here I show that Ras mediates the signal at a point downstream of MyD88, IRAK1/2, and TRAF6, but upstream of MKK3b and MKK6.

I have also shown that the small G protein, Rap, acts as a negative regulator in the activation of p38 MAPK by IL-1, consistent with its role as an inhibitor of Ras function [Chapter 4]. Here I demonstrate that the inhibitory effect of Rap on the activation of p38 MAPK by IL-1 occurs at the same point as Ras, namely downstream of MyD88, IRAK1/2 and TRAF6, but upstream of MKK3 and MKK6.

RESULTS

5.1 MyD88, IRAK1, TRAF6, TAK1, MKK3b and MKK6 all mediate the activation of p38 MAPK by IL-1 in EL4.NOB-1 cells transfected with components of the GAL4-CHOP$^{(1-101)}$ system

5.1i Effect of MyD88 on the activation of p38 MAPK

MyD88 is the adapter protein which, through a carboxy-terminal TIR domain, becomes recruited to the IL-1R and IL-1RAcP following the binding of IL-1. As this adapter protein has been shown to be critical in other IL-1 signalling events, I wanted to investigate the role of MyD88 in the activation of p38 MAPK by IL-1.

As depicted in figure 5.1.1A, cotransfecting EL4.NOB-1 cells with the components of the GAL4-CHOP$^{(1-101)}$ system, together with a dominant negative mutant of MyD88 (ΔMyD88), inhibited the activation of p38 MAPK by IL-1, suggesting that MyD88 plays a critical role in the activation of this pathway. Furthermore, as shown in figure 5.1.1B, overexpression of wild type MyD88 activated p38 MAPK and induced expression of the luciferase reporter gene in this system, providing further evidence for a role for MyD88 in the activation of the p38 MAPK cascade.

5.1ii Effect of IRAK1 and IRAK2 on the activation of p38 MAPK

I next co-transfected EL4.NOB-1 cells with a dominant negative mutant of IRAK1 (DDIRAK1, amino acids 1-211). As seen in figure 5.1.2A, over expression of DDIRAK1 inhibited the activation of p38 MAPK and the expression of the luciferase reporter gene in this system. In addition to this, over expression of wild type IRAK1 caused CHOP to be phosphorylated and the luciferase gene to become expressed, proving that IRAK1 can activate p38 MAPK. The role of IRAK2 in IL-1 signalling is not so well defined. IRAK2 is still required for the activation of NFκB by IL-1. IRAK2 lacks a kinase domain, and does not undergo phosphorylation during signal transduction. I transfected EL4.NOB-1 cells with two different mutant versions of IRAK2, K-IRAK2 (fig 5.1.3A) and dd-IRAK2 (fig 5.1.3B). Both of these IRAK2 constructs have been shown to inhibit IL-1 signalling, acting as dominant negative mutants possibly by competing for downstream signalling components. As seen in figure 5.1.3, both K-IRAK2 and dd-IRAK2 inhibited the

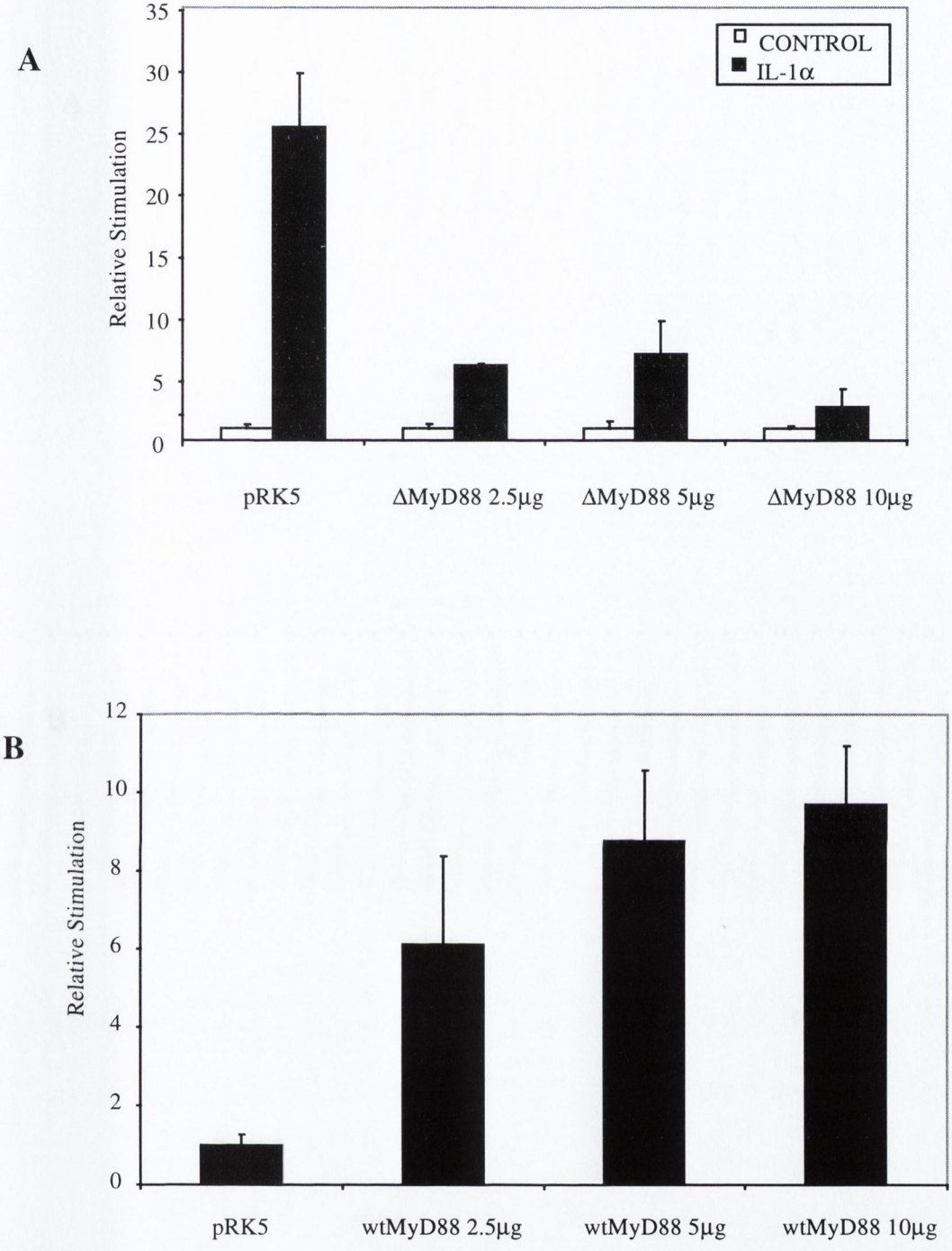


FIGURE 5.1.1

Effects of dominant negative ΔMyD88 and wild type MyD88 on the phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with ΔMyD88 (A) or wtMyD88 (B) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α for 6 hours at 37°C (A, filled bars) or left unstimulated (A, white bars) following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . Results show one representative experiment of four identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

