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Bacterial Products as Activators of NF-$\varkappa$B.

Thesis submitted to the

University of Dublin

For the

Degree of Doctor of Philosophy

By

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Department of Biochemistry

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October 2001

Declaration

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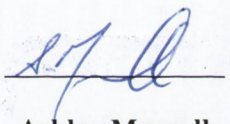

Ashley Mansell

In Italy for 30 years under the Borgia's, they had warfare, terror, murder and bloodshed. They produced Michelangelo, Leonardo Da Vinci, and the Renaissance.
In Switzerland, they had brotherly love and 500 years of democracy, and what did that produce.....the cuckoo clock.
Orson Wells, *The Third Man* (1949)

Anything can be made to work if you fiddle with it long enough.
Anon.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

Abbreviations

ABTS	2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid
AcP	Accessory protein
Akt	Protein Kinase B
AP-1	Activator protein 1
aSMase	acidic Sphingomyelinase
ATIII	Antithrombin III
ATP	Adenosine triphosphate
BMM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
DAG	Diacylglycerol
DTT	Dithiothreitol
ECSIT	Evolutionarily conserved signaling intermediate in Toll/IL-1 pathways
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EMSA	Electrophoretic mobility shift assay
ERK1/2	Extracellular signal-regulated kinase 1/2
FCS	Foetal calf serum
GAP	GTPase activating protein
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GPI	Glycosylphophatidylinostiol
GSH	Glutathione
GST	Glutathione S transferase

GTP	Guanine triphosphate
HEK	Human embryonic kidney
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ehtanesulphonic acid]
HGF	Hepatocyte growth factor
IAP	Inhibitors of Apoptosis
IKK	IκB kinase
IL	Interleukin
IL-1RacP	IL-1 receptor accessory protein
IL-1RI	IL-1 receptor type I
InlA	Internalin A
InlB	Internalin B
IPx	Inositolphosphates
IRAK	Interleukin 1 receptor associated kinase
IκB	Inhibitor of κB
JNK	c-Jun N-terminal kinase
JNK	Jun N-terminal kinase
KDa	Kilodalton
L-ATIII	Latent Antithrombin III
LLO	Listeriolysin O
LPS	Lipopolysaccaride
LTA	Lipoteichoic acid
LTβR	Lymphotoxin β Receptor
Mal	MyD88 adaptor like
MALP-2	Macrophage-activating lipoprotein-2kDa
MAPK	Mitogen activated protein kinase
MEEK1	MAP kinase kinase kinase MEKK1

MyD88	Myloid differentiation factor
NEMO	NF-$\varkappa$B essential modifier
NF-$\varkappa$B	Nuclear factor kappa B
NIK	NF-$\varkappa$B inducing kinase
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PC-PLC	Phosphatidylcholine-specific phospholipase C
PDGF	Platelet derived growth factor
PGI2	Prostaglandin I2
PGN	peptidoglycan
PI(3,4)P2	Phosphatidylinositol-(3,4)-biphosphate
PI(3,4,5)P3	Phosphatidylinositol-(3,4,5)-triphosphate
PI3K	Phosphatidylinositol 3-kinase
PI-PLC	Phosphatitylinositol-specific phospholipase C
PKC	Protein kinase C
PlcA	phospholipase C
PMA	Phorbal myristat acetate
PMSF	Phenylmethylsulphonyl fluoride
PRRs	Pattern Recognition Receptors
RHD	Rel-homology domain
SDS	Sodium dodecyl sulphate
Serpins	Serine Protease Inhibitors
SH	Src homology
SUMO	Small Ubiquitin-like Modifier
TEMED	N, N, N', N',-Tetramethylethylenediamine

TIR	Toll/IL-1R region
TIRAP	TIR Accessory Protein
TLR	Toll-like Receptor
TNF	Tumor necrosis factor
Tollip	Toll/IL-1R interacting protein
TRAF	TNF-receptor associated factor
TRIKA 1/2	TRAF6-regulated IKK activator 1/2

ABSTRACT

The ability of bacterial products to potently activate NF-κB has made this transcription factor one of the most widely studied in the immune response. A greater understanding of the mechanisms and signal transduction pathways that lead to the activation of NF-κB are crucial for our understanding of host-pathogen interactions The aim of this study was to investigate how two bacterial products: Internalin B (InlB) from *Listeria monocytogenes*, and Lipopolysaccharide (LPS) from gram-negative bacteria, activate NF-κB.

Initial studies in the murine macrophage-like cell line, J774, indicated that InlB was a potent activator of NF-κB, a result that was further demonstrated in the murine macrophage cell lines RAW 264, $P388D_1$ and the human epithelial cell line Hep2. The expression of InlB in *Listeria innocua* enhanced its ability to activate NF-κB, while deletion of InlB from wild-type *L. monocytogenes* marginally decreased its effect on NF-κB. The effect of InlB was found to correlate with the rapid degradation of IκBα, and a sustained degradation in IκBβ. InlB found to increase the expression of the pro-inflammatory cytokines IL-6, IL-8 and TNFα. The use of anti-InlB antibodies and shorter domains of InlB demonstrated that InlB-mediated activation of NF-κB was dependent upon the N-terminal 213-amino acid leucine-rich repeat (LRR) domain of InlB, a domain which has previously been shown to be responsible for InlB-mediated *L. monocytogenes* invasion and PI-3 kinase activation. Furthermore, the highly homologous protein Internalin A (InlA) and the InlA-LRR domain, failed to activate NF-κB.

The PI-3 kinase inhibitors LY294002 and Wortmannin inhibited NF-κB activation by InlB. InlB was also found to activate the small G-protein Ras. Inhibition of Ras with the farnesyltransferase inhibitor Manucycin A inhibited NF-κB activation and the recruitment of the p85 subunit of PI-3 kinase, implying that Ras is required for PI-3 kinase activation. InlB also activated the PI-3 kinase downstream effector, Akt as assessed by the increased phosphorylation of Akt on Serine 473. Transfection of Hep2 cells with dominant negative Ras N17 or dominant negative Akt inhibited the induction of a reporter gene linked to the IL-8 promoter by InlB. Furthermore, the Ras inhibitor Manumycin A, the PI-3 inhibitor LY294002, and a recently described Akt inhibitor, all blocked the induction of IL-8 by InlB, as assessed by ELISA. These studies implied that a pathway involving Ras, PI-3 kinase and Akt is activated by InlB, which culminates in NF-κB activation.

In the final part of this study, the more extensively studied bacterial product LPS was examined. In particular, whether an extracellular serine protease was required for LPS action was assessed. LPS rapidly activated a thrombin-like protease in THP-1 monocytes. The serpin Antithrombin III (ATIII), and the thrombin inhibitor Hirudin, both inhibited NF-κB activation by LPS and Lipid A. Inhibition was only observed when cells were pre-treated with ATIII for no more than 5 minutes. Thrombin was not thought to be responsible for the effect seen here, as thrombin failed to activate NF-κB, or potentiate sub-optimal levels of LPS in this system. ATIII and Hirudin were also able to inhibit LPS-induced NF-κB activation in cells stably transfected with the putative receptor for Toll-Like Receptor (TLR) 4. These studies suggest that LPS may drive production of a ligand for TLR4 via a serine protease, in an analogous manner to the activation of Toll by fungal pathogens.

In conclusion, this study has provided the novel observation that an important protein from *L. monocytogenes*, InlB, activates NF-κB via a pathway involving Ras, PI-3 kinase and Akt, and that NF-κB activation by LPS may involve the activation of an extracellular serine protease.

Chapter One

General Introduction

Chapter 1

1. Introduction.

1.1 Innate Immunity and Inflammation

Immunity is a state of protection from infectious diseases that is comprised of non-specific and specific components. The specific component of immunity, termed adaptive immunity is mediated by clonally distributed T and B lymphocytes and is characterised by the specificity of its action and memory. Defense against microorganisms is mediated by the early reactions of innate immunity and the later responses of adaptive immunity (75, 92, 169). Innate immunity can be seen to comprise four types of defensive barriers: anatomic, physiologic, phagocytic and inflammatory (Table 1.1). It is a set of disease resistance mechanisms that are not specific to a particular pathogen. However, it has become increasingly evident that innate immunity has a considerable specificity and is capable of discriminating between self and pathogens. Additionally, the activation of the innate immune response can be a prerequisite for the triggering of adaptive immunity.

One of the most effective components of innate immunity is phagocytosis. Phagocytosis involves the engulfment and destruction of pathogens and particulate matter by the cells of the mononuclear phagocyte system. The cell's plasma membrane expands around the microbe or particulate matter to form large vesicles called phagosomes. The cells that comprise this system are the blood monocytes, neutrophils and macrophage mononuclear cells. Monocytes and macrophages are called professional phagocytes in the sense that their primary role is phagocytosis. These cells comprise the first line of internal defense against invading microbes and particulate matter, responding immediately, and until recently,

Type	Mechanism
Anatomic barriers	
Skin	Mechanical Barrier retards entry of microbes. Acidic environment (pH 3-5) retards bacterial growth.
Mucous membranes	Normal flora compete with microbes for attatchment sites and nutrients. Mucus entraps foreign microorganisms. Cilia propel microbes out of body.
Physiologic barriers	
Temperature	Normal body temperature inhibits growth of some pathogens. Fever response inhibits growth of some pathogens.
Low pH	Acidity of stomach contents kills most ingested microorganisms.
Chemical mediators	Lysomzyme cleaves bacterial walls. Interferons induce antiviral state in uninfected cells. Complement lyses microorganisms or facilitate phagocytosis.
Phagocytotic/endocytotic barriers	Various cells internalise (endocytes) and break down foreign macromolecules. Specialised cells (monocytes, neutrophils and macrophages) internalise (phagocytose), kill, and digest whole microorganisms.
Inflammatory barriers	Tissue damage and infection induce leakage of vascular fluid, containing serum proteins with antibacterial proteins and antibacterial activty, and recruit phagocytotic cells into the infected area.

Table 1.1 Summary of the Innate Immune System.
(adapted from Kuby, Immunology, 4th Ed. 2000)

thought to respond without specificity. Macrophages ingest and digest whole bacteria and even dead and injured cells (figure 1.1). During phagocytosis, macrophages release powerful chemical molecules, including cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), Tumor necrosis factor-α (TNF) and chemokines such as interleukin-8 (IL-8). These monokines activate many non-specific protective effects through the inflammatory response.

Monokines released from macrophages induce a group of reactions that lead to inflammation. The inflammatory response is initiated whenever phagocytosis alone is inadequate to prevent infection or when tissues are injured. The Roman physician Celsus described the four principal signs of inflammation approximately 2000 years ago. The symptoms that accompanied short-term or acute inflammation are redness, swelling, heat and pain. Two hundred years later, another physician Galen, added a fifth symptom: loss of function.

Inflammation collectively involves a series of vascular events that serve as defense mechanisms. This includes (i) clotting, (ii) increased blood flow, (iii) increased capillary permeability, and (iv) enhanced influx of phagocytic cells. At the site of infection, the clotting mechanism in the blood is activated, pathogens are trapped in the clots, the infection becomes localised, and at the same time, immune cells (primarily phagocytes) move to the site in response to chemical signals.

1.2 Nuclear Factor-$\varkappa$B.

The transcription factor Nuclear Factor-$\varkappa$B (NF-$\varkappa$B) plays an important role in the regulation of a number of genes involved in the proinflammatory and immune responses, including

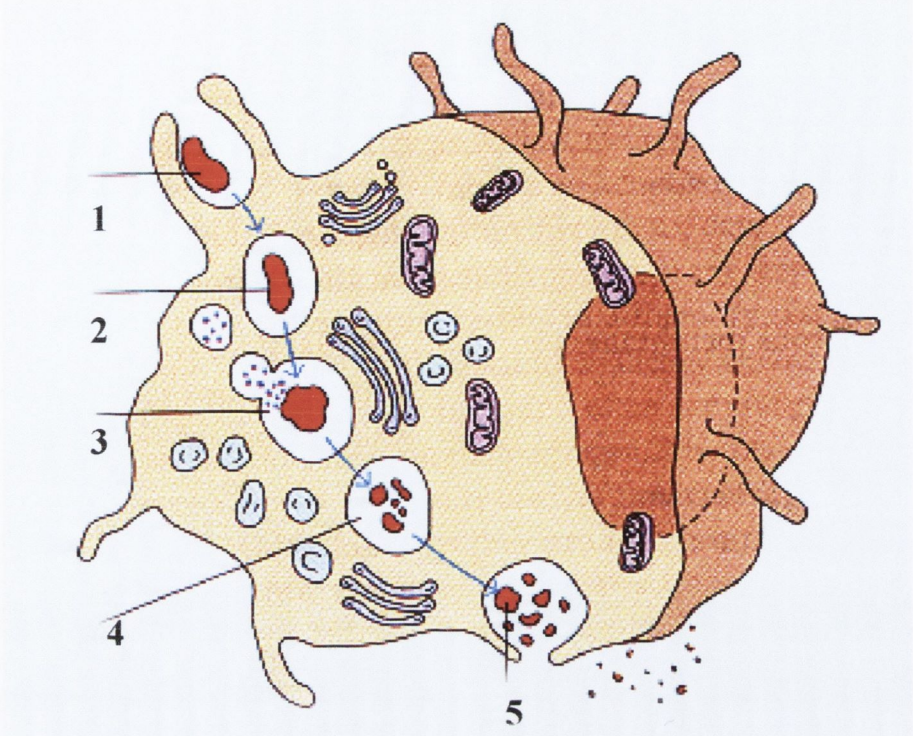


Figure 1.1 Phagocytosis of Microorganism

Cells of the mononuclear phagocyte system are attracted to the site of infection by the factors released by pathogens, damaged host cells, and blood components: (1) the phagosomes engulf the pathogens using pseudopodia, (2) the pathogen is ingested and internalised as membrane-bound organelles (phagosomes) within the phagocyte, (3) phagosomes fuse with lysosomes containing hydrolytic enzymes, (4) pathogen is digested by toxic substances (i) oxygen-dependents products formed by reactive oxygen metaobolites and (ii) oxygen-independent reactants, such as proteases, lactoferrin, and phospholipase A2, and (5) release of digested products from the cell. *Adapted from Kuby 4th ED, 2000.*

many that transcribe cytokines, adhesion molecules, acute phase proteins and cell surface receptors. NF-κB was initially described in B-lymphocytes, but is now recognised as a ubiquitously expressed transcription factor found in a wide range of cell types and tissues. Upon stimulation by an activator, NF-κB binds specifically to a decameric κB motif 5'-GGGACTTTCC-3' originally described in the immunoglobulin kappa light chain enhancer, but now found in a wide range of genes (reviewed in (14)).

The NF-κB family is stimulated by an extensive and ever growing array of activators, emphasizing the importance of the transcriptional response of NF-κB in innate immunity (201). The ever-increasing list of agents capable of activating NF-κB grows regularly, but includes interleukins and growth factors, mitogens, viruses, microorganisms and their products (including lipopolysaccaride (LPS), lipotechoic acid (LTA) and peptidoglycans). Biochemical and genetic analysis has increased our knowledge of the factors and proteins involved in the many convergent signals that initiate NF-κB activation. NF-κB and proteins involved with its regulation are highly conserved from *Drosophila* to humans, though noticeably absent in yeast and *C. elegans* (197).

The multitude of genes regulated by NF-κB increases as rapidly as those stimuli found to elicit the activation, reflecting the importance NF-κB in the host response. The genes regulated by NF-κB have been found to be crucial in all areas of host defense, including both immune and inflammatory responses. NF-κB targeted gene transcription includes cytokines and interleukins, cell adhesion molecules and receptors, and both pro- and anti-apoptotic molecules (57, 201). NF-κB also regulates the expression of different NF-κB subunits,

inhibitors of NF-κB (IκB), and factors that are able to stimulate NF-κB itself. This positive and negative feedback loop within NF-κB transcription facilitates the transient nature of the NF-κB response, thus averting a chronic inflammation response process. Table 1.2 gives a summarised list of NF-κB regulated genes.

1.3 Rel/NF-κB and IκB proteins

NF-κB is sequestered in the cytoplasm of unstimulated cells comprising a transcriptionally active dimer bound to the inhibitory protein IκB (90, 166, 167, 228). Cloning of the NF-κB family members has revealed five distinct subunits of NF-κB, which share a 300 amino acid region of high homology encompassing an amino terminal DNA binding domain and the Rel homology domain (RHD). The RHD provides the dimerisation interface for NF-κB subunits and is so called due to its high degree of homology with the viral oncogene v-Rel (247). The NF-κB family members shown in figure 1.2 can be divided into two subgroups: the first comprising p50 (91, 126) and p52 (32, 187), which are synthesised as the precursor protein p105 and p100 respectively; and the second containing p65 (also termed RelA) (189, 229), RelB (232) and cRel (the proto-oncogene) (281). Both p105 and p100 require proteolytic processing to generate their DNA binding forms (p50 and p52) (202). The second group, RelA, RelB and cRel, do not require processing and possess transactivation domains in their carboxy terminal domains that are responsible for the transcriptional activating potential of NFκB and are essential for mediating interactions with the basal transcription apparatus (238, 239). While the most common form of NFκB is the p50/p65 heterodimer, the subunits freely homo- and hetero-dimerise with the exception of RelB, which will only efficiently form dimers with p50 and p52.

Interleukins and growth factors:	IL-1, IL-2, IL-6, IL-8, TNFα, G-CSF, M-CSF, GM CSF, IFN-β, MGSA/gor-α
Cytokine and cell adhesion:	IL2Rα, I-selectin, ICAM-1, VCAM-1, MadCam-1, BLR1 receptors
Apoptosis-related:	A20, A1, XIAP, c-IAP1, C-IAP2, Fas, TRAF1, TRAF2
Immune modulatory:	MHX-1, and -II, TCR-α and -β, Ig-κ, TAP-1, LMP2, IRF-1, IgE
Others:	Complement factors B, C2,C4, iNOS, COX2, tissue factor, PAI-1, IκBα, NF-κB1, NF-κB2, NnSOD, ferritin (H), p53, MMP-1, MMP-9, HIV-1, wt1, mdr1b, mts1, PTX3, α1-ATS-2, c-myb laminin B2, cyclin D1

Table 1.2 Genes regulated by NF-κB.

(*reproduced from de Martin, et al, Mutation Research, 1999*)

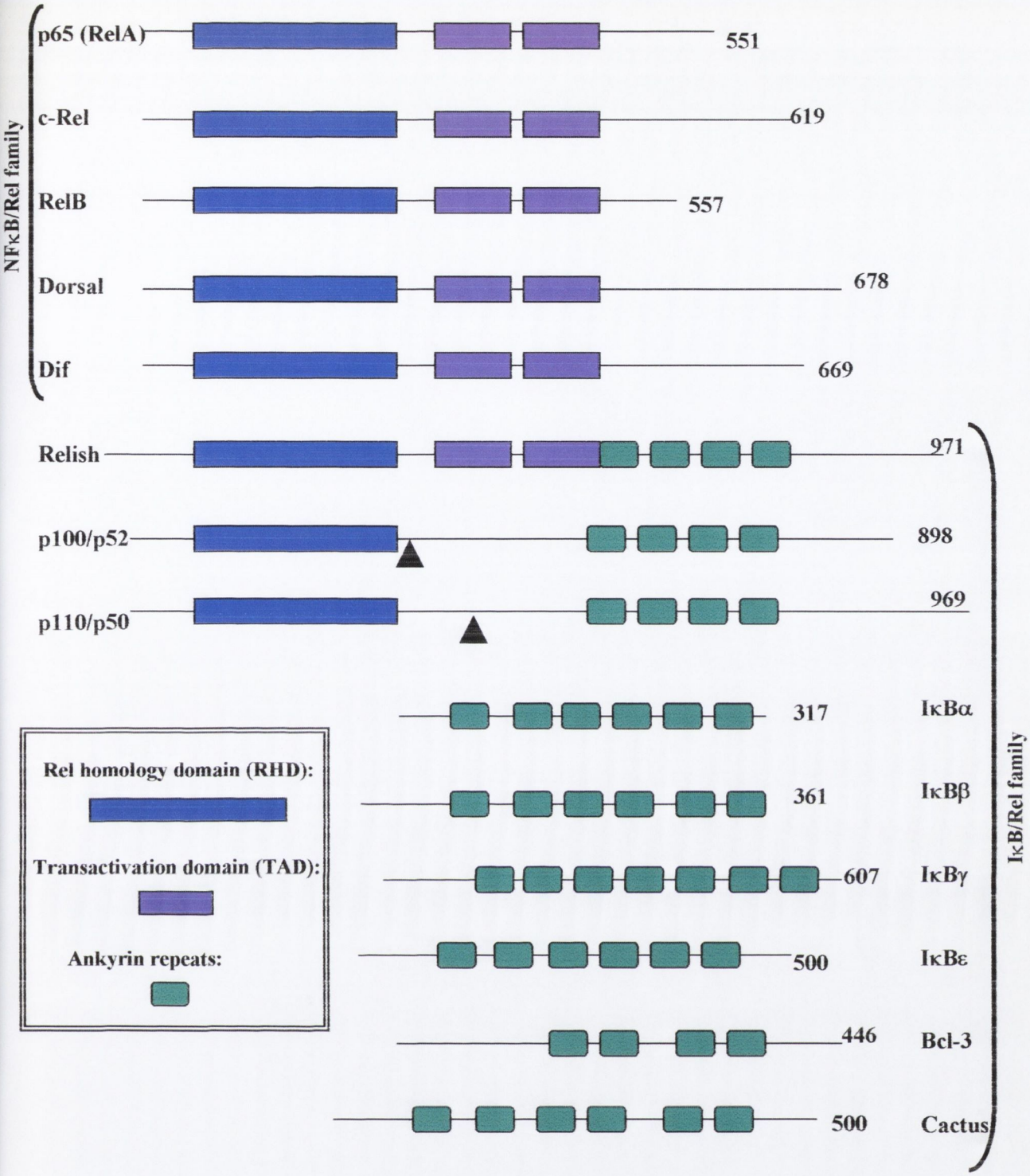


Figure 1.2 Schematic representation of members of the mammalian and Drosophila Rel/NF-κB and IκB families of proteins. The numbering of the mammalian sequences corresponds to the human proteins and the approximate location of the processing sites for p100 and p105 are indicated with an arrow (▲). The key for the regions of homology is given in the box.

Upon stimulation with potent activators of NF-κB, such as the cytokines interleukin-1 (IL-1) or Tumor necrosis factor (TNF), complex signalling pathways are initiated which culminate in the activation of the large molecular weight, serine specific IκB kinase (IKK) complex that rapidly phosphorylates IκB. This targets the inhibitor protein for ubiquitination and subsequent degradation (15, 115, 119). As with the NF-κB protein family, there are several isoforms of IκB protein (including IκBα, IκBβ and IκBε) that bind with varying affinities to a variety of NF-κB complexes, thus conferring different regulation on NF-κB translocation in a tissue specific manner. IκBα was the first identified member and also the most widely studied isoform of the IκB family. This 37kDa protein has high homology between species (ie. human, chicken, mouse, rat and pig) and contains 3 distinct structural domains, a 70 amino acid region, a 205 amino acid internal domain comprising 6 or more ankyrin repeats, and a 42 amino acid C-terminal region rich in proline, glutamic acid, serine and threonine (commonly called a PEST region) (247).

In all members of the IκB family, two serine residues (Ser^{32}, Ser^{36}) are crucial as important sites of phosphorylation by IKKs, and one tyrosine residue (Tyr^{42}) also undergoes phosphorylation (202, 236). Biochemical and structural analysis has also shown that IκBα has multiple interactions with individual NF-κB complexes concealing the DNA localization domain(46, 47). IκBα, β and ε have been demonstrated to preferentially target p65 and cRel containing complexes, masking their nuclear localisation sequence and thus preventing nuclear translocation. IκBα becomes inducibly phosphorylated on serines ^{32} and Ser^{36}, which targets it for ubiquitination at lysine residues Lys21 and Lys^{22} and subsequent proteolytic

degradation (202, 236). Both IκBβ and ε have similarly located serine residues (Ser^{19} and Ser^{23} for IκBβ) and are thought to be regulated in a similar fashion. The selective targeting of particular IκB proteins by various stimuli increases the means by which NF-κB activation is regulated. For example, IL-1, TNF, LPS and the phorbol ester PMA target IκBα, whereas IκBβ is only responsive to IL-1 and LPS. In addition the ability of the p50 and p52 precursor proteins p105 and p100 to inhibit nuclear translocation of NF-κB by means of their ankyrin repeat domains prior to their proteolytic processing demonstrates the complex and varied means by which this pathway is regulated.

IκB binds the nuclear localization sequence of NF-κB dimers, sterically hindering their function causing cytoplasmic retention of NF-κB. The subsequent degradation of IκB generates a free and active NF-κB dimer, permitting the NF-κB complex to translocate to the nucleus where it binds to its conserved site on target genes (197). IκBα also contains a nuclear export sequence which allows newly synthesized IκBα to enter the nucleus and extract NF-κB from the DNA, subsequently exporting the once again sterically hindered complex back into the cytoplasm (233).

1.4 Posttranslational Modification of IκB

Posttranslational protein modifications are a powerful means of modulating protein function, activity or localization after their synthesis. It has already been described how the modification of IκB by phosphorylation and ubiquitination leads to its degradation by the 26S proteasome. This important step in NF-κB activation is controlled by ubiquitin, a small, 76 amino acid protein, which is covalently linked to proteins. Ubiquitin is first activated by

an ubiquitin-activating enzyme E1; followed by the transfer to an ubiquitin-conjugating enzyme E2, which catalyses an isopeptide bond formation between ubiquitin and the target protein (131, 183). This third step is sometimes achieved with the assistance of a third enzyme, E3. The E3 enzymes are considered largely responsible for substrate specificity. The most widely studied mechanism of multi-ubiquitination of proteins is the targeting of the substrate protein for degradation by the 26S proteasome complex (271). However, as will be seen later in this introduction, ubiquitin is also involved in mechanisms other than degradation by the proteasome. It has been suggested that the level of ubiquitination may determine the fate of the modified protein. In the last few years, several proteins have been described as similar to ubiquitin. These proteins fall into two classes. The first class, termed 'ubiquitin-like modifiers' (UBLs) function as modifiers in a manner analogous to that of ubiquitin. The second class are designated 'ubiquitin-domain proteins' (UDPs) (131, 183).

A member of the UBL modifiers termed SUMO (also known as sentrin, GMP-1, UBL1 and PIC1), is the acronym for small ubiquitin-like modifier (96, 131, 183). This highly evolutionary-conserved protein, consisting of 3 family members in humans (SUMO 1,2 and 3), is only 18% homologous to ubiquitin (96), but nuclear magnetic resonance determination and secondary structure predictions shown a common three-dimensional structure that is characterised by a tightly packed globular fold with β-sheets wrapped around one α-helix (23). SUMO also has a short amino-terminal extension absent in ubiquitin. Several studies have shown an important role for SUMO in IκB and NF-κB activation. As previously described, phosphorylation of Ser^{32} and Ser^{36} of IκB triggers multiubiquitination of IκBα on Lys^{21} and Lys^{22} by E1, theUbc4/Ubc5 E2 family members and E3. However, two groups

found that Ubc9, the E2-like SUMO-1 conjugating enzyme, can associate with constituents of NF-κB or with IκB proteins (63, 266). It was determined though that Ubc9 was not involved in IκBα ubiquitination as Ubc9 was shown to conjugate SUMO-1, but not ubiquitin (63). Further analysis of this event showed that a relatively small amount of SUMO-1 is conjugated to IκBα in a number of cell types and that these complexes are resistant to TNFα-induced degradation (61). SUMO-1 appears to conjugate to Lys^{21}, which 'blocks' the ubiquitination site, although Ser^{32} and Ser^{36} phosphorylation is not required for this event to occur. Phosphorylation of these amino acids may also inhibit SUMO-1 conjugation. This specificity was further enhanced by the purification of a SUMO-1 specific E1 enzyme (62). This antagonistic action of SUMO-1 suggests that SUMO-1 creates a pool of IκBα protected from proteasome destruction, suggesting that SUMO-1 conjugation may be a mechanism for controlling the levels of critical signalling proteins within the cytosol, or a general response to genotoxic insult (96).

1.5 The IKK protein family

The enzymes which catalyze the ubiquititation of phosphorylated IκBs are constitutively active, thereby suggesting that the actual phosphorylation of IκB dictates the fate of IκB and as such NF-κB translocation.

A high molecular weight multi-protein complex termed the signalsome with IκB kinase activity was isolated from TNF-treated HeLa cells. Purification of this 900kDa multiprotein complex identified two proteins of 85 and 87kDa that demonstrated IκB kinase activity (56, 176). Microsequencing demonstrated that active IKK is a complex consisting of the initial

formation of IKKα (IKK1) and IKKβ (IKK2) as a heterodimer, followed by the subsequent addition of either a dimer or trimer of IKKγ (also known as NEMO or IKKAP1) proteins. IKKα and IKKβ have similar primary structures with a 52% overall homology, containing N-terminal kinase domains, leucine zippers and helix-loop-helix motifs in the C-terminus. The degree of homology between the IKKs suggests that the two kinases may have functionally redundant roles in the cell. Only by generation of IKKα and β deficient mice have any differences between the two become evident. Briefly, $IKK\alpha^{-/-}$ mice have been found to die within 4 hours after birth and show severe developmental defects consistent with a role for IKKα in regulating epidermal differentiation. In addition studies on these mice showed that IKKα was not essential for IKK complex activation by pro-inflammatory stimuli (105, 259). In contrast, IKK β deficient mice are embryonic lethal as a result of massive liver apoptosis, exhibiting a phenotype identical to that of p65 knockout mice (26, 147, 148, 263). They also demonstrate very little NF-κB activation in response to IL-1 or TNF, indicating that IKK β is predominantly responsible for regulating IKK complex activation in response to IL-1. It has been found that IKKα and IKKβ act as the catalytic subunits of the kinase complex. While IKKγ has no identifiable catalytic domain, it is primarily composed of a leucine zipper inserted in helix-loop-helix motifs acting as the regulatory subunit (119). Figure 1.3 gives a schematic representation of the IKK complex. Interestingly, while IKKα and IKKβ exhibit identical activation kinetics, substrate specificity and phosphorylation exclusively on serines, activation is totally dependent on the presence of IKKγ and the subsequent phosphorylation of its serine and threonine. Currently, however, only the

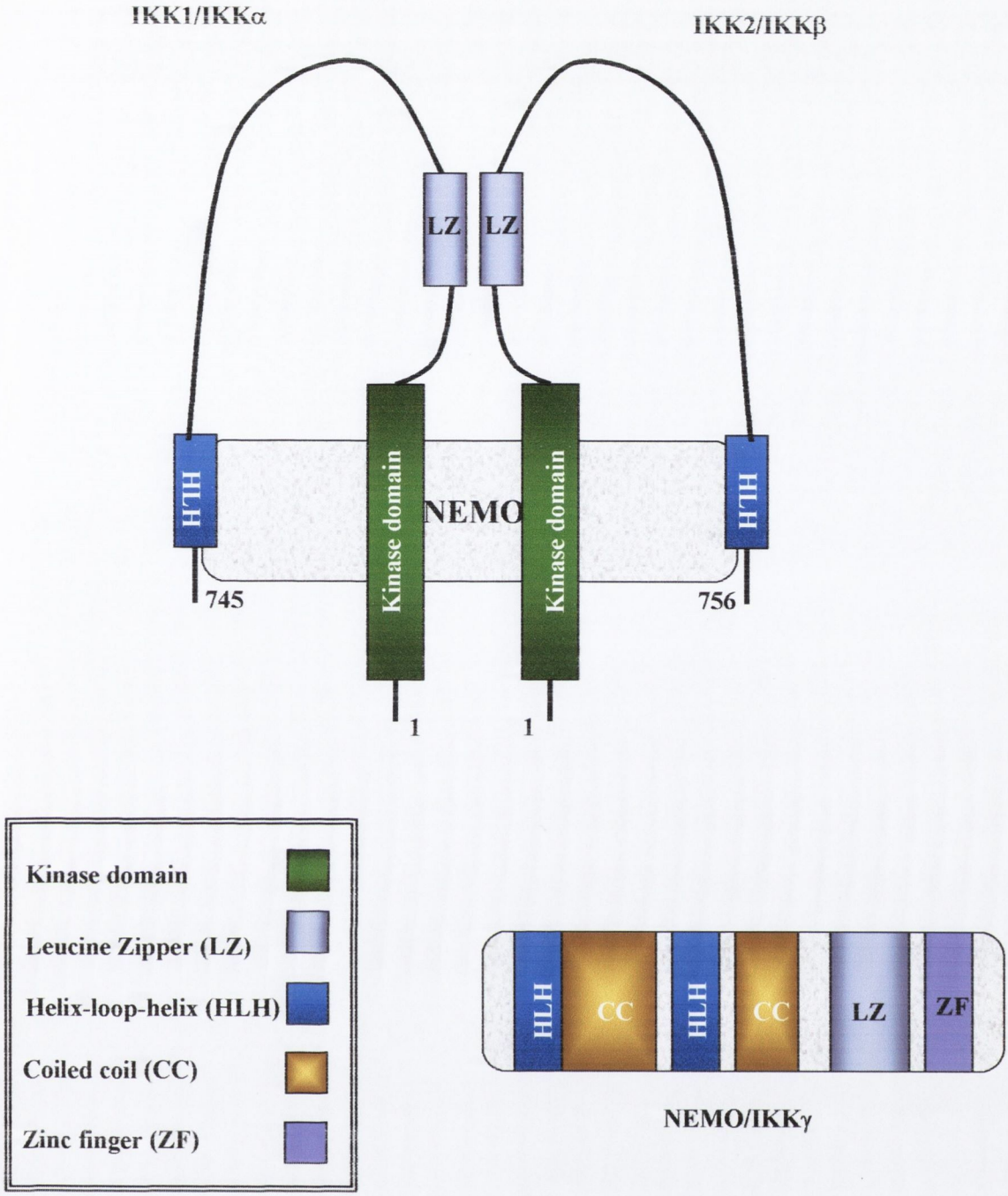


Figure 1.3 Schematic representation of IKK1, IKK2 and NEMO showing the domains important for their activation. IKK1 and 2 heterodimerise via their leucine zipper (LZ) domains and the interaction between their respective kinase domain and helix-loop-helix (HLH) domain is aided by the association of NEMO with the heterodimer. The association of NEMO is essential for the kinase activity of IKK1 and 2.

location of the phosphorylated serines of IKKβ have been biochemically mapped, but it would be assumed that the equivelant sites of IKKα are also phosphorylated.

Recent studies have shown the IKK complex to be the coordinator of NF-κB function and specificity of action in a tissue and cell specific manner (115). Almost all of the convergent signals that culminate in the activation and nuclear translocation of NF-κB are mediated by the IKK complex phosphorylating IκB, initiating the subsequent ubiquitin-dependant degradation. A recent report by Beraud et al (28) however has suggested that PI-3 kinase through interaction of its SH2 domain with phosphorylated Tyr42 of IκBα, could 'pull' IκBα away from complexed NF-κB and thus allow translocation. This tyrosine residue however is not universally conserved amongst IκB proteins, and, as such may not be a ubiquitous response, but tissue or stimulus specific.

Besides its association with IKKβ and IKKγ, IKKα has also been isolated in two-hybrid screens with the MAP kinase kinase kinase MEKK1, which lies on the Jun N-terminal kinase (JNK) pathway (120, 142), and NF-κB inducing kinase (NIK) (220). NIK has been suggested as a potent activator of IKKs, thus acting as an upstream kinase for IKKs, but occurring downstream of adaptor proteins such as TRAF6, in the case of IL-1 signalling. However, recent experiments have cast doubt on this theory and debate still ensues over the actual function and activity of NIK in the NF-κB signalling cascade. Two recent papers however, suggest a role for NIK in NF-κB activation, albeit in a very specific manner. Previously, overexpression of NIK led to enhanced IKK complex kinase activity (282), and caused activation of NF-κB (158, 251). Kinase inactive NIK inhibited ligand stimulated NF-

κB, and recent studies with alymphoplasia mice (*aly/aly*), which express a point mutation of NIK, suggested that NIK may function in a cell- or receptor-specific manner in NF-κB activation. This led Yin and co-workers (286) to generate $NIK^{-/-}$ mice to further explore a role for NIK in NF-κB activation. The mice, while appearing normal, displayed abnormal lymphorganogenesis similar to that observed in *aly/aly* mice and mice lacking the Lymphotoxin β receptor (LTβR). Furthermore, $NIK^{-/-}$ mice treated with IL-1 and TNF displayed no difference in NF-κB activation when compared to wild type mice, but the transcriptional action of NF-κB complex activated by LTβR is severely compromised in $NIK^{-/-}$ mice. Therefore, NIK acts in a receptor-specific manner; its function restricted to promoting transactivation of LTβR activated NF-κB. A further demonstration of the specific nature of NIK action has recently been suggested. It has previously been shown that NIK is required for ubiquitin-dependent processing of NF-κB2 (283), but not required for induction of NF-κB binding activity. Senftleben et al (242) recently demonstrated that the phosphorylation and subsequent processing of the NF-κB2 precursor (p100) requires phosphorylation and activation of the IKKα subunit, which is not required for NF-κB activation. Their conclusion suggested that NIK may be acting upstream of IKKα, as NIK is not involved in NF-κB DNA binding activity (286), but does inhibit NF-κB2 processing (283, 285). Therefore, these papers may suggest that while NIK is not involved in directing NF-κB DNA binding activity, it does have a role to play in receptor-specific responses to LTβR activation. This activation may induce the phosphorylation of the pro-inflammatory inactive IKKα subunit of the IKK complex, thereby leading to ubiquitin-dependent processing of NF-κB2, regulating epidermal differentiation.

The IKK complex has been shown to be the point of convergence for the multitude of signalling cascades that initiate IκB phosphorylation, degradation, and eventual NF-κB translocation. The upstream activators of this complex are therefore of important interest to researchers. Overexpression of a range of protein kinases in wild type or dominant negative forms has affected IKK function. These include Protein kinase Cζ (PKCζ) (136), the previously mentioned NIK and MEKK1, and also, other members of the MAPK kinase kinase family MEKK2, MEKK3 (288), COT/TPL2 (151) and TAK1 (188, 234). Other protein signalling complexes have also been implicated in activating or inhibiting IKK function. Studies have reported that phosphoinositide-3 kinase (PI-3 kinase) induced activation of Protein kinase B (PKB/Akt) is involved in the activation of IKK (28, 199, 227). One report suggests that Akt and NIK are needed for TNF induced activation, and another describes Akt transiently associating with IKK, thus inducing activation in platelet-derived-growth-factor signalling. Figure 1.4 summarises the signals, which converge on the IKK complex.

1.6 DNA-binding of NF-κB

As a result of phosphorylation and subsequent ubiquitin-mediated degradation of IκB, NF-κB is released, allowing translocation to the nucleus where it binds to its consensus sequence upstream of target genes. Although the consensus sequence 5'GGGRNNYYCC3' (where R is a purine, Y a pyrimidine and N any base) has been shown to preferentially bind p50/p65 heterodimers, there is a certain degree of selectivity among the different NF-κB subunits for various κB elements (reviewed in (208)). This ensures that promoters or enhancers containing variant κB elements have the potential to be regulated by specific NF-κB

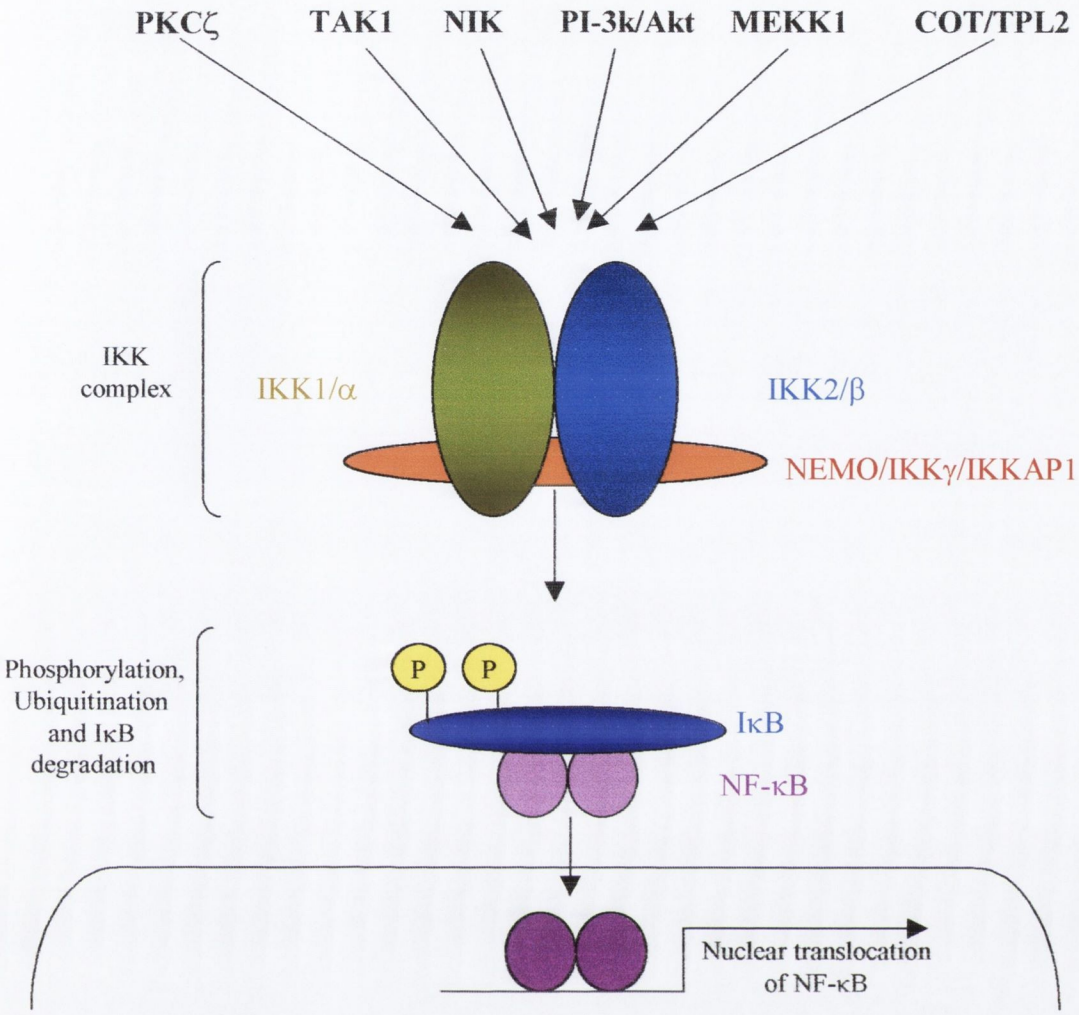


Figure 1.4 Schematic diagram describing IKK activation and function. The diagram illustrates some of the kinases that have been shown to activate the IKK complex, which induces IκB phosphorylation and subsequent degradation are indicated. Abbreviations used are protein kinase C (PKC), TGFβ activated kinase (TAK1), NF-κB inducing kinase (NIK), phosphoinositide-3 kinase (PI-3k), mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1), and the proto-oncongene Cot kinase (COT/TPL2).