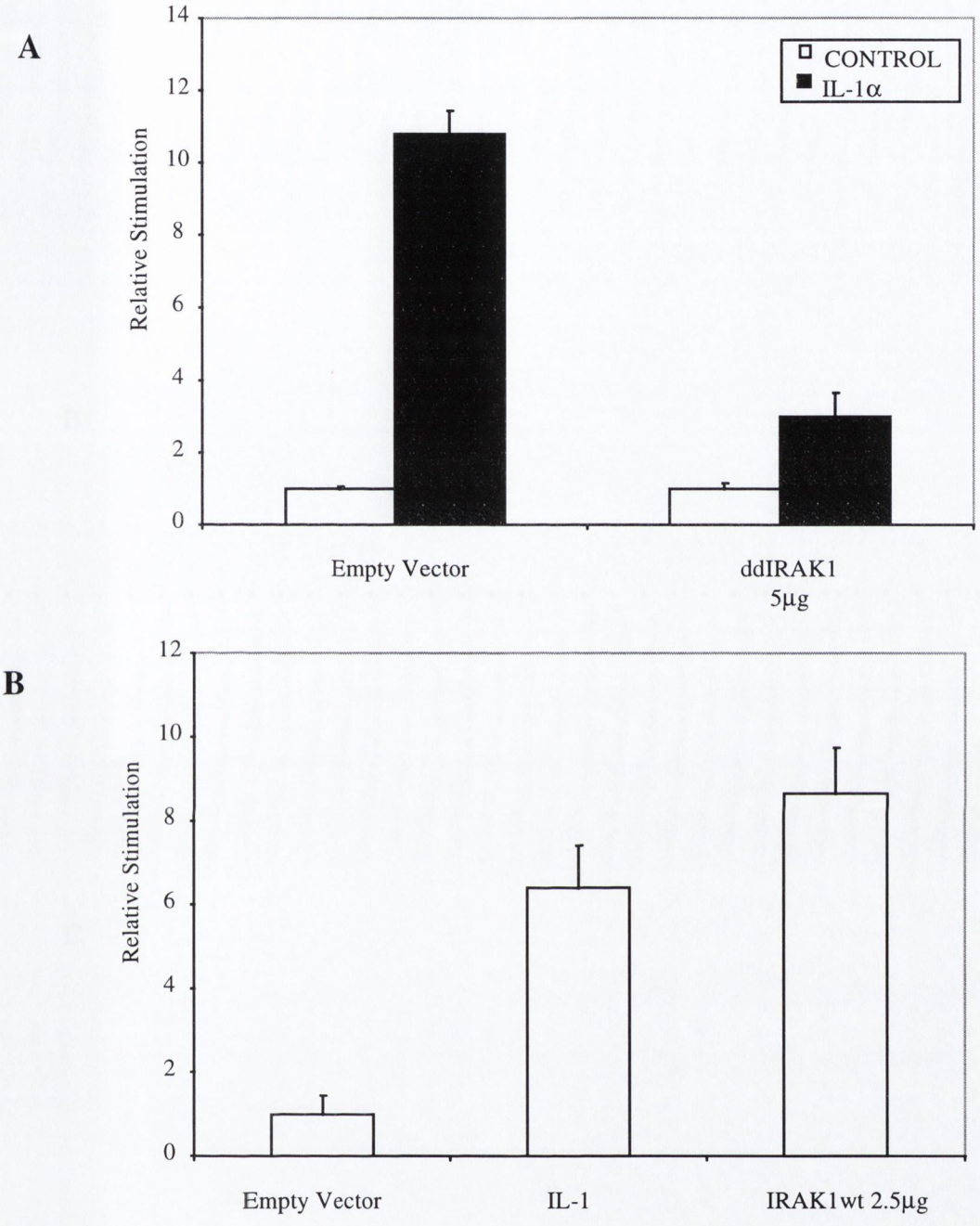


FIGURE 5.1.2

Effects of dominant negative death domain ddIRAK1 (1-211) (A), and wild type IRAK1 (B), on the phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with ddIRAK1 (1-211) (A), or wild type IRAK1 (B) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α for 6 hours at 37°C (A, filled bars) or left unstimulated (A, white bars) following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . Results show one representative experiment of four identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

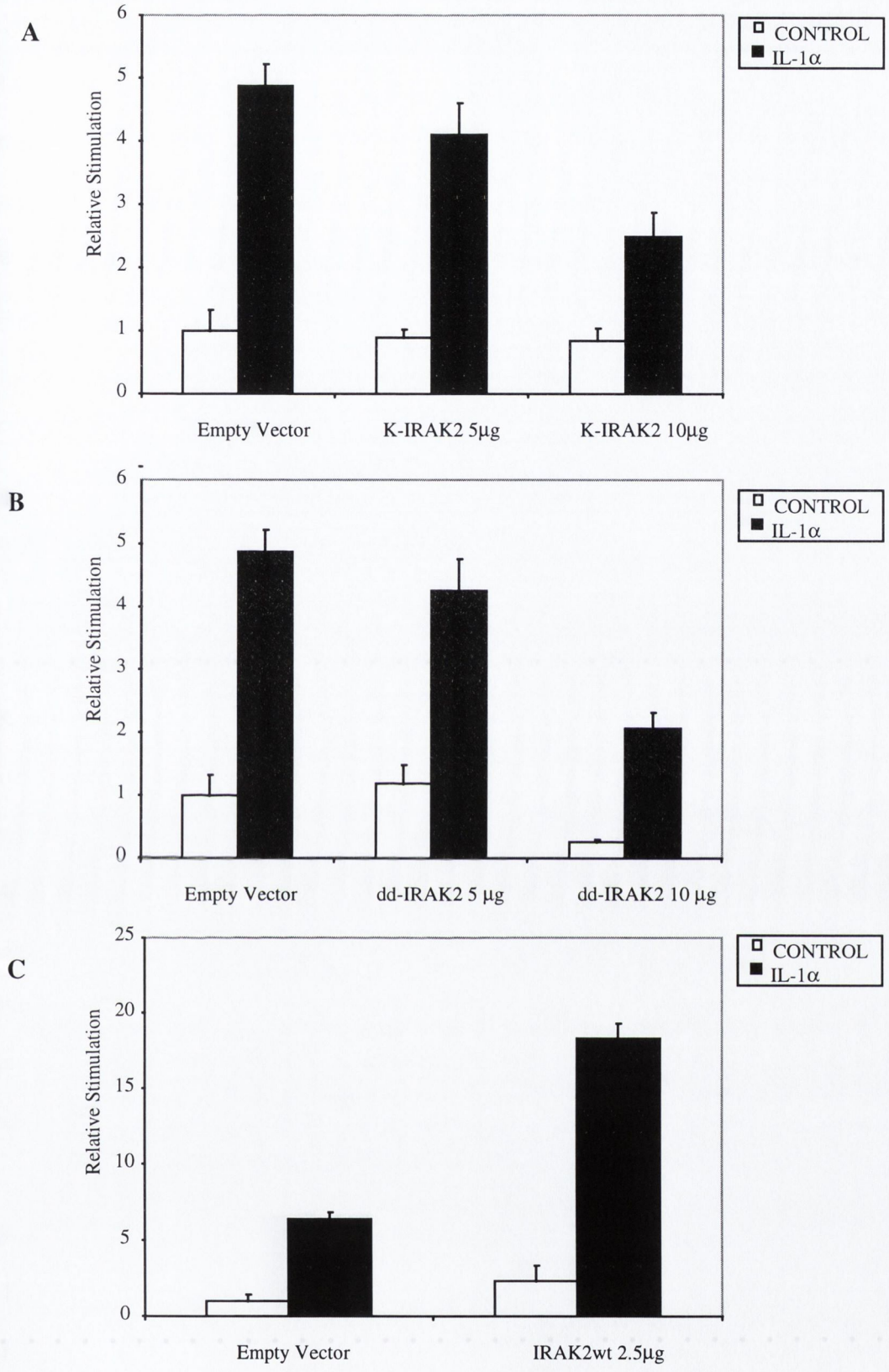


FIGURE 5.1.3

Effects of dominant negative K-IRAK2 (A), dominant negative ddIRAK2 (B) and wild type IRAK2 (C) on the phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with K-IRAK2 (A), ddIRAK2 (B) or wild type IRAK2 (C) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α for 6 hours at 37°C (A, filled bars) or left unstimulated (A, white bars) following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . Results show one representative experiment of four identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

activation of p38 MAPK by IL-1, suggesting that IRAK2 plays a critical role in activating this signalling cascade. However, when overexpressing wildtype IRAK2, a very small increase in luciferase activity can be detected in unstimulated cells. However, if cells overexpressing IRAK2 are stimulated with IL-1, a large potentiation of the IL-1 signal was observed (fig 5.1.3C). A possible reason for this is that IRAK2 cannot signal on its own, but may be limiting in IL-1 signalling, so that an increase in present IRAK2 allows for a stronger response to IL-1.

5.1iii Effect of TRAF6 on the activation of p38 MAPK

I next transfected EL4.NOB-1 cells with a dominant negative mutant of TRAF6 (ΔTRAF6). The results from this assay are depicted in figure 5.1.4A, and show how ΔTRAF6 potently inhibits the activation of p38 MAPK by IL-1 in this system, and in addition, higher doses of ΔTRAF6 caused an inhibition of the basal p38 MAPK activity. As shown in figure 5.1.4B, cells were also transfected with dominant negative TRAF2, as a negative control, with TRAF2 having little effect on the activation of p38 MAPK by IL-1 (Fig 5.1.4B). Furthermore, when over-expressing wild type TRAF6 in these cells, I noted that although at low levels of TRAF6 there was only a small increase in the activation of p38 MAPK, when these cells were stimulated with IL-1, again a potentiation of the IL-1 signal was observed (Fig 5.1.4C). Increasing amounts of TRAF6, up to 10 μg DNA activated p38 MAPK, although the IL-1 response did not increase beyond what was observed in the cells transfected with 2.5 μg wtTRAF6, probably because a threshold had been reached. These results suggest that TRAF6 is involved in the activation of p38 MAPK by IL-1.

5.1iv Effect of TAK1 on the activation of p38 MAPK

Activated TRAF6 associates with the MAPKKK TAK1 on the pathway leading to the activation of NFκB by IL-1, and I therefore wanted to investigate the effect of dominant negative TAK1 on the activation of p38 MAPK by IL-1. Figure 5.1.5A show how transfecting EL4.NOB-1 cells with dominant negative ΔTAK1 inhibited IL-1 induced expression of the luciferase reporter gene, suggesting that TAK1 may play a role in the activation of p38 MAPK by IL-1. Again, the basal p38 MAPK activity is somewhat inhibited by higher

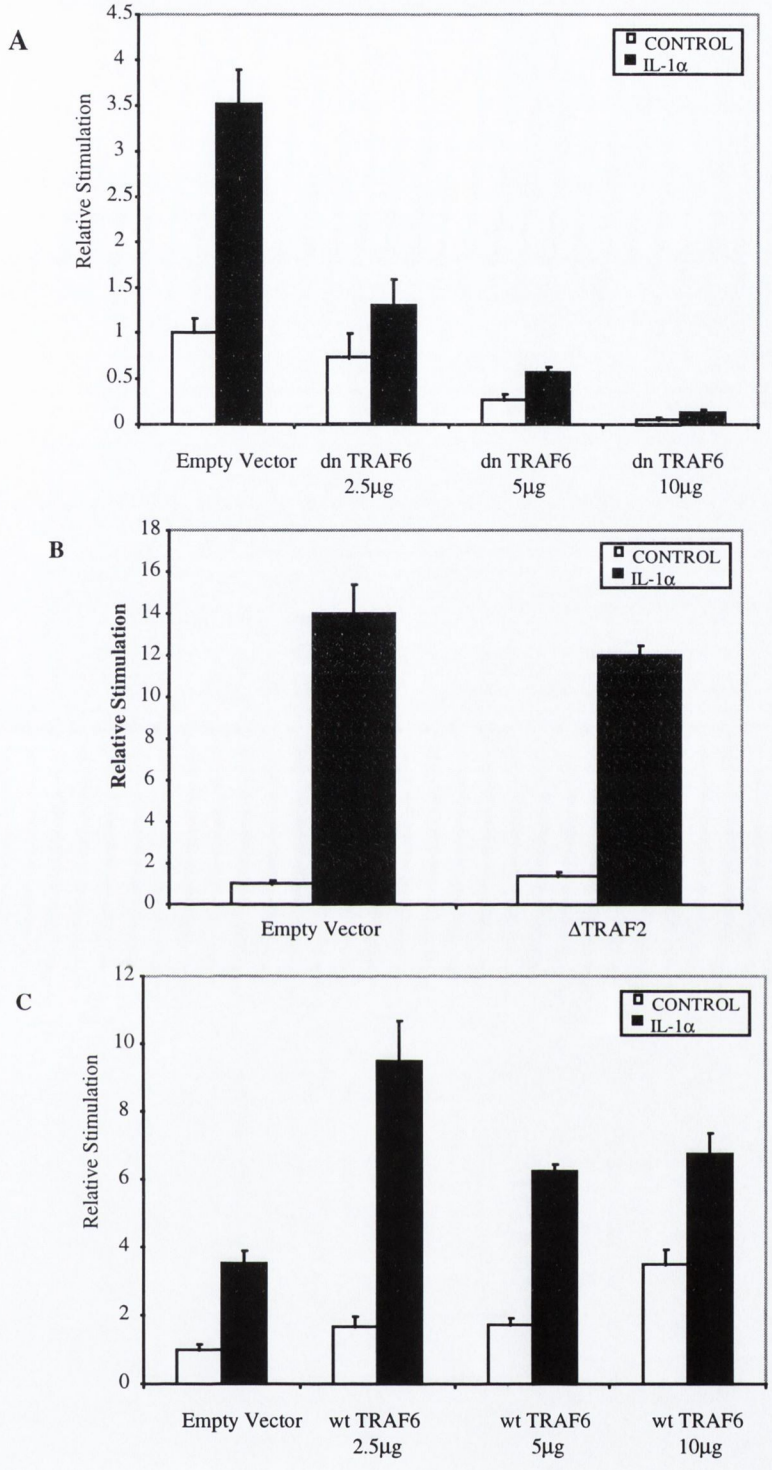


FIGURE 5.1.4

Effects of dominant negative ΔTRAF6 (A), and wild type TRAF6 (B) on the phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with ΔTRAF6 (A), ΔTRAF2 (B) and wild type TRAF6 (C) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α for 6 hours at 37°C (filled bars) or left unstimulated (white bars) following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . Results show one representative experiment of four identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

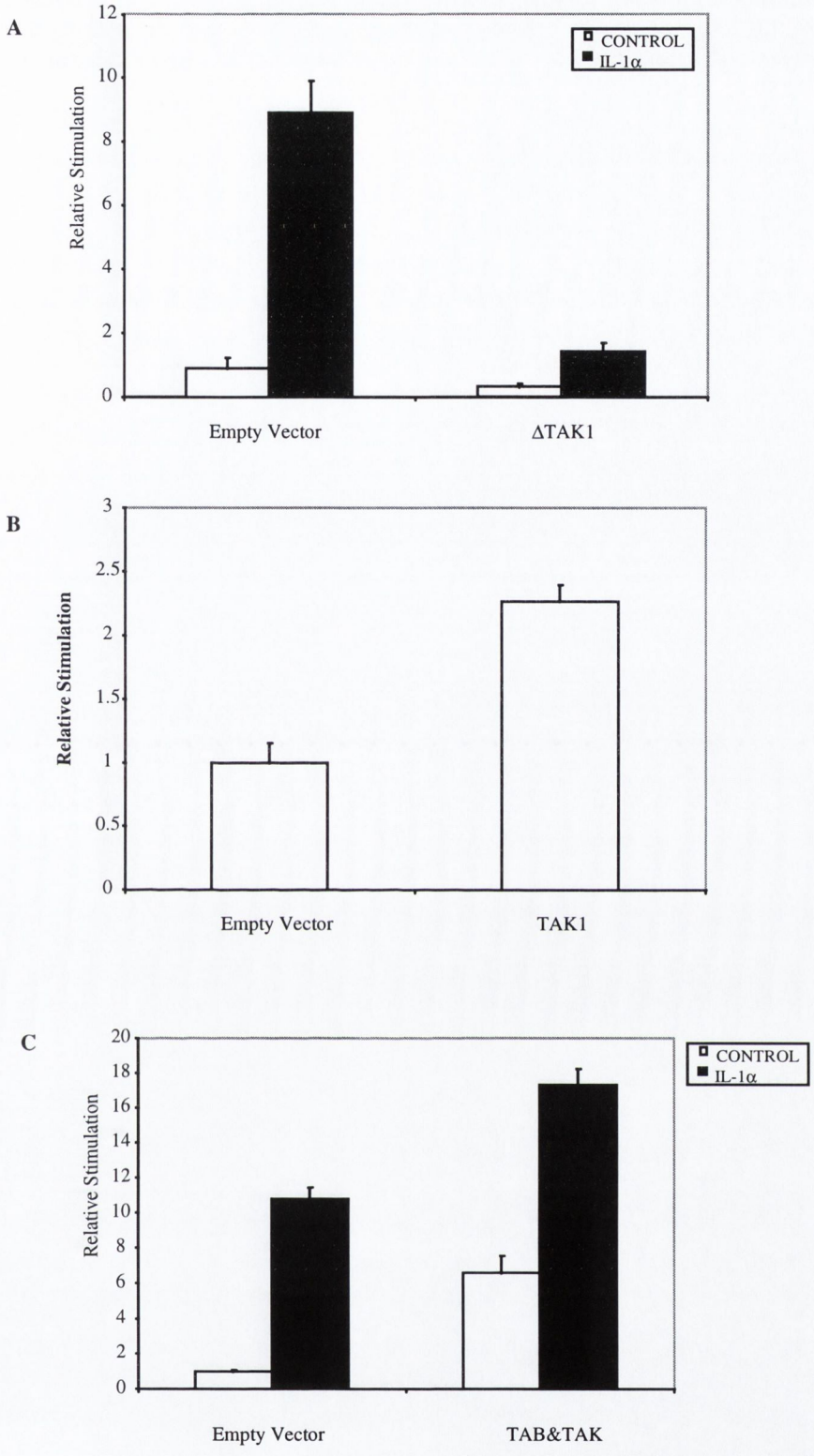


FIGURE 5.1.5

Effects of dominant negative ΔTAK1 (A), and wild type TAK1 and TAB1 (B) on the phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with ΔTAK1 (A), and wild type TAK1 and TAB1 (B) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α for 6 hours at 37°C (filled bars) or left unstimulated (white bars) following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . Results show one representative experiment of four identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

doses of ΔTAK1. TAB1 is an accessory protein constitutively associated with TAK1 in intact cells, a protein which is required for optimal signalling by TAK1. Wild type TAK1, when over-expressed in EL4.NOB-1 cells was able to increase the luciferase activity in this system by about 2-2.5 fold (Fig 5.1.5B). If, on the other hand, wild type TAK1 was expressed in combination with the accessory protein TAB1, the level of activated p38 MAPK was greatly increased (fig 5.1.5C), suggesting that the two proteins act together as important signalling molecules on the pathway leading to the activation of p38 MAPK by IL-1.

5.1v Effect of MKK3b and MKK6 on the activation of p38 MAPK

The two most important immediately upstream kinases which activate p38 MAPK are MKK3b and MKK6. I wanted to investigate the role of these MAPKKs on the activation of p38 MAPK by IL-1, and if possible determine which one was the critical activator. To do this, I transfected EL4.NOB-1 cells with the components of the GAL4-CHOP$^{(1\text{-}101)}$ system, together with dominant negative MKK3b (MKK3b(A)), and as can be seen in figure 5.1.6A, MKK3b(A) inhibited the activation of p38 MAPK by IL-1, suggesting that MKK3b is important for this signal. Furthermore, overexpressing wild type MKK3b activated p38 MAPK to a similar extent as IL-1, supporting a role for MKK3b (fig 5.1.6B).

Dominant negative MKK6 (MKK6(A)), also displayed an inhibitory effect on the activation of p38 MAPK by IL-1, as seen in figure 5.1.7 (A). This points to a role of MKK6 on this pathway, which is further supported by the results shown in figure 5.1.7B, as over-expressing wild type MKK6 also causes p38 MAPK to become activated. As both MKK3b and MKK6 have similar effects on the activation of p38 MAPK, I must conclude that they both play important roles. Although, since neither of the two mutant versions of these proteins causes 100% inhibition, they may work together, both being able to transduce the signal induced by IL-1.