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

1.7 Activation of IKK by IL-1

The most widely studied activation pathways to IKK activation have been those involving the pro-inflammatory cytokines, IL-1 and TNF. Figure 1.5 summarises the currently known components of the IL-1 signalling pathway.

IL-1 binds to the Type I IL-1 Receptor (IL-1R1), which in conjunction with the IL-1 receptor accessory protein (IL-1AP), triggers signal transduction. An adaptor protein MyD88 (108) is then recruited to the receptor complex within the cytoplasm (197, 228).

MyD88 is a 35kDa protein containing two functional domains: an N-terminal death domain and a C-terminal Toll/IL-1R (TIR) domain. The crucial role for MyD88 in cytokine signal transduction was illustrated by the abrogation of IL-1- or IL-18-mediated activation of NF-κB in MyD88$^{-/-}$ mice.

Immunoprecipitation studies have shown that in IL-1 signalling, MyD88 co-precipitates with IL-1R1, the IL-1R-accessory protein and the IL-1R-associated kinase (IRAK). This complex

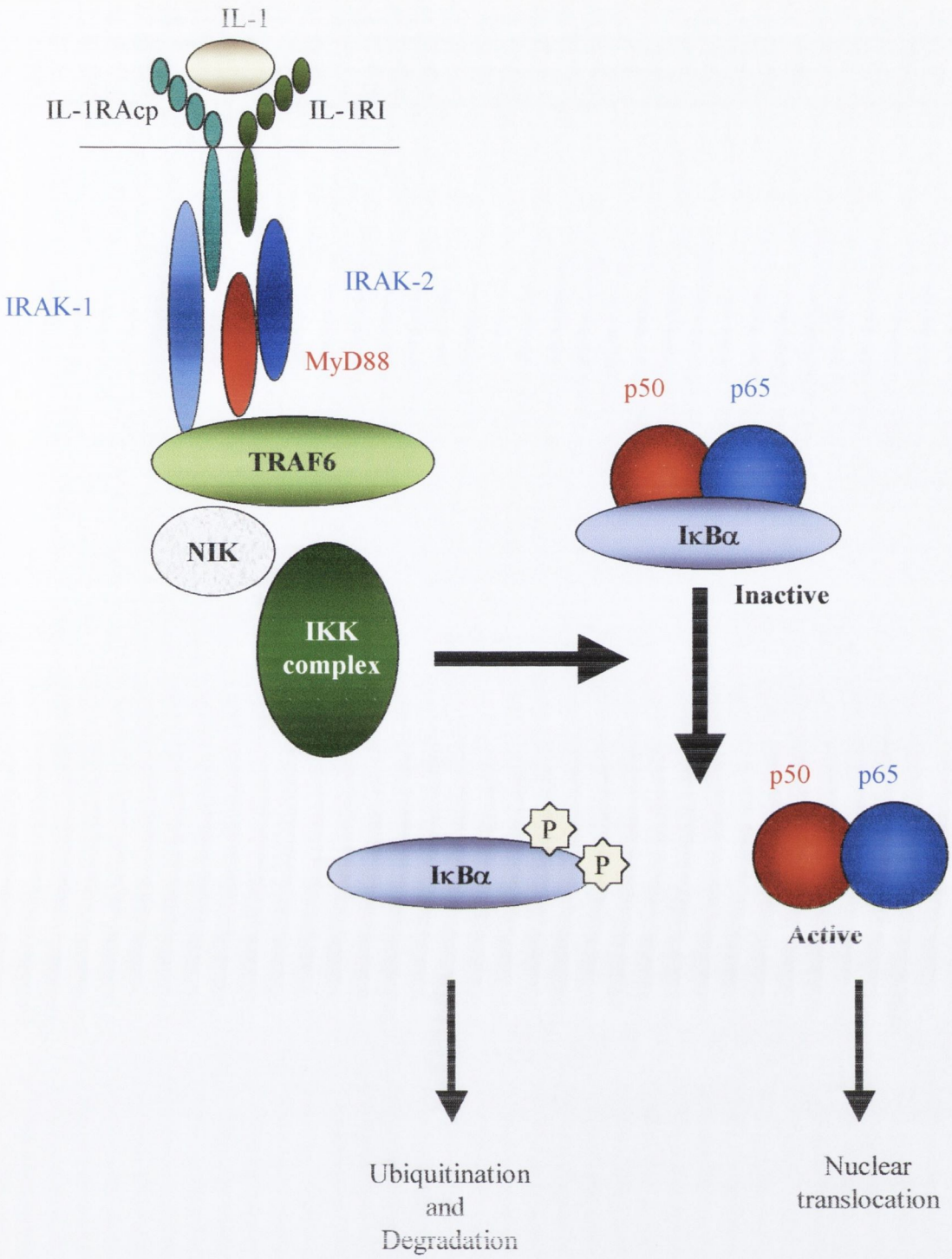


Figure 1.5 Stimulation of NF-κB by IL-1.

Upon binding of IL-1 to the IL-1R and accessory protein, the multimeric signalling complex of MyD88, IRAK1 and IRAK2 engages the IL-1 receptor complex. This promotes the recruitment of TRAF6 to the complex which, upon detachment from the receptor complex, propagates the signal to NIK. This in turn leads to phosphorylation of the IKK signalsome resulting in IκB phosphorylation and subsequent ubiquitination. Phosphorylation and eventual degradation of IκB releases NF-κB, allowing translocation to the nucleus. *Reproduced from O'Neill and Green, J. Leukocyte Biol. 1998.*

of signalling molecules however is ligand dependent and not found associated in un-stimulated cells. The association of MyD88 and IRAK through interaction of their respective death domains allows the autophosphorylation of IRAK through an as yet unknown mechanism. An IRAK homolog IRAK2, that lacks kinase activity, also interacts with the MyD88/IRAK complex through its death domain. The phosphorylation of IRAK appears to allow the disengagement of the IRAKs from the death domain of MyD88, and facilitate the subsequent recruitment of TRAF6 to the complex (reviewed in (197)).

TRAF6 belongs to a family of proteins first described as participants in TNF signalling (40). Characteristics of the family include a C-terminal region of homology known as the TRAF domain, which facilitates TRAF protein oligomerization and interaction with other signalling proteins. TRAF6 however is unique amongst the TRAF protein family in that it is the only protein member that does not directly interact with the receptor complex (41).

How TRAF6 propagates the signal however, is unknown, but immunoprecipitation experiments have shown interaction with the previously described MEKK1 (21) and NIK (251). However, a recent report by Wang et al has suggested a novel role for ubiquitin in mediating TRAF6 signal transduction (275). They found that oligomerisation of TRAF6, in the presence or absence of signalling from the IL-1R complex, triggers its ubiquitination through the action of TRAF6-regulated IKK activator 1 (TRIKA1) consisting of the dimeric ubiquitin-conjugating enzyme complex Ubc13-Uev1A. This is an example of ubiquitination not targeting a protein for proteosomal degradation, but promoting signal transduction. The mechanism for this unusual function may reside in the specific manner in which TRAF6

ubiquitination occurs (78). In TRAF6, the ubiquitin chain polymerises via the Lys^{63} amino acid, in contrast to the degradation ubiquitination signal, which is linked via the Lys^{48} amino acid position. The unique structural and functional assymetrics of the complex may ensure its survival and determine its unique phenotype. Wang et al were also able to demonstrate the purification of TRIKA2, which was found to comprise TAK1, TAB1 and TAB2. It was found that ubiquitinated TRAF6 directly activates TAK1, although this activation mechanism is unknown, which recruits TAB2 from the cell membrane to the cytoplasm. TAK1 is then able to activate IKK through an as yet unknown mechanism. Figure 1.5 gives a schematic representation of the IL-1 induced signalling cascade.

1.8 Bacterial Activation of NF-κB.

Apart from cytokines such as IL-1, bacterial products are extensively studied NF-κB activators. Pathogenic bacteria such as *Shigella flexneri* and heat killed *Staphylococcus aureus*, along with bacterial products such as LPS, LTA, and pneumococcal cell walls (39, 68, 254), have all been shown to activate NF-κB.

This evolutionarily-inherited defense is found in most multicellular organisms and is characterised by the induction of a pro-inflammatory response by the host cells upon pathogen identification based upon pattern recognition receptors (PRRs) (168, 171). The pro-inflammatory response causes the synthesis of cytokines, which in turn causes the synthesis of mediators such as cell lipid derived prostanoids, and endothelial cell adhesion molecules. These events all involve NF-κB activators. The consequences of the synthesis of these mediators when produced in excess are marked by a decrease in heart function, a

lowering of blood pressure, and the blocking of vasculature of the major organs with leukocytes. The PRRs recognize conserved molecular motifs that are shared by a wide range of microorganisms, thus allowing the innate immune system to respond to the microbe and a defense response.

For several decades, research has concentrated on identifying the class of proteins involved in the PRR response. In the last few years' reports have demonstrated that the Toll family of receptors play a crucial role in *Drosophila* and mammalian host response (reviewed in (168, 170, 194, 196)) as discussed below.

1.9 *Listeria monocytogenes*

Much of the work on bacteria and NF-κB has focused on LPS from *E. coli* or heat-killed bacteria. Recently however, the effect of the intracellular bacterium *Listeria monocytogenes* has been studied.

L. monocytogenes is a gram positive, motile, food borne, pathogen, which can be fatal to the host. During the course of infection, it is capable of infecting both professional and non-professional phagocytic cells (50, 67). The ability of *L. monocytogenes* to replicate, and the subsequent infection of other cells from within macrophages and monocytes (156, 157), is an essential facility of this bacterium that ensures the successful infection of the host (73, 74). Indeed, if *L. monocytogenes* were unable to replicate within macrophages, it would be rapidly destroyed and subsequently eliminated by the host.

1.10 Cellular invasion by *L. monocytogenes*.

Entry into the host is generally through the gut following ingestion of *L. monocytogenes,* internalising through the M-cells and Peyers patches, or by direct infection of the epithelial lining. Subsequent infection is found in the regional lymph nodes, where *L. monocytogenes* gains access to the blood stream where it is transported to the liver and spleen (74). Upon reaching these organs, the bacterium is phagocytosed by resident macrophages. In the liver, the Kupffer cells kill the majority of bacteria, however a very small population of bacteria survive and invade the hepatocytes where they rapidly replicate. The replication of bacteria within the hepatocytes is critical in establishing a 'successful' infection (50).

Once the bacterium has been internalised into cells, it is able to rapidly replicate within the cytosol and, coating itself in actin filaments, spread cell-to-cell thus avoiding the immune response extracellularly (137, 267). This invasion process can be divided into two steps: attachment to the cell membrane, and subsequent internalisation (Figure 1.6).

Once *L. monocytogenes* has been incorporated into a membrane vacuole within professional phagocytes, the bacterium lyses the vacuolar compartment using two secreted proteins, listeriolysin O (LLO) and an inositol-specific phospholipase C (PLC) (52). The bacterium then begins to coat itself in actin filaments to form an actin tail utilising the product of the bacterial protein Act A, which propels movement through the cytosol and subsequently cell-to-cell (Fig. 1.6) (49-51). This ability of *L. monocytogenes* to utilise host cell proteins to effect cell motility and avoidance of host defence mechanisms such as circulating antibodies

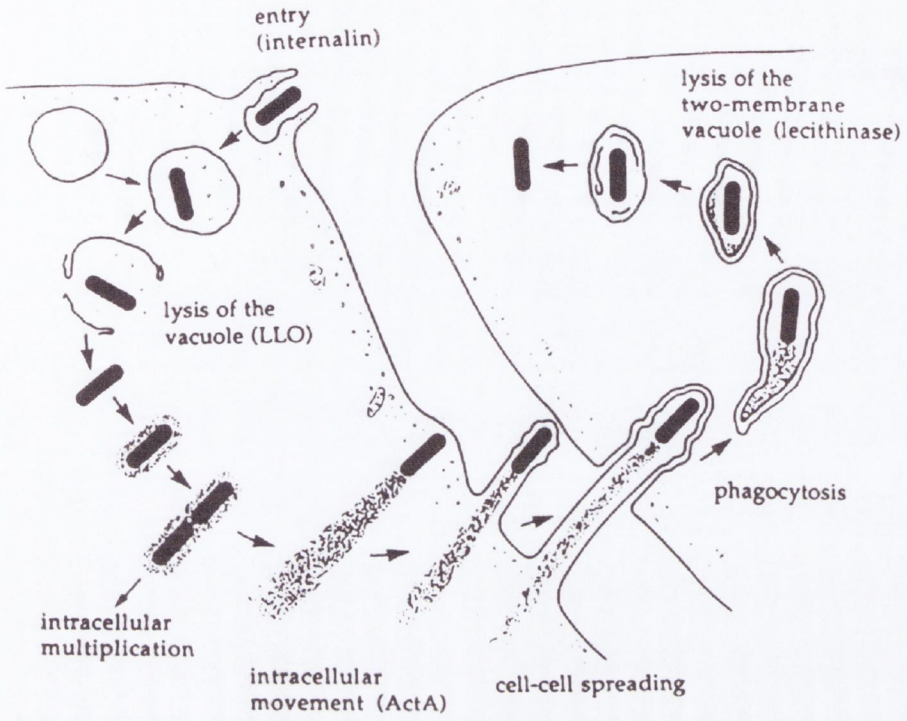


Figure 1.6 The successive steps of *L. monocytogenes* infection.

Proteins in parentheses are those involved in infection process.

Reproduced from Tilney and Portney, 1989.

and complement, explains earlier observations that only T-cell immunity is effective in defence against *L. monocytogenes* (72, 74, 214).

1.11 Mediators of *L. monocytogenes* invasion.

Two proteins of the Internalin family, Internalin A (InlA) and Internalin B (InlB) have been found to be crucial in mediating *L. monocytogenes* cell invasion. Increasing evidence suggests that InlA and InlB both mediate invasion of different cell types (50). InlA is an 800 amino acid protein consisting of 15 highly conserved leucine rich repeats (LRR) containing 22 amino acids, 2 repeats consisting of 70 amino acids and a third of 49 amino acids. The carboxy terminal contains the LPXTG motif, which is found in more that 50% of bacterial surface proteins and is linked through a peptidoglycan linkage to the bacterial cell membrane (83, 84). The receptor for InlA is E-Cadherin (174) and monoclonal antibodies against the N-terminal LRR of InlA have been found to be the most effective in inhibiting *L. monocytogene*- InlA mediated internalization (173). The non-invasive bacteria *Listeria innocua* became able to invade Caco-2 cells when InlA was expressed upon its surface (83) and invasiveness was conferred upon latex beads when coated with recombinant InlA (140). Recently, the use of transgenic mice allowed the demonstration of the importance of InlA-mediated crossing of the gut-barrier, inducing the primary invasion of the host after ingestion (141). In contrast to human E-cadherin, mouse and rat E-cadherins are not receptors for InlA due to a point mutation at the 16th amino acid; hE-cadherin Pro^{16}→Glu m/rE-cadherin (139). Therefore mouse and rat models, while successfully used to study T cell response to intravenous infection with *L. monocytogenes*, are inappropriate for studying InlA function in vivo, as oral infections are not reproducibly lethal due to the low bacterial translocation of

the intestinal barrier. In contrast to this, transgenic mice that express hE-cadherin solely in enterocytes were rendered highly susceptible to oral infection with *L. monocytogenes*, however, InlA mutant *L. monocytogenes* induced zero mortality with the same dose as wild type.

1.12 Internalin B (InlB)

InlB is a 630 amino acid protein consisting of 8 LRR each comprising 22 amino acids with a high degree of homology to InlA, an inter-region (-IR) containing 2 repeats of approximately 70 amino acids, and a 232 amino acid carboxy terminal region of tandem repeats each comprising 80 amino acids, each beginning with a GW motif. InlB is loosely attached to the bacterial surface via this 232 amino acid C-terminal region, which partly releases InlB, such that InlB is found in culture supernatants of wild type *L. monocytogenes* (35, 65). Purified InlB from these cultured supernatants confers invasiveness upon inactive latex beads and also a ΔInlB mutant strain (38). InlB induces phagocytosis through activation of phosphoinositide (PI)-3 kinase within host cells, leading to increased levels of phosphoinositide second messagers (113, 114). PI-3 kinase is involved in many signalling events, including those affecting actin polymerization. Activation occurs through InlB-stimulated tyrosine phosphorylation of adaptor proteins implicated in membrane localization of PI-3 kinase (114). Furthermore, inhibitor studies have shown that InlB-mediated invasion of cells by *L. monocytogenes*, requires the activation of PI-3 kinase, tyrosine phosphorylation and actin polymerization (113, 265). These cell activities were shown to illicited by the N-terminal 213 amino acids comprising the LRR domain of the mature protein (37). The crystal structure of this LRR domain of InlB suggests an elongated, curved surface

containing clusters of residues that are likely to form a bacterial-mammalian complex where the unique binding pattern of calcium suggests a crucial role for this ion in InlB mediated binding (161, 162). The receptor for InlB was first reported to be gC1q-R, the receptor of the globular head of the complement component C1q (36). However, Shen et al (244) recently demonstrated that c-Met, the mammalian receptor for Hepatocyte growth factor (HGF) or 'scatter factor', as a second receptor for InlB.

Further members of the Internalin family have been described (InlC-InlH) (66, 152, 218) which are transcribed by the pleiotropic activator prfA, along with two recently described, smaller, soluble members of the family (InlE-InlF) (76) which are not transcribed in *L. monocytogenes*, but *L. ivanovii*. InlB has been found to be the most strikingly different from other family members in both structure and characteristics (66).

1.13 Activation of NF-κB by *L. monocytogenes*.

Modulation of the host cell functions by L. monocytogenes can occur at six different stages during the infection process: (i) through secreted bacterial substances prior to bacteria-cell contact, (ii) upon adhesion, (iii) during the invasion process, (iv) from inside the phagocytic vacuole, (v) from free bacteria within the cytosol, and (vi) during the process of cell-cell spread (134).

Hauf et al (94) demonstrated that *L. monocytogenes* is capable of activating NF-κB in a biphasic manner proceeding invasion of the macrophage-like cell $P388D_1$. This effect was a rapid transient activation mediated by Lipotechoic acid (LTA), involving IκBα degradation followed by a second more persistent phase characterised by IκBβ degradation. This latent

activation appeared to involve virulence genes including Act A, PC-PLC and PI-PLC, although a direct effect of their products was not demonstrated. Infection of bone marrow derived macrophages (BMM) with *L. monocytogenes* has also been shown to upregulate the cytokines IL-1α, IL-1β and TNFα, after initial infection (60, 132, 133). This classical pro-inflammatory response was also found to be unaffected by cytoskeletal inhibitors which permit bacterial adhesion, but not invasion. This suggests that a transmembrane surface molecule of mammalian cells is able to interact with a bacterial ligand and initiate a pro-inflammatory response.

The pore forming activity of Listeriolysin O (LLO) mediates another signalling event that occurs upon treatment of cells with *L. monocytogenes*. p42/p44 MAP kinase activation was originally thought to be triggered by bacterial adhesion (265), but further studies demonstrated this activity mediated without bacterial contact with the target cells (264, 279). LLO was also found to be a potent activator of NF-$\varkappa$B (123), however the signalling pathway that leads to NF-$\varkappa$B activation has not been elucidated. Figure 1.7 describes some of the cytosolic signalling events mediated by *L. monocytogenes*.

1.14 Bacterial Activation of NF-$\varkappa$B by LPS.

As previously mentioned bacteria such as *Shigella flexneri* and heat killed *Staphylococcus aureus*, and now *L. monocytogenes* (94) have all been shown to activate NF-$\varkappa$B, as have multiple bacterial products.

Perhaps the most widely studied activator of NF-$\varkappa$B and progenitor of the pro-inflammatory response leading to sepsis is LPS. LPS is the principal component of the outer membrane of

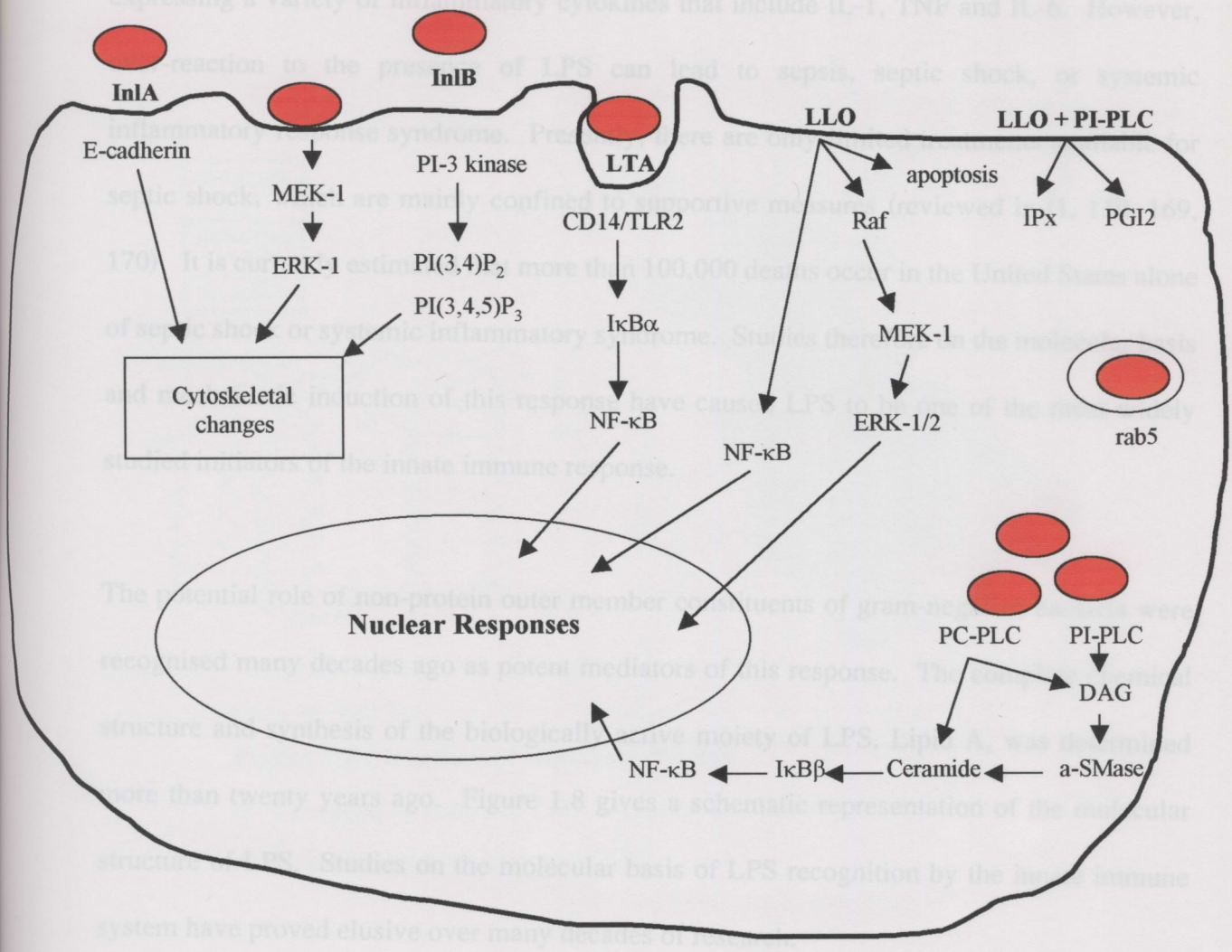


Figure 1.7 Signal Transduction during *L. monocytogenes* infection of Cells.
Abbreviations: aSMase, acidic sphingomyelinase; DAG, diacylglycerol; ERK-1/2, mitogen-activated protein kinase 1 and 2; IPx, inositolphosphates; LLO, listeriolysin; LTA, lipotechoic acid; PGI2, prosaglandin I2; PC-PLC, phosphatidylcholine-specific phospholipase C, PI-PLC, phosphatitylinositol-specific phospholipase C; $PI(3,4)P_2$, phosphatidylinositol-(3,4)-biphosphate; $PI(3,4,5)P_3$, phosphatidylinositol-(3,4,5)-triphosphate; rab5, and small GTPase.
Modified from Kuhn and Goebel, 1998.

gram-negative bacteria. Monocytes orchestrate the innate immune response to LPS by expressing a variety of inflammatory cytokines that include IL-1, TNF and IL-6. However, over-reaction to the presence of LPS can lead to sepsis, septic shock, or systemic inflammatory response syndrome. Presently, there are only limited treatments available for septic shock, which are mainly confined to supportive measures (reviewed in (1, 110, 169, 170). It is currently estimated that more than 100,000 deaths occur in the United States alone of septic shock or systemic inflammatory syndrome. Studies therefore on the molecular basis and mechanistic induction of this response have caused LPS to be one of the most widely studied initiators of the innate immune response.

The potential role of non-protein outer member constituents of gram-negative bacteria were recognised many decades ago as potent mediators of this response. The complete chemical structure and synthesis of the biologically active moiety of LPS, Lipid A, was determined more than twenty years ago. Figure 1.8 gives a schematic representation of the molecular structure of LPS. Studies on the molecular basis of LPS recognition by the innate immune system have proved elusive over many decades of research.

As mentioned above, the evolutionarily inherited defense to microbe infection is found in most multicellular organisms and is characterised by the induction of a pro-inflammatory response by the host cells upon pathogen identification based upon pattern recognition receptors (PRRs) (169, 171).

For several decades, research has concentrated on identifying the PRRs. Most recently, Toll-Like Receptors (TLRs) have emerged as important PRRs.

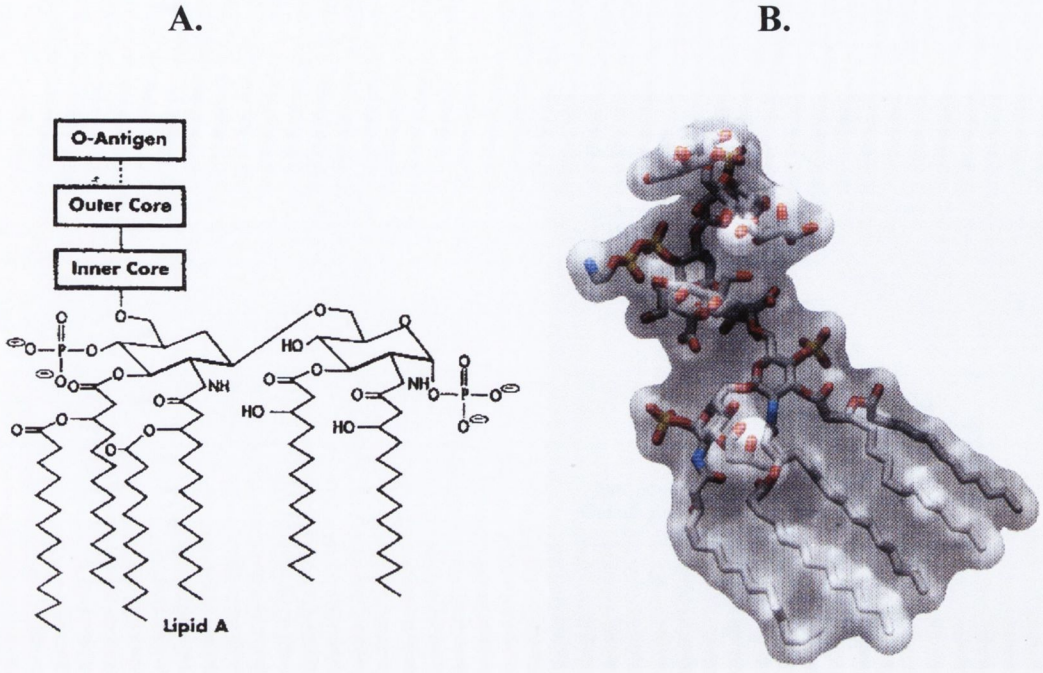


Figure 1.8 Structure of Lipolysaccharide

A. Chemical structure of LPS. *B.* Stick and ball representation of the Lipid A active moiety of LPS.

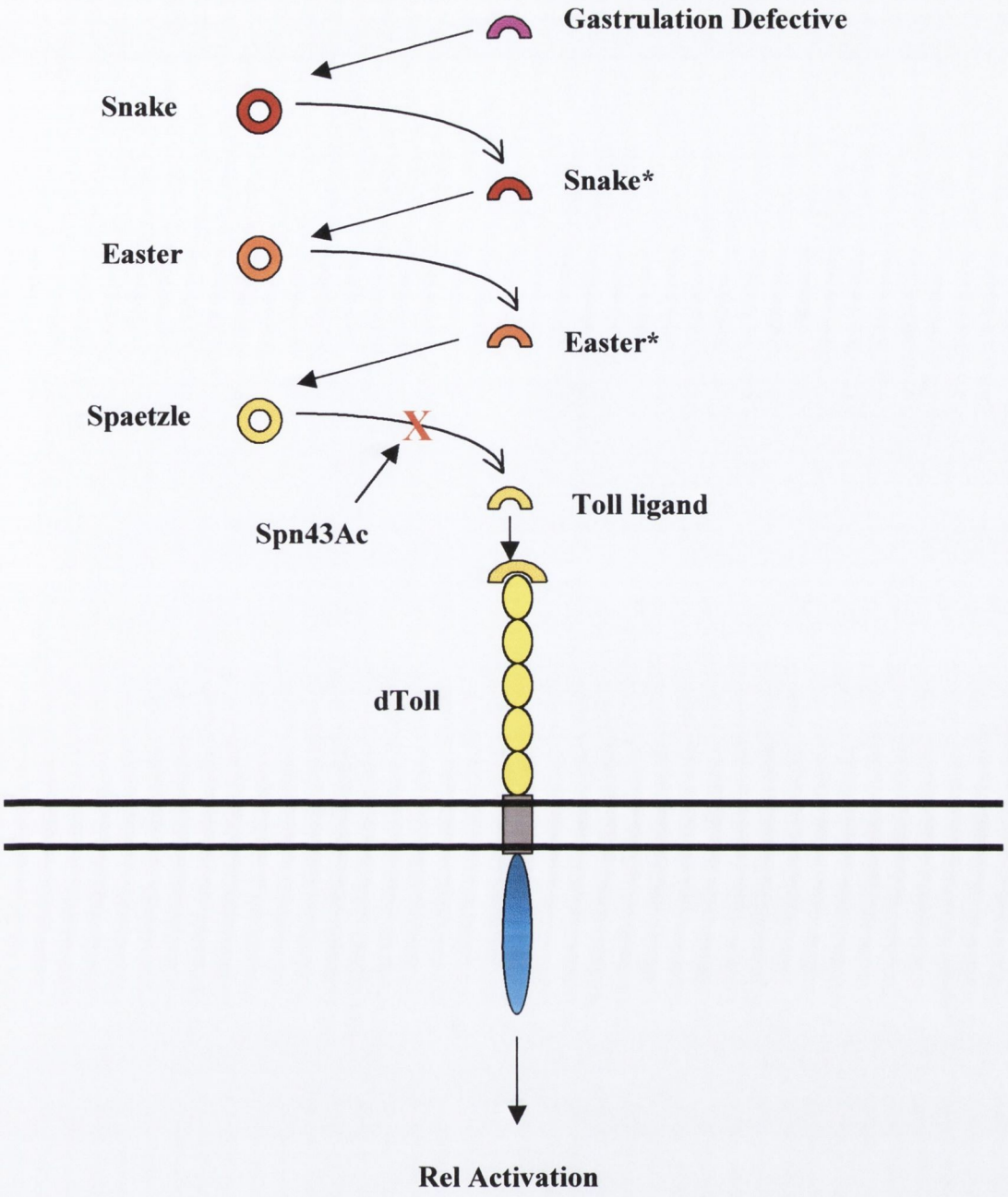


Figure 1.9 Schematic representation of Protease Cascade in Drosophila. The active Toll ligand is produced by proteolytic cleavage of the Spaetzle protein, is rapidly sequestered to the receptor Toll. Spaetzle cleavage is the final step in a protease cascade, in which zymogen forms are sequentially cleaved to yeild an active enzyme. * Represents activated protease.

1.15 Toll receptors in Innate Immunity

In *Drosophila* embryos, the Toll protein, upon binding of its ligand Spaetzle (182), controls dorsal-ventral patterning and activates the transcription factor Dorsal. Spaetzle is a cysteine-knot polypeptide that is proteolytically cleaved following a cascade of zymogens (encoded by the genes *gastrulation defective*, *snake* and *easter*), in the *Drosophila* embryonic system (figure 1.9) (27). Spaetzle is structurally related to the mammalian Nerve growth factor (179). Toll also has a role in host defense in the adult fly. Here, Toll activation induces the synthesis and secretion by the fat body (the *Drosophila* equivalent of the vertebrate liver) of a battery of small cationic polypeptides. Induction of the fat body occurs in response to immune challenge and the peptides induced have potent antimicrobial activities directed against either fungal pathogens (drosomycin, metchnikowin) or bacteria (diptericin, drosomycin, cecropin, attacin, defensin) (102, 103, 124). The genes encoding these peptides have in their upstream regions nucleotide sequence motifs similar to mammalian NF-$\varkappa$B-binding sites (221). Signalling through Toll parallels the signalling pathway induced by the IL-1 in mammalian cells. The IL-1 signalling pathway to NF-$\varkappa$B and its inhibitor I$\varkappa$B, are homologous to Dorsal and its inhibitor Cactus respectively (27). Recent studies provide evidence for an IKK complex in flies (155, 231). Further studies have shown that the Toll family of proteins utilise a homologous signalling cascade to that described earlier regarding IL-1 signal transduction. This is illustrated in figure 1.10. Importantly, the cytoplasmic domain of Toll is homologous to the cytoplasmic domain of IL-1 (85) and is therefore termed the Toll/IL-1 Receptor (TIR) domain.

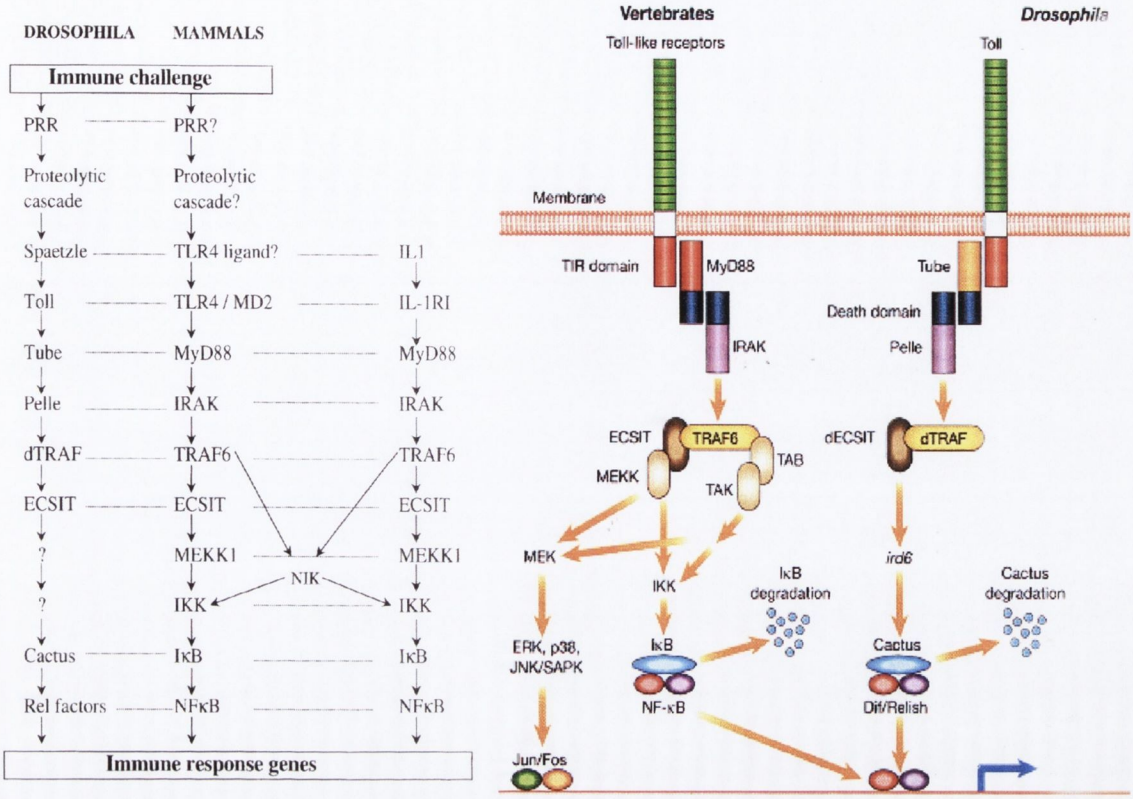


Figure 1.10 Signalling pathways of Drosophila and Vertebrates.

Representation of the signalling pathways activated by the Tolls, TLRs and IL-1 in Drosophila and mammals and the functional homologs shared between pathways. In Drosophila, the Toll pathway is similar to that of the vertebrate TLRs. Toll links to an adapter protein Tube, which is the functional homolog of MyD88 in vertebrates. Tube binds to the kinase Pelle, which is the functional homolog of IRAK. There are a number of other functional homologs shared between the respective pathways, including dTRAF and dECSIT, although a clear role for these proteins is still unclear. Finally, Cactus and Dif/Relish are the Drosophila homologs of IκB and NF-κB respectively. *Reproduced from Bowie and O'Neill, 2000; Aderem and Ulevitch, 2000 respectively.*

Genetic experiments suggest that the zymogens of the embryonic system are largely redundant for the immune response (144). However, immune challenge is rapidly followed by the cleavage of Spaetzle. Furthermore, loss-of-function mutants of a serine protease inhibitor Spn43Ac, exhibit constitutive cleavage of Spaetzle, inducing the constitutive expression of drosomycin in the absence of an immune challenge. These results suggest therefore, that Toll is not a *bona fide* receptor for microbial ligands. It has been suggested that microbial antigens and damaged cell particles interact with recognition receptors upstream of Toll and activate zymogens in the hemolymph (or the extracellular matrix of immune-responsive cells), inducing Spaetzle cleavage to its active ligand form. Spaetzle can form homodimers (10, 59), and may induce the dimerisation of Toll upon ligand binding, although no direct interaction has been shown between the two proteins.

1.16 Toll-like receptors in mammalian Innate Immunity

Evolutionary conservation in innate immunity led Janeway and colleagues to search for mammalian Toll-related proteins. They successfully described the first human homolog of *Drosophila* Toll, initially termed human Toll, but subsequently re-named Toll-like receptor (TLR)-4 (172). The involvement of TLR4 in innate immunity was further supported as constitutively active TLR4 activated NF-κB-dependent genes such as IL-1, IL-6 and IL-8 (172). Furthermore, it was also shown that constitutively active TLR4 induced members of the B7 family of proteins, which are required for the activation of naive T cell by antigen-presenting cells. Rock et al (223) then highlighted the general structural features of the TLR family, including the presence of leucine-rich repeats in the extracellular domain, and the Toll-homology domain found in the cytoplasmic domain of all members of the family. This

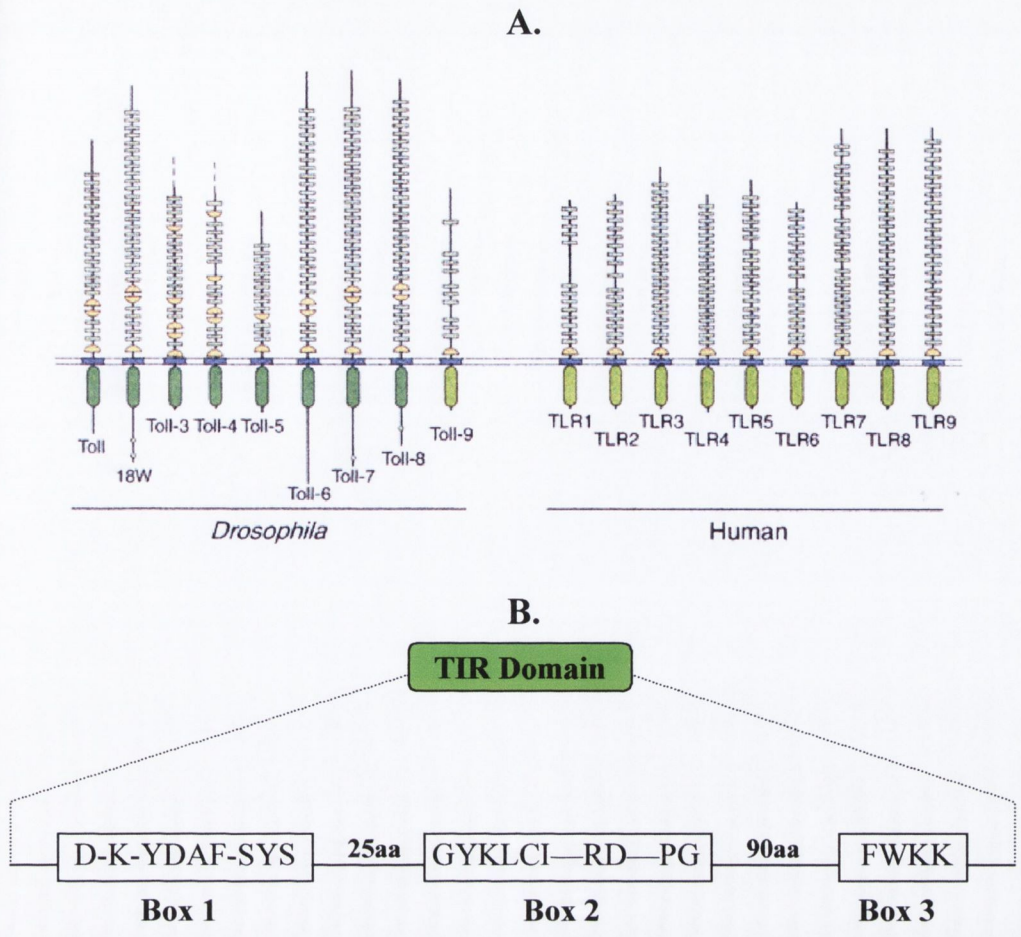


Figure 1.11 The Toll family of receptors in Drosophila and Humans.

A. Schematic representation of the structures of the Toll receptors. Leucine rich repeats are represented by small rectangles and the cysteine-rich carboxy flanking regions are represented half circles. The Toll/IL-1 receptor homology (TIR) domain is represented by the oblong green box. Toll-9 is more closely related to the mammalian TLRs than other Drosophila Tolls. Human TLR10 is not shown. *Reproduced from Imler and Hoffmann, 2001. B.* The consensus sequence for the three conserved regions of the TIR domain.

description of additional family members led to the definition of the IL-1R/Toll-like receptor (TLR) superfamily, based on the homologous cytosolic TIR domain (reviewed in (33, 194, 195)). Presently, the family consists of 10 members of the mammalian TLR family, several members of the plant family, and 9 members of the *Drosophila* Toll family. Figure 1.11 illustrates the members of the Toll families in *Drosophila* and humans.