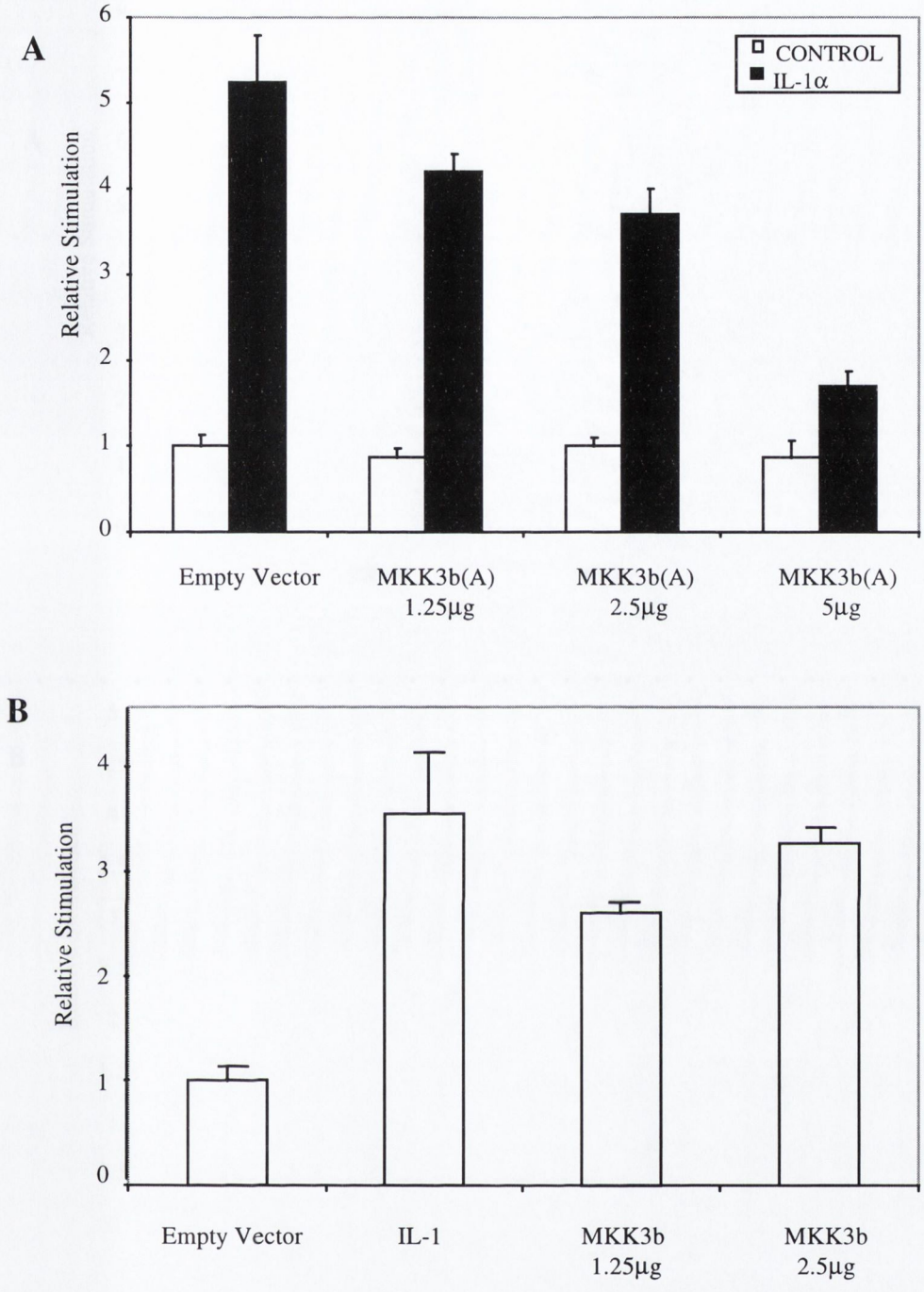


FIGURE 5.1.6

Effects of dominant negative ΔMKK3b(A) (A), and wild type MKK3b (B) on the phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with ΔMKK3b(A) (A), and wild type MKK3b (B) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α for 6 hours at 37°C (filled bars, A) or left unstimulated (white bars, A) following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . Results show one representative experiment of four identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

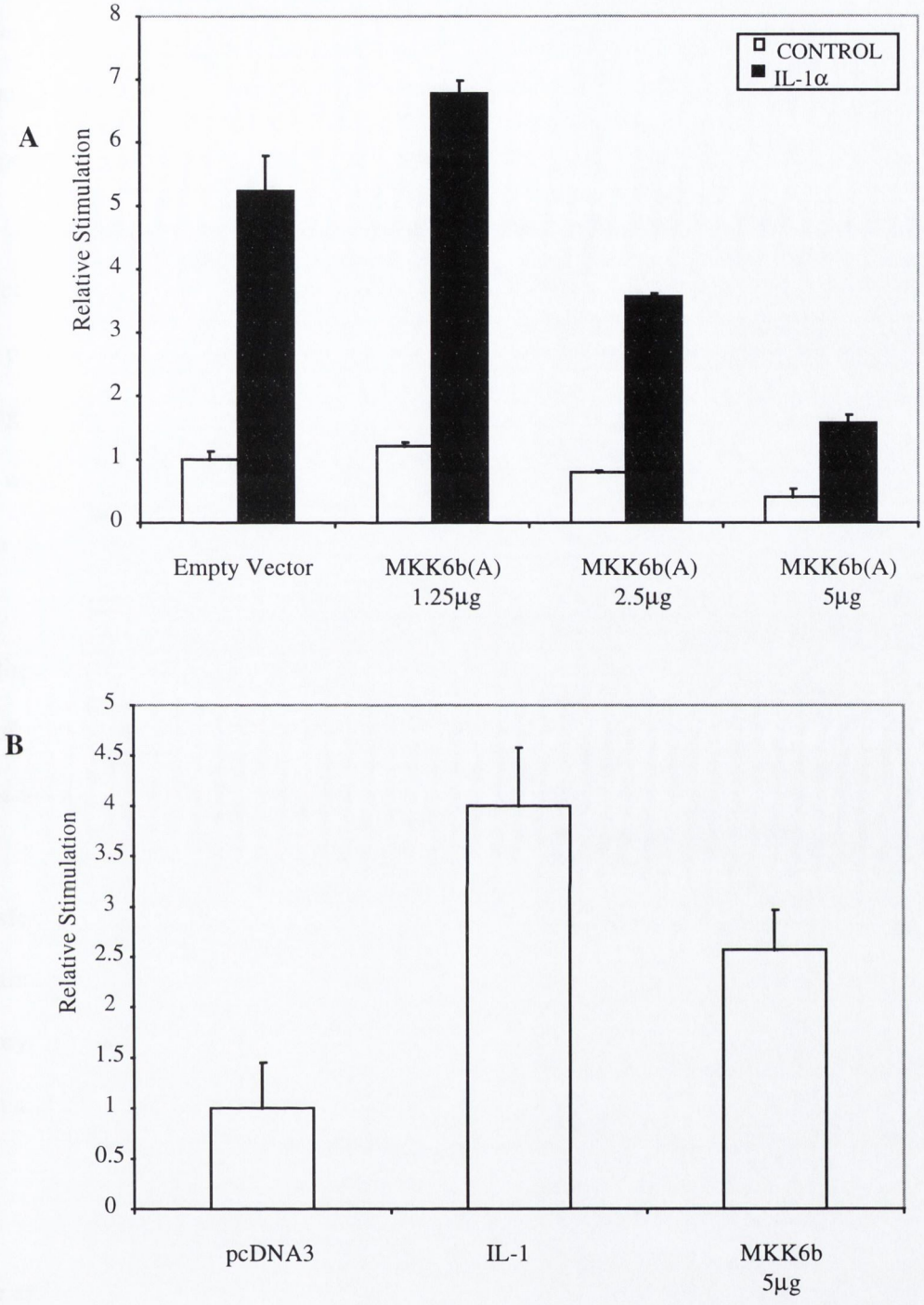


FIGURE 5.1.7

Effects of dominant negative ΔMKK6b(A) (A), and wild type MKK6 (B) on the phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with ΔMKK6b(A) (A), and wild type MKK6 (B) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α for 6 hours at 37°C (filled bars, A) or left unstimulated (white bars, A) following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . Results show one representative experiment of four identical experiments performed expressed as mean of fold stimulation ± SEM for samples assayed in quadruplicate.

5.1vi MyD88, IRAK1, TRAF6, RasVHa, MKK3b and MKK6 all mediate the activation of p38 MAPK by IL-1 as determined in HeLa cells transfected with FLAG-p38 MAPK

To investigate the involvement of these signalling molecules in the activation of p38 MAPK by IL-1, using an independent assay system, constructs encoding dominant negative version of MyD88 (ΔAU1), DD-IRAK1 (1-211), IRAK2 (K), TRAF6 (ΔTRAF6), RasN17, RapV12, MKK3b(A), and MKK6(A) were all cotransfected into HeLa cells together with FLAG-p38 MAPK. As shown in Figure 5.1.8A, IL-1 is unable to activate p38 MAPK in cells expressing any of the above mutant proteins, ranging from 50-100% inhibition, providing further evidence that they are signalling components mediating the activation of p38 MAPK by IL-1. Cells were transfected with a mutant construct of TRAF2 as a negative control, with ΔTRAF2 having little effect on the activation of p38 MAPK by IL-1 (not shown).

Again, the results from the GAL4-CHOP$^{(1-101)}$ assay were confirmed when examining the phosphorylation status of transfected FLAG-tagged p38 MAPK in HeLa cells transfected with wild type signalling components.

Co-transfecting HeLa cells with FLAG-p38 MAPK and wild type MyD88, IRAK1, TRAF6, TAK1 (in combination with the accessory protein TAB1), MKK3b, MKK6 or constitutively active RasVHa, caused phosphorylation and activation of p38 MAPK (fig 5.1.8B). All of these components potently phosphorylated and activated the Flag tagged p38 MAPK (upper band), and to a lesser extent endogenous p38 MAPK (lower band).

5.2 The effects of Ras and Rap occur downstream of MyD88, IRAK1, and TRAF6, but upstream of MKK3 and MKK6 on the pathway leading to the activation of p38 MAPK by IL-1.

At this point in the study, I have gathered evidence for a role of several signalling molecules on the pathway leading to the activation of p38 MAPK by IL-1. The known components of IL-1 signalling, such as MyD88, IRAK and TRAF6, have already been shown to interact during the activation of NFκB by IL-1, and may

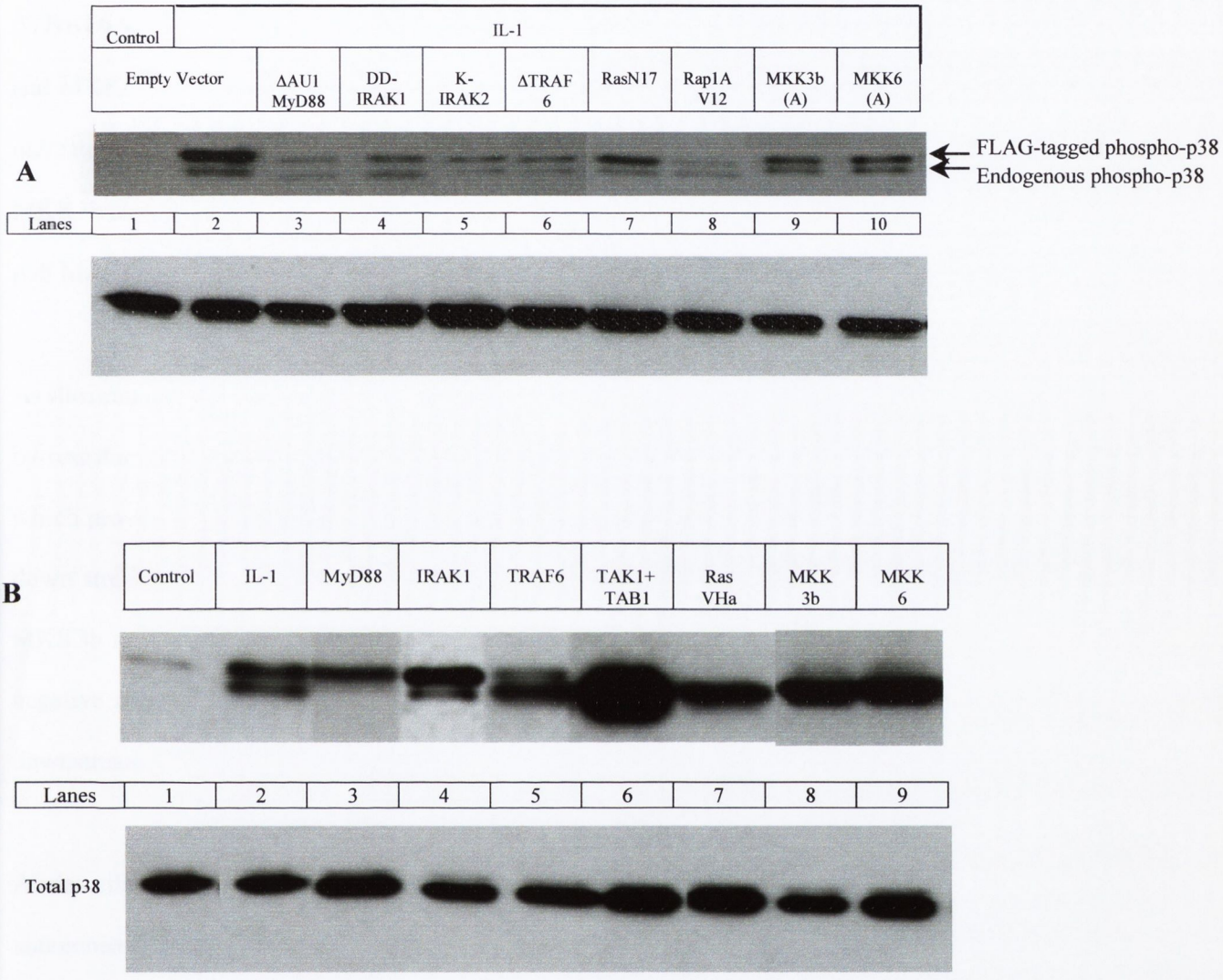


FIGURE 5.1.8

Effects of (A) dominant negative ΔMyD88, DD-IRAK1, K-IRAK2, ΔTRAF6, RasN17, RapV12, ΔMKK3b(A), and ΔMKK6b(A), on IL-1 induced activation of p38 MAPK and (B) effect of wild type MyD88, IRAK1, TRAF6, TAK1, RasVHa, MKK3b, and MKK6 on the activation of p38 MAPK, in HeLa cells transfected with FLAG-tagged p38 MAPK. HeLa cells were seeded at $1.5x10^5$ cells in 3 ml of complete DMEM medium 24 hours prior to transfection. The cells were subsequently co-transfected with FLAG-tagged p38 MAPK and 2.5-5 μg of above indicated dominant negative (A) or wild type (B) signalling component using FuGene™ transfection reagent as described in the methods section. 48 hours after transfection the cells were stimulated with IL-1 where relevant for 10 minutes, harvested and western blotting was performed on the cell extracts, detecting transfected and endogenous p38 MAPK using an anti-phosphorylated p38 MAPK antibody, as described in the methods section.

well do so even in this system. How and where on the pathway Ras and Rap feed in on the other hand is harder to guess. I therefore sought to investigate the relative position of the IL-1 signalling components studied above, in relation to Ras and Rap on the pathway. To do this, I used the GAL4-CHOP$^{(1-101)}$ reporter system to co-transfect constitutively active RasVHa with mutant constructs of MyD88, IRAK1, IRAK2, TRAF6, MKK3b and MKK6. As shown in figure 5.2.1, ΔAU1MyD88, DDIRAK1(1-211), DDIRAK2, and ΔTRAF6 were all unable to inhibit the activation of p38 MAPK by RasVHa. Dominant negative MKK3b(A), and MKK6b(A) however, inhibited the activation of p38 MAPK by RasVHa by more than 65%. This would indicate that Ras signals down stream of MyD88, IRAK1/2, and TRAF6 on this pathway, but upstream of MKK3b and MKK6, as the signalling deficient mutants of these MAPK kinases prevent Ras from activating p38 MAPK.

As illustrated in figure 5.2.2, dominant negative RasN17 potently inhibit the activation of p38 MAPK caused by transfecting EL4.NOB-1 cells with the expression vectors for wild type MyD88, IRAK1, and TRAF6, which provide further evidence that the effect of Ras occurs at a point in the signalling pathway which lies down stream of MyD88, IRAK1 and TRAF6. The activation of p38 MAPK by over expressing wild type MKK3b and MKK6, on the other hand is not inhibited when co transfecting these cells with a dominant negative mutant of Ras (N17). This again points to a position of Ras upstream of the MAPK2 level but downstream of TRAF6, on the pathway.

As described above, Rap has been shown to be a potent inhibitor of Ras signalling, possibly by acting as an antagonist to Ras function, competing for downstream effectors. I have shown an inhibitory effect of Rap on the pathway leading to the activation of p38 MAPK by IL-1, where Ras is a key mediator. To further explore the target for this inhibition, and to investigate where, on the pathway, Rap exerts its inhibitory effect, I again utilised the GAL4-CHOP$^{(1-101)}$ reporter system. By co-transfecting EL4.NOB-1 cells with constitutively active RapV12 together with MyD88, IRAK1, TRAF6, MKK3b, and MKK6, it was possible to demonstrate that RapV12 can inhibit the activation of p38 MAPK by all of the above apart from MKK3 and MKK6, indicating that the inhibitory effect of Rap occurs at a point between TRAF6 and MKK3b/MKK6 (Fig 5.2.3).

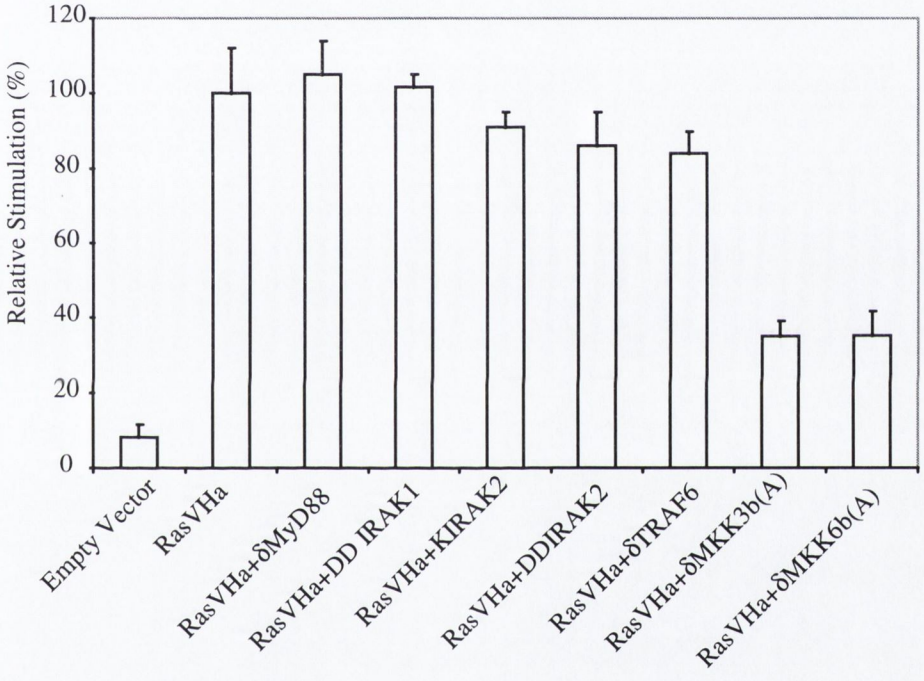


FIGURE 5.2.1

Effects of dominant negative ΔMyD88, DD-IRAK1, K-IRAK2, DDIRAK2, ΔTRAF6, ΔMKK3b(A), and ΔMKK6b(A) on the activation of p38 MAPK by RasVHa in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system and RasVHa together with dominant negative ΔMyD88, DD-IRAK1, K-IRAK2, DDIRAK2, ΔTRAF6, ΔMKK3b(A), and ΔMKK6b(A) as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . For clarity, the stimulation by RasVHa are set at 100%, the inhibition by dominant negative proteins being shown relative to this, expressed as mean of relative stimulation ± SEM for quadruplicate determinations. Similar results were obtained in 3 further experiments..