The combinations of ex vivo studies and knockout mice, or cells derived from these mice, have been widely used to determine the microbial product specificities of various TLRs (reviewed in (4, 5)). These studies have shown that (i) TLR2 is activated primarily by peptidoglycan (PGN) and lipoproteins (260), (ii) TLR4 is predominately activated by LPS and LTA (209, 211, 260), (iii) TLR6 recognises macrophage-activating lipopeptide-2 kDa (MALP-2) from mycobacteria in conjunction with TLR2 (261), and (iv) TLR9 is required for the response to unmethylated bacterial CpG DNA (98). Cells derived from knockout mice demonstrated that TLR2 knockout macrophages respond normally to LPS, but are unresponsive to PGN; TLR4 knockouts do not respond to LPS, but respond to PGN; TLR6 knockout mice are unresponsive to MALP-2, but can discriminate between N-terminal lipoylated structures of MALP-2 from mycobacteria and lipopeptides derived from other bacteria; and macrophages from TLR9 knockout mice are unresponsive to unmethylated CpG DNA, but respond normally to LPS (98, 209, 260, 262) Figure 1.12 summarises some of the known activators of mammalian TLRs.

As expected from proteins involved in the primary innate immune detection and response, TLRs are widely expressed in the cell types involved in the first line of defense such as

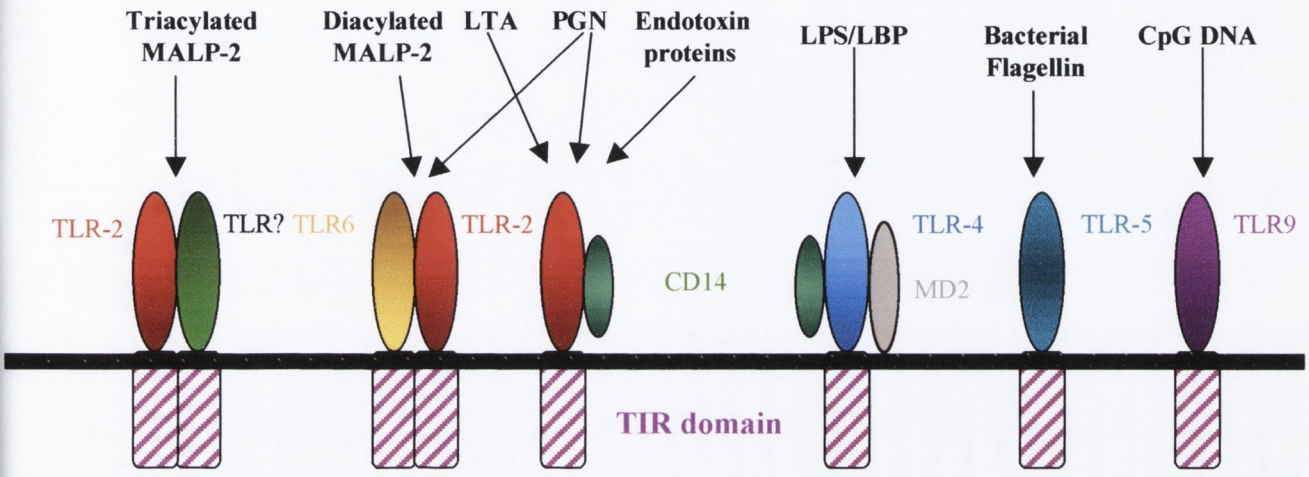


Figure 1.12 Schematic diagram of activators of Toll Like Receptors.
Abbreviations used: MALP-2, macrophage-activating lipopeptide-2 kDa; LTA, lipoteichoic acid, PGN; peptidoglycan, LPS; lipopolysaccaride binding to LBP; LPS-binding protein, TLR; Toll like receptor and TIR; Toll/IL-1 receptor domains.

macrophages, monocytes, neutrophils, gut and lung-derived epithelial cells, and dermal endothelial cells (24, 43, 77, 184, 273). TLRs are probably most widely expressed on Dendritic cells which express a large array of TLRs (99). The role of dendritic cells is to recognise microorganisms and activate the adaptive immune system, which would concur with the important role TLRs have in the recognition of microbes and microbial products. Importantly, TLRs are expressed on T and B lymphocytes (180, 190), are present on the plasma membrane and are recruited to phagosomes upon exposure of the cells to microorgamisms (200, 270). Only TLR9 however, is not widely expressed, and appears to be only expressed on intracytoplasmic vesicles, which would underline its role in sensing unmethylated CpG DNA within cells (98).

Thus TLR receptors and their subsequent activation of NF-$\varkappa$B may represent the most ancient form of host defense to microorganisms found in plants, insects and mammals.

1.17 TLR4 recognises LPS

LPS had long been known to bind LPS-binding protein (LBP), an 80kDa plasma protein, and to CD14, a 55kDa protein with a glycosylphophatidylinostiol (GPI) anchor (269). However, as CD14 has no transmembrane domain, it was apparent there was another component of the signalling complex missing. It had been known for several decades that certain strains of mice were resistant to LPS and that this resistance was the consequence of a genetic defect. Two studies demonstrated that the genetic defect in two mice that are hyporesponsive or non-responsive to LPS was linked to TLR4 (209, 216). The co-dominant *LpsD* allele of the C3H/HeJ strain was a result of a mis-sense mutation in the third exon of TLR4; this mutation was predicted to result in a Pro^{712}→His substitution. Monocytes transfected with this mutant

introduced into wild type TLR4, converted it to a dominant negative phenotype, inhibiting LPS-dependent responses (270) (Underhill 1999). C57B1/10ScCr mice, that are also resistant to LPS, were shown to be homozygous for a null mutation of TLR4 (209, 216). Taken together, these findings suggested the first direct link between TLR4 and the physiological responses to LPS.

However, expression of TLR4 on Human embryonic kidney (HEK) 293 cells did not confer LPS-responsiveness, suggesting that an additional molecule was required for LPS activation of NF-κB via TLR4. This molecule was subsequently identified as the 25kDa secreted accessory molecule MD2 (3, 246). Transfection of cells with either TLR4 or MD2 alone did not confer responsiveness to LPS, but co-transfection of TLR4 with MD2 demonstrated that physical association of both proteins was required for full responsiveness. Interestingly, MD2 can also associate with TLR2, thereby conferring responsiveness to LPS and Lipid A (69, 70). This finding could suggest that association with accessory molecules such as MD2 could enable TLRs to respond to a wider range of microbial stimulants.

It is still unknown nonetheless, if TLR4 binds directly to LPS in mammalian cells, or if TLR4 responds to a ligand generated by microbial-induced proteolytic activation, as described for Toll in *Drosophila*. Two recent reports have suggested that LPS binds to the TLR4/MD2 signalling complex (54, 272), primarily through interaction with MD2. However, it has been suggested that while LPS may be in close proximity to the TLR4/MD2 signalling complex, complementation studies conclude that the complete LPS signalling complex may comprise of at least one additional gene product (240).

Further complexity has recently been added to the signalling pathway initiated by TLR4 activation after stimulation with LPS by several recent studies. LPS stimulated cells derived from MyD88-deficient mice, were still found to activate NF-κB and JNK, although delayed, as was maturation of dendritic cells (121). MyD88 acts as an adapter protein involved in TLR2, TLR4 and TLR9 signalling (185, 280). Therefore, this suggested that an additional adapter is required for a subset of LPS-inducible, TLR4-dependent signals. MyD88-adapter Like (Mal)/TIR-adapter protein (TIRAP) have recently been described as this additional adapter. Fitzgerald et al (79) demonstrated that activation of NF-κB by Mal requires IRAK2, but not IRAK, whereas MyD88 requires both IRAKs. Mal associated with IRAK2 via its TIR domain and dominant negative Mal inhibited LPS-induced NF-κB activation, but had no effect upon NF-κB activation induced by IL-1 and TNFα. Mal was also shown to directly interact with TLR4. Simultaneously, Horng et al (104) demonstrated Mal/TIRAP acting as an adapter for LPS-induced activation of NF-κB via TLR4, also demonstrating Mal/TIRAP involvement in LPS-mediated activation of the interferon-regulated double-stranded RNA activated protein kinase PKR. As TLR4 appears to be the only TLR to signal as a homodimer, and is the only TLR to utilise the specific adapter Mal/TIRAP, where the others signal via MyD88 exclusively, the detection and signalling of LPS in mammals appears far more complex than originally thought. Figure 1.13 illustrates the signalling complex and adapter molecules that regulate LPS-mediated signalling pathways.

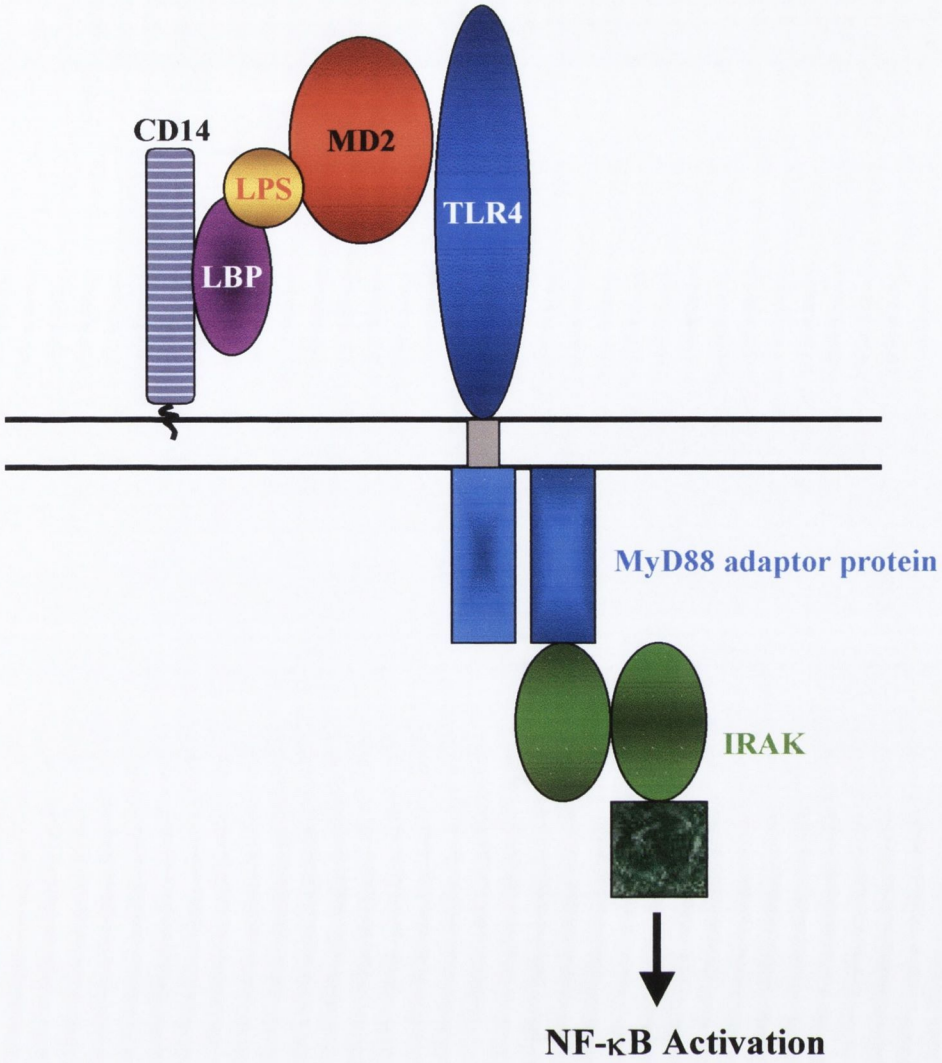


Figure 1.13 LPS recognition complex on Phagocytes.

LPS is bound by LPS-binding protein (LBP), this complex is then recognised by CD14 on the macrophage surface. MD2 then appears able to recongise LPS or the LPS/LBP/CD14 complex and bring this into close proximity of TLR4, to from a signalling complex. TLR4 signals through the adaptor molecule MyD88 and the serine kinase IRAK. The activation mechanism of TLR4 is currently unknown and could be either direct or indirect. Both CD14 and TLR4 contain Leucine rich repeats which may facilitate protein-protein interaction. MD2 is a secreted protein that is required for TLR4-mediated signalling.

1.18 Aims

The primary aim of this study is to investigate NF-κB activation as a signal for two microbial products, InlB from L. monocytogenes, and LPS. The specific aims are as follows:

1. The initial aim of this study is to investigate whether InlB has a role in NF-κB activation by *L. monocytogenes*, as InlB appears not only as a surface bound ligand, but is released into the cellular supernatant. InlB may therefore be another bacterial product, (such as LPS and LTA), capable of activating NF-κB. Secondly, InlB has been shown to activate Phosphoinositide-3 kinase (PI-3 kinase) in cells (113, 114), and a role for PI-3 kinase in NF-κB activation has been proposed (28, 199, 219).

2. To characterize the signalling pathway activated by InlB, thereby assisting in the determination of the mammalian receptor for InlB

3. To examine the recognition of LPS by the innate immune system. TLR4 has been demonstrated to act as the transmembrane signalling receptor for LPS (209), in conjunction with MD2 (246). Given the evolutionary homology between the *Drosophila* and human innate immune systems. I wished to determine if the activation of NF-κB via TLR4 would be susceptible to serpin inhibition, in an analogous fashion to Spaetzle inhibition by Spn43Ac in *Drosophila*.

This study identifies InlB as an activator of NF-κB and has mapped the signalling pathway induced. Secondly, the inhibitory effect of the serpin, Antithrombin III, on NF-κB activation by LPS suggests that a serine protease may be required to generate a ligand for TLR4.

Chapter Two

Materials and Methods

Chapter 2

2.1 Materials

The murine macrophage-like cell line J774 was kindly provided by Kingston Mills (National University of Ireland, Maynooth) and was grown in 10% heat inactivated Fetal Calf Serum (FCS) (Poole, Sigma, UK) in RPMI 1640 (Poole, Sigma, UK) which was supplemented with 2 mM L-Glutamine (Hyclone, UK), 5% CO_2. The human epithelial cell line Hep2 was a kind gift from Pascale Cossart (Institut Pastuer, Paris, France) and was grown in 10% FCS, DMEM (Gibco, UK), 2mM L-Glutamine at 10% CO_2. The human monocyte cell line THP-1 and the murine macrophage cell line RAW 264 were purchased from European Collection of Cell Cultures (ECACC). The stably transfected cell line HEK 293-TLR4 was a kind gift from Prof. Douglas Golenbock (Boston Medical Centre, Boston, MA, USA). Manumycin A, Calphostin C, Geldanamycin, and Tyrophostin AG490 (Calbiochem, UK), LY294002 (Poole, Sigma, UK), and 1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-Methyl-3-*O*-octadecylcarbonate] (Alexis Biochemicals, UK) were all dissolved in dimethyl sulfoxide. The 22 base pair oligonucleotide containing the consensus sequence for NF-$\varkappa$B (5'-AGTTGAGGGGACTTTCCCAGGC-3') was purchased from Promega (Madison, WI, U.S.A) as was the T4 polynucleotide kinase kit. [γ-32P]-labelled ATP was purchased from Amersham (Nycomed Amersham plc, Amersham Place, Buckinghamshire, UK). Poly(dI.dC) was supplied by Pharmacia (Uppsala, Sweden). The transfection reagant SuperFect was purchased from Quigen (Crawley, UK) as were the Midi- and Maxi-plasmid purification kits. The DuoSet human IL-8 ELISA development system was purchased from R&D Systems (MN, USA).

Vectors pPL-IL-8-pLuc and pTK-rLuc were kind gifts from Dr E. Kiss-Toth (University of Sheffield, UK). The expression vectors encoding dominant negative Ras N17 (described previously, (31)), empty vector RSV, and the expression vector encoding amino acids 1-149 of human c-Raf1 in pGex-KG *ie*. Glutathione-S-transferase (GST)-Ras binding domain (RBD), were kind gifts of Dr Doreen Cantrell (Imperial Cancer Research Fund, London, UK). The expression vector encoding dominant negative Akt was donated by Dr Stephan Ward (Bath University, UK).

The α-IκBα monoclonal antibody was a kind gift from Prof. Ron Hay (St Andrews University, UK). Phospho-Akt, phospho-p38, Akt, and p38 antibodies were obtained from New England Biolabs (Beverly, MA, USA). Anti-pan Ras antibody was purchased from Oncogene Research Products (Cambridge, MA). Polyclonal antiserum against rat p85α, and monoclonal antibodies against phosphotryrosine (Tyr(P)) (clone 4G10) used for immunoprecipitation, were purchased from Upstate Biotechnology (Lake Placid, NY). Purified mouse IgG, goat anti-mouse and anti-rabbit IgG peroxidase conjugates were purchased from Sigma (Poole, UK) or New England Biolabs (Beverly, MA, USA). The recombinant domains of InlB and the monoclonal antibodies raised against these domains were purified as described (35) and were a kind gift from Pascale Cossart (Institut Pastuer, Paris, France).

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

2.2 Cell Culture

The murine macrophage-like cell line J774 was grown in 10% heat inactivated Fetal Calf Serum (FCS) (Poole, Sigma, UK) in RPMI 1640 (Poole, Sigma, UK) which was supplemented with 2 mM L-Glutamine (Hyclone, UK), 5% CO_2. Cells were passaged every 2-3 days by physical agitation. Cells were seeded at a density of 5.10^4 cells/ml for experiments unless otherwise stated.

The human epeithelial cell line Hep2, was grown in 10% FCS (Poole, Sigma, UK), Dulbecco's Modified Essential Medium (DMEM) (Gibco, UK), supplemented with 2mM L-Glutamine and incubated at 5% CO_2. Cells were passaged every 2-3 days, cells were removed from plasticware by incubated of cells with Trypsin/EDTA solution, and seeded at $1x10^5$ cells/ml.

THP-1 cells were maintained in RPMI 1640 (Poole, Sigma, UK), supplemented with 10% Fetal Calf Serum (Poole, Sigma, UK) and 2 mM L-Glutamine (Gibco, UK), and incubated at 5% CO_2. Cells were passaged every 2-3 days, ensuring that cell density did not exceed $1x10^6$ cells/ml as THP-1 cells are known to differentiate into macrophages at this concentration. Stably transfected HEK 293-TLR4 were maintained in RPMI 1640,

10% FCS, 2mM L-Glutamine, and incubated at 5% CO_2 supplemented with selection antibiotic 25μg/ml puromycin. Cells were passaged every 2-3 days, removal from plasticware achieved by physical agitation, and seeded at $1x10^5$ cells/ml for experiments.

In all cases, cell viability was determined by Trypan blue dye exclusion assay, which is excluded from healthy cells, however, non-viable cells are permeable to the dye. Cells were counted using a hemocytometer and viewed with an inverted light microscope.

2.3 Bacterial strains, growth conditions and bacterial stimulation.

L. monocytogenes and *L. innocua* were both grown in brain-heart infusion (BHI) at 37°C. For *L. innocua* strains containing pAT28 derivatives, erythromycin was added to a final concentration of 5μg/ml. The strains used are described in Table 2.1.

For bacterial stimulation of J774 cells with bacterial cultures, 1ml of expotential-phase cultures (OD_{600}=0.8-1.0) of strains *L. monocytogenes* and *L. innocua* were washed three times with PBS, pelleted and resuspended in 1ml of PBS. Bacteria were added to mammalian cells (~$1x10^6$ cells total) to give an Multiplicity of Infection (MOI) of either 20:1 (*L. monocytogenes*) or 50:1 (*L. innocua*) and incubated for a further 60 minutes, after which cells underwent nuclear extract preparation for NF-κB analysis.

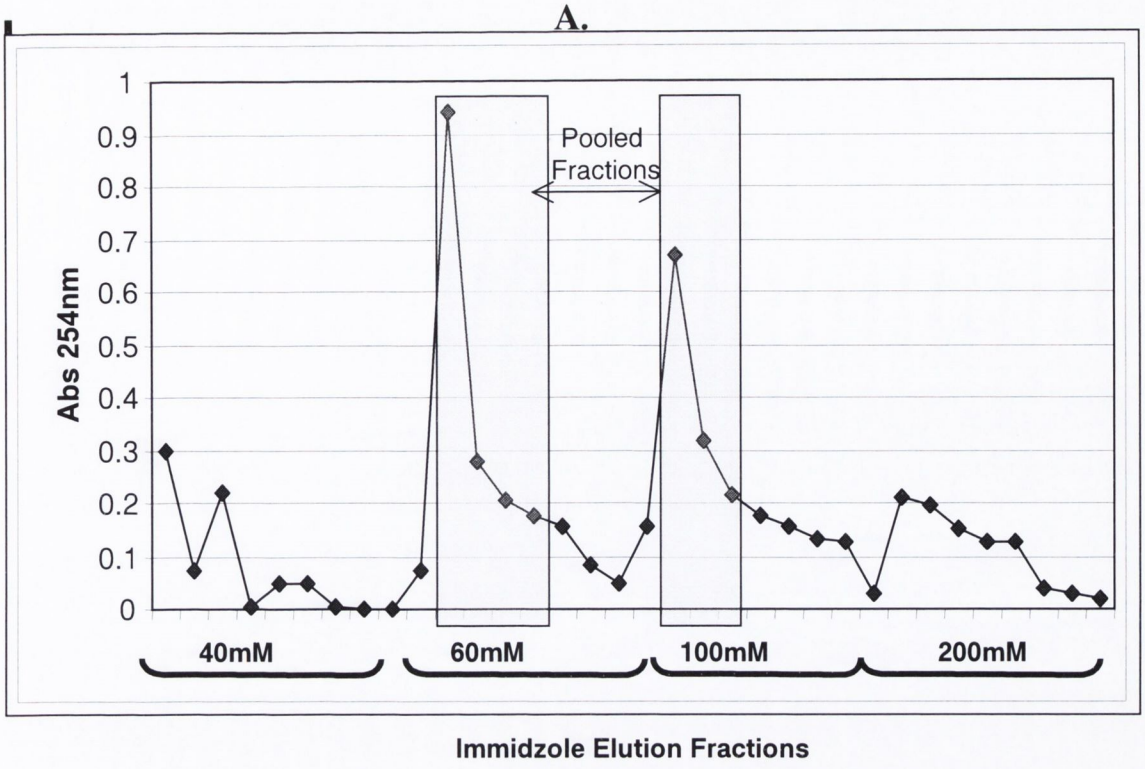
2.4 Purification of InlB.

The construction of a bacterial strain encoding full-length InlB containing N-terminal 6xHis tag termed BUG 1348, has previously been described (35). BUG 1348 was grown

Bacterial Strain	Genotype/properties	Reference
BUG600	*L. monocytogenes*: Wild Type, serotype 1/2a	Mackness (1962)
BUG1047	*L. monocytogenes*: EGDΔInlB	Dramsi et al. (1995)
BUG994	*L.innocua*: pAT28 (vector)	Dramsi et al. (1995)
BUG1533	*L.innocua*: (InlB)	Jonquières (1999)

Table 2.1 *Listeria* bacterial strains used.

at 37°C in LB broth containing kanamycin (30μg/ml), with shaking until early log-phase (OD_{600}~0.8-1.0) and expression of InlB-6xHis induced by addition of 1mM isopropyl-b-D-thiogalactopyranoside (IPTG). After 3 hours induction, bacteria were harvested by centrifugation, resuspended in column binding buffer (5mM Imidizole, 500mM NaCl and 50mM Hepes (pH 7.4)) and disrupted with a French Press. Debris was removed by centrifugation and protein was batch mixed with metal affinity matrix (Ni-ATA, Quigen, USA) overnight. Affinity matrix was poured into chromotography column and extensively washed with 40mM imidizole, 500mM NaCl and 50mM Hepes (pH 7.4). InlB-6xHis was eluted from Ni-ATA column by addition of 10mls of 60mM imidizole, 500mM NaCl and 50mM Hepes (pH 7.4), 1ml fractions collected and analysed for protein elution using a spectrotometer (OD_{254}). Further elutions were carried out using 10ml of 100mM and 200mM imidizole added to 500mM NaCl, 50mM Hepes (pH 7.4) respectively, and further 1ml fractions collected and analysed. Figure 2.1 illustrates the elution profile for InlB-6xHis, and the fractions pooled for further purification shown. Pooled fractions were diluted to ~200mM NaCl with 50mM Hepes, pH 7.4, and loaded onto a HiTrap SP cation exchange column (Parmacia, Uppsala, Sweden). Protein was eluted by NaCl concentration gradient (0.2-1M NaCl), and fractions collected according to peak detection by spectrophormetic readings (OD_{254}). Collected fractions were analysed for purity by SDS-PAGE and subsequent Commassie blue protein staining. Figure 2.1A demonstrates the OD_{254} elution profile, collected fractions illustrated, while figure 2.1B demonstrates the purity of InlB at each purification step. InlB-6His was concentrated to desired volume using centriprep 50 devices (Amicon, USA), and then



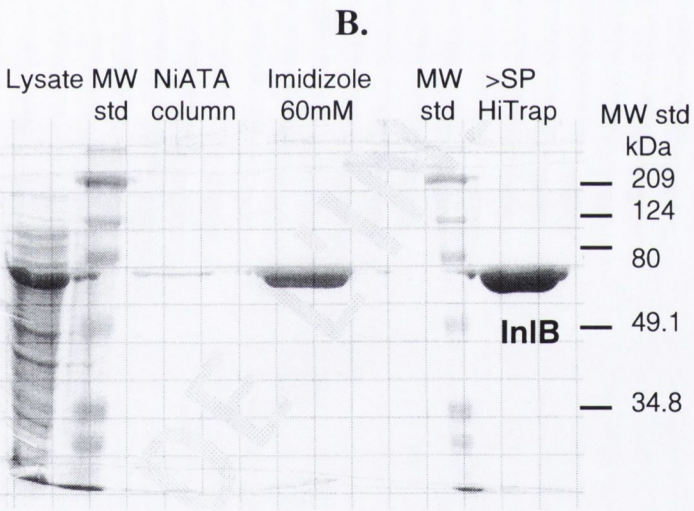


Figure 2.1 Purification of InlB

stored in 500mM NaCl, 50mM Hepes pH 7.4 at –80°C. Protein concentration was determined by Bradford method (34).

2.5 Activation Assays and Nuclear Extract Preparation.

Murine macrophage-like cells J774 were seeded at $1x10^5$ cells/ml in 6 well plates (3 ml volume), 48 hours prior to stimulation and incubated at 37°C, 5% CO_2. InlB was diluted in 35 mM Tris, 75 mM NaCl, 1 mM EDTA, pH 7.5. Relevant concentrations of proteins were added to cells and incubated for 1 hour at 37°C.

Nuclear extracts were prepared using a modified version of the method of Osborn *et al* (198). Stimulation was terminated by aspiration of media from cells and replacement with 1 ml of ice-cold hypotonic buffer (10 mMHepes buffer, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and 0.5 mM phenylmethylsufonyl fluoride (PMSF)), cells scraped and centrifuged (5 minutes, 13,200rpm, 4°C). The subsequent cell pellet was lysed in 0.1% Igepal CA-630 in hypotonic buffer and held on ice for 10 minutes. Centrifugation (10 minutes, 13,200 rpm, 4°C) led to a cell debris pellet from which nuclear associated proteins were extracted by addition of 20 mM Hepes, pH 7.9, containing 420 mM NaCl, 1.5M $MgCl_2$, 0.2 mM EDTA, 25% glycerol, and 0.5mM PMSF and maintained on ice for 20 minutes. After centrifugation (10 minutes, 13,200 rpm, 4°C), the supernatant was removed and mixed with 10mM Hepes, pH 7.9, containing 50 mM KCl, 0.2M EDTA, 20% glycerol, 0.5 mM dithiothreitol and 0.5 mM

PMSF. Protein concentrations were determined using the method of Bradford (Bradford, 1970) and extracts stored at $-20^{O}C$.

2.6 Labelling of oligonucleotides for Electrophoretic mobility shift assay.

5' end-labelling of the 22 base pair oligonucleotide containing the NF-κB consensus sequence was based on the method of Sambrook et al (235).

The reaction mixture containing 10pmol of NF-κB DNA, 50pmol (150μCi) [γ-^{32}P] ATP, 20U T4 polynucleotide kinase and 5μl of 10x kinase buffer (100mM $MgCl_2$, 50mM DTT, 1mM spermidine, 500mM Tris-HCl, pH 7.5) was made up to 50μl with H_2O and incubated at $37^{o}C$ for 10 minutes. The reaction was terminated with 2μl of 0.5M EDTA. 50μl of a phenol 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 minute, the solution was centrifuged at 13,000g for 2 minutes. The resulting top aqueous layer was transferred to a fresh tube, 2μl of 5M NaCl added, vortexed and 100μl of ethanol added. This was incubated at $-30^{o}C$ for 30 minutes to effect ethanol precipitation of DNA. After centrifugation (13,000g, 5 minutes), the supernatant was carefully removed and discarded. The pellet was air dried at 37oC, and resuspended in 50μl TE buffer (1mM EDTA, 10mM Tris-HCl, pH 8). 1μl of this solution was counted in 5ml scintillant fluid, and the stock solution subsequently diluted to 10,000cpm/μl immediately before use in the electrophoretic mobility shift assay.

2.7 Electrophoretic Mobility Shift Assay (EMSA).

Nuclear extracts (4 μg) were incubated with 10,000 cpm of a 22-base pair DNA fragment oligonucleotide containing the NFκB consensus sequence that had previously been labeled with [γ-^{32}P]ATP (10 mCi/mmol) by T4 polynucleotide kinase. Incubations were performed for 30 minutes at room temperature, in the presence of 2 μg poly(dI.dC) as nonspecific competitor and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 4% glycerol, and 100 μg/ml nuclease free bovine serum albumin. For competition studies, unlabeled wild type or mutant NFκB oligonucleotides were added to the binding reaction 30 minutes prior to addition of the radiolablled probe. In experiments involving antisera to NF-κB subunits, 0.5 μl of specific antibodies to p50, p65, and c-Rel were incubated with nuclear extracts for 20 minutes on ice prior to the binding reaction. All incubation mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels (Table 2.2), which were subsequently dried and autoradiographed using Kodak X-OMAT diagnostic film at –70°C in an autoradiography cassette with intensifying screens.

2.8 Immunoblotting of Proteins (Western Blot)

2.8.1 Preparation and running of SDS-PAGE gels.

Samples were resolved by electrophoresis using Sodium Dodecyl Sulphate polyacrylamide gels (SDS-PAGE). Table 2.2 shows the volumes of gel components required to prepare the various percentage gels mentioned in experimental descriptions, however, as and example, a 10% resolving gel was prepared using 5.3ml of 30% bisacrylamide solution (Protogel), 6.7ml 1.5M Tris, 0.05% w/v SDS (ph 8.8), 0.035ml

Component	Volume/Amount added
40% Acrylamide/bisacrylamide (29:1)	3.125ml
10x TBE (0.89M Tris, 20nM EDTA)	2.5ml
H2O	19.4ml
1M DTT	5μl
N,N,N',N'-Tetramethylethylenediamine (TEMED)	15μl
Ammonium persulphate	0.05g

Table 2.2 Composition of native polyacrylamide gel used for EMSA.

10% ammonium persulphate, and 0.0125ml of TEMED, made up to 16ml with autoclaved water. The stacking gel consists of 1.6ml 30% Protogel, 2.5ml 0.5M Tris, 0.05% w/v SDS (ph 6.8), 0.035ml ammonium persulphate and 0.0125ml TEMED, made up to 10ml with autoclaved water. Samples were electrophorised at constant voltage (150V). The components of the SDS-PAGE gels used is given in table 2.3.

2.8.2 Transfer of proteins to membrane

The resolved proteins were transferred to either nitrocellulose or polyvinylidene difluoride (PVDF) using a wet transfer system with all components soaked beforehand in transfer buffer (25mM Tris-HCl (pH 8.0), 0.2M glycine, 20% methanol). Briefly, the gel was placed on a layer of filter paper and sponge and overlaid with nitrocellulose paper. A second piece of filter paper was placed on top followed by the second sponge. The entire assembly was placed in a cassette and an electric current of 150mA was applied for 60 minutes.

2.8.3 Detection of protein of interest

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

Stacking Gel	5% Gel
Acrylamide/bisacrylamide (37.5:1)	800µl
500mM Tris, pH=6.8, 0.4% w/v SDS	1.25ml
H_2O	2.85ml
N,N,N',N'-Tetramethylethylenediamine (TEMED)	12.5µl
10% w/v Ammonium persulphate	30µl

Running Gel	7%	10%	15%
30% Acrylamide/bisacrylamide (37.5:1)	3.8ml	2.7ml	8.0ml
1.5M Tris, pH=8.8, 0.4% w/v SDS	4.0ml	4.0ml	4.0ml
H_2O	8.2ml	6.3ml	4.0ml
N,N,N',N'-Tetramethylethylenediamine (TEMED)	12.5µl	12.5µl	12.5µl
10% w/v Ammonium persulphate	30µl	30µl	30µl

Table 2.3 Composition of stacking and resolving SDS-PAGE gels.

subsequently washed twice with TBS-T (5 minutes each) and once with TBS (5 minutes). Blots were developed by enhanced chemiluminescence (ECL) according to manufacturers instructions (Amersham).

2.9 IκB Immunoblot Analysis.

Murine macrophage-like cells J774 were seeded at $1x10^5$ cells/ml in 6 well plates (3 ml volume), 48 hours prior to stimulation and incubated at 37^oC, 5% CO_2. InlB (100 ng/ml) was added and stimulation was terminated at relevant time points by aspiration of reaction media and subsequent addition of 1 ml ice cold PBS. After aspiration of PBS from the cells, 200 μl of ice-cold RIPA buffer (1% Igepal CA-630, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate in PBS, containing 10mg PMSF, 7 μg aprotinin and 1 mM sodium vanandate) was added. Plates were shaken on ice for 15 minutes and cell scraped to ensure lysis. Following further disruption of cells by passage through a 21-guage needle (10 strokes), a further 0.1 mg/ml PMSF was added to the samples, which were incubated for 45 minutes. Samples were then centrifuged for 20 minutes at 13,200 rpm at 4^oC, and the supernatant was removed from the cell debris and assayed for protein by Bradford method (34). Equal amounts of protein (4μg) were mixed with 5x sample buffer (5ml glycerol, 10ml 10% SDS, 10mg bromophenol blue, 6.25ml 1M Tris (pH 6.8) and 28.75ml autoclaved water; 20μl/ml of β-mercaptoethanol was added fresh), resolved by SDS-PAGE and transferred to nitrocellulose.

2.10 Protein Concentration Determination.

Protein determination was carried out as described by Bradford (34). Briefly, samples were diluted 1:5 (nuclear extract samples) or 1:10 (cell lysis samples) in autoclaved water, to give a final volume of 20μl. 200μl of Bradford reagant (0.01% w/v Coomassie Brilliant Blue G-250, 4.7% v/v ethanol, 8.5% v/v orthophosphoric acid) was added to each sample and incubated for a further 5 minutes. Plates were read at OD_{570nm} and corrected at OD_{405nm} using a Dynatech MR5000 plate reader. Protein concentrations were determined using a standard curve constructed using BSA the concentration range of 0-20μg/20μl. A typical standard curve is shown in figure 2.2.

2.11 TNF and IL-6 Gene Expression Analysis.

J774 cells were seeded at $5x10^5$ cells/ml in 24 well plates (1 ml volume), 24 hours prior to stimulation, and incubated at 37^oC, 5% CO_2. Cells were stimulated with 100 ng/ml and 1 μg/ml of InlB, in triplicate, and incubated for a further 24 hours. Supernatant was aspirated and centrifuged (1600 rpm, 5 minutes) to remove any detached cells, samples were stored (-70^oC) or analysed. Protein concentrations were determined as described by Loscher et al (154).

2.12 p85 recruitment assay.

Cells were seeded at $7x10^4$ cells/ml in 100mm culture dishes 48 hours prior to stimulation with InlB (500ng/ml). Inhibitors were pre-incubated with cells for indicated times where necessary. After stimulation, cells were washed with cold PBS, lysed in 1ml of ice-cold immunoprecipitation buffer (50mM NaCl, 50mM Tris (pH 8.0), 1% NP 40, 1mM EDTA,

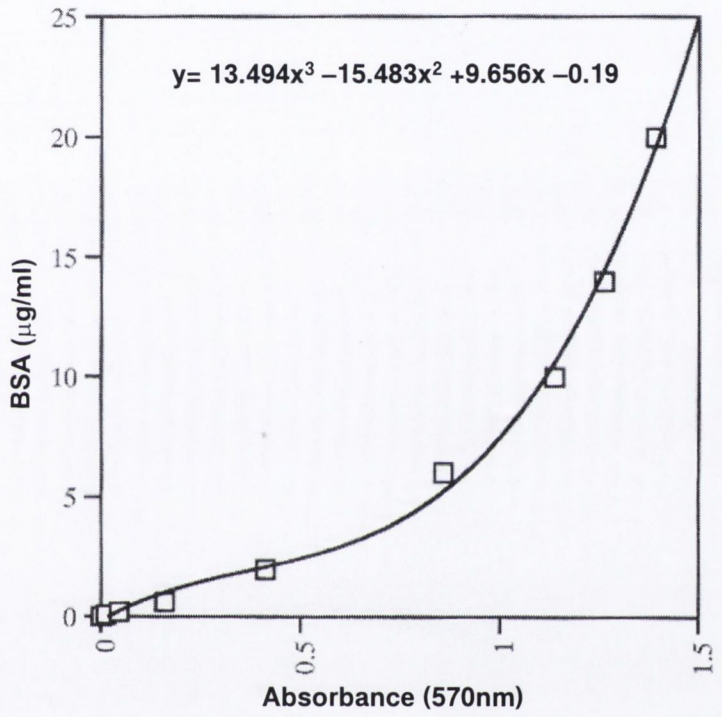


Figure 2.2 Bradford standard curve relating to protein concentration to absorbance at 570nm.

Absorbance was measured five minutes following addition of Bradford reagant to concentrations of Bovine Serum Albumin (BSA) in the range 0.1-20µg/ml.

1mM phenylmethylsulfonyl fluoride (PMSF), 3mM sodium vanadate, 5μg/ml each of leupeptin, pepstatin and aproptinin) for 10 minutes, cell scraped and centrifuged at 13,200g, 10 minutes 4°C, to remove nuclei and cell debris. Cleared lysates were assayed for protein as Bradford (34), and pre-cleared with 30μl of protein A-sepharose beads, (50% slurry equilibrated in lysis buffer) (Poole, Sigma, UK) for 30 minutes at 4°C. The beads were pelleted at 2500g, 3 minutes, 4°C and the lysates removed. Lysates were incubated with rotation for 60 minutes at 4°C, with anti-phosphotyrosine antibody 4G10 (UBI, USA) at 1.0μg/mg of total protein for each sample. Protein A-sepharose beads (30μl of 50% slurry in lysis buffer) is added and incubated with rotation for a further 120 minutes, 4°C. The beads were pelleted at 2500g, 3 minutes, the lysate removed and the beads washed a further 3 times at 4°C, with lysis wash buffer (0.2% NP 40, 50mM NaCl, 50mM Tris (pH 8.0), 1mM EDTA, 1mM PMSF, 3mM sodium vanadate, 5μg/ml each of leupeptin, pepstatin and aproptinin). Beads were resuspended in SDS-PAGE sample (60mM Tris, pH 6.8, 25% glycerol, 2% SDS, 14.4mM 2-mercaptoethanol , 0.1% bromophenol blue) buffer, boiled for 5 minutes, centrifuged at 2500g for 3 minutes to remove the beads, then undergone electrophoresis (7% SDS-PAGE). Proteins were transferred to nitrocellulose, probed with anti-p85α polyclonal antibody, detected with rabbit immunoglobulin G and visualised with chemiluminescence system (Amersham, UK).

2.13 Akt immunoblot analysis

J774 cells were cultured at $5x10^4$/ml in 6 well plates (3ml) for 48 hours prior to stimulation in 0.5% FCS, RPMI 1640. Media was replaced with serum-free media, 2

hours prior to stimulation with 500ng/ml InlB, for the indicated stimulation times. Cells were aspirated, washed with ice cold PBS, and lysed with 100μl of SDS-PAGE sample buffer. The lysate was sonciated, boiled for 5 minutes, and centrifuged for 10 minutes, 13,200g, to remove nuclei and cell debris. Proteins were seperated by electrophoresis (SDS-PAGE, 10%), transferred to nitrocellulose and immunoblotted with antibodies against Phospho-Akt(ser473) antibody (New England Biolabs, USA). Secondary antibody rabbit immunoglobulin G was applied and detected by chemiluminescence according to manufacturers recommendations (Pierce, USA). Nitrocellulose membranes were stripped of previous antibodies (washed 3 time with 50mM glycine, pH 2.0), and re-probed with Akt antibody (New England Biolabs, USA) to determine protein loading.

2.14 Ras activation assay

2.14.1 Purification of fusion proteins

500ml of L-broth containing ampicillin (LB-Amp) was innoculated with a 5ml overnight culture which had been inoculated with BL21 strain of *E.coli* transformed with the GST-C-Raf-1 RBD (residues 1-149) fusion protein to be purified and grown overnight at 37°C in an orbital incubator. The following day 50ml of the culture was added to 500ml of LB-Amp and incubated for 1 hour at 37°C after which time the fusion protein was induced by the addition of IPTG (100μM) to the culture. Following incubation of the culture for three hours the bacteria were pelleted by centrifugation (15 minutes, 4000xg). The pellet was resuspended in 1/50th the original culture volume with ice-cold lysis buffer (20mM Tris, 100mM NaCl, 1mM EDTA, pH 8.0, 0.5% NP40, 1mg/ml lysozyme, 10μg/ml PMSF, 30μl/ml aprotonin and 10μg/ml sodium orthovanadate). The bacteria were lysed by

sonication on ice and the debris pelleted by centrifugation (10,000xg, 5 minutes, 4°C). The supernatant was added to 1ml of glutathione-S-agarose beads (70% mixture in lysis buffer) and incubated with rotation at 4°C for 30 minutes. The beads were washed three times with lysis buffer and the GST-fusion protein coupled to the beads stored at 4°C as 50% slurry in lysis buffer.

2.14.2 Ras activation assay

J774 cells were cultured at $5x10^6$ in 100mm (10mls) culture dishes for 24 hours in 10% FCS/RPMI 1640, then a further 24 hours in serum-free RPMI 1640, prior to InlB stimulation (500ng/ml). Cells were washed with ice-cold PBS, and lysates extracted with Ras lysis buffer (50mM Hepes, pH 7.4, 10mM NaF, 10mM iodoacetamide, 75mM NaCl, 1% NP-40, 10mM $MgCl_2$, 1mM PMSF, 1mM sodium vanadate, 1mg/ml β-glycerol phosphate) for 20 minutes on ice. Equal protein amounts were incubated for 2 hours at 4°C with C-Raf-1 RBD (residues 1-149) pre-coupled to glutathione agarose beads (50% slurry). Only activated Ras-GTP will bind to beads, so that activated protein can be pelleted with beads by centrifugation 2500g, 3 minutes, 4°C. Beads were boiled in SDS-PAGE sample buffer for 5 minutes and separated on 15% SDS-PAGE. Proteins were transferred to nitrocellulose, and immunoblotted with Ras antibody, anti-mouse IgG peroxidase conjugated antibody, and visualised by chemiluminescence. Equal protein loading was also determined by staining transferred PAGE gel with Commassie Blue stain.

2.15 Transient Transfections

2.15.1 Preparation of competent cells for transformation

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

2.15.2 Plasmid transformation

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

2.15.3 Plasmid purification procedure

Plasmids were purified using QIAGEN Midi- or Maxi-prep Plasmid purification protocol based on a modified alkaline lysis procedure. Volumes for Maxi kits are given in brackets. 25ml (100ml) of L-broth (amp) was inoculated with a single colony from single purification step above. Cells were grown to a density of OD_{600} 1-1.5 ($1x10^9$ cells) at 37°C in an orbital shaker (approximately 16 hours of growth). Cells were harvested by centrifugation at 6000xg for 10 minutes at 4°C. Pellets were resuspended in 4ml (10ml) chilled resuspension buffer (50mM Tris-HCL, pH 8; 10mM EDTA; 100µg/ml RNaseA).

Cells were lysed for 5 minutes in 4ml (10ml) lysis buffer (200mM NaOH, 1% SDS) at room temperature and the reaction stopped using 4ml (10ml) neutralisation buffer (3M potassium acetate, pH 5.5), incubating the cells at 4°C for 15 minutes. To avoid localised potassium dodecyl sulfate precipitation, the solution was mixed thoroughly immediately after the addition of neutralisation buffer by inverting the tube several times. The sample was then centrifuged at 20,000xg for 45 minutes at 4°C. The supernatant was removed immediately and applied to a QIAGEN column, which had been equilibrated with 4ml (10ml) equilibration buffer (750mM NaCl; 50mM MOPS, pH 7; 15% ethanol). The column was washed 3 times with 4ml (10ml) wash buffer (1M NaCl; 50mM MOPS, pH 7; 15% ethanol) and the bound plasmid eluted using 2.5ml (15ml) elution buffer (1.25M NaCl; 50mM Tris-HCl, pH 8.5; 15% ethanol), passed twice through the column in the case of the Midi-prep kit. The DNA was precipitated using 0.7 volumes of room temperature isopropanol and collected by centrifugation for 30 minutes at 12,000xg. The pellet was washed twice with 70% ethanol and the DNA resuspended in a suitable volume of sterile H_2O (approx. 300µl for Midi and 1ml for Maxi-prep). The concentration and purity of the DNA was determined on a 0.8% agarose gel stained using ethidium bromide.

2.15.4 Transient transfections.

Hep2 cells ($1.5\text{-}2x10^4$) were seeded into 96 well tissue culture plates 24 hours prior to transfection. Transfections were preformed with SuperFect (Quigen, UK) according to manufacturers instructions; each well receiving 500ng of pPL-IL-8 (which comprises the IL-8 promoter linked to luciferase, which requires NF-κB for its activation) (Dower paper), 100ng of pTK-rLuc for normalization of transfection efficiency, indicated

amounts of either dominant negative Akt or Ras N17 for relevant experiments, and pRSV empty vector was used to maintain constant amounts of DNA dose. After transfection (3 hours incubation) cells were washed and 100ml of fresh medium added. Cells were transfected in triplicate for each sample. Twenty-fours hours later, cells were both pre-treated, and then stimulated with 500ng/ml of InlB. Six hours following stimulation, cells were washed with PBS and measured for luciferase activity Normalised IL-8-promoter driven activity is the ratio of firefly to *Renilla* luciferase activity.

2.16 Determination of IL-8-luciferase reporter gene activity

Cells transfected with IL-8-luciferase were stimulated with InlB for indicated time. Cells were centrifuged (1200xg) for 5 minutes at room temperature and washed once with PBS. After removal of PBS cells were lysed using the appropriate amount (50µl for 96 well plates) of a 1:5 dilution of passive lysis buffer (Promega) in water according to manufacturer's instructions and incubated at room temperature with vigorous shaking. Following centrifugation of plates for 10 minutes (2500xg) at room temperature, 50% of the supernatant was used to determine luciferase activity and an equivalent amount retained for TK-Renilla-luciferase activity. Luciferase activity was assayed by the addition of 40µl of luciferase assay mix (20mM tricine, 1.07mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT, 270mM coenzyme A, 470mM luciferin, 530mM ATP) to the sample and luminesence read using Mediators PHL luminometer. Luminesence readings were corrected for TK-Renilla-luciferase activity and expressed as fold increase over unstimulated control values.

2.17 IL-8 ELISA

2.17.1 Preparation of samples

Cells were seeded at a density of $4x10^4$ cells/ml in a 96 well plate, then pre-treated or un-treated with inhibitors for 1 minute, followed by stimulation with LPS for 6 hours. The cells were centrifuged at 250g and 100µl of supernatants removed for IL-8 determination.

2.17.2 IL-8 ELISA protocol

A 96 well microtitre plate is coated with 100µl/well of mouse anti-human IL-8 antibody (4µg/ml in PBS) and incubated overnight at room temperature. The antibody solution is aspirated and wells washed 3 times with PBS containing 0.05% v/v Tween 20 (wash buffer). Plates had all excess moisture removed by pounding dry on paper towelling as a final step. The microtitre plate was blocked by incubating each well (300µl) with 1% w/v BSA, 5% w/v surcrose in PBS, pH 7.4, for 2 hours. Decant the block buffer and wash plate 3 times with wash buffer and pounded dry. IL-8 standards (in the range 1-2000pg/ml) and cell culture supernatant samples were added at 100µl and incubated for 1 hour at room temperature. Decant the standards and samples and wash plate 3 times with wash buffer and pound dry. Biotynilated goat anti-human IL-8 antibody was diluted to 20ng/ml in PBS and 100µl added per well, then incubated for 1 hour at room termperature. Decant detection antibody and wash plate 3 times with wash buffer and pound dry. Add 100µl of streptavidin conjugated to horseradish-peroxidase (1:200 dilution diluted in PBS) to each well and incubate for 20 minutes in the dark at room temperature. Decant the contents of the plate and wash 3 times with wash buffer then pound dry. 100µl of freshly prepared substrate solution (10ml 0.11M sodium acetate

pH5.5, 167µl tetramethylbenzidin (TMB) (6mg/ml in DMSO) and 10µl of 3% v/v hydrogen peroxide) is added to each well and incubated for 20 minutes in the dark at room temperature. Adding 50µl of 2M H2SO4 stops the development of the reaction, and the optical density of each well read at OD_{405}, with a reference wavelength OD_{570} to correct for optical imperfections in the plate, using a Dynatech MR5000 plate reader. A standard curve was constructed relating to IL-8 concentration to absorbance at 405nm, and was used to determine the IL-8 concentration in unknown samples. A typical standard curve is shown in figure 2.3.

2.18 Protease Assay.

THP-1 cells ($7.5x10^4$/ml) were resuspended in freshly prepared RPMI 1640 (phenol-red free) containing 2% FCS in 96 well plates. Cells were stimulated with 10µg/ml of LPS for 3 minutes. 30µl samples were removed and diluted into 170µl of Chromozym TH (1.25mg/ml) dissolved in Phenol-red free RPMI 1640, 2mM L-Glutamine. Chromozym TH is a tosyl-glycyl-prolyl-arginine-4 nitranilide acetate compound, a substrate that is cleaved by serine proteases (specifically members of the 'thrombin-like' proteases) into residual peptide and free 4-nitroaniline, which is measured at 405nm. Absorbance readings were taken at the indicated time points. Results are displayed as arbitrary absorbance units, displayed as increase in absorbance units in regard to time zero, per time point.

2.19 Statistical Analysis

Statistical significance was determined using the Students *t-test* for unpaired data.

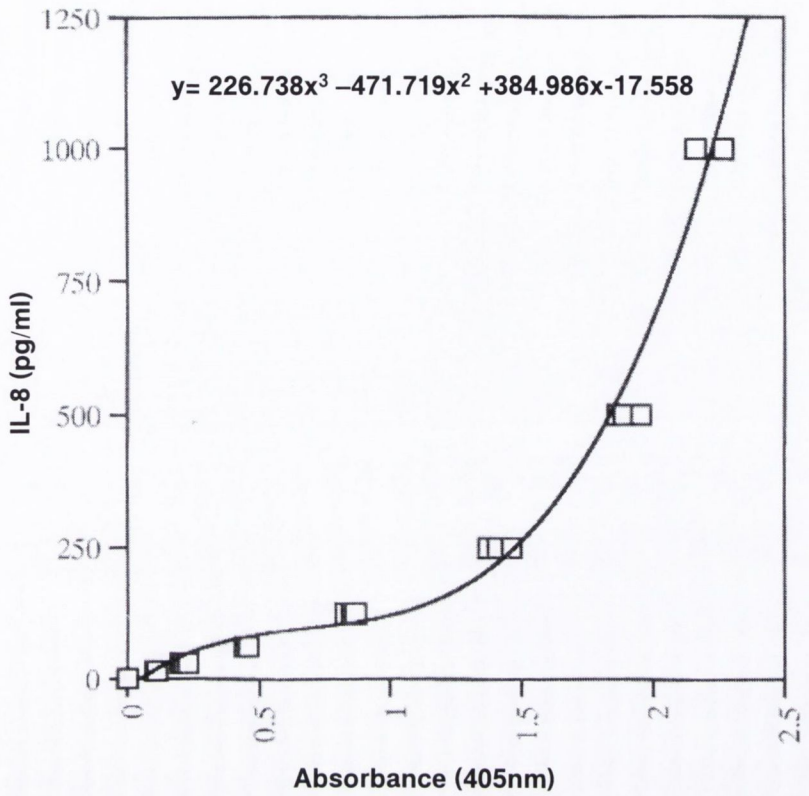


Figure 2.3 IL-8 Standard curve relating to IL-8 concentration to absorbance at 405nm.

A standard curve was constructed as described in the methods section and was used to determine IL-8 concentrations from unknown samples. A representative standard curve is shown.

Chapter Three

Characterisation of InlB-mediated NF-κB activation.

Introduction.

Two surface proteins of *Listeria*, internalin (InlA) and InlB, are crucial in mediating cell invasion by *L. monocytogenes* (50, 65, 113). InlA is an 800 amino acid protein consisting of 15 highly conserved 22 amino acid leucine rich repeats (LRRs), an inter-repeat region (IR), and a second repeat region consisting of 2 repeats of 70 amino acids and a third of 49 amino acids (B repeats). The receptor for InlA is E-cadherin, a transmembrane protein normally involved in cell-cell adhesion (138, 174).

InlB is a 630 amino acid protein consisting of 8 LRRs, each comprising 22 amino acids with a high degree of homology to InlA LRRs, and a 232 amino acid carboxyl terminal region beginning with a series of GW repeats. InlB is loosely attached to the bacterial surface via the so-called GW repeats, and is found in culture supernatants of wild type *L. monocytogenes* (35, 65). InlB has also been shown to activate phosphoinositide-3 kinase (PI-3 kinase) in Vero cells causing a rapid and transient increase in the lipid products of the PI 3-kinase p85-p110, tyrosine phosphorylation of the mammalian adaptor proteins Gab1, Cbl, and Shc, and association of these proteins with p85 (113, 114). The receptor for InlB has recently been identified as c-Met (244). At the outset of this project, the receptor was unknown, one of the inspirations for this work was that a TLR may be a receptor for InlB, possibly interacting with InlB via a homotypic LRR-LRR interaction.

The first aim of this investigation was to test whether the *Listeria* invasion proteins, InlA and InlB have a role in NF-κB activation.

To examine if InlB is able to mediate NF-κB activation, this study will rely on two techniques, which directly relate to assaying NF-κB activation, based on the known effects of stimuli on IκB and NF-κB. Firstly, un-treated or stimulated cells can be lysed, and their nuclear proteins extracted. By incubating these proteins with a radiolabled κB-specific oligonucleotide, followed by separation by polyacrylamide gel electrophoresis, it is possible to assay for stimuli-induced nuclear translocation of NF-κB. Secondly, immunoblotting for IκBα allows the degradation of IκBα to be measured.

Initially, this study will begin by assessing the ability of two strains of *Listeria* to induce NF-κB nuclear translocation. Wild type *L. monocytogenes* (EGD) contains, besides both Internalin proteins, several known inducers of NF-κB including LTA and LLO. This wild type strain will be compared to a strain of *L. monocytogenes* containing an in-frame deletion of InB termed EGDΔInlB. This mutant *L. monocytogenes* strain has previously been used to show the requirement of InlB expression for *L. monocytogenes* invasion of hepatocytes (65). The effect of InlB on NF-κB can be further assessed by the use of the *L. innocua* strain. This bacterial strain does not express InlB or LLO. This strain will be stably transformed with a plasmid expressing InlB and will be tested for stimulatory effect on NF-κB induced by InlB. A final approach will be to test the ability of recombinant purified InlB to stimulate NF-κB. Shorter, truncated recombinant proteins of InlB will also be tested and monoclonal antibodies raised against the respective truncated proteins, will be used for inhibitory studies. The use of these bacterial strains, recombinant proteins and antibodies will enable the comprehensive characterisation of the effect of InlB on NF-κB.

3.2 Results

3.2.1 InlB contributes to Listeria-induced activation of NF-κB.

I first tested two species of *Listeria* to determine whether InlB was contributing to the activation of NF-κB by *L. monocytogenes*. EGDΔInlB, which does not express the InlB protein, and wild type EGD *L. monocytogenes*, were used to stimulate J774 cells for 60 minutes, at a multiplicity of infection (MOI) of 20:1 bacteria to eukaryotic cells. NF-κB activation was analysed using the Electrophoretic Mobility Shift Assay (EMSA), which is described in Materials and Methods. As shown in figure 3.2.1, while both strains of EGD bacteria are able to potently activate NF-κB, there is a discernable difference in the intensity of activation between EGD and the mutant EGDΔInlB (compare lane 4 to 5). The high level of NF-κB activation induced by EGDΔInlB is likely to be due to two previously described potent activators of NF-κB produced by Listeria, LTA and LLO. Therefore, the difference in overall NF-κB activation induced by both EGD and EGDΔInlB is slight.

I next tested the avirulent species *Listeria innocua*, which does not express either InlB or LLO, but still contains LTA, and a transformed strain of *L. innocua* (InlB), which expresses surface associated InlB. J774 cells were stimulated for 60 minutes with both strains, at an MOI of 50:1 bacteria to eurkaryotic cells. As illustrated in figure 3.2.1, *L.innocua* (InlB) clearly activates NF-κB at a higher intensity than that of *L. innocua* alone (compare lane 6 to 7). Finally, treatment of J774 cells with purified recombinant InlB at 100ng/ml (lane 2) for 60 minutes also activated NF-κB.

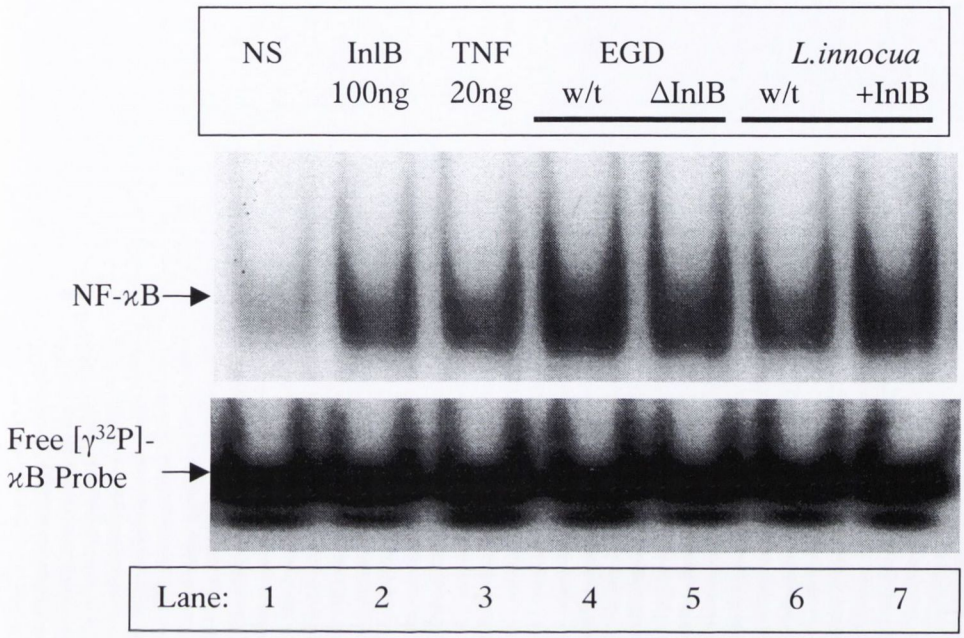


Figure 3.2.1 InlB participates in NF-κB activation by *L. monocytogenes*

J774 cells were seeded at $5x10^4$ cells/ml in six well plates, 48 hours to prior stimulation with bacteria. Cells were stimulated with wild type EGD strain(lane 4) and EGΔDInlB strain (lane 5) at a MOI of 20:1, bacteria to eukaryotic cells. *L.innocua* strains (lanes 6 and 7) were used at MOI of 50:1. Nuclear extracts were assayed by EMSA (see Materials and Methods) for their ability to activate NF-κB. Lanes 2 and 3 demonstrate the effect of treatment of cells for 60 minutes with recombinant InlB (lane 2) at 100ng/ml, and TNF-α (lane 3) at 20ng/ml. NF-κB-DNA complexes are shown in each case, with unbound DNA also shown in the lower panel. Results shown are representative of four independent experiments.

These results therefore clearly indicate that InlB can activate NF-κB, either alone or when expressed in *L. monocytogenes* or *L. innocua*.

3.2.2 Characterisation of NF-κB activation by InlB.

I further investigated the effects of purified recombinant InlB on NF-κB activation. Firstly, cells were tested with a range of concentrations of InlB for 60 minutes. As can be seen in figure 3.2.2, activation of NF-κB was initially observed at 5 ng/ml InlB (lane 3), reaching a maximal response at 50ng/ml InlB (lane 5). Therefore it was decided to use 100ng/ml of InlB for further stimulations. There is some variation in the strength of the response as a result of differences in the basal activation of NF-κB, which varies between experiments. Various efforts to decrease this high basal activation such as serum starving and cell density did not lead to consistently lower basal levels. In spite of the varying basal levels, InlB activated NF-κB in all experiments.

Next, cells were stimulated with 100ng/ml of InlB over a broad time range. As shown in figure 3.2.3, InlB-mediated NF-κB activation was initially observed at 30 minutes post-stimulation, peaking at 60-120 minutes after stimulation, with activation persisting for at least 4 hours after stimulation. From this data it was determined that a concentration of 100ng/ml InlB, together with a 60 minute stimulation time would be used for further experiments.

The subunit composition of the activated complex was next examined. The activated complex consisted of p50 and p65, as both subunits were supershifted upon incubation of

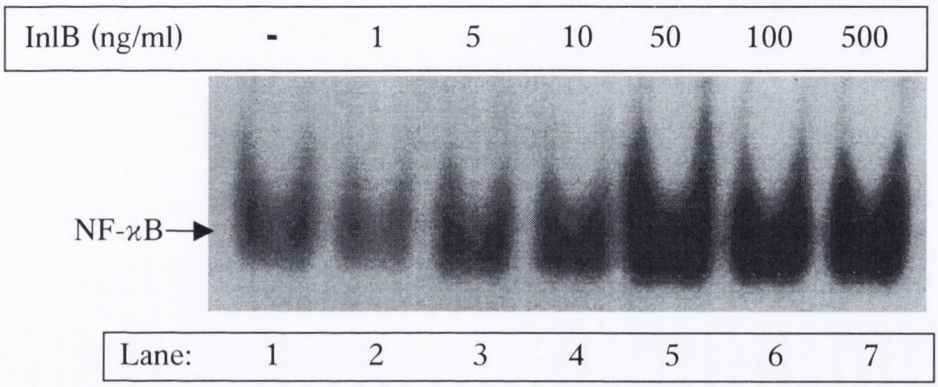


Figure 3.2.2 InlB activates NF-κB in a dose-dependent manner

J774 cells were seeded at $1x10^5$/ml in 6 well dishes 48 hours prior, such that final concentration of cells was ~ $3x10^6$/well. Cells were subsequently stimulated with the indicated concentrations of InlB, increasing from 1ng/ml to 500ng/ml (lanes 2-7) for 60 minutes. Nuclear extracts were assayed for their ability to stimulate NF-κB activation by EMSA when compared to non-stimulated cells (lane 1). NF-κB-DNA complexes are shown in each case. Results shown are representative of 3 experiments.

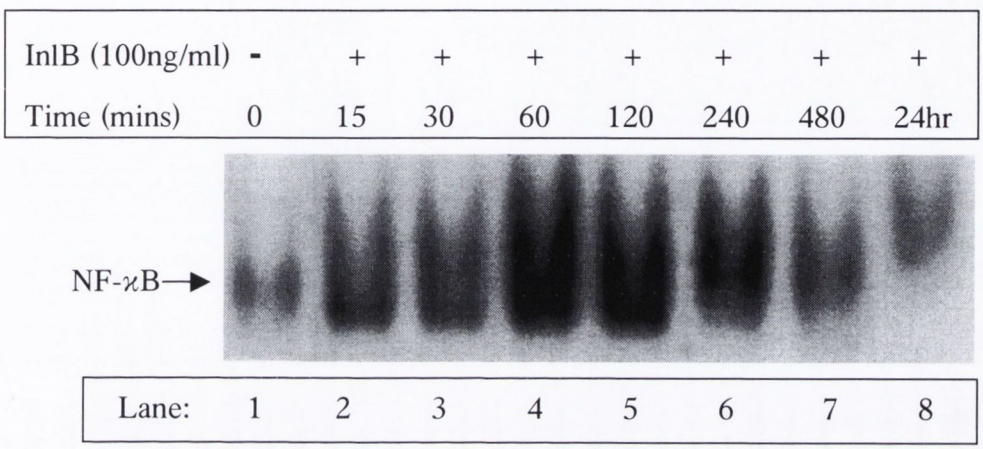


Figure 3.2.3 Time dependent activation of NF-κB by InlB

J774 cells were seeded at $1x10^5$/ml in 6 well dishes 48 hours prior to stimulation with 100ng/ml of InlB for indicated time ranging from 5 minutes to 8 hours (lanes 2-7). Nuclear extracts were assayed by EMSA for NF-κB activation . NF-κB-DNA complexes are shown in each case. Results shown are representative of 3 experiments.

nuclear extracts from InlB-treated cells, with the relevant anti-p50 (lane 2) and anti-p65 (lane 3) antibodies. The anti-c-rel antibody (lane 4) had no effect (Figure 3.2.4A). The antibodies used were not directly comparable in terms of titre or avidity; therefore the result is qualitative rather than quantitative.

The specificity of the binding of NF-κB to its radiolablled κB consensus sequence was verified by pre-incubation of nuclear extracts prepared from InlB-treated cells with an oligonucleotide containing unlabeled wild-type κB sequence. Binding was inhibited (compare lanes 2 and 1 in Figure 3.2.4B), but remained unaffected by pre-incubation with an oligonucleotide containing a mutant κB sequence (compare lane 4 and 5 with lane1 in Figure 3.2.4B).

Taken together, these results indicate that InlB is a potent activator of NF-κB and that the activated complex contains p50 and p65 subunits.

3.2.3 NF-κB activation in different cell types by InlB.

InlB was next tested for its ability to activate NF-κB in a broad range of cell types. Table 3.1 describes the types of cells tested for InlB responsiveness. It was determined that the murine macrophage cell line $P388D_1$, the mouse macrophage cell line RAW 264, and the human epithelial cell line Hep2 were the only cells tested that were responsive to InlB. As shown in figure 3.2.5, RAW 264 cells, $P388D_1$ cells and Hep2 cells were all less responsive to InlB than J774 cells, however, requiring up to 1000ng/ml of InlB to induce NF-κB activation, as compared to only 100ng/ml of InlB required for NF-κB activation in

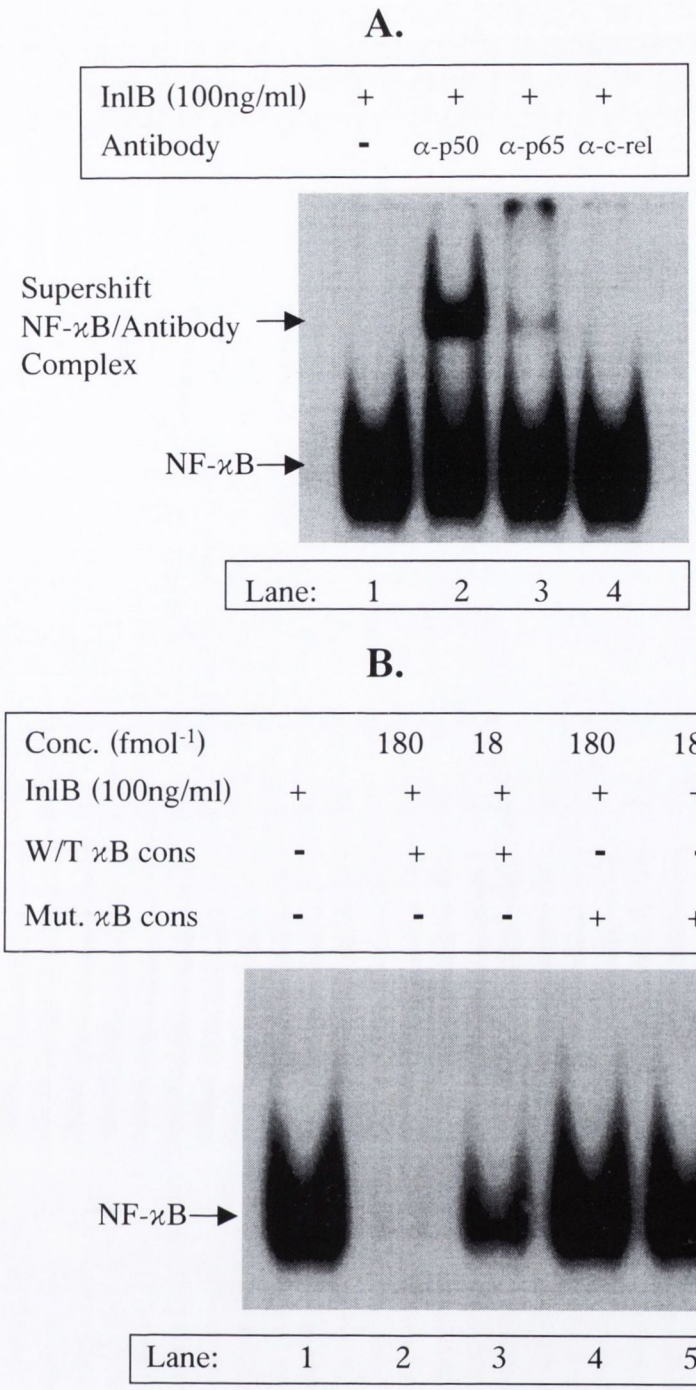


Figure 3.2.4 Specificity of InlB-mediated NF-κB activation

A. The composition of the NF-κB complex was determined by incubating pre-stimulated nuclear extracts (2μg/sample) from InlB treated cells, with antibodies against the indicated members of the Rel family (lanes 2-4), and compared to the non-incubated extracts (lane 1). Samples were then assayed for NF-κB by EMSA. Supershifted complexes corresponding to p50 (lane 2) and p65 (lane 3) indicated p50 and p65 subunits are present in InlB-mediated NF-κB activation. *B*. Nuclear extracts (2μg/sample) samples were incubated with wild-type (W/T) (lanes 2 and 3) or mutant (Mut) (lanes 4 and 5) non-radiolabled κB-consensus oligonucleotide, as indicated, at 18 and 180 fmol/ml. Samples were than assayed for NF-κB by EMSA. NF-κB-DNA complexes are shown in each case.

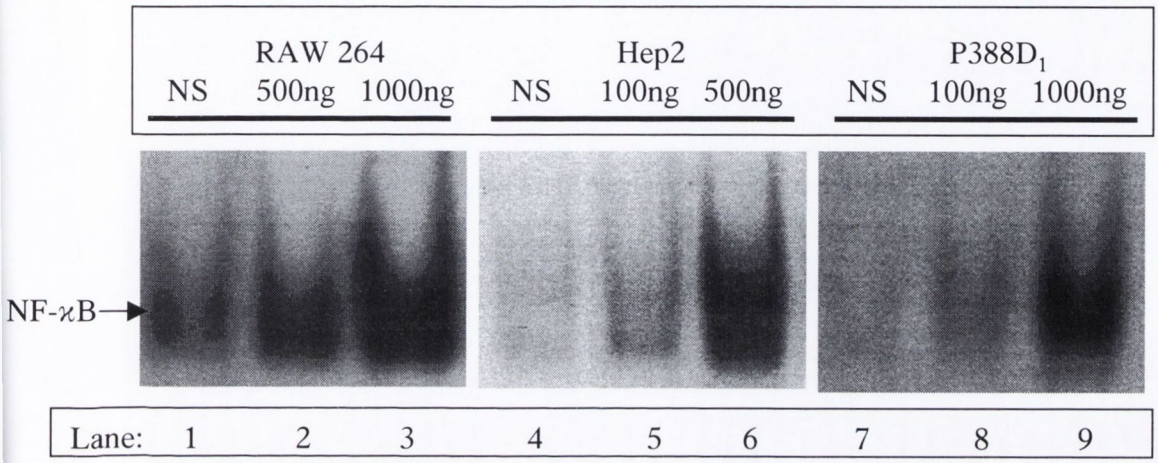


Figure 3.2.5 NF-κB activation by InlB in other cell types

Cells were seeded at $5x10^4$/ml in 6 well dishes, 48 hours prior to stimulation with indicated concentrations of InlB. Lanes 1, 4 and 7 correspond to non-stimulated (NS) cells for each cell line. NF-κB-DNA complexes are shown in each case. Results are representative of 2 experiments for each cell type indicated.

Cell Name	Origin	Cell Type	InlB responsive
J774	Mouse	Macrophage-like	Yes
$P388D_1$	Mouse	Macrophage	Yes
Hep2	Human	Epithelial	Yes
RAW 264	Mouse	Macrophage	Yes
Vero	Monkey	Fibroblast-like	No
HeLa	Human	Epithelial	No
LoVo	Human	Epithelial	No
ECV 304	Human	Endothelial	No
Hep G2	Human	Epithelial	No
EL4.NOB-1	Mouse	Lymphoblast	No
CaCo-2	Human	Epithelial	No
U937	Human	Lymphoblast	No
THP-1	Human	Monocyte	No
Jurkat	Human	Lymphoblast	No
HEK 293	Human	Epithelial	No

Table 3.1. Cell Lines tested for InlB-mediated NF-κB activation.

J774 cells. The human epithelial cell lines HeLa, LoVo, Hep G2, and CaCo-2 were all found to be non-responsive to InlB, as was also found with the monkey fibroblast-like cells Vero, human endothelial ECV 304 cells, the lymphoblast cell lines mouse EL4.NOB-1, human U937 and Jurkatt, and human monocyte THP-1 cells. These results suggest cell type specificity in the effect of InlB on NF-κB activation. Subsequently, J774 cells were used for all future stimulation studies with InlB unless otherwise stated.

3.2.4 InlB induces degradation of IκBα and IκBβ.

The phosphorylation and subsequent degradation of IκB is an integral event allowing the translocation of NF-κB to the nucleus. Therefore I next determined if InlB could induce the degradation of IκB.

Treatment of J774 cells with InlB caused the degradation of both IκBα and IκBβ as illustrated in figure 3.2.6. The degradation of IκBα, commenced within 5 minutes of InlB stimulation (compare lane 3 to 1), reaching maximal degradation between 60-120 minutes post-stimulation (lanes 4 and 5), and returning to basal levels after 480 minutes. In contrast, IκBβ degradation, began within 30-60 minutes post-stimulation (compare lanes 3-4 to 1), and persisted for up to 480 minutes after InlB stimulation.

3.2.5 InlB-mediated NF-κB Activation is not LPS dependent.

Since the recombinant InlB was prepared from *E. coli*, it was possible, though unlikely given the purification protocol used, that the effect of InlB activation was caused by contamination of sample with LPS. To examine this, InlB was incubated with the LPS

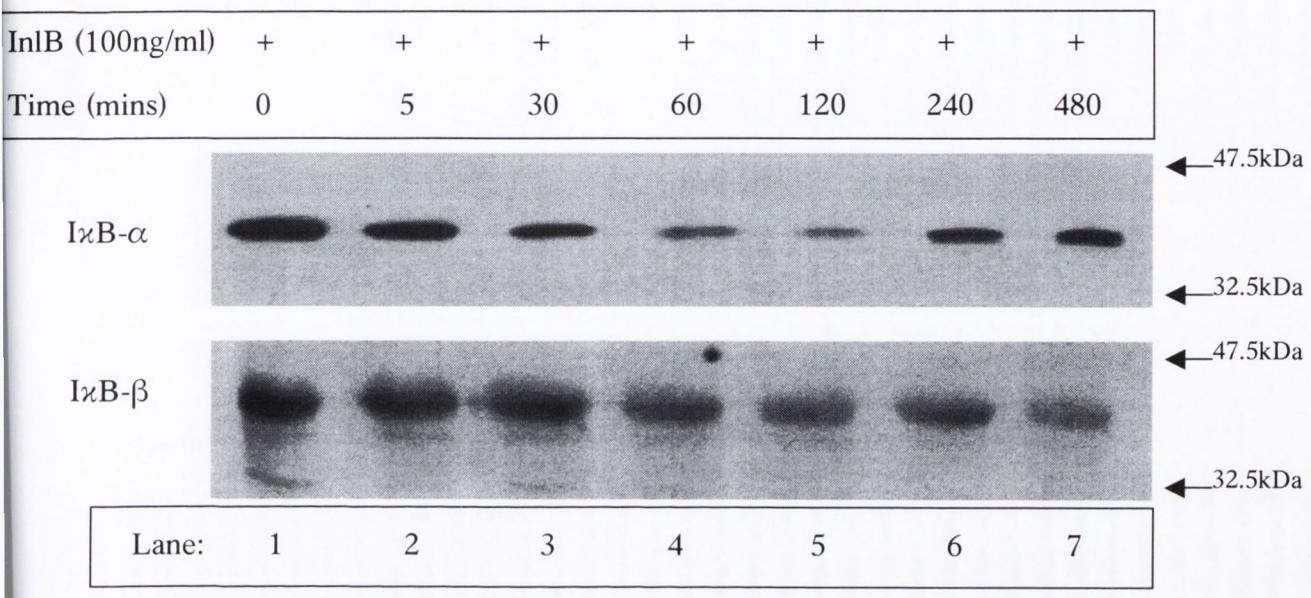


Figure 3.2.6 The effect of InlB on the degradation of IκBα and IκBβ

J774 cells were seeded at $1x10^5$ cells/ml in six well plates, 48 hours prior to stimulation with 100ng/ml of InlB for indicated time periods. Cells were lysed and assayed for IκBα and IκBβ by Western Blot analysis. Only one band was detected at 37kDa, corresponding to each IκB. A similar result was obtained in a further experiment.

neutralising agent polymyxin B (255). As shown in Fig. 3.2.7, polymyxin B at concentrations of up to 10 μg/ml had no effect on the InlB response (lanes 3-8). Furthermore, LPS at a concentration of 100ng/ml was required to observe an activation of NF-κB in J774 cells comparable to that of InlB (lane 9). Polymyxin B at 1μg/ml blunted this effect (lane 10).

3.2.6 NF-κB activation is caused by the N-terminal LRR region of InlB.

To further understand the stimulatory effect of InlB on NF-κB activation, truncated forms of InlB were used to stimulate J774 cells. Figure 3.2.8A illustrates a schematic diagram of the different domains of InlB used in this study. As can be seen in figure 3.2.8B, the LRR domain activated NF-κB almost as effectively as full-length InlB (compare lane 3 and 2). Interestingly, the C-terminal domain had some activity, although it was much less effective than either full-length or LRR domain InlB (compare lane 4 to 2). These results are shown for the response of 100ng/ml of each protein, which corresponds to 1.4nM InlB, 3.3nM of InlB-LRR, and 3.75nM of C-terminal InlB. Equimolar concentrations of each protein were also tested, and there was no difference in their ability to activate NF-κB as compared to that achieved by 100ng/ml of each protein (results not shown).

Further evidence for a role of the LRR region was obtained using a series of anti-InlB monoclonal antibodies, as shown in figure 3.2.9. Figure 3.2.9A gives a schematic representation of the regions of InlB against which the antibodies were raised against. The monoclonal antibody D23.1, which recognises the LRR domain responsible for invasiveness of InlB into Vero cells (37), blocked the effect of InlB (compare lanes 3 to

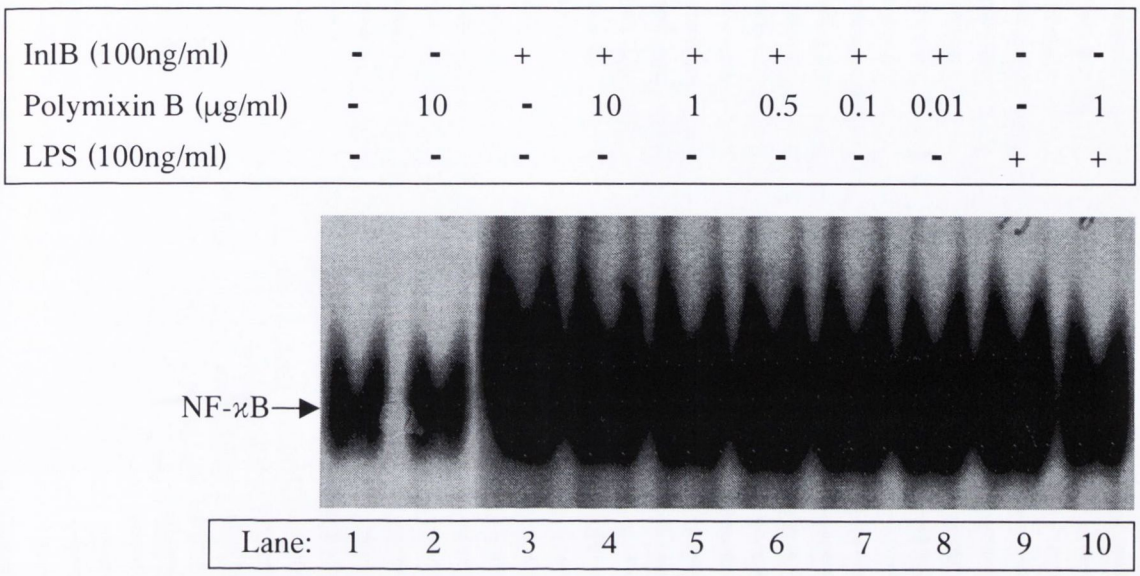


Figure 3.2.7 LPS contamination is unlikely to be responsible for the activation of NF-κB by InlB

J774 cells were seeded at $1x10^5$/ml in 6 well dishes for 48 hours. Cells were then left un-treated (lane 1) or treated with polymyxin B (10μg/ml, lane 2), InlB (100ng/ml, lane 3), InlB (100ng/ml) pre-incubated with polymyxin B for 30 minutes (lanes 4-8), LPS (100ng/ml, lane 9) or LPS pre-incubated for 30 minutes with 1mg/ml polymyxin B (lane 10), and incubated for 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained from a further experiment.

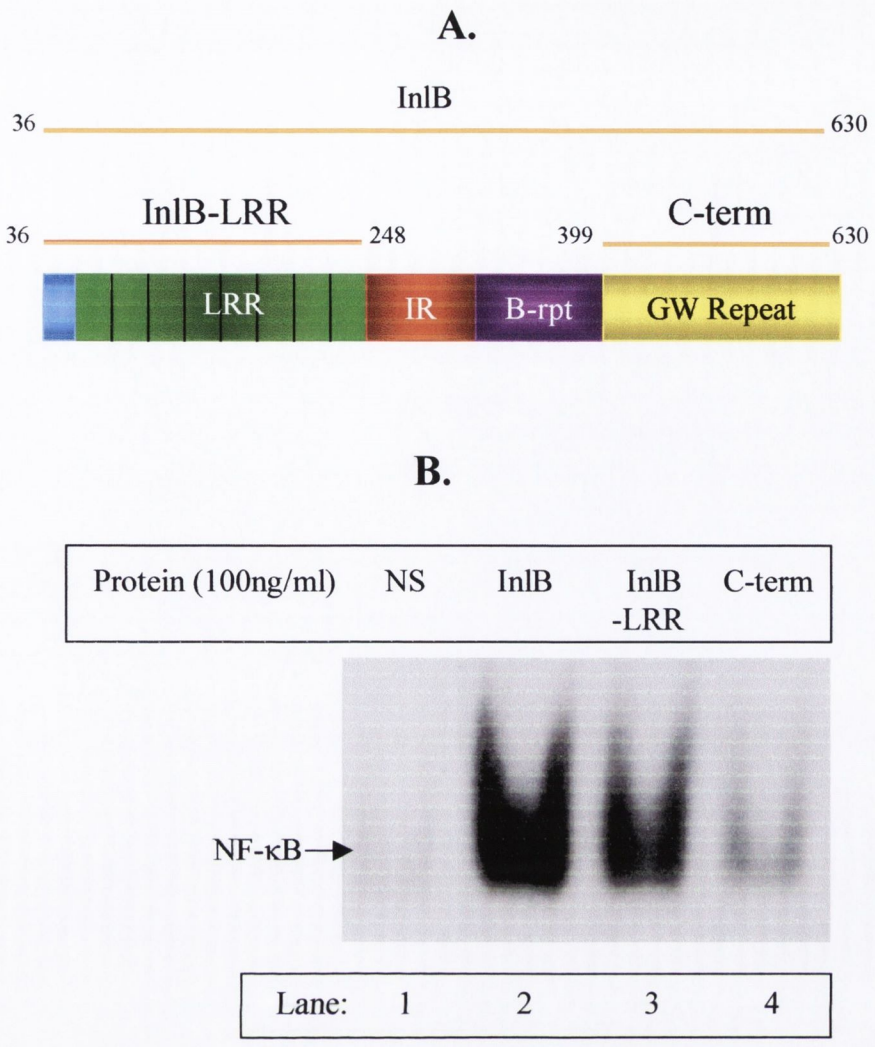


Figure 3.2.8 Domains of InlB responsible for InlB-mediated NF-κB activation

A. Schematic diagram representing the different constructs of InlB used for stimulation of J774 cells. Wild-type InlB and truncations (InlB-LRR and C-terminal) of InlB are indicated by numbers corresponding to amino acid positions relative to wild-type InlB. *B.* J774 cells were seeded at $5x10^4$ cells/ml in 6 well plates, 48 hours prior to stimulation with 100ng/ml of either wild-type InlB (lane 2), InlB-LRR (lane 3) or C-terminal InlB (lane 4) for 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained from a further experiment.