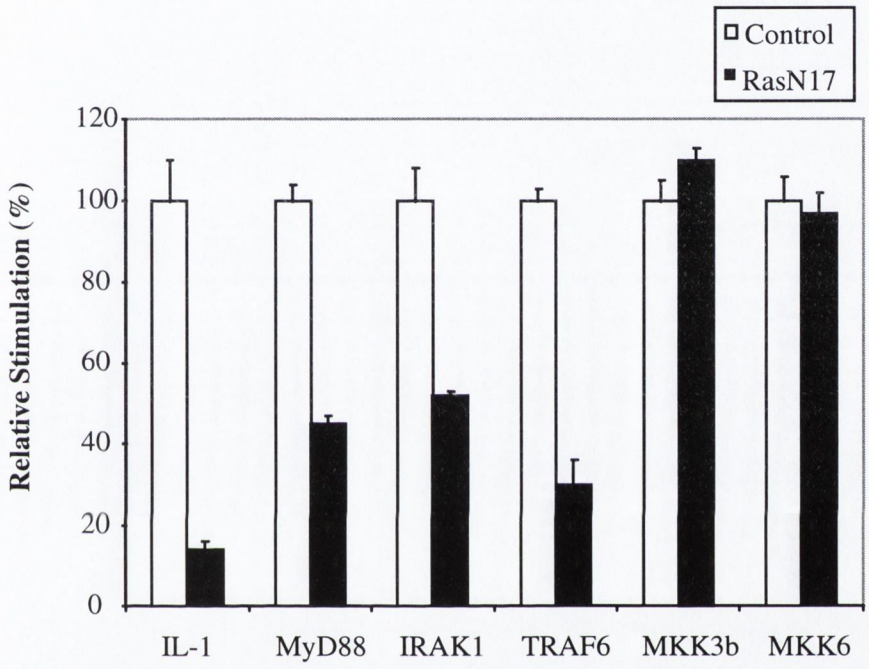


FIGURE 5.2.2

Effect of RasN17 on the activation of p38 MAPK by wild type MyD88, IRAK1, TRAF6, MKK3b, and MKK6 in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system and RasN17 together with wild type MyD88, IRAK1, TRAF6, MKK3b, and MKK6 as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α where required for 6 hours at 37°C or left unstimulated following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section . For clarity, the stimulation by IL-1, or transfected wild type proteins were set at 100%, the inhibition by RasN17 being shown relative to this, expressed as mean of fold stimulation ± SEM for quadruplicate determinations. Similar results were obtained in 3 further experiments.

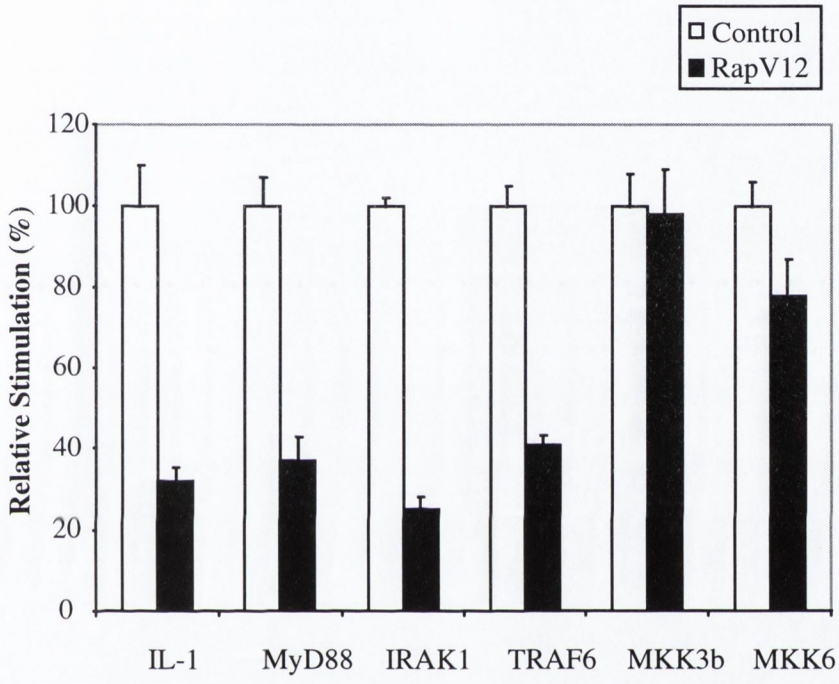


FIGURE 5.2.3

Effect of RapV12 on the activation of p38 MAPK by wild type MyD88, IRAK1, TRAF6, MKK3b, and MKK6 in EL4.NOB-1 cells. 1.4 x 10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system and RapV12 together with wild type MyD88, IRAK1, TRAF6, MKK3b, and MKK6 as described in Experimental Procedures. Transfected cells (5 x 10^5 in 0.25 ml per sample) were stimulated with IL-1α where required for 6 hours at 37°C or left unstimulated following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalised for transfection efficiency by a β-galactosidase assay as described in the methods section. For clarity, the stimulation by IL-1, or transfected wild type proteins were set at 100%, the inhibition by RapV12 being shown relative to this, expressed as mean of fold stimulation ± SEM for quadruplicate determinations. Similar results were obtained in 3 further experiments..

This fits in with the model of Rap being a direct antagonist to Ras function, as the inhibitory effect of Rap would occur at the same point on the pathway as that of Ras.

Dominant negative TAK1, as well as wild type TAK1 in combination with TAB1, were cotransfected into EL4.NOB-1 cells together with RasVHa, and RasN17/RapV12 respectively, although the results obtained were inconclusive, and further experiments using additional MAPKKKs may prove more conclusive.

DISCUSSION

Even though IL-1 is a well-known activator of p38 MAPK, the signalling cascade leading to p38 MAPK activation by IL-1 has not been fully elucidated. The small G protein Ras has been shown to play a key role in the activation of p38 MAPK by multiple stimuli, including hemopoietic cytokines, platelet-derived growth factor and fibroblast growth factor (Rausch and Marshall, 1999) (Tan *et al.*, 1996). I have demonstrated that Ras also plays an important role in the activation of p38 MAPK by IL-1 with Rap having an inhibitory role. In the final part of this study I have set out to further investigate the role of Ras in the activation of p38 MAPK.

In addition to Ras, several other signalling components are known to be important for the activation of p38 MAPK by IL-1. Using IRAK-deficient mouse fibroblast cells, Kanakaraj et al (Kanakaraj *et al.*, 1998) implicated IRAK-1 in the activation of p38 MAPK by IL-1. Furthermore, overexpressing TRAF6 proved to activate p38 MAPK, in the absence of an extracellular stimuli, and the oligomerization of the effector domain of TRAF6 was shown to induce IL-1 responsive genes (Baud *et al.*, 1999), implicating TRAF6 in the activation of p38 MAPK by IL-1. In this study I attempt to resolve the cascade leading from the IL-1R to the activation of p38 MAPK and to determine the roles and relative positions for Ras and the negative regulator Rap on this pathway.

Dominant negative mutants of the IL-1 signalling components MyD88, IRAK1/2 and TRAF6 all inhibited the activation of p38 MAPK by IL-1. This indicates that the IL-1R signalling complex assembled in response to IL-1, on the pathway leading to the activation of NFκB, also participates in the activation of p38 MAPK. In the case of NFκB, this complex has been shown to involve the recruitment of MyD88 and IRAK by IL-1R and IL-1RAcP, causing IRAK to become phosphorylated and released from the complex, with subsequent association with TRAF6. Furthermore, overexpressing wild type MyD88, IRAK1/2 and TRAF6 caused an increase in p38 MAPK activity, supporting a role for these molecules in the activation of p38 MAPK.

Overexpressing constitutively active RasVHa in the GAL4-CHOP$^{(1-101)}$ system, or together with FLAG-tagged p38 MAPK in HeLa cells, activated p38 MAPK (also shown in chapter 4), and this activation was inhibited by overexpressing dominant negative MKK3b(A) or MKK6b(A). This suggested, not only that Ras activates p38 MAPK from a point in the pathway that occurs up-stream of the MAPKK level, but that both MKK3b and MKK6b may play an important role in the activation of p38 MAPK by IL-1. On the other hand, dominant negative MyD88, IRAK1/2 and TRAF6, which all act as potent inhibitors of the activation of p38 MAPK by IL-1, were unable to inhibit the activation of p38 MAPK by RasVHa. Accordingly, over-expression of dominant negative RasN17 not only inhibited the activation of p38 MAPK by IL-1, but also inhibited the activation caused by over expressing wild type MyD88, IRAK1 and TRAF6. Finally, MKK3b and MKK6b both activated p38 MAPK. Their effect was not however inhibited by over expressing RasN17. Taken together, these results show that IL-1 activates p38 MAPK on a pathway involving MyD88, IRAK1/2, TRAF6, Ras, MKK3b and MKK6b, with Ras feeding in at a point downstream of TRAF6 but up stream of MKK3b and MKK6b, as shown in figure 5.3 below.

Expression levels of the various mutant proteins were not monitored, as relevant antibodies were not available to me. I considered tagging the mutant constructs, although as the results obtained were of such nature, and correlated well with published data, and since tagging of all the above constructs would be very time consuming, tagging was not performed. In addition, dose responses were performed for all mutant constructs, and reflected the decreased/increased expression of the inhibitory or activating constructs. Furthermore, personal communications with Andrew Bowie of our laboratory have revealed that expression of constructs inserted into the vectors used in this study are not expected to differ significantly in either EL4.NOB-1 or HeLa cells.