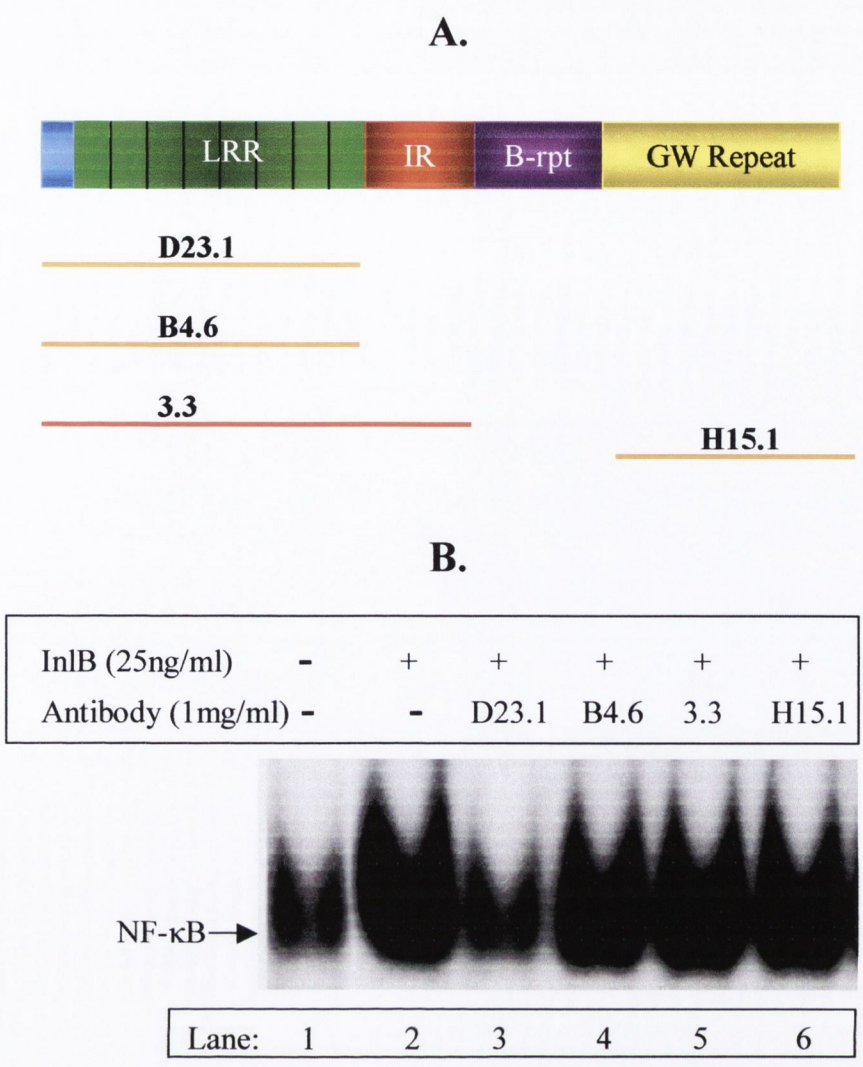


Figure 3.2.9 The LRR of InlB is responsible for InlB-mediated NF-κB activation

A. Schematic diagram indicating regions of InlB recognised by antibodies. Lines represent truncated recombinant proteins used to raise antibodies. *B.* J774 cells were seeded at $5x10^4$ cells/ml,48 hours prior to stimulation. InlB (25ng/ml) was pre-incubated for 60 minutes at 4°C, with 1 μg/ml of indicated antibodies. The antibodies were D23.1 (anti-InlB-LRR, lane 3), B4.6 (anti-InlB-LRR, lane 4), 3.3 (anti-InlB-LRR-IR, lane 5), and H15.1 (anti-InlB-C-terminal, lane 6). Cells were stimulated with InlB-antibody mix for 60 minutes and nuclear extracts assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained from a further experiment.

2). The antibody B4.6, which recognises another part of the LRR domain of InlB, but is unable to inhibit invasion of Vero cells, had no effect on stimulation, as were antibodies to the inter-repeat region and the C-terminal domain of InlB (lanes 4-6). Interestingly, the antibody directed against the LRR and/or inter-region of InlB, mAb 3.3, which has been found to inhibit InlB mediated invasion of Vero cells (37), had no effect upon NF$\varkappa$B activation (lane 3).

Taken together these results suggest that the LRR domain, which is also required for InlB-induced internalisation, is also responsible for NF-$\varkappa$B activation by InlB.

3.2.7 InlA does not activate NF-$\varkappa$B.

As the LRR domain of InlB is highly similar to that of InlA, both the native InlA, and the shorter LRR domain of InlA, were tested for their ability to activate NF-$\varkappa$B. Figure 3.2.10A gives a schematic representation of the domains of both InlA and InlB with their respective amino acid numbers used in this study. As shown in figure 3.2.10B, both the InlA and InlA-LRR proteins were unable to induce activation of NF-$\varkappa$B, as compared to the activation achieved by InlB and InlB-LRR (compare lanes 4-5 to 2-3). The molar concentrations tested were 1.16nM InlA and 2.1nM InlA-LRR. Again, stimulation of J774 cells with equimolar amounts of InlA, InlA-LRR, as compared to InlB and InlB-LRR, had no effect on NF-$\varkappa$B activation intensity (data not shown).

These results suggest therefore, that the InlB-LRR domain specifically mediates the activation of NF-$\varkappa$B by InlB.

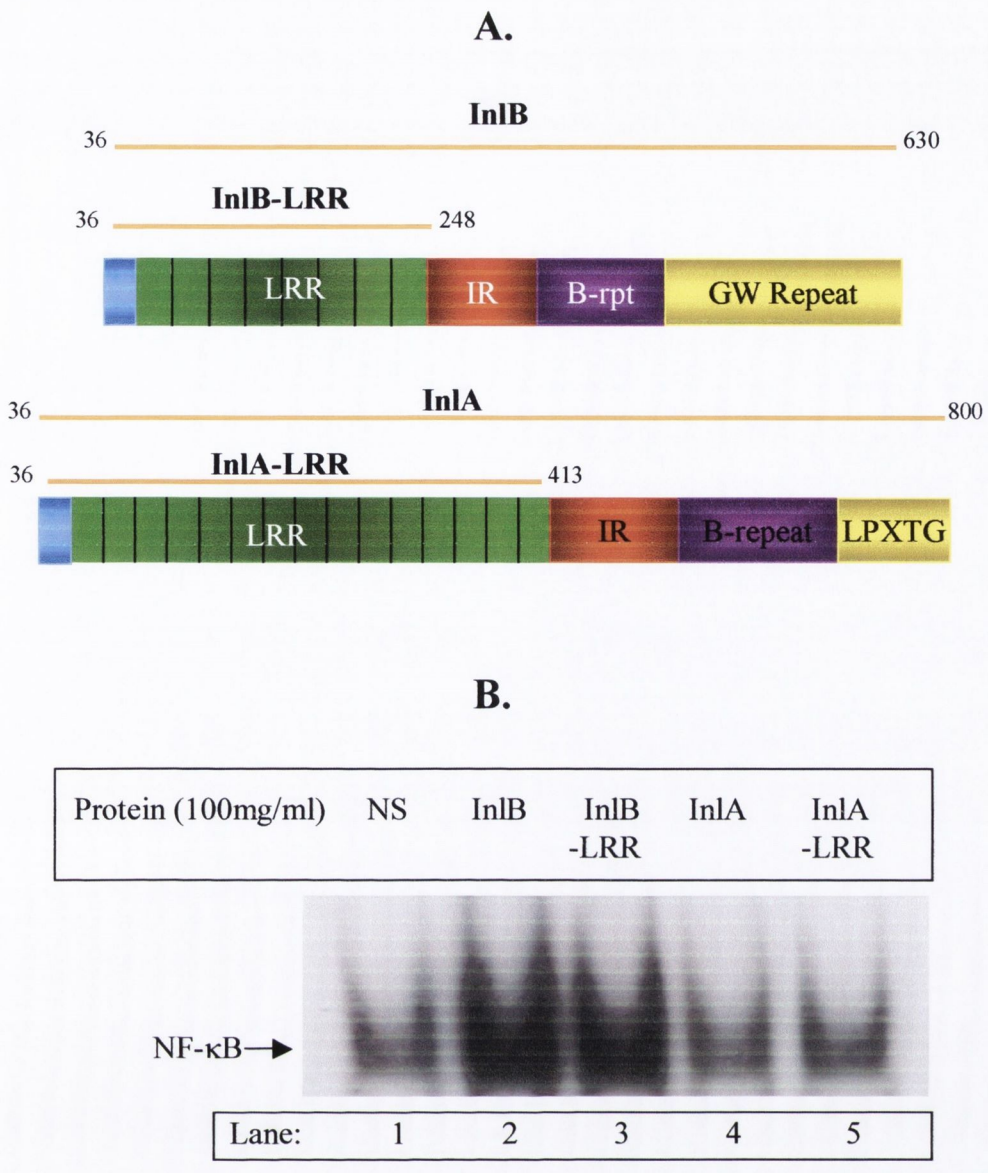


Figure 3.2.10 Neither InlA or LRR-InlA induces activation of NF-κB

A. Schematic diagram representing the constructs of InlA and InlB used for stimulation of J774 cells. *B*. J774 cells were seeded at $5x10^4$ cells/ml in 6 well plates 48 hours prior to stimulation. Proteins (100ng/ml) corresponding to InlB (lane 2), InlB-LRR (lane 3), InlA (lane 4), and InlA-LRR (lane 5) were assayed for their ability to activate NF-kB by EMSA as compared to unstimulated cells (lane 1). NF-κB-DNA complexes are shown in each case. A similar result was obtained from a further experiment.

3.2.8 InlB induces the expression of TNFα and IL-6 in J774 cells.

Finally, whether InlB could induce expression of NF-κB-regulated genes was examined. The expression of both TNFα and IL-6, was therefore determined. J774 cells stimulated for 24 hours with InlB were shown to increase both TNFα and IL-6 protein levels in a dose-responsive manner over untreated cells (Figure 3.2.11).

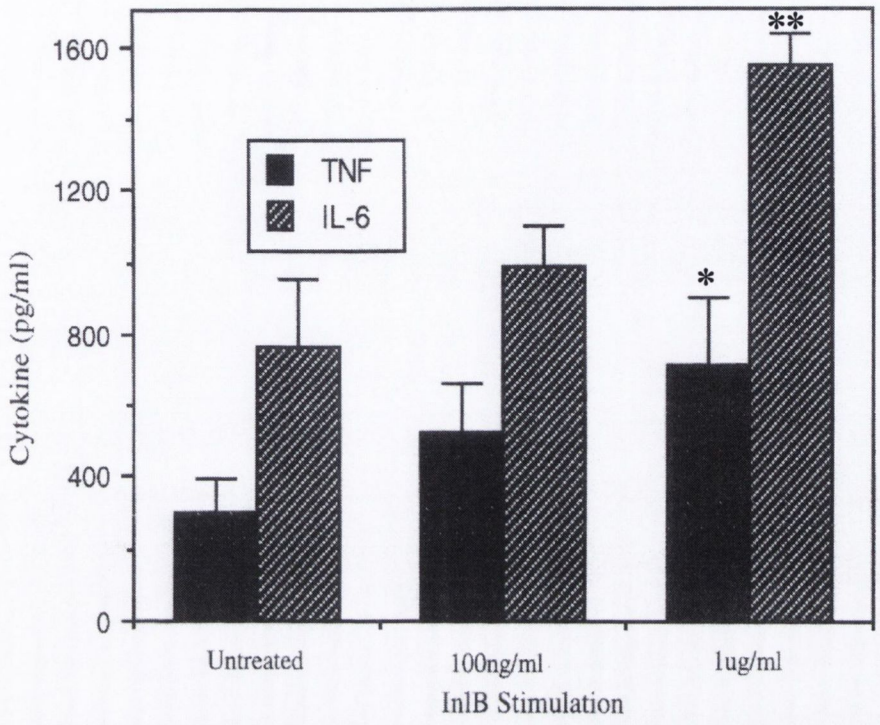


Figure 3.2.11 Effect of InlB Stimulation upon κB-dependent gene expression

J774 cells ($1x10^6$ cells/ml) were seeded in 24 well plates 24 hours prior to stimulation. Cells were stimulated with either 100ng/ml or 1μg/ml of InlB and incubated for a further 24 hours. Control data are unstimulated cells. Supernatants were removed, centrifuged (5 minutes at 13,200 rpm) to remove detatched cells, and assayed for IL-6 and TNF-α as described in Materials and Methods. Results are shown as cytokines (pg/ml) and are expressed as means ãS.D. (n=3). Data indicate significant differences (*P=0.05, **P=0.007) when compared to control values.

3.3 Discussion.

In this chapter, I have demonstrated that the invasion protein InlB from *L. monocytogenes* activates NF-κB in the murine macrophage-like cell line J774, causing the expression of two cytokines TNF-α and IL-6, which are part of a pro-inflammatory response. The effect is sustained, and involves the transient degradation of IκBα and the sustained degradation of IκBβ. The LRR domain of InlB mediates this activation, a region recently shown to be required for internalisation in epithelial cells and activation of PI-3 kinase (37).

Infection of bone marrow derived macrophages with *L. monocytogenes* has previously been shown to upregulate cytokines IL1α, IL-1β and TNF-α, after initial infection (60). This classical pro-inflammatory response was found to be unaffected by cytoskeletal inhibitors which prevent bacterial invasion, but not adhesion. These results suggested that secreted or surface molecules of mammalian cells are able to interact with a bacterial ligand and initiate a pro-inflammatory response. Given that InlA and InlB are cell surface proteins, and may also be found in culture supernatants, we felt they may be excellent candidates for activation of NF-κB, and thus contribute to, or promote the pro-inflammatory response previously observed in macrophages, as has been observed by other soluble bacterial products such as LPS and LTA (13, 94). Our results show that this is the case for InlB.

Initially I was able to demonstrate the contribution of InlB to *L. monocytogenes* activation of NF-κB, by the use of two strains of *Listeria*. Wild type *L. monocytogenes*, termed

EGD, contains components which have previously been shown to mediate NF-κB activation. Hauf et al (94) demonstrated that a phenol-extracted, purified fraction of LTA from *L. monocytogenes*, termed LTAII, rapidly activates NF-κB via IκBα degradation in the mouse macrophage cell line P388D$_1$, whereas the LTAI fraction had no effect. Further analysis determined that LTAII contained a glycolipid anchor substituted with a phosphatidyl residue that is absent in LTAI. A second sustained activation mediated by IκBβ degradation also seemed to occur and required the expression of the PrfA-dependent listerial phospholipases PI-PLC and PC-PLC. Indeed, Hauf et al found that mutant forms of *L. monocytogenes*, which lack these PrfA-dependent phospholipases, and the non-invasive *L. innocua*, did not induce this sustained activation of NF-κB. The phospholipase mutants still probably express a full length InlB which also could contribute to the activation of NF-κB.

Another report demonstrated that LLO also induced NF-κB activation in endothelial cells and stimulated cell adhesion molecule expression (123). This finding further demonstrated that *L. monocytogenes* expresses several factors that are potent NF-κB activators, and in many checkpoints of the infection process, may use different components to trigger similar signalling events.

J774 cells stimulated with the EGD strain strongly induced NF-κB activation. However, J774 cells stimulated with a mutant strain of EGD that has an in-frame deletion of InlB, termed InlBΔEGD (65), demonstrated a marginally lower level of NF-κB activation when compared to that mediated by EGD alone. Indeed, this result is in contrast to the findings

of Hauf et al (95) who also used an in-frame deletion InlB mutant EGD strain in their study, but found this mutant had no effect on the ability of *L. monocytogenes* to activate NF-κB in $P388D_1$ cells. The Hauf study however, used an MOI of 50:1 bacteria to eukaryotic cells, as compared to an MOI of 20:1 in my study. The higher concentrations of NF-κB activators present in both strains of EGD at an MOI of 50:1, such as LLO and LTAII, could have 'masked' the decrease of NF-κB activation induced by InlB. Also, it was later found that InlB is buried within the peptidoglycan layer (118) of *L. monocytogenes*. If soluble InlB is required for NF-κB activation, or only a small percentage of InlB is present on the bacterial surface, it is possible that this form of InlB was not present in the Hauf et al experiments. It is also possible, that InlB expression or activity was very low in their bacterial cultures.

To further probe the role of InlB in *Listeria*-mediated NF-κB activation, I used a second strain of *Listeria* to probe this effect. *L. innocua* is a non-invasive strain of *Listeria*, that does not express Internalins or LLO. Using a MOI of 50:1 bacteria to eukaryotic cells, *L. innocua* was able to clearly activate NF-κB. However, this activation was lower in potency when compared to that induced by EGD. This activation would presumably be due to factors such as LTAII and phospholipases. However, J774 cells stimulated with *L. innocua* stably transformed with a plasmid expressing InlB, increased NF-κB activation above levels observed for *L. innocua* alone. Therefore, the removal of NF-κB activators such as LLO, enabled the stimulatory effect of InlB to be noticeable in this assay. This result, in conjunction with the decrease in NF-κB activation observed with the InlBΔEGD

bacterial strain, therefore suggested a role for InlB in the activation of NF-κB by *L. monocytogenes*.

The availability of purified recombinant InlB allowed further study of this effect, free from the interference of the NF-κB activators present in *L. monocytogenes*. Incubation of J774 cells with recombinant InlB demonstrated a potent NF-κB stimulatory effect mediated by InlB, achieving a maximal activation response at 50ng/ml. InlB-mediated NF-κB activation occurred within 30 minutes of stimulation and persisted for up to 4 hours. It was further found that InlB induced the transient degradation of the NF-κB inhibitory protein IκBα within 30-120 minutes, and the further sustained degradation of IκBβ from 60 minutes up to 4 hours.

It was possible that as InlB was prepared from *E. coli*, purified InlB could be contaminated with trace amounts of LPS. As LPS is a known potent activator of NF-κB in macrophages, it was necessary to determine if LPS was responsible for the effect seen here. Polymyxin B, a polycationic cyclic peptide, is known to neutralize most of its activities (255). Pre-incubation of InlB with polymyxin B however, was found to have no effect upon InlB-induced NF-κB activation. LPS-mediated NF-κB activation however, was inhibited by polymyxin B. This further supported the suggestion that InlB was responsible for the NF-κB activation observed in this study.

Using a combination of shorter domains of InlB, and monoclonal antibodies directed against some of these regions, I was able to demonstrate that the LRR domain mediates

activation of NF-κB. This region activated NF-κB at concentrations comparable to native InlB, and activation was inhibited by an antibody raised against a region of LRR involved in InlB-mediated invasion of Vero cells, but was unaffected by an antibody raised against another LRR motif of InlB that is unable to inhibit invasion. Taken together, these results indicate that the LRR motif of InlB involved in invasion (37) is critical for NF-κB activation. Interestingly however, the antibody 3.3, which is also raised against the LRR- and/or IR region of InlB and is able to inhibit InlB-induced invasiveness of Vero cells, was unable to inhibit InlB-mediated NF-κB activation in J774 cells. As these antibodies have not been epitope-mapped, and therefore the exact regions of recognition are unknown, it is difficult to draw direct comparisons between the ability of the antibodies D23.1 and 3.3 in their inhibitory effects upon InlB function. However, the discrepancy between the ability of antibody 3.3 to inhibit InlB-mediated invasiveness, but not NF-κB activation, may suggest that different regions of InlB within the LRR and/or IR domains are able to induce a different response from cells, or that InlB-induced invasiveness may require a more specific ligand-receptor interaction in Vero cells, than that required to induce NF-κB activation in J774 macrophages.

InlA shares with InlB a similar LRR region (67), as do the other members of the internalin family (66). Yet my investigation has shown that native InlA, and its LRR region, was unable to activate NF-κB in J774 macrophages. Thus the activation was specific for the LRR of InlB. The difference in the LRR domains of InlA and InlB are sufficient to allow both proteins to bind different receptors. InlA binding its receptor, E-cadherin, has previously been shown to require the LRR region of InlA (173, 174).

However, it has been found that E-cadherin does not bind InlB. The results presented here are thus in perfect agreement with the fact that E-cadherin is absent in J774 macrophages. The first receptor described for InlB, gC1q-R (36), is one of the receptors for the complement protein C1q. It does not contain a transmembrane region or glycosylphosphatidylinositol (GPI) linkage sequence (86-88). This would suggest that gC1q-R is acting as a co-receptor for a transmembrane signalling protein capable of initiating signal transduction. A possible candidate has been recently described by Shen et al (244) who demonstrated that c-Met, the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF), or 'scatter-factor', can bind InlB. Similar to InlB, HGF has been shown to activate PI-3 kinase and InlB can induce epithelial cell scattering. Both InlB (160) and HGF (237) also activate the transcription factor NF-$\varkappa$B.

c-Met is expressed in many cell types. In this study, InlB did not activate NF-$\varkappa$B in all of the cells tested. One of the cells that were unresponsive, Vero, express' c-Met and respond to InlB in terms of PI-3 kinase activation. Why these cells do not respond in terms of NF-$\varkappa$B is therefore unclear. One explanation may be that c-Met may induce different responses in different cells. This is further discussed in chapter 4. HGF was initially described as a growth factor for both liver cells, and epithelial cells (178, 186, 230), an epithelial morphogen (181), an effector of epithelial movement and cell-cell interactions (89, 256, 278), an inhibitor of tumor growth (245), and as a chemoattractant for motorneurons (71). The ability of c-Met, the single receptor for HGF, to induce motility stimulation, proliferation, morphogenesis and tumor inhibition, suggests that the signal transduction pathway initiated by HGF, utilises several signalling mediators to

induce its effect, and that these mediators are cell specific (257). Upon ligand binding, c-Met undergoes autophosphorylation of two critical tyrosines within a so-called 'multi-docking site', at positions Tyr1349 and Tyr1356 (29, 268). Both phosphotyrosines recruit key adaptor proteins to the multi-docking site, including Grb2, Gab1, SHC, PI-3 kinase and PLCγ, which act downstream of c-Met (reviewed in (29, 257). Several studies have established that motility responses induced by HGF, requires activation of PI-3 kinase, thereby activating the Ras-Rac/Rho pathway, whereas growth responses requires activation of the Ras-mitogen-activated protein (MAP) kinase pathway via recruitment of the Grb2-SOS adapter complex. From these studies, it can be concluded that HGF/c-Met coupling induces recruitment of either PI-3 kinase or Ras-driven intracellular signalling pathways, leading to either mitogenesis, scattering or morphogenesis. It has been suggested that most cells types respond to HGF by developing a mixed phenotype, and that the induction of any particular phenotype requires the involvement of specific intracellular mediators that are either particular to, or highly abundant in that particular cell line (257). Therefore, it has been suggested that PI-3 kinase and Grb2, via Ras, seem to provide a signalling pathway 'platform', which alone does not induce a specific response. Downstream of these effectors, intracellular signalling mediators are recruited, activated, and induce further signalling effectors, which leads to the explicit signal that is specific for that phenotopic response.

Making the assumption that InlB is inducing the same ligand-induced autophosphorlyation of c-Met within the multi-docking site, it could be suggested therefore, that the specificity of InlB-mediated NF-κB activation in J774, P388D$_1$, Hep2

and RAW 264 cell lines, could be due to the involvement of specific intracellular mediators unique to, and/or expressed at a higher concentration, to the other cell types used in this study, which did not induce the same response. Although InlB may activate PI-3 kinase in Vero, this may not be sufficient to activate NF-κB. This effect will be further examined in chapter 4, however, the effect could be similar to that observed for Akt activation of the IKK complex after stimulation by TNF (199). Ozes et al have found that Akt-dependent activation of the IKK complex is dependent upon the amount of Akt present in the cells, the activity of Akt and maybe the presence or increased expression of mediators that induce Akt activation of the IKK complex. As Akt is a well-known downstream effector of PI-3 kinase, a similar effect could be responsible for what I have observed here.

What are the downstream consequences of NF-κB activation in infected cells? As NF-κB has been implicated in many aspects of the pro-inflammatory response to bacterial invasion (11, 12, 14), it is possible that the InlB-mediated NF-κB activation observed mainly represents a host defence response to the bacterium. Another possibility however is that NF-κB activation may be beneficial to the bacterium. PI-3 kinase can also generate an anti-apoptotic signal via protein kinase B/Akt (165). If PI-3 kinase and NF-κB activation are linked, NF-κB activation could be another anti-apoptotic signal activated by PI-3 kinase in response to InlB. Interestingly, PI-3 kinase has been shown to have a role in NF-κB activation by the cytokine IL-1 (219, 249). Since previous studies (37, 113, 114) have implicated InlB in activation of PI-3 kinase, this observation, and our present results lead us to speculate that PI-3 kinase may be a component of the activation

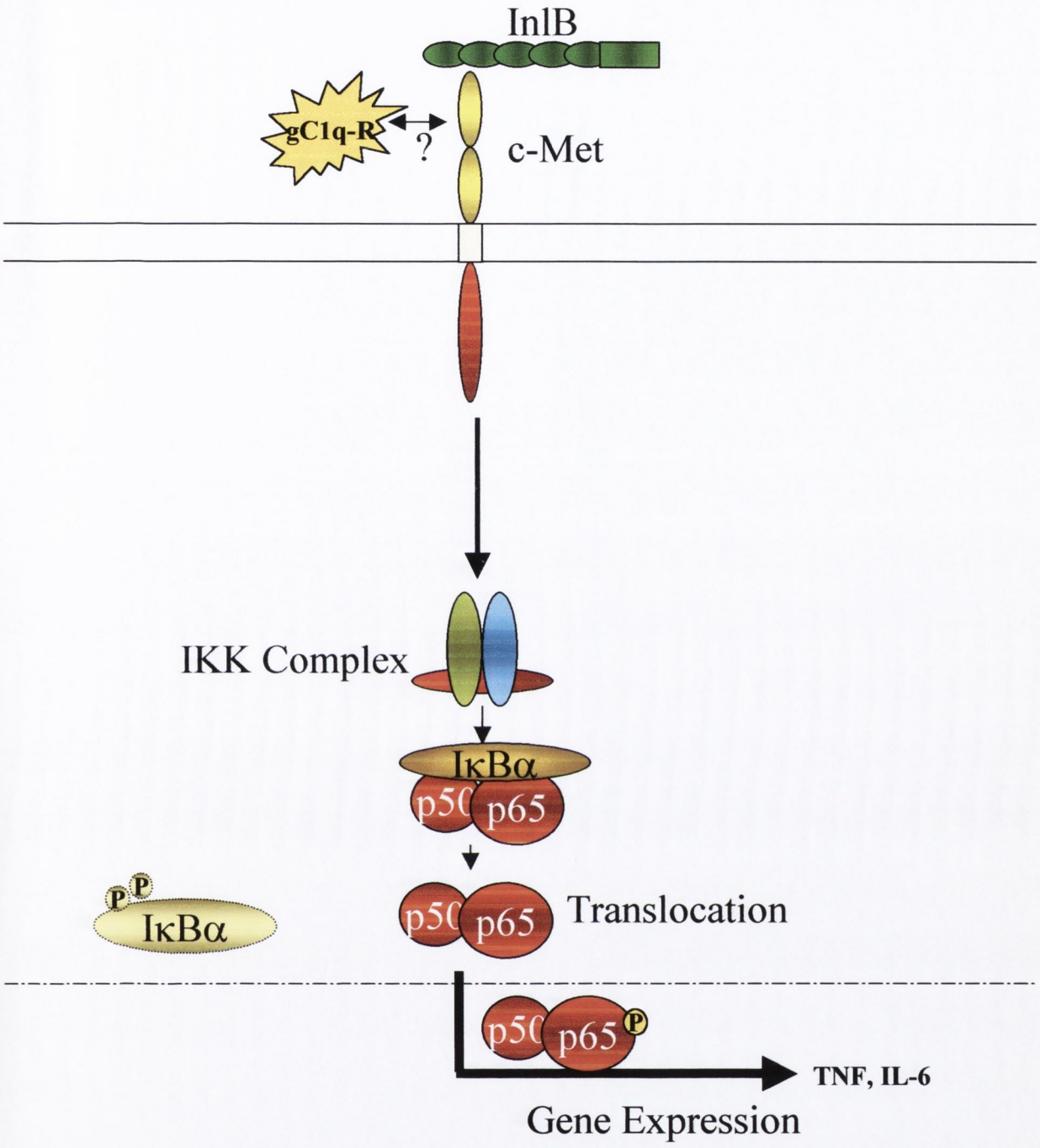


Figure 3.3.1 Schematic representation of InlB-mediated signalling pathway that leads to NF-κB activation

of NF-κB by InlB. The role of PI-3 kinase in the pathway to NF-κB is controversial however, as discussed in the next chapter. InlB was also found to increase IL-6 and TNF production, implying that NF-κB is participating in a pro-inflammatory response.

In conclusion, the results of this study so far indicate that InlB is not only involved in *L. monocytogenes* invasion. It also appears that InlB activates NF-κB. Thus, NF-κB activation during *Listeria* infection seems to be mediated by a series of components including LTA, phospholipids, LLO and InlB.

Chapter Four

InlB activates NF-κB via PI-3 kinase, Ras and Akt.

Chapter 4.

4.1 Introduction

In the previous chapter, the ability of the invasion protein InlB from *L. monocytogenes* to activate the pro-inflammatory transcription factor NF-κB was described. The first receptor described for InlB, gC1q-R, is one of the receptors for the complement protein C1q (36). It does not contain a transmembrane region or glycosylphosphatidylinositol (GPI) linkage sequence (88). This would suggest that gC1q-R is acting as a co-receptor for a transmembrane signalling protein capable of initiating signal transduction, as the finding that InlB activates a cytoplasmic transcription factor, combined with Ireton et al's description of PI-3 kinase activation by InlB (113, 114) suggested a transmembrane signalling cascade.

Therefore, there was considerable interest in continuing the search for this signalling receptor, and the aim of this study was to further examine the signalling pathway initiated by InlB, resulting in NF-κB activation, as the first step in identifying the receptor. As mentioned in the previous chapter, during this research, Shen et al (244), demonstrated that c-Met, the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF), or 'scatter-factor', can bind InlB. Similar to InlB, HGF has been shown to activate PI-3 kinase and InlB can induce epithelial cell scattering. Both InlB and HGF (237) also activate the transcription factor NF-κB.

InlB has previously been shown to activate PI-3 kinase and induce the recruitment of the adapter proteins Gab-1, Grb-2 and Shc (114). HGF has also been found to induce

activation of PI-3 kinase, requiring recruitment of several key adapter proteins including Gab-1 and Grb-2, mediating critical cellular regulation pathways such as proliferation or morphogensis (212, 213, 222). As it appears that InlB is utilising a mammalian growth factor receptor to initiate cellular invasion of the host, understanding the signalling pathway induced by this interaction, and the mediators involved, could suggest critical insights into subcellular processes mediated by InlB binding c-Met. These events could have important consequences for both bacterium and host.

In this chapter I have investigated the signalling pathway activated by InlB leading to NF-κB activation. I have that that a pathway involving Ras, PI-3 kinase, and Akt is involved, correlating well with previous studies of HGF, further strengthening the role of c-Met as the mammalian receptor for InlB.

4.2 Results

4.2.1 PI-3 kinase is involved in InlB-mediated NF-κB activation.

I first investigated the effect of the PI-3 kinase inhibitors, LY294002 and Wortmannin, on InlB-mediated NF-κB activation. J774 cells were pre-treated with a range of concentrations of both compounds for 20 minutes prior to stimulation with 100ng/ml of InlB, for 60 minutes. As shown in figure 4.2.1, both LY294002 (lanes 3-6) and Wortmannin (lanes 7-10) were able to decrease InlB-mediated NF-κB activation in a dose-dependent manner. Figure 4.2.2 illustrates that the PI-3 kinase inhibitor LY294002 (50μM) also inhibited the induction of IκBα degradation by InlB (compare lanes 6 and 7 to lanes 3 and 4). Taken together, these results suggest that PI-3 kinase is involved in the signalling pathway activated by InlB, which leads to activation of NF-κB.

4.2.2 InlB-induces the recruitment of the PI-3 kinase subunit, p85.

I next assessed for PI-3 kinase activation in response to InlB, by assaying p85 recruitment to phosphotyrosine following InlB activation. As shown in figure 4.2.3, InlB induced the rapid recruitment of the p85 subunit of PI-3 kinase to a complex containing tyrosine-phosphorylated proteins in J774 cells. For this, cell lysates prepared from InlB stimulated J774 cell were immunoprecipitated with an anti-phosphotyrosine antibody, then blotted with anti-p85 polyclonal antibody as described in Braun et al (37). Recruitment was observed 1 minute post-stimulation (lane 2), reaching a maximum between 5 and 15 minutes (lanes 3-4), and began declining at 30 minutes (lane 5). InlB has previously been shown to stimulate p85 recruitment in Vero cells after one minute, decreasing rapidly

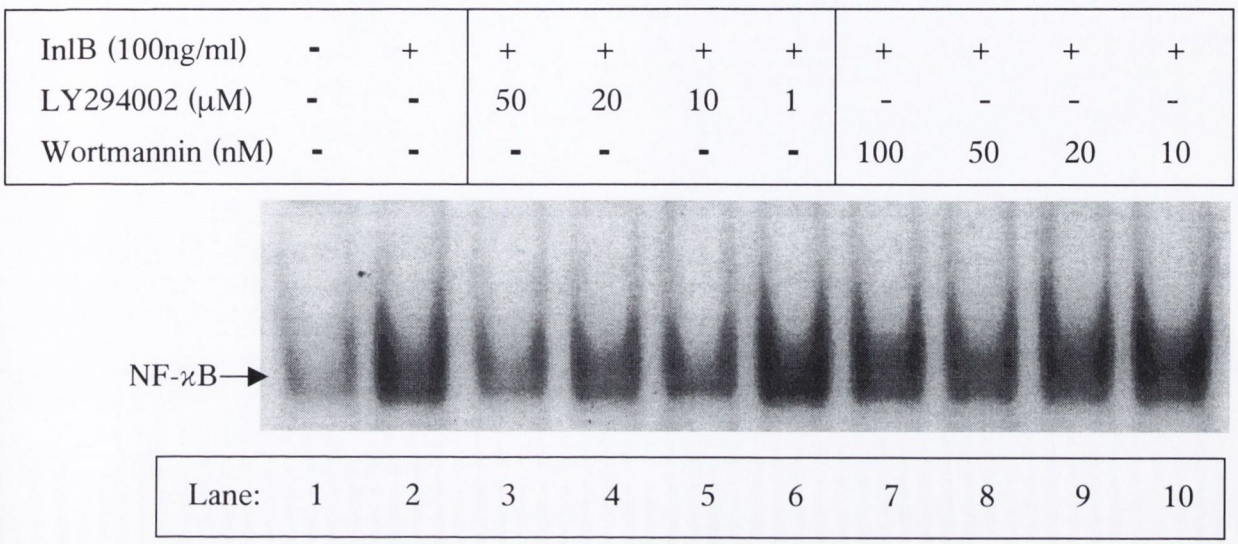


Figure 4.2.1 Effect of PI-3 kinase inhibitors on InlB-mediated NF-κB activation

J774 cells were seeded at $5x10^4$ cells/ml in 6 well plates, 48 hours prior to treatment. Cells were pretreated for 20 minutes with a concentration range of 50-1μM LY294002 (lanes 3-6), or 100-10nM of Wortmannin (lanes 7-10). Cells were then stimulated with InlB (100ng/ml) for 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. Results shown are representative of 3 experiments.

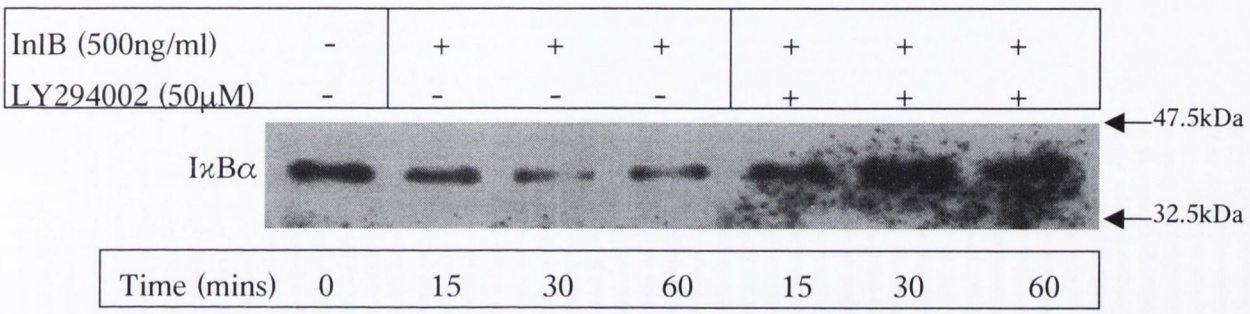


Figure 4.2.2 The PI-3 kinase Inhibitor LY294002 blocks InlB-induced IκBα degradation

J774 cells were seeded at $1x10^5$ cells/ml in six well plates, then incubated for a further 48 hours. Cells were un-treated or pre-treated for 20 minutes with 50μM LY294002, then stimulated with 500ng/ml InlB for a further indicated times. Cells were lysed and assayed for IκBα and IκBβ by Western Blot analysis. Only one band was detected at 37kDa, corresponding to each respective IκB. Results shown are representative of 3 experiments.

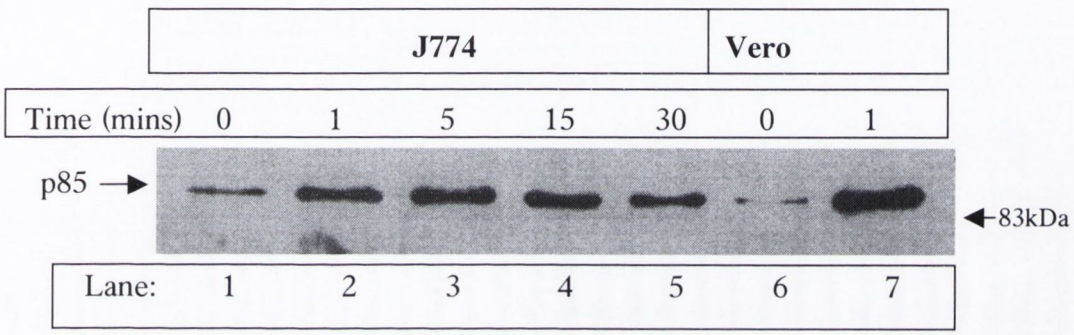


Figure 4.2.3 InlB-mediated p85 recruitment

J774 (lane 1-5) and Vero (lane 6 and 7) cells were seeded at $7x10^4$/ml in 100mm dishes 48 hours prior to stimulation with InlB. Cells were stimulated for indicated times with 500ng/ml of purified InlB. Lysates were centrifuged to remove cell debris, and supernatants probed with α-phosphotryosine antibody 4G10, then immunoprecipitated with a 50% slurry, Protein-A Sepharose. Beads were pelleted, boiled and separated on 10% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes and immunoblotted with polyclonal α-p85 antibody. No other bands were detected. Results shown are representative of 3 experiments.

thereafter (37, 114). Vero cells were therefore used as a positive control, a sample from cells treated for 1 minute with InlB being shown (lane 7).

Interestingly, when J774 cells are treated with LY294002, prior to stimulation with InlB, the inhibitor was found to repress the degree of p85 recruitment (Fig. 4.2.4). LY294002 is known to act as an inhibitor of the p110 catalytic subunit of PI-3 kinase, which covalently binds to the subunit, rendering it catalytically inactive (274). The basis for the inhibition observed here is unclear, but suggests that p85 recruitment depends on p110 catalytic activity.

The PI-3 kinase involvement in NF-κB-dependent transcriptional activity induced by InlB was next investigated. The PI-3 kinase inhibitor LY294002 was used at a range of concentrations previously shown to inhibit InlB-mediated NF-κB activation (figure 4.2.1), to determine if the induction of a reporter gene, luciferase, under the control of the IL-8 promoter (which is NF-κB dependent), could be inhibited. These experiments were carried out in the cell line Hep2, since it was found that J774 cells could not be transfected to a high enough efficiency (not shown). Previously in this report, it has been shown that Hep2 cells are responsive to InlB in terms of NF-κB activation. InlB induced a 2-2.5-fold increase in luciferase activity. This was the maximum response achieved in these cells. As shown in figure 4.2.5 LY294002 inhibited this response, with 100μM having a significant effect.

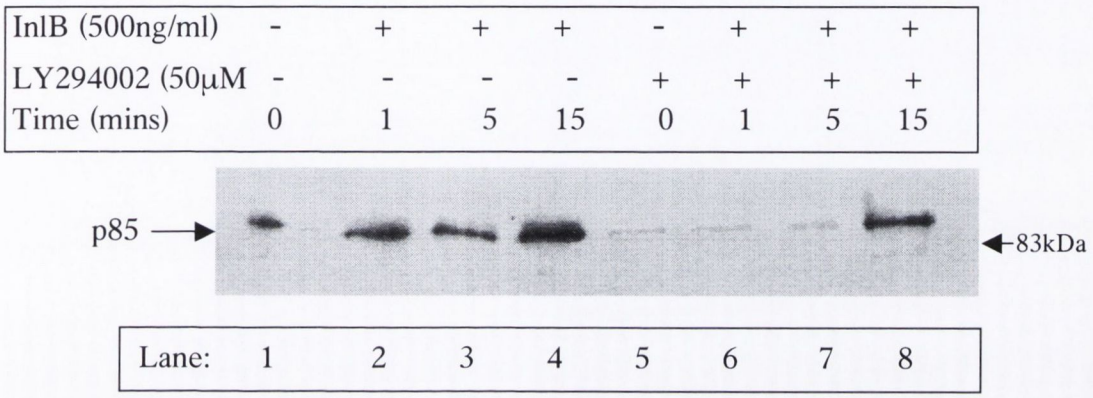


Figure 4.2.4 LY294002 inhibits InlB mediated p85 recruitment

J774 cells were cultured 48 hours prior to treatment at $7x10^4$ cells/ml in 100mm culture dishes. Relevant samples were pre-treated for 20 minutes with 50μM LY294002 in DMSO, cells were stimulated with InlB (500ng/ml) for indicated times, lysed and cell debris removed by centrifugation. Supernatants were probed with α-phosphotryosine antibody 4G10, then immunoprecipitated with a 50% slurry, Protein-A Sepharose. Beads were pelleted, boiled and separated on 10% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes and immunoblotted with polyclonal α-p85 antibody. No other bands were detected. A similar result was obtained in a further experiment.