The activity of Ras is regulated through a GDP/GTP cycle. Guanine-nucleotide exchange factors (Ras-GRF1/2, mSos1/2) cause the dissociation of GDP from GDP•Ras replacing it with GTP, forming active GTP•Ras (reviewed in (Rebollo and Martinez, 1999)). GTP-ase activating proteins (p120GAP, NF1) accelerate the intrinsic GTPase activity of Ras to promote the formation of inactive GDP•Ras. When bound

to GTP, Ras undergoes conformational changes, allowing it to interact with multiple downstream effectors. Membrane localisation allows Ras to form a dimer, which is essential for activation of downstream effectors (Inouye *et al.*, 2000). Ras is a potent activator of the classical p42/p44 MAPK pathway and the function of Ras on this pathway has been studied intensely. The best understood means of activating the p42/p44 pathway through Ras is that used by receptor tyrosine kinases in for example growth factor signalling. Ligands cause receptors to auto-phosphorylate, and bind SH2 domains of adapters such as Grb2 (growth factor receptor bound protein 2), which can then recruit guanine nucleotide exchange factors for Ras(Izquierdo Pastor *et al.*, 1995). When activated, the effector domain of Ras interacts with the Ras binding domain of Raf-1(Zhang *et al.*, 1993), which leads to the binding of the C-terminal cysteine-rich domain of Raf-1 to Ras, thereby recruiting Raf-1 to the membrane. The catalytic domain of Raf-1 can now interact with the down stream kinases MKK1 and MKK2 (Kyriakis *et al.*, 1992). MKK1 and MKK2 activate p42 and p44 MAP kinases through phosphorylation of tyrosine and threonine at the Thr-Gly-Tyr phosphorylation motif (Zhang *et al.*, 1993). Although IL-1 signalling differs from growth factor signalling in that no receptor tyrosine phosphorylation occurs (Bird *et al.*, 1991), the data presented in this study may suggest a somewhat similar model for the activation of p38 MAPK by IL-1. IL-1 activates Ras, which occurs down stream of TRAF6. Raf-1 occupies a role as a MAPKKK on the pathway leading to the activation of p42/p44 by Ras, and although I have shown the importance of the MAPK kinases MKK3b and MKK6 on the p38 pathway, the effector downstream of Ras remains to be identified.

MAPKKKs that have been shown to play a role in p38 MAPK signalling include PAK(Bagrodia *et al.*, 1995), TAK1(Moriguchi *et al.*, 1996), ASK1(Ichijo *et al.*, 1997), and mixed-lineage kinase MLK-3(Rana *et al.*, 1996), and future investigations may reveal if these play a role in the activation of p38 MAPK by IL-1. The effect of TAK1 on Ras induced activation of p38 MAPK was tested but inconclusive results indicate that the role of a MAPKKK on this pathway is more complex. This is currently under investigation.

Rap is a small G protein belonging to the same sub-family of small G proteins as Ras. The two proteins possess high sequence homology, most significantly, they are virtually identical in region amino acids 28-45

(H-Ras) constituting the effector binding domain (reviewed in (Bos, 1998)). Several studies have suggested an antagonistic role for Rap in Ras signalling. I have, in chapter 4, demonstrated an inhibitory effect of active Rap on the activation of p38 MAPK by IL-1 and I have also demonstrated that IL-1 can activate Rap, as well as Ras. Rap can bind to Ras specific effectors, but is unable to induce a downstream signal. My present study suggests that the inhibitory effect of Rap occurs at the same level as that of Ras since active RapV12 blocked MyD88, IRAK1, and TRAF6 but not MKK3 and MKK6. Of the dominant negative versions of these proteins, only MKK3b and MKK6 inhibited the stimulatory effect of dominant negative RapN17.

In conclusion, my study identifies a novel signalling pathway leading to the activation of p38 MAPK by IL-1. The pathway involves the activity of MyD88, IRAK1, IRAK2, TRAF6, Ras, MKK3b and MKK6b, with a negative down-regulatory role for Rap, occurring at the level of Ras on the pathway. I have therefore illustrated a signalling pathway for the p38 MAPK response, which is critical for the induction of inflammatory proteins by IL-1.

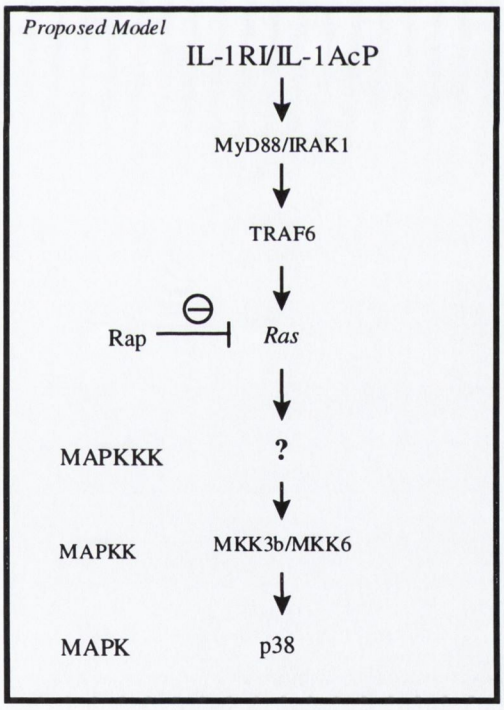


Figure 5.4 Proposed model of the roles for Ras and Rap in the activation of p38 MAPK by IL-1. Further details in the text.

CHAPTER 6

General Discussion, final conclusions and comments

IL-1 signalling is an area that has been studied in great detail since IL-1 was first identified. Partly due to the fact that it was the first Interleukin described, but also due to its pleiotropic effects. Practically all cell types respond to IL-1, the major effects elicited being pro-inflammatory. IL-1 signals through the IL-1RI and the IL-1RAcP, which are linked by the adapter protein MyD88 via a homotypic interaction to downstream signalling components such as IRAK1, Tollip and IRAK2, as well as TRAF6 and TAK-1, on the signalling pathway leading to the activation of the transcription factor NFκB (described in detail in Chapter 1).

An involvement of a small G protein in IL-1 signalling has been implicated for the past 10 years, although the suggested roles for these have been somewhat inconclusive. The first evidence came from O'Neill et al who demonstrated an increased GTPase activity in membranes prepared from EL4 cell stimulated with IL-1 (O'Neill *et al.*, 1990). Rac and Cdc42 have both been implicated in the activation of JNK as well as p38 MAPK by IL-1 (Coso *et al.*, 1995; Minden *et al.*, 1995), although other groups have disputed this, since results using dominant negative constructs of Rac have been without effect when examining the activation of JNK and p38 MAPK by IL-1 (Davis *et al.*, 1999). Furthermore, a role for the small G protein Ras in IL-1 signalling has emerged recently. Induction of collagenase promoter by IL-1 in chondrocytes has been shown to require Ras, as has induction of the brain natriuretic peptide in myocytes (Grumbles *et al.*, 1997; He and LaPointe, 1999). Others, however, have failed to demonstrate activation of Ras by IL-1 (Bird *et al.*, 1994).

In this study I have further investigated the role of small G proteins in IL-1 signalling, and the first approach I took involved the Clostridial virulence factors LT and Toxin B. These are related glucosyltransferases, which through an N terminal region [amino acids 364 through 516 responsible for substrate specificity], glucosylate and inhibit a distinct subset of small G proteins (Hofmann *et al.*, 1998). These G proteins include Ras, Rap, Rac and Ral for LT and Rac, Cdc42 and Rho for Toxin B. The functional consequences of this monoglucosylation of the substrate G protein within its effector domain (amino acid 35, Ha-Ras) have been shown to involve a blocking of Ras-effector coupling, which results in complete inhibition of downstream signalling (Herrmann *et al.*, 1998).

While an effect of LT was observed on IL-1 induced expression of IL-2, and on the increased transcription of a reporter gene under the control of NFκB, concerns were raised regarding the toxic effects of LT. The reasons for this included an increased uptake of trypan blue stain, indicating a reduction of viable cells. When exposed to these virulence factors for a prolonged period of time, or if high doses (up to 5 μg LT) are used, cells undergo morphological changes. Toxin B induces cell shrinkage, which results in cell adhesion points resembling neurite-like extensions, whereas LT merely causes rounding of cell bodies (Bette *et al.*, 1991). All of these effects are due to inactivation of small G proteins, causing destruction of the actin filament system.

Prolonged exposure may therefore cause other, secondary effects, and with this in mind I turned the study to investigating short-term responses to IL-1, including the activation of p42/p44 and p38 MAPK. Although, from the literature, the doses of toxins used here, as well as the short exposure times, should not cause non-specific effects, great effort was made to verify that the results obtained were true. These efforts included thorough glucosylation studies, usage of a UDP-glucose and therefore toxin resistant fibroblast cell line, as well as studies of other phosphorylation dependent events, ensuring that these were not affected by the toxin under the conditions used here. Furthermore, throughout this study, cell viability was closely monitored by measuring trypan blue uptake.

The results obtained, after optimising the treatment times with LT and Toxin B, revealed that a small G protein was indeed involved in IL-1 signalling. This G protein was inhibited after pretreatment with LT but not Toxin B, which caused me to draw the first conclusion of this report, namely that Ras, Rap or Ral (or a novel G protein not yet identified to be a target of LT), but not Rac, Rho, or Cdc42 is involved in the activation of p38 and p42/p44 MAPK by IL-1.