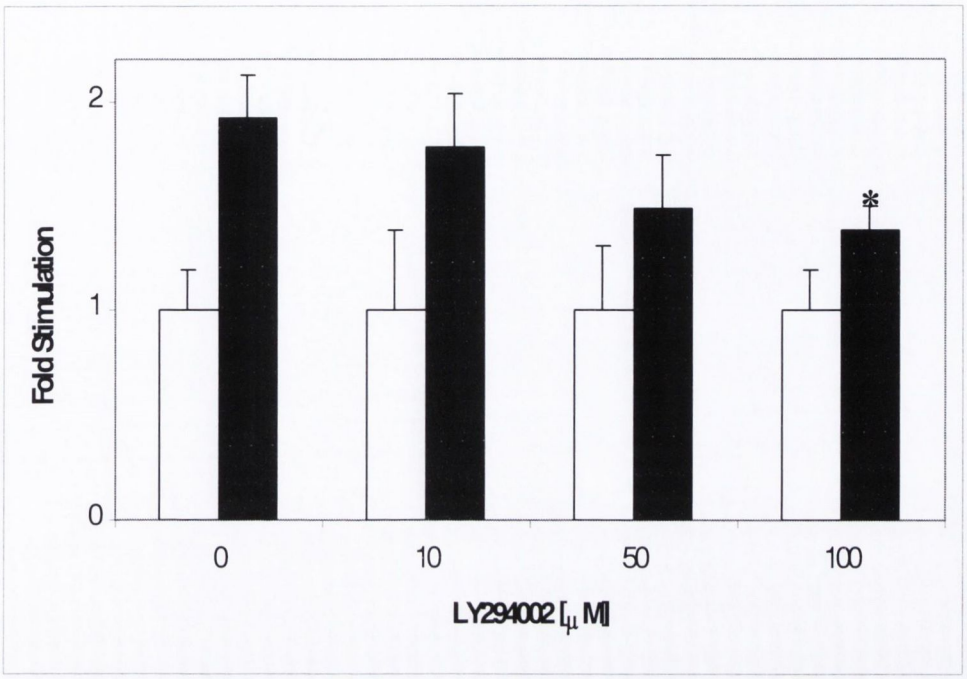


Figure 4.2.5 The PI-3 kinase inhibitor LY294002 blocks induction of an NF-κB-linked reporter gene by InlB

Hep2 cells (1.5-2.0x10^4) were transfected with reporter plasmids for IL-8 luciferase and TK-renilla-luciferase for 24 hours, pre-treated with the indicated concentration of LY294002, then unstimulated (open boxes) or stimulated with InlB (500ng/ml) (closed boxes). Luciferase activity was assayed for each sample. Readings are normalised for each sample relative to constitutively expressed TK-renilla-luciferase and plotted as fold stimulation. Results are means and ±S.D. for triplicate determinations. A similar result was obtained in a further experiment. Data indicate significant differences (*P=0.023) when compared to control values.

To further investigate the ability of InlB to induce NF-κB-dependent transcriptional activity, I next assayed the ability of InlB to induce IL-8 protein expression by ELISA. Once again, these experiments were carried out in Hep2 cells, J774 cells do not express IL-8 (not shown). As can be seen in figure 4.2.6, InlB induced a significant increase in IL-8 expression. LY294002 inhibited the expression of IL-8 in a dose-dependent manner, with 50μM and 100μM both reducing expression to basal concentrations.

These results indicate that PI-3 kinase is involved in the activation of NF-κB by InlB, at a point upstream of IκBα degradation.

4.2.3 InlB induces phosphorylation of Akt.

PKB/Akt is a critical downstream target of PI-3 kinase (64, 80, 252) that has been shown to transiently associate with, and activate, the IκB kinase complex leading to NF-κB activation (199, 227). To determine if InlB was able to activate Akt was next determined. As shown in figure 4.2.7, InlB induced phosphorylation of Akt as assessed by immunoblotting whole cell lysates with a phospho-specific antibody that recognizes Akt only when phosphorylated on Ser473. Initially these experiments were unsuccessful due to fetal calf serum inducing increased basal phosphorylation of Akt. However, method development allowed beneficial completion of these experiments. Accordingly, increased phosphorylation was observed within 15 minutes (lane 3), and was optimal 30 minutes post-stimulation (lane 4), returning to basal phosphorylation levels at 45 minutes (lane 5). Pre-treatment of the cells with the PI-3 kinase inhibitor LY294002, as shown in figure 4.2.8, blocked the effect (compare lane 3 to 1and 2).

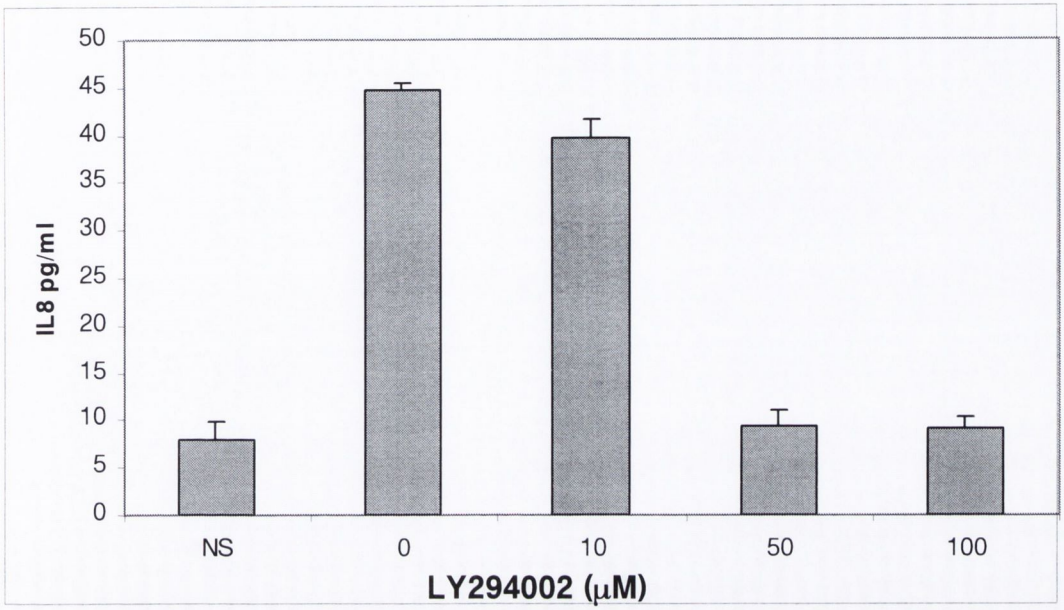


Figure 4.2.6. The PI-3 kinase inhibitor LY294002 blocks InlB-mediated IL-8 protein expression

$1x10^4$ Hep2 cells were seeded in 96 well plates 24 hours prior to treatment with the indicated concentrations of LY294002 and stimulated with InlB (500ng/ml), then incubated for a further 24 hours. Cell supernantants were removed and assayed for IL-8 by ELISA. IL-8 concentrations are calculated by comparison to a standard curve of IL-8 concentrations and plotted as pg/ml. DMSO vehicle in NS had no effect. Results are means and ±S.D. for triplicate determinations. A similar result was obtained in a further experiment.

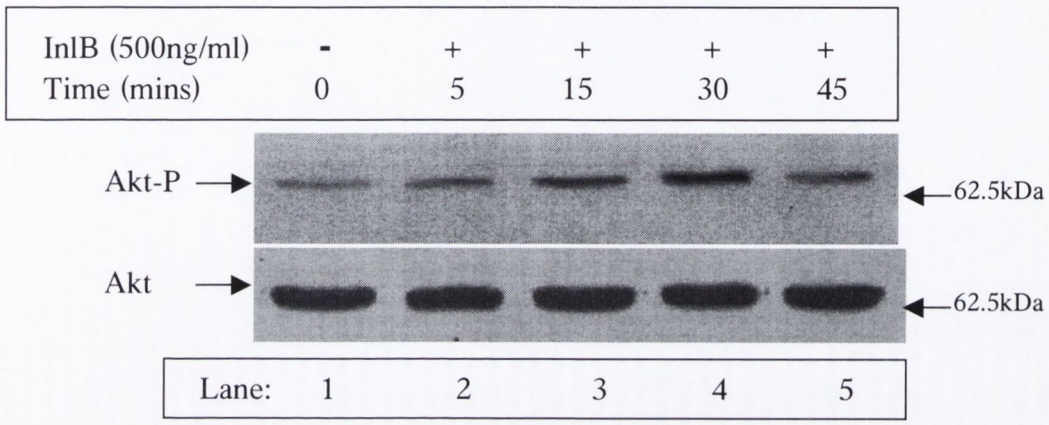


Figure 4.2.7. InlB-induced phosphorylation of Akt

$7x10^5$ J774 cells were grown in serum free media, 48 hours prior to stimulation with 500ng/ml InlB for indicated incubation times. Cells were lysed with Laemelli sample buffer, cell debris removed by centrifugation, then supernatants separated by 10% SDS-PAGE. Samples were transferred to nitrocellulose, then immunoblotted with anti-phospho-Akt antibody (Ser 473). Membranes were stripped and further probed with anti-Akt antibody to determine equal protein loading for each sample. Results shown are representative of 3 experiments.

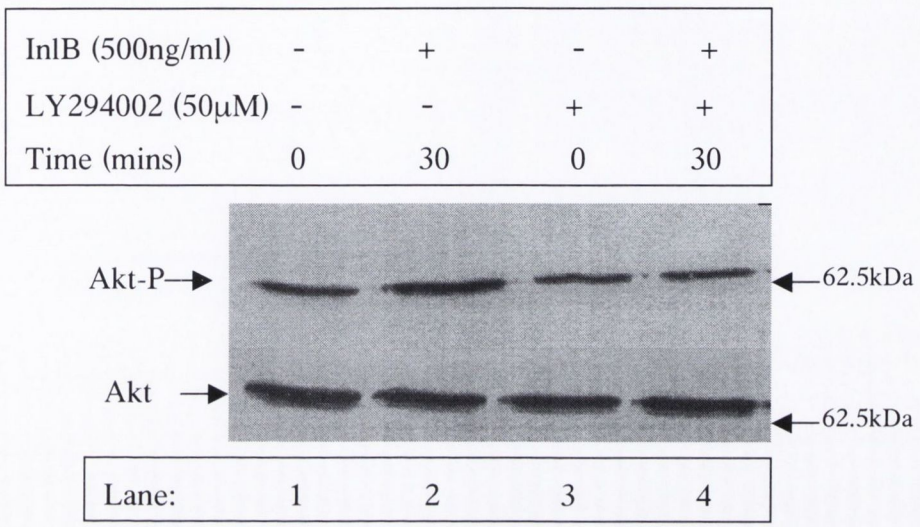


Figure 4.2.8. LY294002 inhibits InlB-induced Akt phosphorylation

J774 cells ($7x10^4$/ml in100mm dishes) were grown for 48 hours in serum free medium, treated or not-treated with 50 μM LY294002 (lane 3) for 20 minutes prior to InlB (500ng/ml) stimulation. Cells were lysed, cell debris removed by centrifugation, then supernatants separated by 10% SDS-PAGE. Samples were transferred to nitrocellulose, then immunoblotted with anti-phospho-Akt antibody (Ser 473). Membranes were stripped and further probed with anti-Akt antibody to determine equal protein loading for each sample. Similar results were obtained from a further experiment.

To further examine a role for Akt in InlB-mediated signal transduction resulting in NF-κB activation, the effect of a plasmid encoding a dominant negative mutant of Akt on expression of the IL-8 promoter was tested. Again these transfection experiments were conducted in Hep2 cells due to their increased capacity for transient transfection as compared to J774 cells. As can be seen in figure 4.2.9, transient transfection of the dominant negative mutant inhibited the response induced by InlB, in a dose-dependent manner, with 50-100ng of plasmid reducing the effect. This result indicates that Akt activation is required for NF-κB activation by InlB.

To further investigate Akt involvement in InlB-mediated NF-κB transcriptional activation, the effect of the recently described Akt inhibitor 1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-Methyl-3-*O*-octadecylcarbonate (106, 107) on InlB-mediated IL-8 gene expression was assayed by ELISA. As shown in figure 4.2.10, InlB induced a significant increase in IL-8 protein expression. Akt inhibitor inhibited the expression of IL-8 in a dose-dependent manner, with 10μM and 20μM both having an inhibitory effect, reducing expression by up to 50%.

Taken together, these results suggest that Akt is involved in InlB-mediated NF-κB activation.

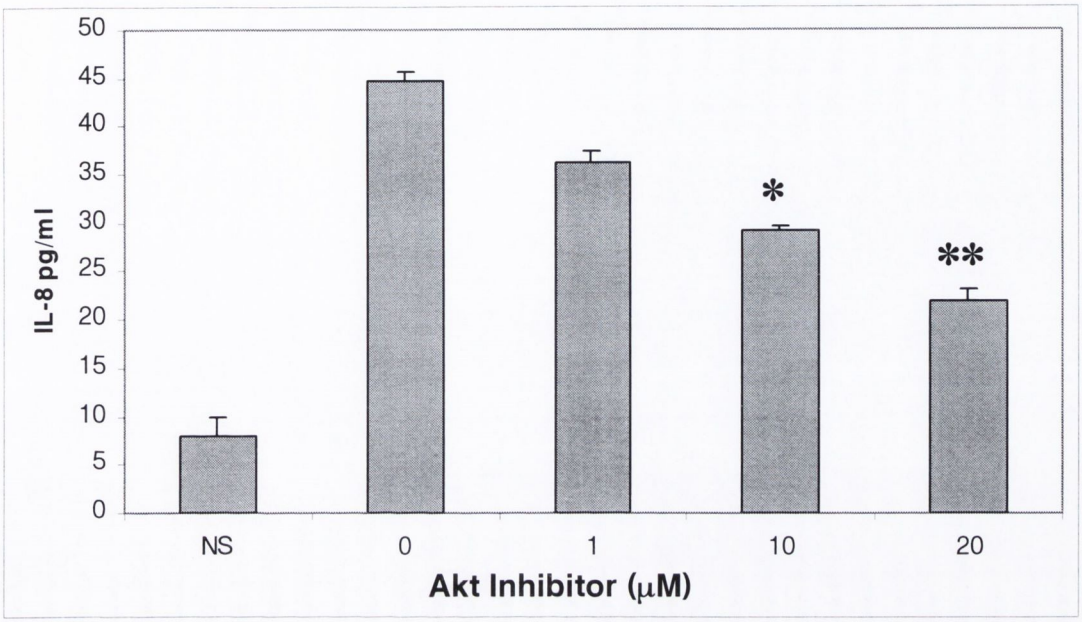


Figure 4.2.10. The Akt inhibitor blocks InlB-mediated IL-8 protein expression

$1x10^4$ Hep2 cells were seeded in 96 well plates 24 hours prior to treatment with the indicated concentrations of Akt Inhibitor and stimulated with InlB (500ng/ml), then incubated for a further 24 hours. Cell supernantants were removed and assayed for IL-8 by ELISA. IL-8 concentrations are calculated by comparison to a standard curve of IL-8 concentrations and plotted as pg/ml. DMSO vehicle in NS had no effect. Results are means ±S.D. for triplicate determinations. A similar result was obtained in a further experiment. Data indicate significant differences (*P=0.004, **P=0.005) when compared to control values.

4.2.4 Testing of signalling inhibitors for abrogation of InlB-mediated NF-κB activation.

As can be seen in figure 4.2.11A, the highly specific Protein kinase C inhibitor Calphostin C, had no effect upon InlB-mediated NF-κB activation, as did either the $p60^{c\text{-}src}$ and Raf-1 complex inhibitor Geldanamycin (Fig. 4.2.11B); or Tyrophostin AG490 (Fig. 4.2.11C), which is a potent inhibitor of Epidermal Growth Factor (EGF) receptor kinase autophosphorylation and also JAK2.

4.2.5 Inhibitors of the small G-protein Ras block InlB mediated NF-κB activation.

The low molecular weight G protein Ras is an important regulator of PI-3 kinase, which has also been shown to play a role in NF-κB activation (31, 159). I therefore next investigated a role for Ras using the farnesyltransferase inhibitor Manumycin A (93), which impedes Ras activity. As can be seen in Figure 4.2.12A, Manumycin A inhibits InlB-mediated activation of NF-κB, in a dose dependent manner.

Manumycin A also prevented InlB-induced degradation of IκBα as shown in figure 4.2.12B. InlB induced IκBα degradation from 15 minutes, most of the IκBα being degraded by 60 minutes. Manumycin A inhibited this response (compare lanes 8-10 to lane 3-5).

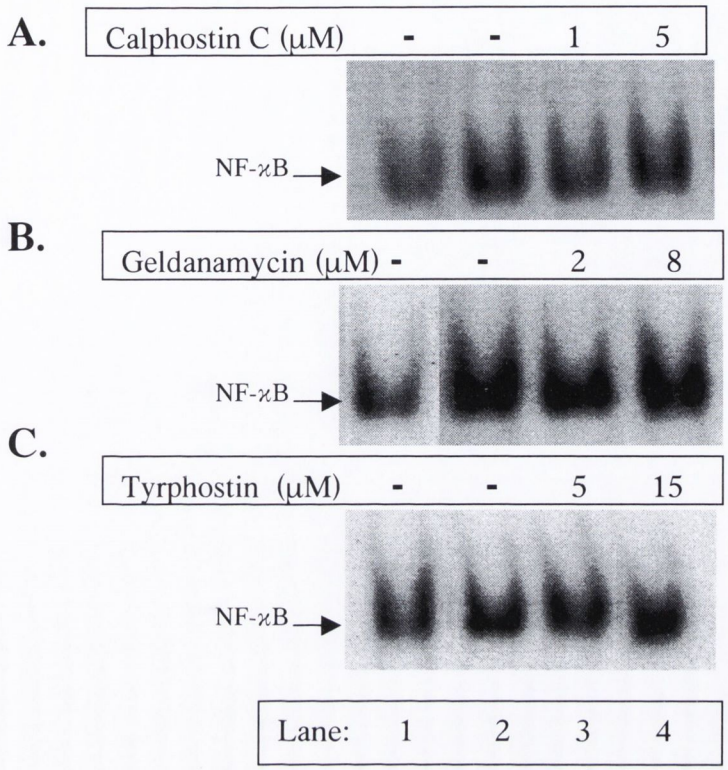


Figure 4.2.11. Effect of Signalling pathway inhibitors

J774 cells were cultured at $5x10^4$ cells/ml for 48 hours prior to stimulation. Cells were un-treated or pre-treated with indicated concentrations of inhibitors (lanes 3 and 4) for 45 minutes, then stimulated with InlB (100ng/ml) (lanes 2-4) or left untreated (lane 1), for a further 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case.

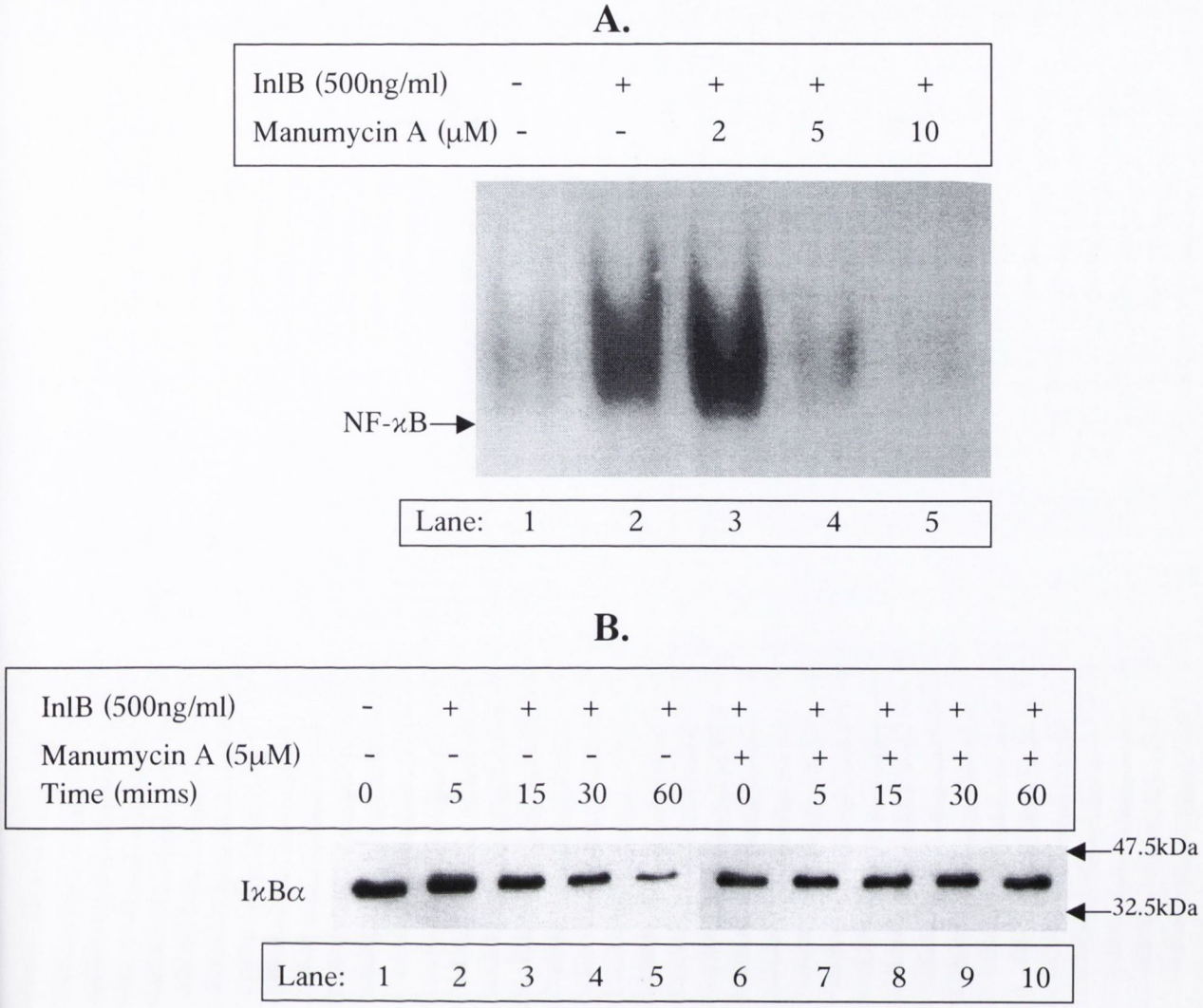


Figure 4.2.12. InlB-mediated NF-κB activation involves Ras

A. 5x10^4 cells/ml J774 cells were grown for 48 hours, pretreated for 60 min with Manumycin A (2-10μM) (lane 3-5), and stimulated with 200ng/ml InlB for 60 min. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. Inhibition shown is representative of 3 experiments. *B.* 5x10^4/ml J774 cells were grown for 48 hours, pretreated with Manumycin A (lane 6-10) or media as control for 60 min and stimulated with 500 ng/ml InlB for 60 min. Equal amounts of protein from cell lysates were separated on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with monoclonal antibody against IκBα. Only one band was detected at 37kDa. A similar result was obtained in a further experiment.

4.2.5 InlB induces the activation of Ras.

Manumycin A is not a specific Ras inhibitor, but a more generic inhibitor of farnesyltransferase transferase reactions. Therefore it was necessary to attempt to implicate Ras activity in InlB signal transduction by assaying for Ras activation.

Ras activation can be assayed by immunoprecipitating the active form of Ras (ie. GTP-bound Ras) from cell lysates using an affinity matrix containing the Ras-binding domain of Raf, which only recognises GTP-bound Ras. Samples separated by SDS-PAGE are immunoblotted with an anti-pan-Ras antibody to show activated Ras. As can be seen in figure 4.2.13, InlB induced activation of Ras in a time-dependent manner. Maximal activation was detected after 1 minute of stimulation (lane 2), and the response continued until 5 minutes post-stimulation (lane 4). The effect was transient, returning to basal activation levels 15 minutes post-stimulation (lane 5).

The involvement of Ras in acting as a mediator of InlB-induced signalling, leading to NF-$\varkappa$B activation, was tested by the effect of a plasmid encoding a dominant negative mutant Ras N17 (31), on activation of the IL-8 promoter induced by InlB. Again, Hep2 cells were used for these experiments. Transfection of Hep2 cells with 10ng and 50ng of plasmid encoding Ras N17, inhibited InlB-induced expression of IL-8 luciferase reporter gene, with 50ng of plasmid achieving a maximal inhibition (Figure 4.2.14).

To further illustrate Ras involvement in InlB-mediated NF-$\varkappa$B gene transcription activity, the ability of Manumycin A to inhibit InlB-induced IL-8 protein expression was assayed

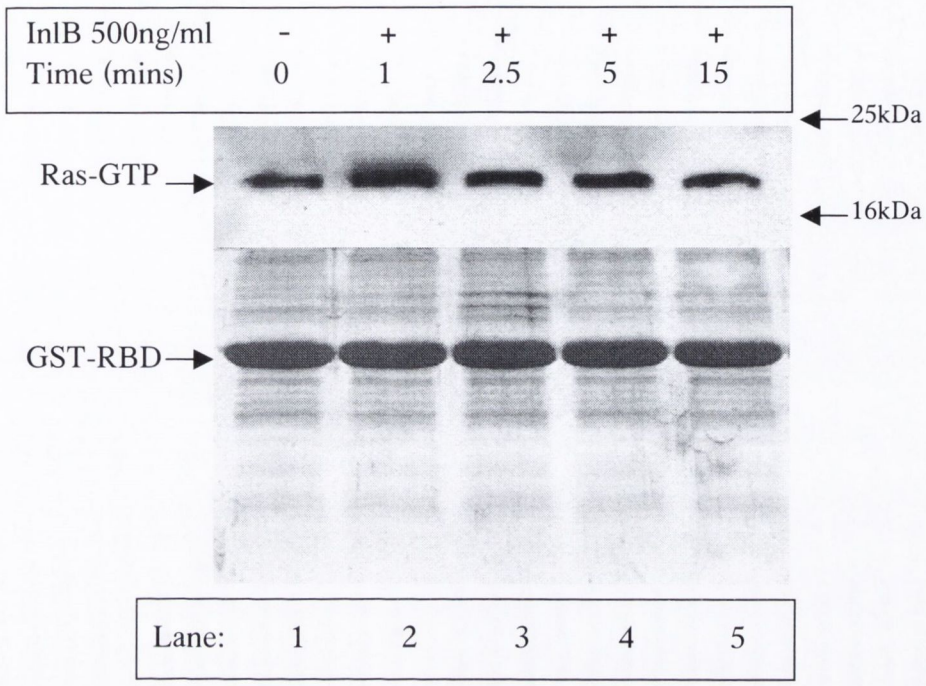


Figure 4.2.13 InlB induces activation of the small G-protein Ras

$5x10^6$ J774 cells were grown for 24 hours and serum starved for 24 hours prior to stimulation with 500 ng/ml InlB in serum-free media for the indicated time course (lane 2-5). Activated Ras present in cell lysates was immunoprecipitated, as described in Materials and Methods. Proteins were separated on 15 % SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-pan Ras antibody. Results shown are representative of 3 experiments. Lower band shows GST-RBD and confirms equal protein loading as assayed by Commassie blue staining of the proteins remaining on the SDS-PAGE after transfer.

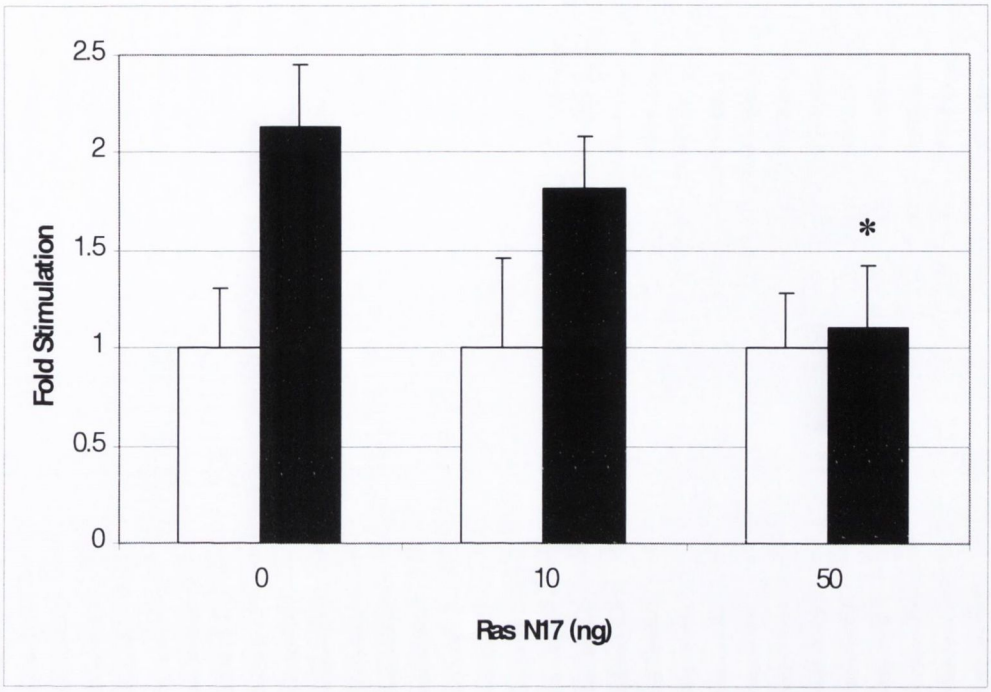


Figure 4.2.14. Dominant negative Ras inhibits induction of an NF-κB-linked reporter gene by InlB

Hep2 cells ($1.5\text{-}2x10^4$) were transiently transfected with IL-8-luciferase (500ng), TK-Renilla-luciferase, and indicated amounts of Ras N17 for 24 hours prior to stimulation with InlB (500ng/ml, 6 hours)(closed boxes), or untreated (open boxes). Cell Extracts were analysed for luciferase activity. Readings are normalised for each sample relative to constitutively expressed TK-renilla-luciferase and plotted as fold stimulation. Results are means and ±S.D. for triplicate determinations. A similar result was obtained in a further experiment. Data indicate significant differences (*P=0.050) when compared to control values.

by ELISA. As shown in figure 4.2.15, Manumycin A inhibited IL-8 induction in a dose dependent manner, 50nM and 100nM being the most effective inhibitory doses, decreasing expression to basal levels. Higher concentrations of Manumycin A (1-5μM) appeared to have a toxic effect on Hep2 cells after 24 hours incubation leading to cell death (data not shown).

These results imply that Ras is involved in mediating NF-κB activation via InlB.

4.2.6 InlB-induced activation of Ras occurs upstream of PI-3 kinase.

We next wished to determine the relationship between Ras and PI-3 kinase in InlB-mediated signalling. Ras is known to act as both an upstream and also, downstream mediator of PI-3 kinase signalling, in both a cell-type and stimulus-specific manner. Therefore, to determine the relationship in regard to signalling regulation by either Ras or PI-3 kinase, the PI-3 kinase LY294002 and the Ras inhibitor Manumycin A were used to probe the respective mediators of InlB-induced effects.

J774 cells were pre-treated with LY294002 for 20 minutes, stimulated with 500ng/ml of InlB, and Ras activation determined. As can be seen in figure 4.2.16, LY294002 had no effect on InlB-induced Ras activation (compare lanes 2 and 4) in regard to non-stimulated cells (lanes 1 and 2 respectively). Similar to non-treated cells, Ras activation occurred at 2.5 minutes stimulation, which was also evident in LY294002 treated cells.

I next determined if the Ras inhibitor Manumycin A could block recruitment of p85. J774 cells were pre-treated with 5μM Manumycin A for 60 minutes, stimulated with

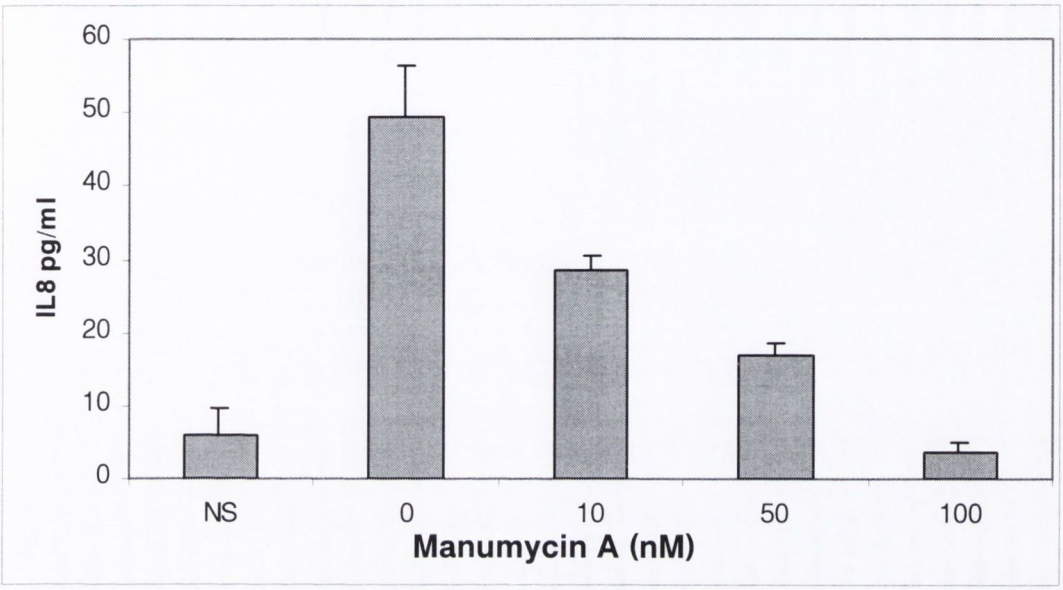


Figure 4.2.15. The Ras inhibitor Manumycin A blocks InlB-mediated IL-8 protein expression

$1x10^4$ Hep2 cells were seeded in 96 well plates 24 hours prior to treatment with the indicated concentrations of Manumycin A and stimulated with InlB (500ng/ml), then incubated for a further 24 hours. Cell supernantants were removed and assayed for IL-8 by ELISA. IL-8 concentrations are calculated by comparison to a standard curve of IL-8 concentrations and plotted as pg/ml. DMSO vehicle in NS had no effect. Results are means and ±S.D. for triplicate determinations. A similar result was obtained in a further experiment.

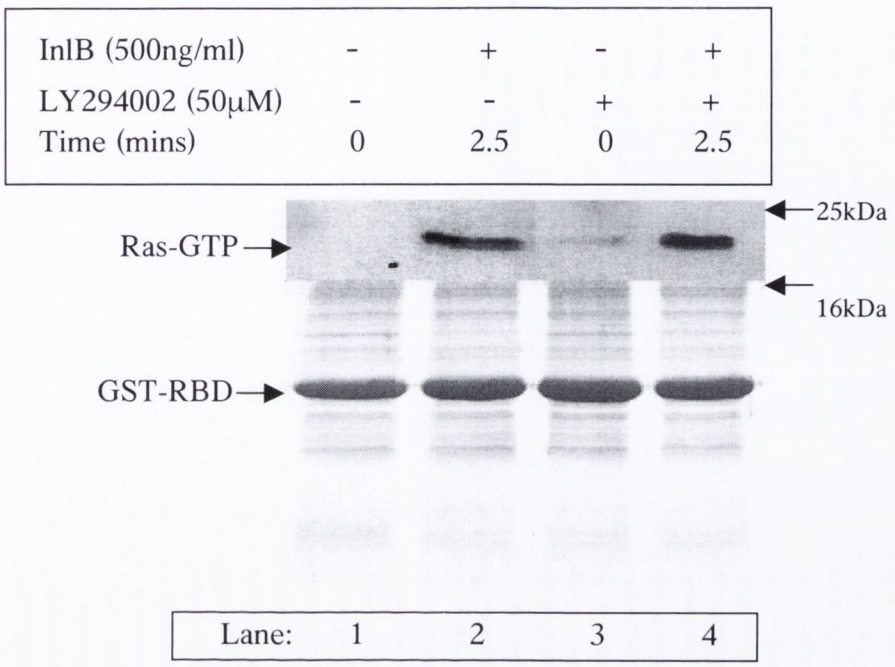


Figure 4.2.16. InlB-induced activation of Ras occurs upstream of PI-3 kinase

J774 ($5x10^6$) cells were grown for 24 hours in 10 % FCS/RPMI, serum starved for 24 hours, treated for twenty minutes with serum free media containing 50 μM LY294002 (lane 3 and 4), or untreated (lane 1 and 2) prior to InlB stimulation (500 ng/ml). Activated Ras present in cell lysates was immunoprecipitated, as described in Materials and Methods. Proteins were separated on 15 % SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-pan Ras antibody. Similar results were obtained in a further experiment. Lower band shows GST-RBD and confirms equal protein loading as assayed by Commassie blue staining of the proteins remaining on the SDS-PAGE after transfer.

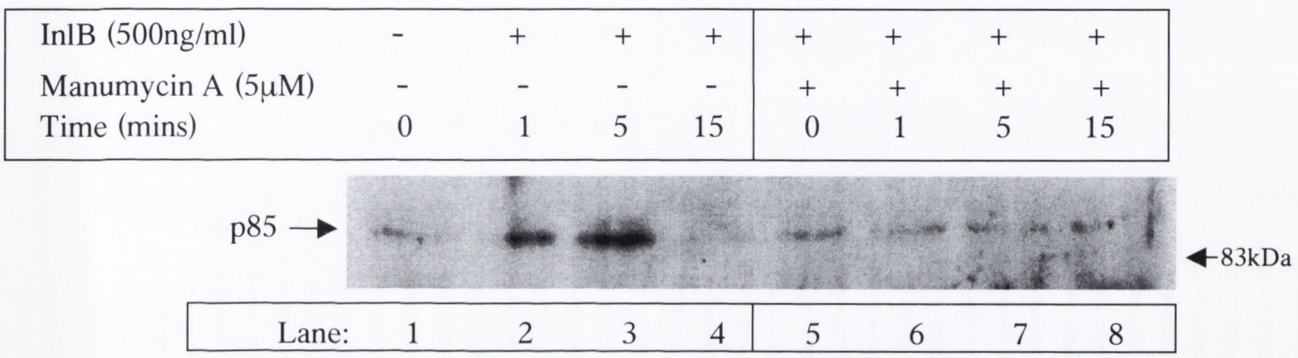


Figure 4.2.17. InlB-mediated recruitment of p85 requires Ras

$7x10^4$ J774 cells were grown for 48 hours, treated with Manumycin A (lane 5-8) for 60 minutes prior to stimulation with 500ng/ml InlB. Lysates were centrifuged to remove cell debris, and supernatants probed with α-phosphotryosine antibody 4G10, then immunoprecipitated with a 50% slurry, Protein-A Sepharose. Beads were pelleted, boiled and separated on 10% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes and immunoblotted with polyclonal α-p85 antibody. No other bands were detected. Results shown are representative of 3 experiments.

500ng/ml of InlB, then analysed for p85 recruitment. Figure 4.2.17 illustrates that Manumycin A was able to totally abrogate the recruitment of p85 to the InlB-mediated phosphorylated complex (compare lanes 6 and 7, to lanes 2 and 3). This implies that Ras activation occurs upstream of PI-3 kinase in InlB-induced signalling, a conclusion supported by the earlier finding that the timing of Ras activation by InlB precedes recruitment of p85 to the signalling complex.

4.3 Discussion

In the previous chapter it had been established that InlB was able to induce the activation of NF-κB. Activation was mediated by the InlB-induced degradation of IκBα and IκBβ, which led to the expression of cytokines whose promoters are controlled by NF-κB. The aim of this chapter was to establish the signal transduction pathway induced by InlB binding its transmembrane receptor, which culminates in IκB degradation and subsequent NF-κB translocation to the nucleus. InlB had previously been shown to activate PI-3 kinase (37, 113, 114), a response required for internalisation of *L. monocytogenes* (113). Here, we have shown that the activation of PI-3 kinase occurs downstream of Ras activation, and that Akt is activated following PI-3 kinase. These findings are the first demonstration of a bacterial protein utilising Ras, PI-3 kinase and Akt as signalling mediators that result in NF-κB activation and suggests that PI-3 kinase-mediated internalisation may require Ras.

During the course of this research, Shen et al (244) demonstrated that c-Met, the heterodimeric receptor for HGF was the mammalian transmembrane receptor for InlB. The first reported receptor for InlB was gC1q-R (36), a protein that binds the complement protein C1q. This protein lacks a transmembrane domain and also lacks an identifiable cytoplasmic region. Its role in InlB signal transduction remains presently unclear, although it is possible that it interacts with c-Met. The identification of c-Met as a receptor for InlB provides a molecular explanation for previously reported InlB signals. These signals include recruitment of Gab-1 and Cbl to InlB-induced phosphotyrosines. The binding of HGF to c-Met triggers dimerisation and auto-phosphorylation of the

receptor, instigating recruitment of adaptor proteins to the receptor, thereby initiating several different signalling pathways, including recruitment of Gab-1, Cbl, p85 and Gab-2, the last signal leading to Ras activation reviewed in (29, 257). HGF also activates NF-$\varkappa$B, although its signalling pathway is yet to be determined (237). The research presented here that InlB activates NF-$\varkappa$B, via Ras and Akt, add two further common signals to the list mediated by InlB and HGF, further strengthening the role of c-Met in InlB signal transduction. The finding that PI-3 kinase activation by InlB requires Ras, concurs with other studies on growth factor signalling, where, although p85 can be recruited directly to growth factor receptors, initiating specific cellular mechanisms, activation of PI-3 kinase can also involve upstream Ras.

In describing the involvement of PI-3 kinase in mediating InlB-induced NF-$\varkappa$B activation, I used the PI-3 kinase inhibitors Wortmannin and LY294002. Wortmannin is a fungal metabolite that demonstrates a high specificity for PI-3 kinase compared to other lipid kinases, especially when used at low concentrations. Wortmannin binds covalently to the p110 catalytic subunit of PI-3 kinase, thereby inhibiting the subsequent signalling pathway following PI-3 kinase activation (203, 204). However, Wortmannin is also known to be unstable in medium, and I found that results could be inconsistent using this inhibitor. For this reason, I predominately used the second PI-3 kinase inhibitor, LY294002. LY294002 is a chemically synthesised substrate of a previously known PI-3 kinase inhibitor, Quercetin, which are both competitive inhibitors of the ATP-binding site of PI-3 kinase (274). LY294002 however, also inhibits the catalytic subunit of DNA-activated protein kinase. However, LY294002 was found to be a more specific inhibitor

of PI-3 kinase than quercetin, as it had no effect on PI-4 kinase, EGF receptor kinase, MAP kinase, Protein kinase C, S6 kinase, c-Src, PDGF receptor-mediated tyrosine phosphorylation, and several ATP-requiring enzymes at 50μM.

Since Wortmannin and LY294002 my not be absolutely specific for PI-3 kinase however, the next alternative was to demonstrate direct recruitment of the p85 adapter subunit of PI-3 kinase to InlB-mediated tyrosine phosphorylation. This technique has previously been successfully used to demonstrate InlB-induced recruitment of p85 to phosphotyrosines in the Green Monkey Kidney cell line Vero (37). Interestingly, while both my study, and the numerous studies by the Cossart laboratory have clearly established that InlB activates PI-3 kinase in Vero cells, I have never been able to demonstrate InlB-mediated NF-κB activation in that cell type. Therefore, there is a clear difference in the downstream cellular response to PI-3 kinase activation in both Vero and J774 cells. In the previous chapter I remarked on the broad range of responses that HGF could induce upon binding and subsequent autophosphorylation of the 'multi-docking' site within the β-chain of c-Met. It was suggested that this ligand-induced autophosphorylation mediated the recruitment of key adapter proteins, which further recruited signalling mediators such as PI-3 kinase and Ras. These two key signalling transducers then provided a signalling 'platform' to intracellular signalling pathways thus inducing specific intracellular mediators that are either highly abundant, or particularly expressed, in that particular cell line. I would suggest that this phenomenon might explain the results here. It appears that there could be a signalling mediator expressed in J774, P338D$_1$, RAW 264 and Hep2 cells line that is either unique to, or more highly

abundant in, these cell lines in comparison to Vero and the other cell lines that demonstrated no InlB-mediated NF-κB activation.