IL-1 is a potent activator of p38 MAPK, but merely increases the basal activity of p42/p44 MAPK by 2-3 fold. This caused problems when studying the activation of p42/p44 MAPK by IL-1 in this system. Basal activity however was reduced when the incubation times were cut down to 1 hour and although LT was

shown to inhibit this activation, I decided for practical reasons, to examine in depth, the pathway leading to the activation of p38 MAPK.

Several studies have demonstrated a role for the small G proteins Rac and Cdc42 in the activation of p38 MAPK by stimuli other than IL-1. While, as mentioned above, a role for these G proteins in the activation of p38 MAPK by IL-1 has been suggested, other studies have disputed this, and a role for Ras in p38 MAPK activation has been demonstrated. Since the results using LT suggested that a small G protein inhibited by LT is critical for IL-1 induced p38 MAPK activation, I next studied the effects of dominant negative constructs of Ras, Rac and Rap on p38 MAPK activation by IL-1. The approaches used involved transient transfections, firstly in a reporter assay system, and secondly in a western blotting assay. One could conclude from this that Rac indeed did not play a role in the activation of p38 MAPK by IL-1, although over expressing constitutively active Rac caused activation of p38 MAPK. This suggests that, as Rac can activate p38 MAPK, Rac may be involved in the activation of this pathway by stimuli other than IL-1. Recent evidence from our group has demonstrated a role for Rac in another IL-1 dependent signal. Jefferies et al (Jefferies and O'Neill, 2000) demonstrated that dominant negative RacN17 inhibited IL-1-driven p65-mediated transactivation of NFκB, proposing a role for Rac in transactivation, independent of IκB regulation. Interestingly, this study suggested that both p38 MAPK and p42/p44 lie downstream of Rac1 on the pathway leading to p65 mediated transactivation of NFκB. This suggests that, while Rac1 is not critical for the activation of p38 MAPK by IL-1, as I have shown here, p38 MAPK may be critical for Rac1 mediated transactivation of NFκB.

Ras however appears to have a critical role in the activation of p38 MAPK by IL-1. This could be demonstrated in both the CHOP reporter assay, as well as in the p38 transfection based western blotting assay. Interestingly, dominant negative Rap activated p38 MAPK in the GAL4-CHOP$^{(1-101)}$ system, and furthermore constitutively active Rap was a potent inhibitor of the activation of this pathway by IL-1. An antagonistic role of Rap towards Ras function has been discussed above, and appears indeed to be the role of Rap on this pathway. Apparent activation of p38 MAPK by over-expression of dominant negative RapN17

suggests that a tonic inhibition of Ras by Rap occurs in resting cells and by removing this inhibition, p38 MAPK is allowed to become activated by the basal activity of the pathway.

Activation of Ras occurred after a few minutes in the EL4.NOB-1 cell system, peaking at 5 minutes, and finally reaching basal levels again after 10-15 minutes. This was the time when the activation of Rap by IL-1 reached a maximum, suggesting that Rap was involved in down-regulating the effect of Ras. p38 MAPK activation by IL-1 peaks at 10 minutes, but reaches basal levels again rapidly, within 40 minutes. I was able to demonstrate that dominant negative Rap could delay this transient response of p38 MAPK to IL-1. I therefore propose that Rap acts down regulatory on this pathway, possibly playing a part in maintaining the transient nature of the p38 MAPK response to IL-1. As Ras is critical for the activation of this pathway, and as Rap has been shown to compete with Ras for downstream effectors, I also propose that Rap plays an antagonistic role towards Ras function on the activation of p38 MAPK by IL-1.

As the signalling complex assembled in response to IL-1 on the pathway leading to the activation of NFκB has been described in detail (O'Neill and Greene, 1998), I wanted to investigate the role of the different IL-1 signalling components on the activation of p38 MAPK. I here show that MyD88, IRAK1, IRAK2, TRAF6 and TAK-1 are all critical in the activation of p38 MAPK by IL-1. The next question naturally involved the role and position of Ras and Rap in relation to these. Using combination transfections I demonstrated that Ras as well as Rap feed in downstream of TRAF6 on this pathway. The role for Ras in the activation of the classical p42/p44 MAPK pathway has been described in detail and a correlation to this pathway can be made here. Ras becomes activated through tyrosine kinase receptors by growth factors. Specific Ras-GAP proteins cause GTP to become bound to Ras, enabling Ras to bind the downstream effector and MAPKKK: Raf-1(Zhang *et al.*, 1993). Raf-1 in turn phosphorylates and activates the cascade leading from the MAPKKs: MKK1 and MKK2, to the activation of p42/p44 MAPK (described in detail in Chapter 1). The next step for me therefore may involve identifying the MAPKKK activated by Ras in response to IL-1. As the preliminary results with TAK-1 proved inconclusive, the search goes on. Several MAPKKKs involved in the activation of p38 MAPK have been identified, including TAK-1, ASK, PAK etc, although there is no evidence for an

interaction of any of these with Ras. Ras has, on the other hand, been shown to interact with MEK kinase, Bcl-2, REKS (Ras-dependent extracellular signal-regulated kinase kinase stimulator) and KSR (kinase suppressor of Ras or ceramide-activated protein kinase)(Chen and Faller, 1996; Russell *et al.*, 1995; Shimizu *et al.*, 1994; Therrien *et al.*, 1995). An interaction between Ras and a MAPKKK is the most plausible mechanism for completing the pathway, although the activation of p38 MAPK by IL-1 may not be a direct reflection of the activation of the p42/p44 MAPK pathway by growth factors. This is an area where further research is needed.

$p130^{CAS}$ (Crk-associated substrate) is a protein which was initially identified as a highly tyrosine-phosphorylated protein in cell transformed by v-Crk or v-src. It forms stable complexes with both of these oncogenic products through its SH2 and SH3 domains. It has since been shown to be a signalling molecule that can bind the GEF C3G as well as inducibly associate with focal adhesion complex. Recently, a $p130^{CAS}$ binding protein, AND-34, was identified as an mRNA transcript upregulated following CD3ε cross-linking and induction of apoptosis in immature CD4+CD8+ thymocytes(Cai *et al.*, 1999). The cDNA for AND-34 encodes a carboxy-terminal domain with homology to the Ras/Rap GEFs: Sos, Ral-GEF, and C3G, with a homology of 60% in the 61 conserved residues of these 4 GEFs analysed. Interestingly, the open reading frame of full length AND-34 cDNA also predicts an amino-terminal Src homology 2 (SH2) domain. This protein is therefore the first RasGEF protein carrying both a SH2 domain and a GEF domain, possibly providing a short-cut in signalling to Ras proteins, by-passing the need for an adapter such as Grb2. As described in the general introduction, the RasGEF Sos can be found in the cytoplasm of unstimulated cells, complexed to the SH3 domain of the adapter protein Grb2. Upon activation, the Sos/Grb2 complex associates with the autophosphorylated cytoplasmic domain of receptor tyrosine kinases by interactions with the SH2 domain of Grb2, allowing the GEF Sos to become activated(Buday and Downward, 1993; Egan *et al.*, 1993).

AND-34 share high homology with C3G which functions as a GEF for both Rap1 and R-Ras. Interestingly, IL-1 up-regulates levels of AND-34, providing a link between Ras signalling and IL-1(Cai *et al.*, 1999).

Focal adhesion is necessary for some IL-1 signalling, such as the activation of p42/p44 MAPK by IL-1(Lo *et al.*, 1998). Since AND-34 has been shown to associate with $p130^{CAS}$, a component of the focal adhesion complex(Cai *et al.*, 1999), and since IL-1RIs are enriched at focal adhesion sites(Qwarnstrom *et al.*, 1988), this further suggests a link between this RasGEF and IL-1 signalling. Investigating the role of AND-34 in the activation of p38 MAPK by IL-1 may provide a missing link on this pathway.

Furthermore, I have show in this study that the MAPKKs: MKK3b and MKK6 are both involved in the activation of p38 MAPK by IL-1 and that Ras lies upstream of these kinases on the pathway leading to the activation of p38 MAPK by IL-1. In agreement with this, Guan et al (Guan *et al.*, 1999) demonstrated that overexpression of either of the two kinase-dead mutant forms of MKK3 or MKK6 inhibited IL-1beta-induced p38(MAPK) (but not JNK/SAPK) phosphorylation and iNOS expression. Furthermore, at later times of HCMV infection, p38 activation is due to increased activity of both MKK3 and MKK6 (Johnson *et al.*, 2000). On the other hand, FAS-induced apoptosis is selectively mediated via MKK6, but not MKK3, suggesting that MKK3 and MKK6 can be specifically activated by extracellular stimuli, and that they have distinct roles (Huang *et al.*, 1997).

In conclusion, this study has brought us closer to understanding the role of small G proteins in IL-1 signalling. Upon binding to the IL-1RI, IL-1 activates p38 MAPK on a pathway that involves MyD88, IRAK1, IRAK2, TRAF6, Ras and MKK3b/MKK6, with Rap having an antagonistic effect at the level of Ras. This inhibitory effect of Rap appears to play a role in down-regulating the activation of p38 MAPK by IL-1, although the precise mechanism of this has yet to be determined. This provides the first evidence for a role for the small G protein Ras in IL-1 signalling and also provides the first results where it is shown that IL-1 can activate Ras and Rap. Although the general signalling cascade of the p38 MAPK activation pathway by IL-1 has been presented in this study, further experiments are needed to investigate interactions between Ras or Rap and up-stream or down-stream signalling elements.

Proving how Ras is activated by IL-1, and the particular isoforms of Ras involved is a key goal in future work.

CHAPTER 7

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Full papers

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