PI-3 kinase is recruited to phosphotyrosines via the SH2 domain of the p85 subunit. This recruitment alleviates the inhibitory effect upon the catalytic p110 subunit induced by p85, thus allowing full activation of PI-3 kinase (53). The demonstration of the p110 inhibitor LY294002 blocking recruitment of p85 to phosphotyrosines suggests a more complex relationship between p110 and p85. It has previously been shown that the small G-Protein Ras can interact with the catalytic p110 subunit of PI-3 kinase directly, in a GTP-dependent manner (128, 225, 226). This interaction occurs through the effector region of Ras, and there is evidence that Ras can stimulate PI-3 kinase activity, which is required for optimal activation in response to growth factors. There is also the possibility that Ras-GTP and the tyrosine phosphoprotein interactions with p85/p110 synergise to give full activation of PI-3 kinase. As I have shown a role for activated Ras acting upstream of PI-3 kinase in mediating InlB-induced NF-κB activation, and since InlB is the ligand for a growth factor receptor, I would suggest that the effect described above is responsible for the ability of LY294002 to inhibit the recruitment of p85 to phosphotyrosines as the p110 subunit was unable to be recruited initially to membrane bound Ras-GTP. This effect would further support the finding that Ras acts upstream of PI-3 kinase as LY294002 had no effect upon Ras activation, but the Ras inhibitor Manumycin A blocked p85 recruitment to phosphortyrosines. This would suggest that Ras recruits PI-3 kinase initially via the p110 subunit to the membrane; this then allows

the p85 subunit to interact with phosphotyrosines on the c-Met β chain. This complex interaction may be required to achieve maximal activation of PI-3 kinase in this system.

A critical requirement of Ras activation is its association with the inner face of the plasma membrane. The C-terminus is essential for this interaction and a conserved Cysteine (Cys186) within the motif CAAX is required to initiate posttranslational modification. The cysteine is first modified by a C_{15} polyisoprenyl (farnesyl) moiety attatched to it. The three C-terminal amino acids are proteolytically cleaved and the newly farnesylated Ras C-terminus is carboxymethylated leading to a more hydrophobic protein with a higher affinity for the plasma membrane (reviewed in (42)). Inhibition of farnesylation with Manumycin A (205), a metabolite from *Streptomycies parvulus*, a protein farnesyltransferase inhibitor, revealed the requirement of Ras in mediating InlB-induced degradation of IκBα and the subsequent activation of NF-κB. As previously mentioned, this inhibitor was also used to indicate that Ras acts upsteam of PI-3 kinase, and also inhibited InlB-induced NF-κB-dependent gene transcription of the chemokine IL-8. Interestingly, while concentrations such as 1-5μM of Manumycin A were required to observe inhibition of InlB-mediated NF-κB activation by EMSA, these concentrations appeared to be toxic to Hep2 cells which were incubated with the inhibitor for 24 hours in the IL-8 ELISA (data not shown). Inhibition was still achieved however, at much lower concentrations. The reason for this toxic effect is unclear, however, while Manumycin A may have no toxic affect in the relatively short incubation time of 2 hours for EMSA analysis, the 24-hour incubation period could effect other intracellular process' within Hep2 that results in apoptosis. To further support the role for Ras in transducing InlB-

mediated NF-κB activation, transient transfection of Hep2 cells with dominant negative Ras N17 inhibited InlB-induced NF-κB-dependent luciferase-reporter gene transcription. Ras N17 has a point mutation of Thr^{17} to Asn^{17} (31) rendering Ras constitutively bound in its Ras-GDP form, the G-protein exchange factors (GEFs) unable to activate Ras to its Ras-GTP form, thereby blocking Ras signalling.

In addition to the role of Ras and PI-3 kinase, the downstream effector of PI-3 kinase, Akt, was found to act as a downstream effector in the pathway, following PI-3 kinase activation. This effect was initially observed by immunoblotting of whole cell lysates with antibodies specific for phosphorylated Akt. These findings were further supported by the ability of the Akt inhibitor 1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-Methyl-3-*O*-octadecylcarbonate] (106, 107), to block InlB-mediated NF-κB-dependent IL-8 protein expression as assayed by ELISA. This chemically synthesised compound acts as a substrate for Akt, and interacts in a complementary fashion with the positively charged pocket formed by the β1-β2 and β3-β4 loops of the PH domain of Akt (106, 107). The axial hydromethyl group in 1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-Methyl-3-*O*-octadecylcarbonate] is anchored through the hydrogen bonds with Arg25 of Akt. This specificity of binding accounts for the high selectivity of this compound to Akt, rather than the p110 subunit of PI-3 kinase, which has been found for several other members of this substrate family.

Further evidence for the involvment of Akt in InlB-mediated NF-κB activation was demonstrated by the use of a dominant negative mutant of Akt, which is kinase dead, and

its ability to inhibit InlB-induced NF-κB-dependent luciferase-reporter gene transcription. Kinase dead Akt is unable to induce signal transduction by the downstream phosphoryalation of target proteins. Two studies have shown the downstream target of Akt that leads to IκBα degradation and subsequent NF-κB activation is the IKK signalsome. Romashkova and Makarov (227) showed that platelet-derived growth factor (PDGF), caused activation of NF-κB via the IKK signalsome. By the use of dominant negative proteins, they showed a direct association between Akt and one of the IKK subunits, IKKβ. In contrast, Ozes et al (199) showed that TNFα activates NF-κB via Akt, but their Akt associates with, and phosphorylates another IKK subunit, IKKα. Transfection of cells with a mutant of IKKα which could not be phosphorylated, demonstrated the inhibition of TNFα-mediated IkBα degradation and the subsequent nuclear translocation of NF-κB. The inability of the kinase dead mutant of Akt to mediate InlB-induced activation of NF-κB would suggest that this pathway is responsible for the effect observed here.

Controversy however, has ensued over Akt's ability to act as a direct upstream effector of IKK. Others propose that Akt only promotes transactivation by NF-κB, thus mediating gene transcription. Several studies have shown direct involvement of Akt in transactivation of NF-κB, but failed to observe Akt's involvment in signal transduction leading to NF-κB nuclear translocation. Indeed, one group trying to replicate the findings of Ozes et al (199), failed to observe the phosphorylation of IKKα by TNFα, and suggested that the effect they observed was Akt-mediated phosphorylation of the nuclear bound p65 subunit of NF-κB, or transactivation (58). Ozes et al retorted that this study

only examined one cell line, HeLa, a cell line whose properties are known to vary greatly amongst different laboratories, and that their further studies upon several cell lines found that the amount of Akt, the extent to which it is activated by TNFα, and its effects on the IKKα subunit are cell-type specific.

Our data would suggest that Akt is having an effect upon IKK, as inhibition of Ras and PI-3 kinase by specific inhibitors blocked IκBα degradation. The kinase dead Akt also blocked NF-κB-driven gene transcription, further supporting Akt signal transduction inducing NF-κB activation. However, the possibly of InlB-inducing transactivation of NF-κB via Akt cannot be discounted.

In this study, the use of transiently transfected Hep2 cells allowed the evaluation of dominant negative mutants of Ras and Akt, in conjunction with LY294002, to further dissect the InlB-induced signalling pathway. A problem encounted with this technique however, was the low degree of stimulation achieved by InlB in these cells, at best achieving only 2-2.5-fold stimulation over non-stimulated cells, longer stimulation times have only a marginal effect in increasing stimulation (data not shown). This low stimulation meant that the effect of the inhibitors (namely LY294002, DN Akt and DN Ras N17) was difficult to evaluate, although the results were consistent. It is difficult to explain why gene expression was so low in Hep2, as InlB does mediate NF-κB activation in these cells. EMSA data indicated that NF-κB activation by InlB required 500ng/ml, approximately 10-fold lower than the activation induced in J774 cells, however, increased amounts of InlB failed to increase fold stimulation, and increased stimulation times of 24

hours had a minimal effect. The difference in the ability of InlB to activate NF-κB in J774 cells in comparison to Hep2, could in part be due to the level of expression of a signalling mediator between c-Met and IKK. This theory has already been touched on earlier, where different cells may express different concentrations of specific signalling mediators that may be required to transduce the signal. Indeed, as Ozes et al pointed out, the amount of Akt, the extent to which it is activated, and its effects on IKK, are cell-type specific. As I have shown a role for Akt in mediating the effect reported here, this could well be responsible for the low NF-κB-dependent luciferase reporter stimulation observed in Hep2 cells. The obvious way to overcome this problem would be to use cells known to respond potently to InlB, such as J774 cells. However, J774 cells being macrophage-like, are extremely difficult to transfect, their transfection efficiency too low to allow meaningful results to be achieved (data not shown).

Considerable interest however, has centred on the anti-apoptotic effects of Akt. Numerous substrates have been reported in recent years. Different studies have shown that HGF/c-Met signalling triggers either anti-apoptotic (16, 237) or pro-apoptotic (8, 48) signals in different cell lines, and that their mechanisms are not fully understood. It has been suggested this duplicity of action could be due to the expression, or availability of co-receptors or downstream regulators, both of which may determine the phenotype presented by the cell. Reports have now demonstrated that HGF/c-Met exerts its anti-apoptotic actions via the PI-3 kinase/Akt pathway (153, 287), while recently Xiao et al (284) demonstrated the anti-apoptotic properties of the c-Met signalling pathway required the activation of both the MAPK pathway via Ras, and PI-3 kinase/Akt. Akt is able to

exert its anti-apoptotic effects in a variety of ways, including the already described phosphorylation of IKK, which leads to NF-κB activation. NF-κB activation has been shown to be anti-apoptotic in TNF-induced cell death (25, 276) and also via the induction of genes encoding anti-apoptotic proteins such as IAPs (135). The ability of activated Akt to induce phosphorylation or sequestering of proteins of the Forkhead and Bcl-2 families, causes the retention of these proteins in the cytoplasm, thereby preventing pro-apoptotic transcription events or mitochondrial death signals (reviewed in (55, 81, 164)). Another notable substrate of Akt is Caspase 9. Akt-induced phosphorylation of caspase 9 decreases apoptosis by directly inhibiting the apoptotic protease activity of caspase 9.

In conclusion, these results identify Ras and Akt as novel signals activated by InlB, which are required for NF-κB activation, with Ras acting upstream of PI-3 kinase. These results correlate well with the discovery of Met as the mammalian receptor for InlB, further illustrating the ability of a pathogenic bacterium to subjugate mammalian cellular proteins to allow successful invasion and subsequent survival in the host. Figure 4.3.1 schematically illustrates the signal transduction pathway initiated by InlB binding to c-Met. How InlB induces this effect in phagocytotic cells warrants further investigation, and the discovery of InlB as the first bacterial product to activate NF-κB via Akt could suggests a novel role for InlB promoting an anti-apoptotic response from these cells. These effects could have a significant consequence on the bacterium's ability to successfully survive and replicate in host mammalian cells, or conversely, represent the host cells innate immune response to bacterial infection.

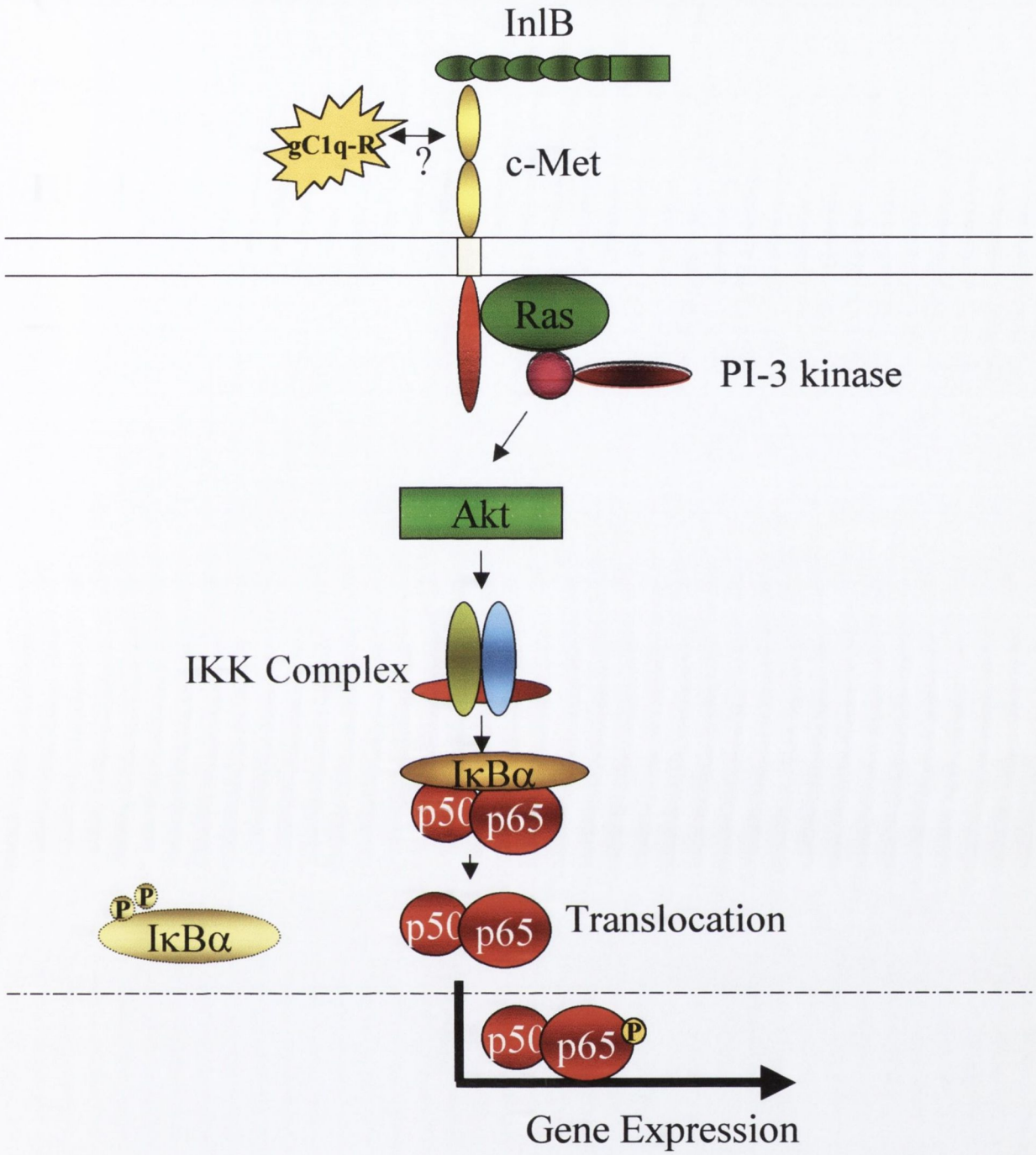


Figure 4.3.1 Schematic representation of InlB-mediated signalling pathway that leads to NF-κB activation

Chapter Five

The serine protease inhibitors Antithrombin III and Hirudin inhibit LPS-induced activation of NF-κB.

Chapter 5

5.1 Introduction

In chapter 3 and 4 I described data on a bacterial component, InlB from *L. monocytogenes*, which I found was an activator of NF-κB. In this chapter, I have investigated another much more widely characterised bacterial activator of NF-κB, Lipopolysaccharide (LPS). LPS is a powerful activator of the innate arm of the immune system.

Vertebrates have developed a sophisticated immune system to defend themselves against microbial infection, involving both innate and adaptive components. The adaptive immune system has been widely researched for several decades, recently however attention has turned to the innate immune response due to recent discoveries regarding the innate immune components of the fruit fly *Drosophila melagonaster*. In *Drosophila*, the Toll family of proteins mediates the immune response (reviewed in (109, 112, 144)). Toll-like receptors (TLR) have been found to occur in humans, where 10 have been found. Similar to *Drosophila*, all of these TLRs possess a Toll/IL1 Receptor (TIR) cytoplasmic domain, which is responsible for signal transduction via a highly homologous signalling pathway shared by both *Drosophila* and mammals (2, 111). TLRs and Toll both activate Rel family transcription factors, which are involved in host defense gene expression (27).

The role of Toll in Drosophila is to initiate a host response to fungal pathogens. Mammalian TLRs were therefore felt to be receptors for pathogen-derived products.

A crucial discovery in the innate immune response of mammals was the finding that TLR4 (209, 216) was the long sought for putative receptor for LPS, from gram-negative bacteria. Further studies have now found several other pathogens that act as agonists for several of the mammalian TLRs: TLR2 responds to Peptidoglycan and Lipotechoic acid (150, 241, 260), TLR5 responds to *L. monocytogenes* flagellin (97), TLR6 responds to macrophage-activating lipopeptide-2kDa (MALP-2) in conjunction with TLR2 (261), and TLR9 responds to bacterial CpG-DNA (98). Because of this wide range of responses to pathogen-derived products, the TLR family have been identified as pattern recognition receptors which sense pathogen associated molecular patterns (PAMPs).

Despite the high degree of homology between the *Drosophila* and mammalian Toll responses, there is the suggestion of a difference between them. In *Drosophila*, an extracellular protease cascade generates a proteolytically cleaved peptide, Spaetzle, the putative ligand for Toll, in response to fungi (145, 182). This cascade is inhibited by the serpin, Spn43Ac (146). In mammals, LPS is described as the putative ligand for TLR4. Whilst no direct evidence has been presented showing direct interaction between TLR4 and LPS, several genetic and complementation studies suggest that this is the case in mammals (210). It has been established for some time that both LPS-binding protein (LBP) and CD14 act as co-receptors for LPS . A recent report demonstrated direct binding interaction between LPS and another protein MD2, independent of either LBP or CD14 (272). This data further supported an earlier study using cross-linking studies that demonstrated a close proximity of LPS to the

MD2 and TLR4 signalling complex (54). However, antibodies directed against TLR4, previously shown to inhibit TLR4-dependent LPS stimulation, were unable to inhibit LPS cross-linking to the MD2/TLR4 complex, suggesting that there is an additional component involved in the signalling response.

Due to the high degree of similarity between *Drosophila* and mammalian Toll immune responses, and the suggestion that all components of the TLR4 signalling complex have yet to be elucidated, the aim of this study was to investigate whether the LPS response in humans could also be sensitive to inhibition by a serpin. I have found that the serpin Antithrombin III (ATIII) and the thrombin inhibitor Hirudin were able to inhibit NF-$\varkappa$B activation induced by both LPS and Lipid A, the active moiety of LPS, in the human monocyte cell line THP-1. ATIII may therefore be acting in a similar way to Spn43Ac in the fly, possibly inhibiting a serine protease involved in LPS action extracellulary.

5.2 Results

5.2.1 Characterisation of LPS-mediated NF-κB activation in THP-1 Cells.

LPS is a known potent activator of NF-κB in monocyte and macrophage cell lines. To characterise this response in THP-1 cells, the ability of LPS to induce NF-κB over a concentration range was tested.

Firstly, THP-1 cells were stimulated with a range of concentrations (1-500ng/ml) of LPS and NF-κB assayed by EMSA. As can be seen in figure 5.2.1A, LPS activated NF-κB in a dose dependent manner, activation initially observed at 5ng/ml (lane 5), achieving a maximal response at 100-500ng/ml (compare lanes 6 and 7 with lane 1). To further support this finding, whole cell lysates prepared from THP-1 cells stimulated with the same concentration range of LPS, indicated a similar dose dependent LPS-induced degradation of IκBα, another means of assaying for NF-κB activation (Fig. 5.2.1B). From these results, it was concluded that the optimal dose of LPS to be used in further studies to ensure sufficient activation of NF-κB was 100ng/ml.

Next, THP-1 cells were treated over a range of stimulation times to determine the incubation time necessary to achieve optimal LPS-mediated NF-κB activation. Cells were treated with 100ng/ml of LPS for times ranging from 5 minutes to 2 hours post-stimulation. As illustrated in figure 5.2.2A, LPS activated NF-κB in a time dependent manner, initial activation observed 15 minutes post-stimulation (lane 3) achieving a maximal response between 60-120 minutes post-stimulation (lanes 5 and

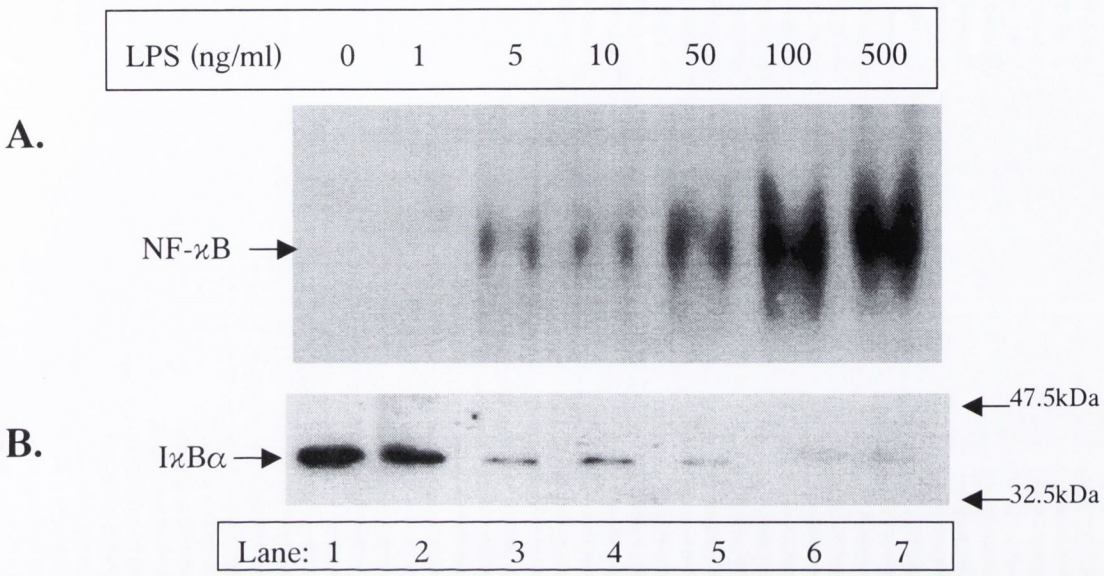


Figure 5.2.1 Dose dependent activation of NF-κB by LPS

A. The human monocytic cell line THP-1 were resuspended in serum free RPMI 1640 at $1x10^6$/ml in 24 well plates. Cells were stimulated with the indicated concentrations of LPS, increasing from 1ng/ml to 500ng/ml (lanes 2-7). Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment. *B.* THP-1 cells were treated as above for NF-κB, assessed for IκBα levels by western blotting as described in Materials and Methods and seperated by 10% SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with monoclonal α-IκBα antibody. Only one band was detected at 37kDa. A similar result was obtained in a further experiment. Different extracts were used for EMSA and western blots.

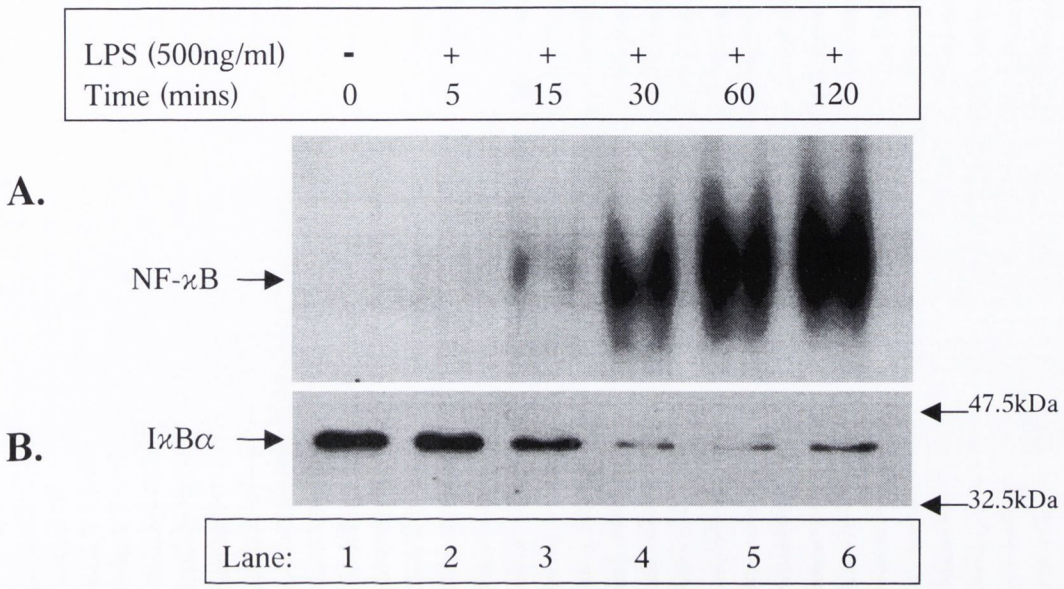


Figure 5.2.2 Time dependent activation of NF-κB by LPS

A. The human monocytic cell line THP-1 were resuspended in serum free RPMI 1640 at $1x10^6$/ml in 24 well plates. Cell were then stimulated with LPS (500ng/ml) for indicated times ranging from 5-120 minutes (lanes 2-7). Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment. *B.* THP-1 cells were treated as above for NF-κB, assessed for IκBα levels by western blotting as described in Materials and Methods and seperated by 10% SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with monoclonal α-IκBα antibody. Only one band was detected at 37kDa. A similar result was obtained in a further experiment. Different extracts were used for EMSA and western blots.

6), when compared to non-stimulated cells (lane 1). Once again, these findings were further supported by western blot analysis of cytoplasmic extracts, which indicate that LPS-induced IκBα degradation occurred over a similar time course as that observed for NF-κB activation determined by EMSA. Figure 5.2.2B shows that IκBα degradation induced by LPS was initially observed after 15 minutes (lane 3), maximal degradation occurring 30-60 minutes post-stimulation (lanes 4-5). These results concur well with the EMSA data, the slight time differences consistent with IκB degradation occurring upstream of NF-κB translocation to the nucleus.

Taken together, these results suggest that optimal activation of NF-κB induced by LPS is observed when cells are stimulated with 100ng/ml of LPS for 60 minutes. These parameters were used in all further experiments regarding LPS.

5.2.2 Characterisation of Lipid A-mediated NF-κB activation in THP-1 Cells.

Lipid A, the active moiety of LPS (217), was next examined for its ability to activate NF-κB in THP-1 cells. As Lipid A is a synthetic compound, it does not contain lipoproteins or other contaminants that have previously been shown to induce NF-κB activation (100). Once again, THP-1 cells were stimulated with range of Lipid A concentrations and analysed for NF-κB activation by EMSA. As can be seen in figure 5.2.3A, Lipid A-activated NF-κB in a dose dependent manner, activation initially observed at 500ng/ml (lane 4), reaching maximal activation at 2-5μg/ml (compare lanes 6 and 7 to lane 1), as compared to non-stimulated cells. To further support this finding, IκBα degradation western blot analysis (Fig. 5.2.3B) indicate

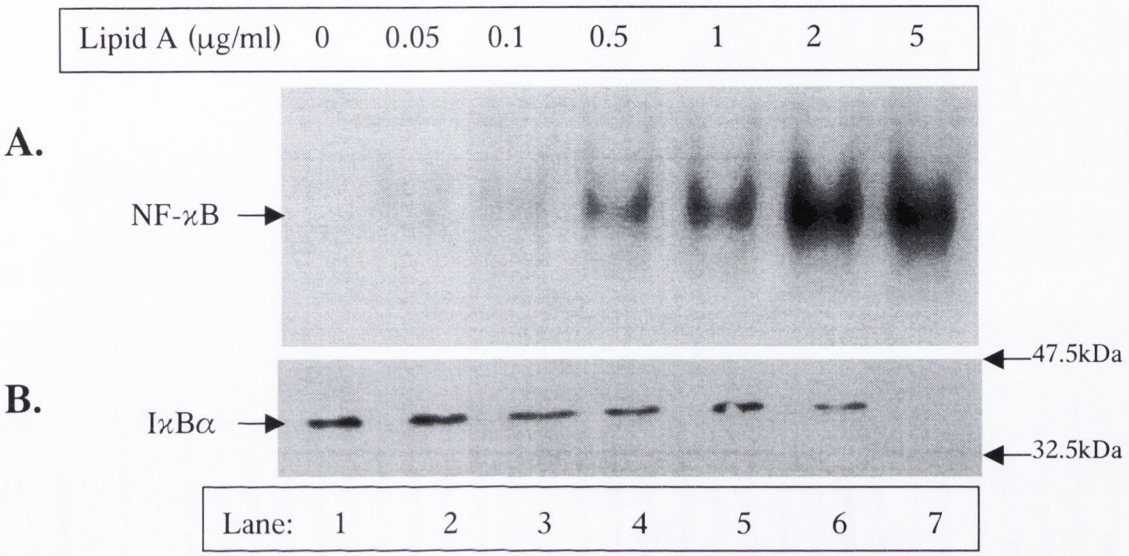


Figure 5.2.3 Dose dependent activation of NF-κB by Lipid A

A. The human monocytic cell line THP-1 were resuspended in serum free RPMI 1640 at $1x10^6$/ml in 24 well plates. Cells were stimulated with the indicated concentrations of Lipid A, increasing from 50 ng/ml to 5 μg/ml (lanes 2-7). Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment. *B.* THP-1 cells were treated as above for NF-κB, assessed for IκBα levels by western blotting as described in Materials and Methods and seperated by 10% SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with monoclonal α-IκBα antibody. Only one band was detected at 37kDa. A similar result was obtained in a further experiment. Different extracts were used for EMSA and western blots.

that initial degradation of IκBα is observed at 100ng/ml of Lipid A (lane 3), however, maximal degradation is not achieved until cells are stimulated with 2-5μg/ml (lanes 6 and 7), as compared to un-stimulated cells (lane 1).

THP-1 cells were next stimulated with 2μg/ml of Lipid A over a series of stimulation times, to determine the time dependence of Lipid A-mediated NF-κB activation. Figure 5.2.4A illustrates that Lipid A activates NF-κB in a time dependent manner, activation first observed 30 minutes post-stimulation (lane 4), reaching maximal activation 60 minutes post-stimulation (lane 5), then beginning to return to basal levels after 2 hours stimulation (lane 6), when compared to non-stimulated cells (lane 1). Figure 5.2.4B indicates the induction of IκBα degradation by Lipid A concurs with the NF-κB activation data, degradation being evident 15 minutes post-stimulation (lane 5) and continuing up to 2 hours (lane 6).

These results suggest that optimal NF-κB activation is achieved by stimulating THP-1 cells with 2μg/ml of Lipid A for 60 minutes. The time dependent data concurs well with the results observed for LPS-mediated NF-κB activation, which was expected.

5.2.3 Antithrombin III inhibits LPS- and Lipid A-mediated NF-κB activation.

As the aim of this study was to determine if a serpin could inhibit activation of mammalian cells by LPS, the ability of Antithrombin III, a plasma inhibitor of thrombin (22) was next examined for its ability to inhibit LPS-mediated NF-κB

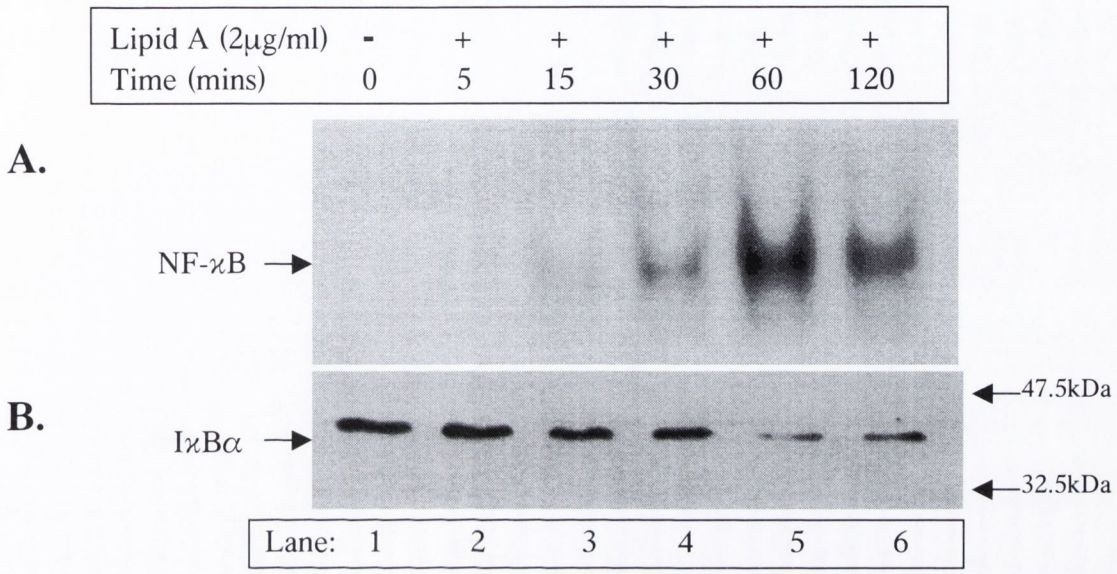


Figure 5.2.4 Time dependent activation of NF-κB by Lipid A

A. The human monocytic cell line THP-1 were resuspended in serum free RPMI 1640 at $1x10^6$/ml in 24 well plates. Cells were then stimulated with Lipid A (2 μg/ml) for indicated times, ranging from 5-120 minutes (lanes 2-7). Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment. *B.* THP-1 cells were treated as above for NF-κB, assessed for IκBα levels by western blotting as described in Materials and Methods and seperated by 10% SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with monoclonal α-IκBα antibody. Only one band was detected at 37kDa. A similar result was obtained in a further experiment. Different extracts were used for EMSA and western blots.

activation. ATIII has previously been shown to inhibit LPS-induced expression of IL-6, an NF-κB dependent gene (253).

Firstly, as a control, ATIII alone was tested for effects on NF-κB. As can be seen in figure 5.2.5, ATIII was unable to induce activation of NF-κB as determined by EMSA over a variety of concentrations, ranging from 500ng-20μg/ml (lanes 2-6), however, 100ng/ml of LPS strongly activated NF-κB (lane 7), when compared to non-stimulated cells (lane 1).

Next, the ability of ATIII to inhibit LPS-mediated NF-κB activation was investigated. Cells were pre-treated over a range of times with 10μg/ml of ATIII, then stimulated with 100ng/ml of LPS for 60 minutes. As shown in figure 5.2.6, ATIII was effective in reducing LPS-mediated NF-κB activation if the cells were pre-treated with ATIII for no more than 1-5 minutes (compare lanes 4 and 5 with lane 2) when compared to LPS-stimulated cells. Simultaneous addition of ATIII had no effect on LPS-mediated NF-κB activation (lane 3), while longer pre-treatment times (15 and 30 minutes)(lanes 6 and 7) also were found to be ineffective in reducing NF-κB activation. This result suggests that the pre-treatment time with ATIII was critical in mediating the inhibitory effect seen here. This may be due to endogenous serine protease activity of the cells, which is inactivating ATIII. The inability of co-incubation to have an effect is this assay suggests that the activation of the protease by LPS proceeds more quickly than the inhibitory effect of ATIII.

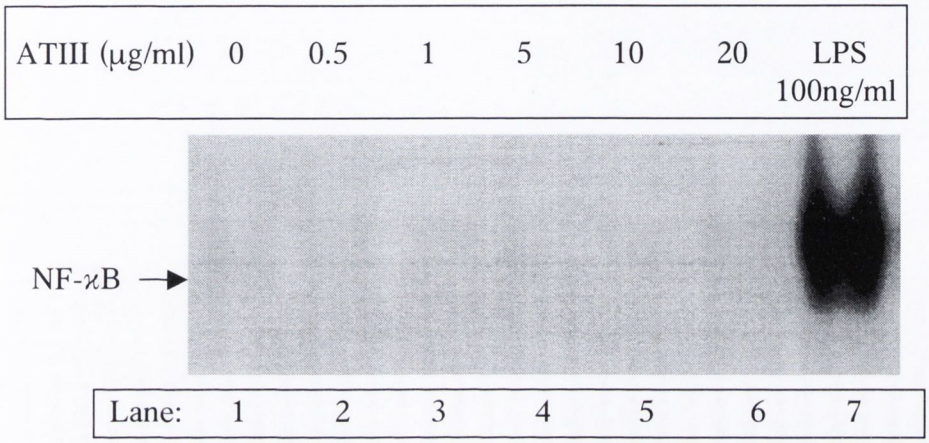


Figure 5.2.5 The serpin ATIII does not activate NF-κB

THP-1 cells were resuspended in serum free RPMI 1640 at $1x10^6$/ml in 24 well plates. Cells were then stimulated with the indicated concentrations of ATIII, increasing from 50ng/ml to 20μg/ml (lanes 2-6). NF-κB activation is compared to control LPS (100ng/ml) stimulation (lane 7). Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case.

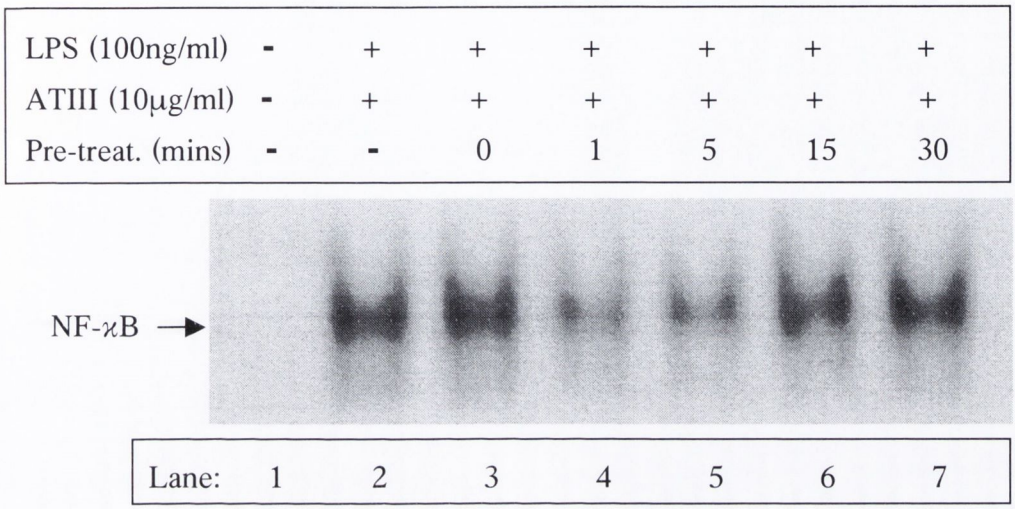


Figure 5.2.6 Pre-treatment of THP-1 cells with ATIII inhibits NF-κB activation by LPS

THP-1 cells ($1x10^6$/ml) were resuspended in serum free RPMI 1640 in a 24 well plate, non-treated or pre-treated with 10μg/ml of ATIII (lanes 3-7) for indicated times. Cells were then stimulated with 100ng/ml of LPS and incubated for a further 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. Inhibition shown is representative of 3 experiments.

Using a pre-treatment time of one minute optimised above, cells were pre-treated with a range of concentrations of ATIII, then stimulated with LPS. As can be seen in figure 5.2.7A, while ATIII was able to inhibit LPS-mediated NF-κB activation over a broad concentration range of 0.5-20μg/ml (lanes 3 to 7), optimal inhibition was achieved with 10μg/ml when compared to activation achieved by LPS alone (compare lane 6 with lane 2).

To further examine the effect, ATIII was also tested for its ability to inhibit Lipid A-mediated NF-κB activation. Figure 5.2.7B clearly illustrates the ability of ATIII to almost totally abolish Lipid A-induced NF-κB activation over the same concentration range as that observed for LPS (compare lanes 3-7 with lane 2).

5.2.4 ATIII inhibition is specific for LPS-mediated NF-κB activation.

As protease inhibitors are known to inhibit cytoplasmic proteasomal activities, which are critical in transducing signalling events, it was important to determine the specificity of the ATIII effects in respect to LPS. Therefore, the effect of ATIII on two other NF-κB activators, IL-1 and TNF-α (15), were tested. These two cytokines have previously been shown to activate NF-κB, and in the case of IL-1, induce a similar signalling pathway as that required for transducing LPS-induced nuclear translocation of NF-κB via TLR4. As shown in figure 5.2.8, an optimal dose of ATIII was unable to inhibit NF-κB activation induced by either IL-1 (lane 2 and 3) or TNF-α (lane 4 and 5). This result would suggest that the inhibitory effect of ATIII is

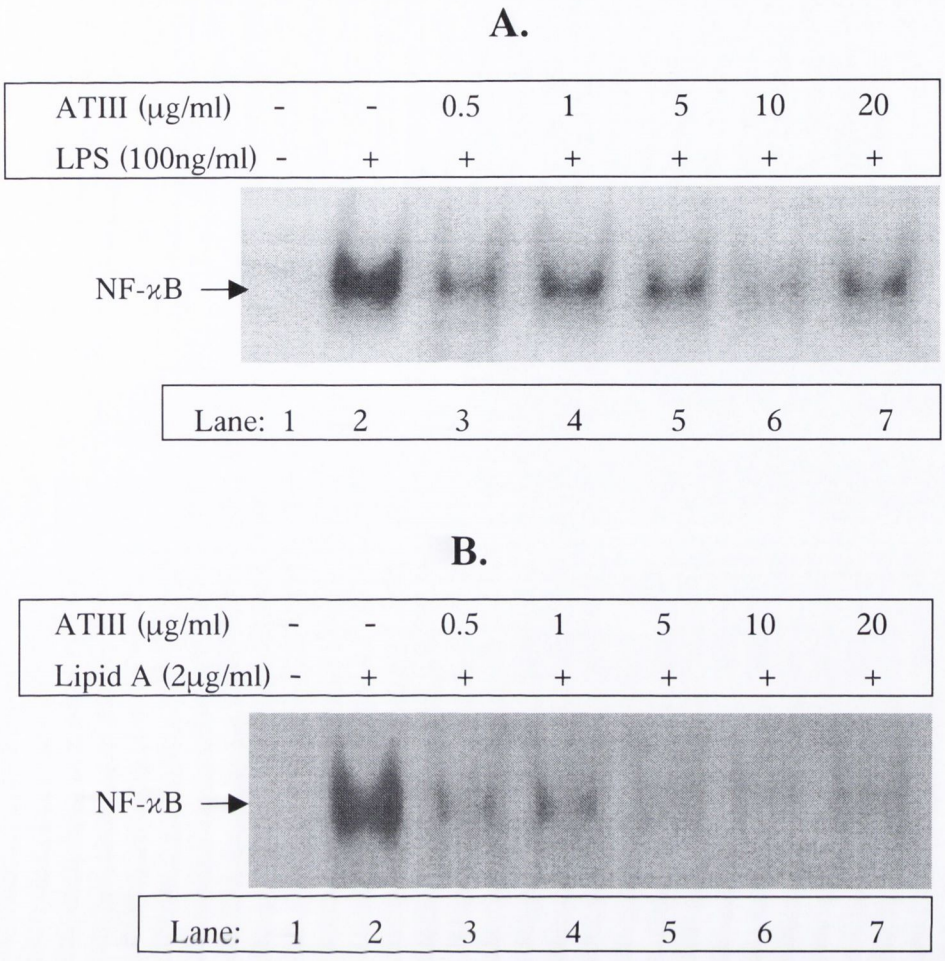


Figure 5.2.7 ATIII inhibits both LPS- and Lipid A-mediated NF-κB activation

THP-1 cells ($1x10^6$/ml) were resuspended in serum-free media, and pre-treated with 0.5-20μg/ml ATIII (lane 3-7) for one minute. Cells were stimulated with either 100ng/ml LPS (*A*) or 2μg/ml Lipid A (*B*) and incubated for a further 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. Inhibition shown is representative of 3 experiments.

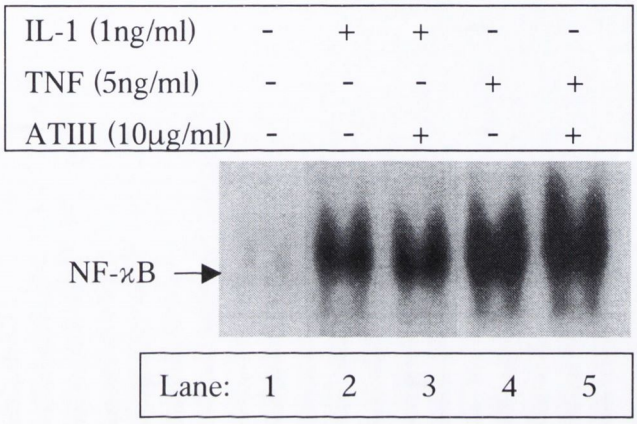


Figure 5.2.8 ATIII does not inhibit NF-κB activation by IL-1 or TNFα

THP-1 cells ($1x10^6$/ml) were resuspended in serum free RPMI 1640 in 24 well plates, then non-treated or pre-treated with 10μg/ml of ATIII (lanes 3-7) for one minute. Cells were then stimulated with TNFα (5ng/ml) or IL-1 (1ng/ml), then incubated for a further 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment.

not affecting common signalling events leading to NF-κB activation within the cytosol.

Next, the requirement of the serpin activity of ATIII was examined. Cells were pre-treated for one minute with an optimal dose of ATIII or an inactive latent form of ATIII (L-ATIII), the inhibitory loop of which is inserted into the A-beta sheet and is thus inaccessible to proteases (277). Figure 5.2.9A demonstrates that L-ATIII at 10μg/ml (lane 4) had no inhibitory effect upon LPS-mediated NF-κB activation (lane 2) as compared to the inhibitory effect achieved by the equivalent amount of native ATIII (lane 3). Higher concentrations of L-ATIII also had no effect (Fig. 5.2.9B).

Taken together, these results suggest that the inhibitory effect of ATIII observed here is specific for LPS-mediated NF-κB activation, and that the inhibitory effect of ATIII requires its serpin activity.

5.2.5 Heparin does not potentiate the ATIII inhibitory effect.

In plasma, ATIII binds the glycoprotein heparin, thereby increasing the ability of ATIII to inhibit thrombin protease activity up to 1000 times (44, 117). Whether Heparin increases the inhibitory effect of ATIII observed here was next studied. A sub-inhibitory amount of ATIII was pre-incubated with an equivalent amount of Heparin for 60 minutes, then incubated with THP-1 cells prior to stimulation with LPS. Figure 5.2.10 demonstrates that Heparin was unable to promote inhibition of

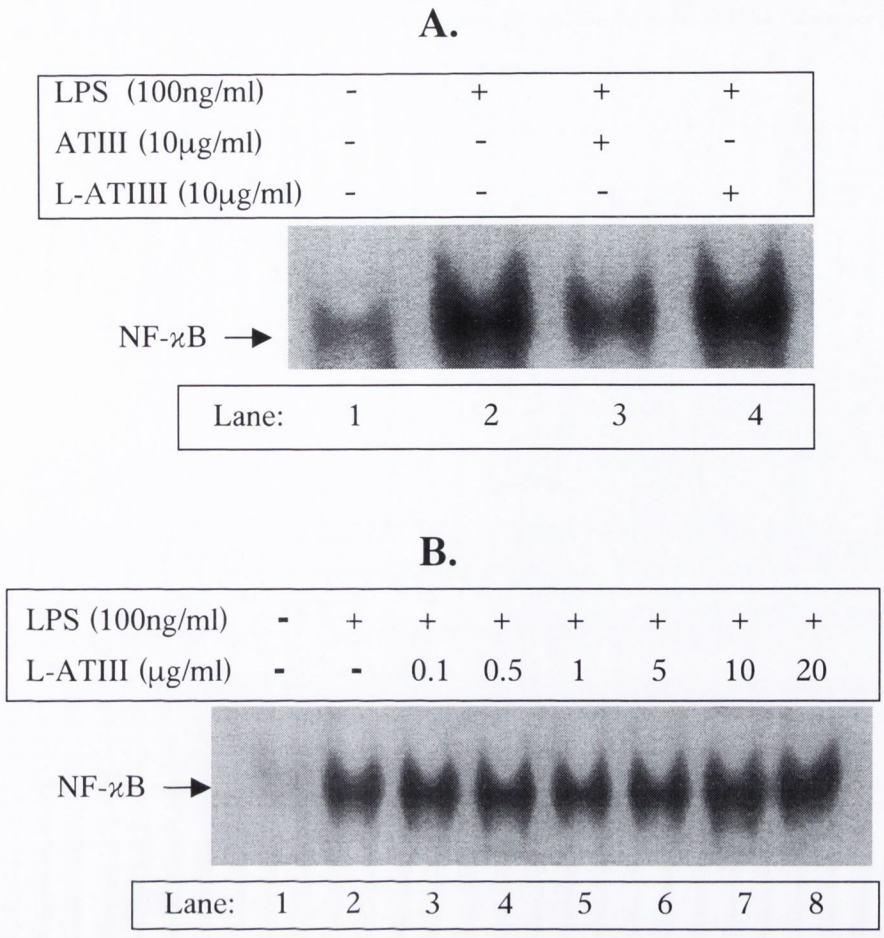


Figure 5.2.9 The effect of ATIII requires its serpin activity

A. THP-1 cells ($1x10^6$ cell/ml) were resusupended in serum free RPMI, and un-treated or pre-treated with 10μg/ml of ATIII (lane 3) or L-ATIII (lane 4) for one minute. Cells were then stimulated with LPS (100ng/ml) and incubated for a further 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. Inhibition shown is representative of 3 experiments. *B.* THP-1 cells ($1x10^6$ cell/ml) were resusupended in serum free RPMI, and un-treated or pre-treated with indicated amounts of L-ATIII (lanes 3-8) for 1 minute. Cells were then stimulated with 100ng/ml of LPS, and incubated for a further 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment.

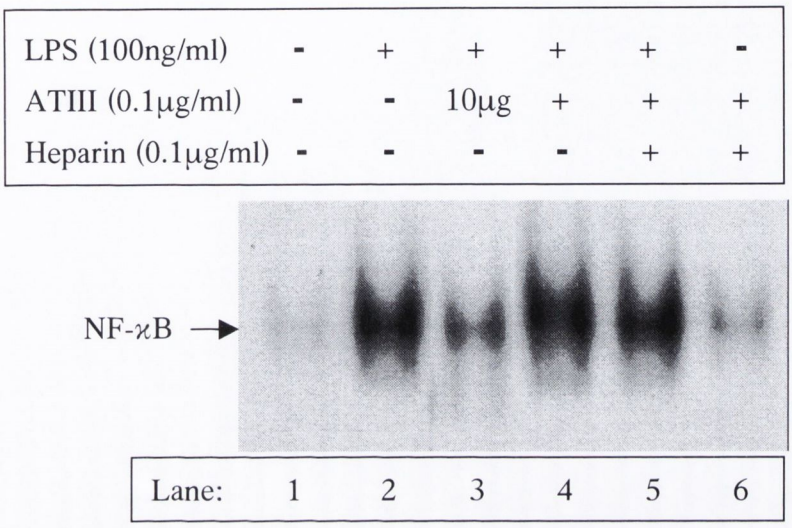


Figure 5.2.10 Heparin does not increase the ability of ATIII to inhibit LPS-mediated NF-κB activation

ATIII (0.1 μg/ml) was pre-incubated with Heparin (0.1 μg/ml) for 60 minutes. Cells (THP-1) were resuspended at $1x10^6$ cells/ml in serum free RPMI and either un-treated or pre-treated with ATIII/Heparin complex for 1 minute (lane 4-5). Cells (lane 3) were also pre-treated for 1 minute with ATIII at 10μg/ml. Cells were then stimulated with LPS (100ng/ml) (lane 2-5) for 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment.

ATIII on NF-κB activation by LPS (compare lane 4 with lane 2), where-as 10μg/ml of ATIII was able to abrogate LPS-induced activation (lane 3) as noted before.

5.2.6 The thrombin inhibitor Hirudin blocks LPS-mediated NF-κB activation.

As ATIII is known to act as an inhibitor of several proteases, the thrombin-specific inhibitor Hirudin (163) was next tested for its ability to inhibit LPS-mediated NF-κB activation. As shown in figure 5.2.11A, Hirudin inhibited LPS-induced NF-κB activation in a dose dependent manner over a concentration range of 1-20U/ml (compare lanes 4-7 with lane 2), with a maximal effect obtained using 10U/ml of Hirudin (lane 6). Additionally, Hirudin was able to inhibit Lipid A-mediated NF-κB activation as potently as that observed for LPS (compare lanes 3-7 with lane 2), however, it appears that Hirudin was effective over a broader concentration range as compared with LPS, inhibiting Lipid A at the lower concentration of 0.5U/ml (compare lane 3 of A with lane 3 of B) (figure 5.2.11B).

5.2.7 Thrombin does not activate NF-κB in THP-1 cells.

Thrombin has previously been shown to activate NF-κB (7). Therefore it was of interest to determine if thrombin was responsible for the effect observed here. However, as can be seen in figure 5.2.12, thrombin failed to induce the activation of NF-κB over a broad concentration range (50-1000μg/ml), and one that exceeds the amounts of LPS used to stimulate THP-1 cells in this study (5-500ng/ml) (compare lane 2 with lanes 3-6). Also, it has previously been reported that thrombin is able to potentiate NF-κB activation at sub-optimal LPS concentrations (101). However, as

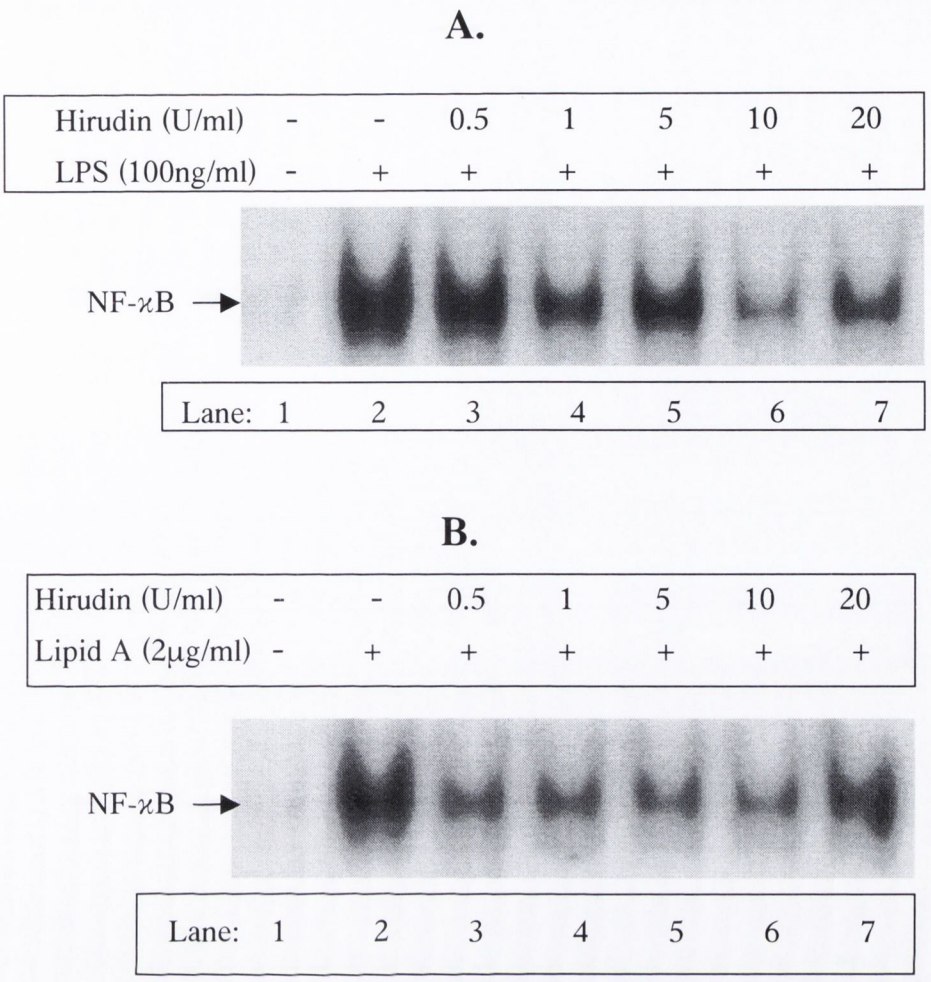


Figure 5.2.11 The thrombin inhibitor Hirudin blocks both LPS- and Lipid A-mediated NF-κB activation

THP-1 cells ($1x10^6$/ml) were resuspended in serum-free media, and pre-treated with 0.5-20 U/ml Hirudin (lane 3-7) for one minute. Cells were stimulated with either 100ng/ml LPS (*A*) or 2μg/ml Lipid A (*B*) and incubated for a further 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. Inhibition shown is representative of 3 experiments.

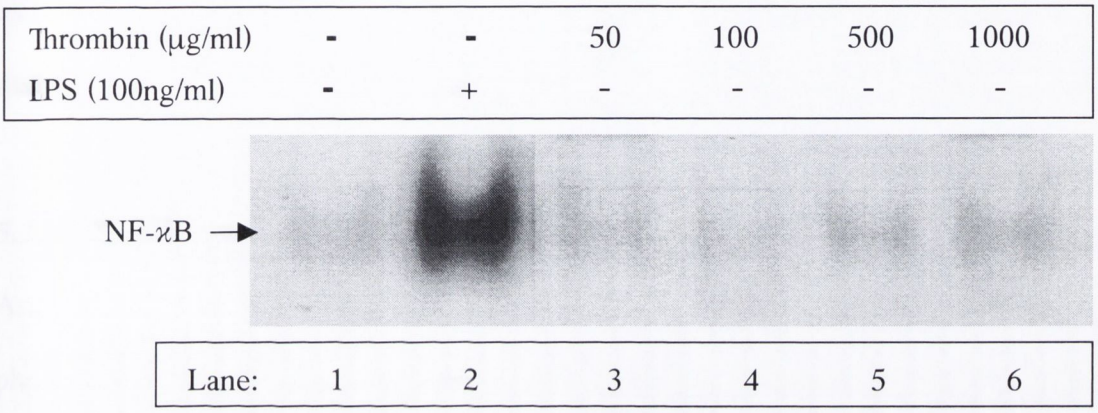


Figure 5.2.12 Thrombin does not activate NF-κB in THP-1 cells

THP-1 cells ($1x10^6$ cell/ml) were resuspended in serum free RPMI and stimulated with a range of concentrations of Thrombin (50-1000ng/ml) (lanes 3-6) and incubated for a further 60 minutes. Lane 2 was also stimulated with 100ng/ml of LPS as a positive control. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment.

shown in figure 5.2.13, it was found that thrombin failed to potentiate sub-inducible levels of LPS (10ng/ml) pre-incubated with a range of concentrations of thrombin (10-1000μg/ml) (compare lanes 4-5 with lane 3). Activation achieved by 100ng/ml of LPS (lane 2) is included for comparison.

From this data it can be concluded that thrombin may not responsible for the effect seen here, and that activation of NF-κB by LPS requires the activation of a protease that is inhibited by ATIII and Hirudin.

5.2.8 LPS-induced phosphorylation of p38.

Another signalling pathway initiated by LPS is that which induces the phosphorylation of p38 MAP kinases (45, 130). For that reason, it was felt that this could be another assay for determining the inhibitory effects of ATIII within a pathway independent of NF-κB activation. Initially, LPS was examined for its ability to induce phosphorylation of p38 in order to determine optimal assay conditions. As can be seen in figure 5.2.14, THP-1 cells stimulated with LPS was optimal at 30-45 minutes post-stimulation (lanes 6 and 7).

The ability of ATIII and Hirudin to inhibit this effect was next examined. Figure 5.2.15 illustrates that ATIII pre-treatment inhibited LPS-induced p38 phosphorylation partially at 10μg/ml as compared to LPS (lane 2); Hirudin also had an inhibitory effect, whilst L-ATIII had a minimal effect.

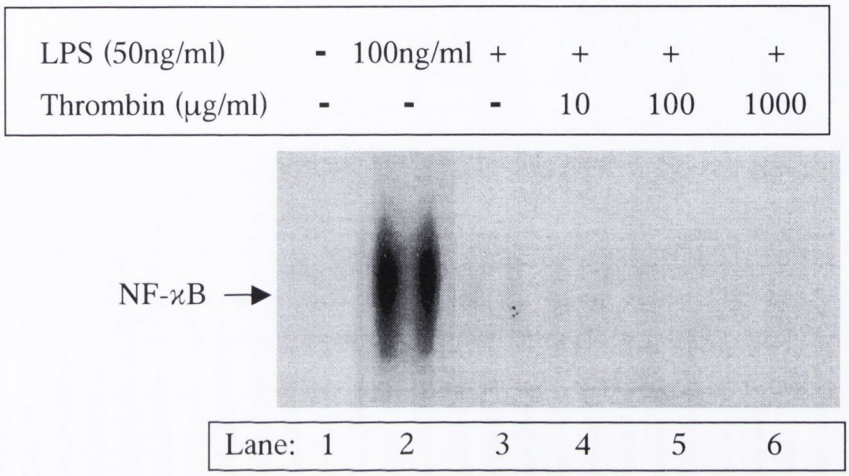


Figure 5.2.13 Thrombin does not potentiate LPS-mediated NF-κB activation

LPS (10ng/ml) was pre-incubated with a range of Thrombin concentrations (10-1000μg/ml) for 60 minutes. THP-1 cells ($1x10^6$/ml) were resuspended in serum free RPMI 1640 in 24 well plates. Cells were then stimulated with LPS (100ng/ml, lane 2; 50ng/ml, lane 3) or the LPS/thrombin mixtures (lanes 4-6) indicated for 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment.

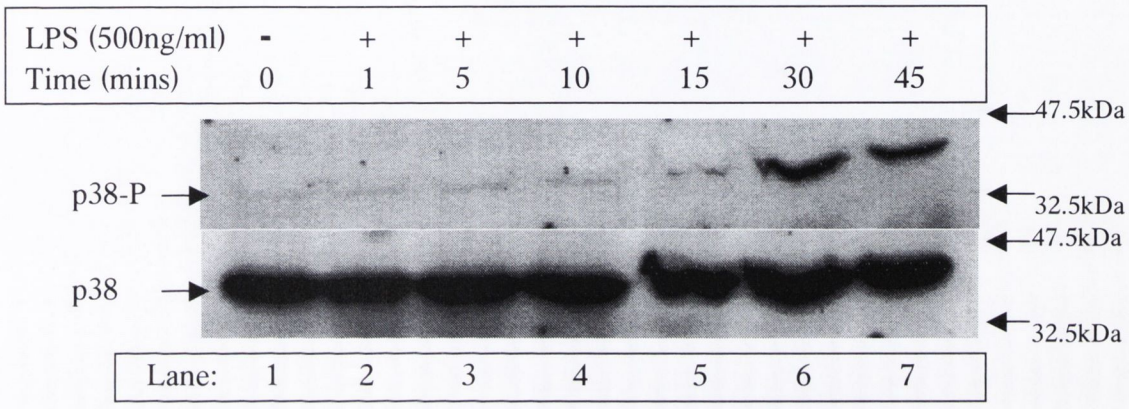


Figure 5.2.14 Time-dependent activation of p38 by LPS

THP-1 cells were resuspended at $3x10^6$ cells/ml in serum free media and stimulated with LPS (500ng/ml) for indicated times. Cells were lysed, centrifuged and and supernatants assessed for p38 levels by western blotting as described in Materials and Methods and seperated by 10% SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with polyclonal phospho-p38 antibody. Only one band was detected at 38kDa, corresponding to each respective phospho-p38. Membranes were stripped and further probed with anti-p38 antibody to determine equal protein loading for each sample. Only one band was detected at 38kDa, corresponding to each respective p38. A similar result was obtained in a further experiment.

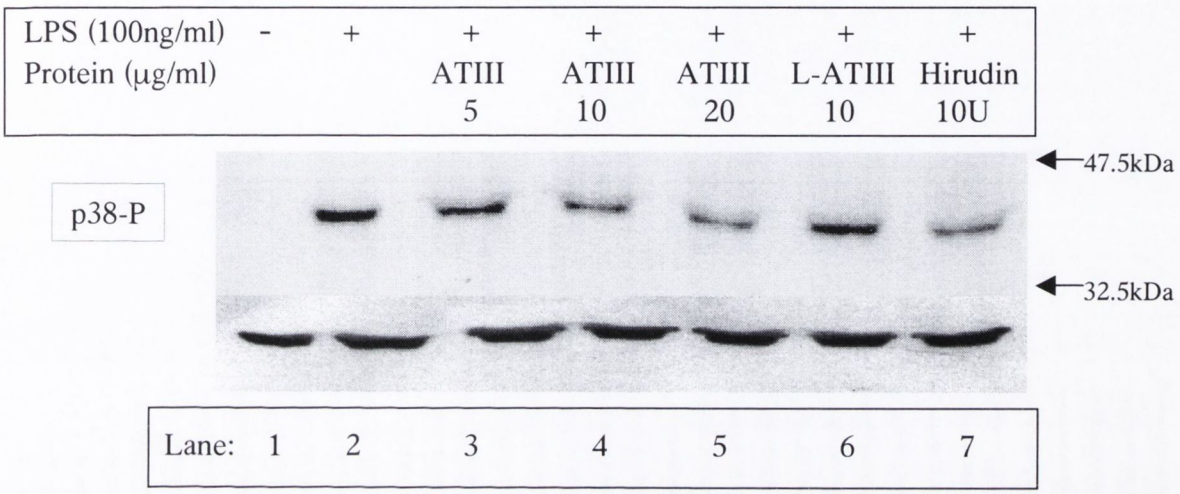


Figure 5.2.15 ATIII and Hirudin inhibit LPS-induced p38 phosphorylation

THP-1 cells ($3x10^6$ cells/ml) in serum free media were pre-treated with indicated proteins for 1 minute. Cells were then stimulated with 500ng/ml of LPS and incubated for a further 30 minutes. Cells were lysed, centrifuged, and assayed for Phospho-p38 levels by western blotting as described in Materials and Methods and seperated by 10% SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with Phospho-p38 antibody. Membranes were stripped and further probed with anti-p38 antibody to determine equal protein loading for each sample. Only one band was detected at 38kDa, corresponding to each respective form of p38. A similar result was obtained in a further experiment.

This result, provided further evidence that ATIII inhibition of LPS signalling.

5.2.9 LPS induces activation of a Thrombin-like protease.

As activation of Toll in *Drosophila* requires the activation of an extracellular serine protease cascade (145), the ability of LPS to induce the rapid activation of a protease in mammalian cells was next determined. THP-1 cells were therefore treated with LPS and protease activity monitored using a substrate that is specifically cleaved by thrombin-like proteases. As shown in figure 5.2.16, while THP-1 cells demonstrate considerable basal protease activity in non-stimulated cells, there is an increase in protease activity observed with 7.5 minutes post-stimulation. This protease activity was significantly enhanced above non-stimulated basal activity between 15-30 minutes ($p=0.016$ and 0.021 respectively) post LPS treatment. After 60 minutes however, no difference was observed between untreated and stimulated cells.

This result suggests that LPS can induce a thrombin-like protease activity over basal protease activity, in a time dependent manner, and further supports the earlier suggestions of basal cellular protease activity inactivating ATIII in a time dependent manner.

ATIII and Hirudin were examined for their ability to inhibit this LPS-induced increase in thrombin-like protease activity; however, their inhibitory effect was inconsistent (data not shown). This may have been because of the small increase in protease activity observed.

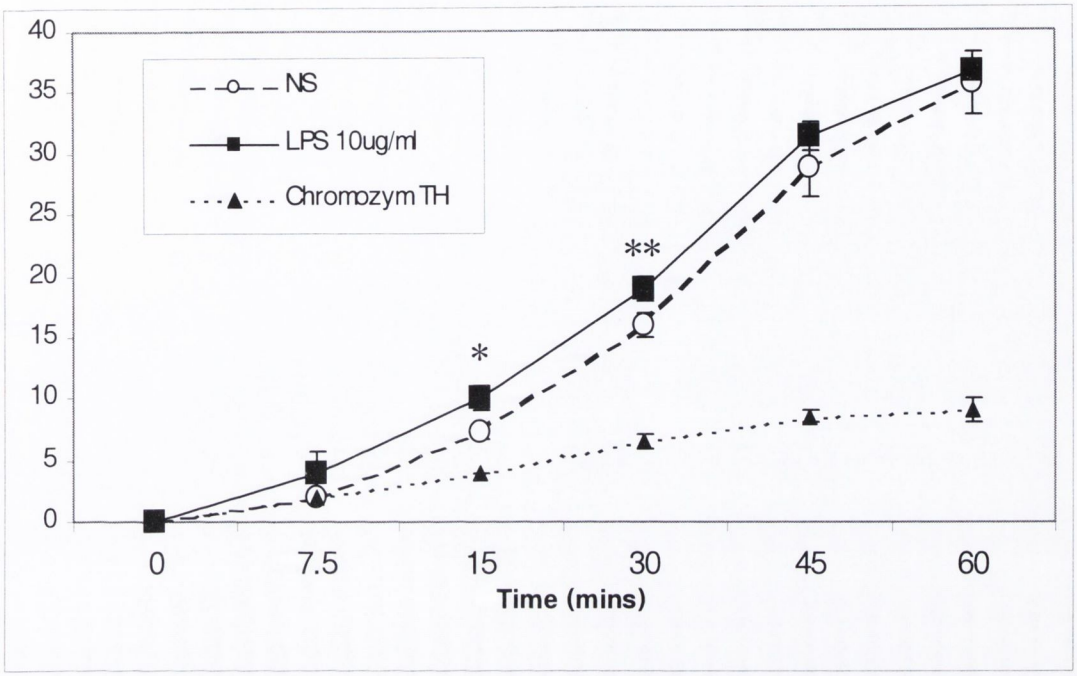


Figure 5.2.16 LPS-induces Thrombin-like protease activity

THP-1 cells ($7.5x10^4$/ml) were resuspended in freshly prepared RPMI 1640 (phenol-red free) containing Chromozym TH in 96 well plates. Cells were than stimulated with 10μg/ml of LPS. 30μl of cell suspension is removed and diluted into 170μl of RPMI 1640 (phenol-red free) containing Chromozym TH solution in 96 well plates. Samples are spectrometrically assayed at relevant time points. Results are shown as absorbance units per individual sample and are expressed as means ± S.D. (n=3). NS= not stimulated. Chromozym TH data represents substrate degradation in cell-free media. Data indicate significant differences (*P=0.016 and **P=0.021) when compared with non-stimulated control values. Results shown are representative of 4 experiments.

5.2.10 ATIII and Hirudin inhibit LPS-mediated NF-κB activation via TLR4.

Previous studies have determined that LPS activates NF-κB via TLR4 and its co-receptors CD14 and MD2 (reviewed in (1, 2, 192, 193). Further studies have also shown that contaminants of commercial LPS preparations are able to activate NF-κB via TLR2 (100). Therefore it was important to establish that the ability of ATIII and Hirudin to inhibit LPS-mediated activation of NF-κB was specific for a LPS-TLR4 response, and not inhibiting contaminants signalling via TLR2.

Therefore, the ability of LPS to stimulate NF-κB in HEK 293 cells stably transfected with TLR4 was next examined. Parental HEK 293 cells do not respond to LPS and do not express TLR2. Cells were stimulated with a range of concentrations of LPS, and assayed for its ability to induce translocation of NF-κB by EMSA. As can be seen in figure 5.2.17, activation of NF-κB was initially observed at 50ng/ml LPS (lane 2), reaching a maximal response at 200ng/ml (lane 4) of LPS, as compared to non-stimulated cells (lane 1). From this result, it was determined to use 200ng/ml of LPS for further stimulations.

The cells were pre-treated with ATIII, L-ATIII and Hirudin, and then stimulated with LPS. As shown in figure 5.2.18, ATIII was able to inhibit LPS-mediated NF-κB activation at 10μg/ml (compare lane 3 with lane 2). Hirudin at 10U/ml was also able to reduce this effect (compare lane 5 with lane2), however, NF-κB activation was unaffected by L-ATIII (10μg/ml) (compare lane 4 with lane 2).

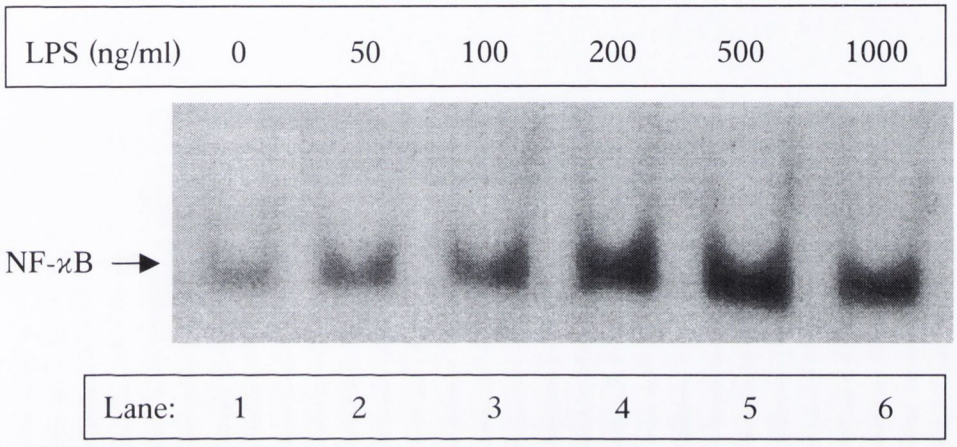


Figure 5.2.17 Dose dependent effects of LPS on NF-κB activation of HEK 293 cells stably transfected with TRL4

THP-1 cells were resuspended in serum free RPMI 1640 at $1x10^6$/ml in 24 well plate. Cells were subsequently stimulated with the indicated concentrations of LPS, increasing from 50ng/ml to 1000ng/ml (lanes 2-6). Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. A similar result was obtained in a further experiment.

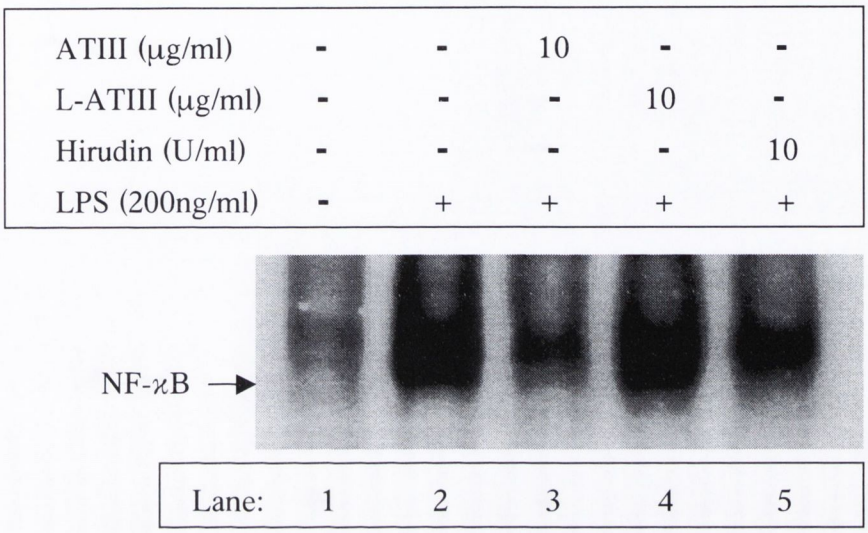


Figure 5.2.18 ATIII and Hirudin Inhibit LPS-induced NF-κB activation via TLR4

Stably transfected HEK 293-TLR4 cells ($5x10^4$/ml) were seeded 48 hours prior to treatment. Cells were pre-treated for one minute with indicated serine protease inhibitor, then stimulated with 200μg/ml LPS and incubated for a further 60 minutes. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB-DNA complexes are shown in each case. Inhibition shown is representative of 3 experiments.

This result would suggest that the effects of ATIII and Hirudin responsible for inhibiting LPS-mediated NF-κB activation are blocking LPS signalling acting via TLR4.

5.3 Discussion

Structural features of the innate immune response are highly conserved throughout evolution. The high degree of homology between the *Drosophila* and mammalian Toll-Dorsal/IL-1R-NF-κB pathway has led to several discoveries in the field of mammalian innate immune response. Crucially, the discovery of the mammalian TLR family relied heavily upon genomic searching of the mammalian database for Toll homologs. In *Drosophila*, Toll responds to fungal pathogens (109-111). Genetic evidence suggests the activation of Toll occurs via the binding of microbial cell components to as yet undetermined pattern recognition receptors, which activate blood protease zymogens. This results in the proteolytically generated peptide Spaetzle (182), the putative ligand for Toll. Proteolytic processing of Spaetzle requires the activities of six preceding proteases (reviewed in (145)). Loss of function mutation of the serpin, Spc43Ac, leads to constitutive Spaetzle and Toll activation (145, 146, 182). Though mammalian TLR4 has been described as the putative receptor for LPS (209, 215, 216), no direct binding studies have been performed. Prompted by the high degree of similarity between *Drosophila* and mammalian innate immune response, and the inhibitory effect of the serpin Spc43Ac, the aim of this study was to investigate the effect of the serpin ATIII on LPS-mediated NF-κB activation in the human monocyte cell line THP-1. ATIII has previously been shown to protect baboons from LPS-induced septic shock (177), and a recent study found that ATIII could inhibit LPS-induced expression of Interleukin (IL)-6, an NF-κB-dependent gene (253).

This study has demonstrated the ability of ATIII and Hirudin, a thrombin specific inhibitor (163), to specifically inhibit both LPS- and Lipid A (the active moiety of LPS (217)) -induced NF-$\varkappa$B activation in a dose dependent manner. Crucially, the ability of ATIII and Hirudin to inhibit LPS activation of NF-$\varkappa$B, was found to be time dependent, requiring short pre-incubation times for the inhibitory effect to be observed. Further research demonstrated the activation of a thrombin-like protease in THP-1 cells when stimulated with LPS. This activity was transient in nature and required above normal concentrations of LPS. Though the effect was subtle, this would be expected if LPS were activating a specific serine protease, generating a specific ligand. This assay also demonstrated a high basal activity of thrombin-like proteases, which may explain the need for short pre-treatment times necessary to observe the effect described here, as endogenous protease activity would quickly inactivate ATIII. Importantly, the effect of ATIII required its serpin activity, as a latent form of ATIII was unable to inhibit LPS-mediated NF-$\varkappa$B activation. Also, ATIII was found to have no effect upon NF-$\varkappa$B activation induced by the cytokines IL1 and TNFα, of which IL1 shares the same cytoplasmic signalling pathway as that initiated by LPS (33, 194, 195), further supporting the specificity of ATIIIs inhibitory effect seen here.

ATIII is a 58kDa glycoprotein that inhibits thrombin in two kinetically distinct steps; the formation of a weak initial complex, followed by rapid conversion to a stable complex (191). The inhibitory action of ATIII occurs upon recognition and cleavage of the Arg^{393}-Ser^{394} bond in an exposed loop of ATIII, which induces a conformational change in ATIII resulting in the trapping of the enzyme either as a

tetrahedral intermediate, or as an acyl-enzyme. The thrombin and ATIII complex is stable and resistant to hydrolysis. Although ATIII acts predominantly as a plasma inhibitor of thrombin, it also has potent effects on a variety of active serine proteases, including factors IXa, Xa, Xia, XIIa, and FVIIa bound to tissue factor. Hirudin however, is thought to act as a specific inhibitor of thrombin (30, 258). As both ATIII and hirudin are capable of inhibiting LPS activation of NF-κB, it would be reasonable to assume that the protease involved in the extracellular action of LPS which leads to NF-κB activation, could be thrombin. It is generally recognized that activation of coagulation is closely linked to immune and inflammatory responses. Thrombin is known to act as a potent proinflammatory agonist of endothelial cells, and also has receptor-mediated chemotactic and mitogenic activities for monocytes and macrophages (17-19). A unique family of tethered ligand-type receptors, termed PAR 1,3-4, mediates these biological activities of thrombin. The receptor is proteolytically cleaved by thrombin near the N-terminus region, resulting in a new N-terminus that serves as a tethered peptide ligand. Thrombin has previously been shown to activate NF-κB via PAR1, inducing the up-regulation of IL-8 and E-selectin in endothelial cells (7). At sites of inflammation, thrombin cleaves fibrinogen to yield fibrin monomers, stimulating local fibrin(ogen) deposits. Fibrin(ogen) has previously been reported to promote CD11b/CD18-dependent NF-κB activation and IL-1β expression by monocytes (207, 248). Furthermore, a recent study found that macrophages and monocytes stimulated with fibrinogen and LPS induced chemokine secretion. This induction required the expression of functional TLR4 (250) and did not require thrombin-mediated proteolysis of fibrinogen,

although they have yet to formally demonstrate fibrinogen signalling directly through TLR4. Interestingly, under certain experimental conditions, full leukocyte response to LPS required the expression of both TLR4 and CD11b/CD18 (206). Smiley et al (250) postulated that fibrinogen, Spaetzle, and coagulogen are functional homologues, each polymerizing upon activation of an upstream protease cascade.

The protease activity however, is unlikely to involve thrombin, as thrombin itself was unable to initiate NF-κB activation, nor as previously reported, could it potentiate sub-optimal concentrations of LPS to activate NF-κB, over a broad concentration range. Furthermore, in plasma, ATIII binds Heparin, thereby increasing its ability to inhibit thrombin by several hundred-fold over ATIII alone (44, 117). However, Heparin had no effect upon the ability of sub-inhibitory levels of ATIII to block LPS-mediated NF-κB in this study, suggesting that thrombin is not the substrate for ATIII in the effect reported here. It is possible that Hirudin is targeting another protease here, but there is the possibility that membrane bound thrombin could be responsible for the effect seen here, that the addition of free thrombin fails to mimic. Additionally, thrombin was commercially sourced, and the specific activities of commercial preparations are quite variable and their purity is not always satisfactory (243). Furthermore, commercial preparations often contain the autolytic derivatives of thrombin that do not cleave fibrinogen. Thrombin is also known to precipitate and absorb to glass and plastic surfaces at low concentrations and ionic strength, these effects remedied by addition of poly(ethylene glycol) or bovine serum albumin (243). As my experiments were carried out with a commercial thrombin preparation and

also in serum free media, these effects could account for the inability of thrombin to induce NF-κB activation, or potentiate LPS stimulation.

ATIII and Hirudin were able to inhibit LPS-induced activation of NF-κB in HEK 293 cells that were rendered LPS responsive by stably transfecting them with TLR4. This result suggests the possibility that the protease activated by LPS generates a ligand that is able to activate TLR4 specifically.

Though no explicit evidence has been shown supporting direct binding between LPS and TLR4, several studies have been published suggesting such an interaction via genetic and complementation studies (210). Lipid IVa, a partial struture of the active LPS moiety Lipid A, acts as an agonist of mouse TLR4, however, it acts as an antagonist in humans (149). Transfection of mouse macrophages with human TLR4 conferred an antagonist effect to Lipid IVa, while human macrophages transfected with mouse TLR4, detected Lipid IVa as an agonist. These findings suggest that TLR4 alone is able to determine the response to Lipid IVa. A recent report however, suggests that MD2 is responsible for this species-specific pharmacology (122). Taxol, a plant derived anti-tumor agent, has been found to mimic many LPS-like activities, requiring TLR4 and MD2 to induce NF-κB activation in murine macrophages, but not on human LPS-responsive cells including macrophages (6, 143). Kawasaki et al (122) recently demonstrated that this species-specific responsiveness was due to MD2. Taxol-induced signalling was mediated by mTLR4-mMD2 complex but not by hTLR4-hMD2, suggesting specific taxol

recognition within mTLR4-mMD2 that is absent in the human complex. Human and mouse TLR4 and MD2 share 69% and 66% homology respectively, the relatively low homology could account for the species-specificity seen for taxol recognition. Kawasaki et al further demonstrated that mMD2 was important for mediating taxol signalling, but not hMD2. Although the structures of taxol and LPS are quite different, these findings seem to suggest that the mouse and human TLR4/MD2 signalling complex can identify distinct stimuli and respond to them accordingly.

Two recent studies have illustrated the close proximity of LPS to the TLR4/MD2 signalling complex (54, 272). The first study crosslinked these components with radioiodinated LPS in transfected human HEK 293 cells. Though LPS was found to reside within close proximity of the signal complex, anti-TLR4 antibodies that inhibit LPS-induced NF-κB dependent gene expression were unable to reduce radiolabeling of TLR4 and MD2. This study concluded that while LPS was indeed in close proximity of TLR/MD2, additional steps were required for cell activation. The authors suggest that the antibody may interfere with receptor oligomerisation. Another possibility is that the antibody blocks the binding of an endogenous ligand. The second report found that MD2 was able to specifically bind LPS in the absence of either LBP or CD14, and in the case of CD14, actively compete out CD14 binding LPS. The functional effects of added MD2 however had varied effects, mediating NF-κB activation in TLR4 transfected hamster CHO cells, yet in human U373 cells, and whole blood, MD2 inhibited LPS responses. Yet again, there is the suggestion that MD2 is responsible for the species-specific response, but also that proximity of

TLR4 to MD2, either bound or soluble, may not be sufficient to induce NF-κB activation.

The data presented here suggests that the additional step required for TLR4 activation could be the activation of a protease, which proteolytically generates a ligand. It is possible that LPS may be required to form a complex with CD14/MD2 and TLR4, and that this complex initiates or participates in the protease activity described, the resulting ligand acting in an analogous manner to that seen with Spaetzle and Toll in *Drosophila*.

In conclusion, I have shown that NF-κB activation induced by both LPS and Lipid A in the monocytic cell line THP-1, is sensitive to inhibition by the serpin ATIII. Furthermore, activation of NF-κB by LPS via TLR4-mediated signalling was also sensitive to serpin inhibition. These findings would suggest that extracellular activity by LPS in mammals is susceptible to inhibition of a serine protease, analogous to inhibition of pathogen activation of Toll in *Drosophila* by the serpin Spc43Ac. Interestingly, the ability of the thrombin-specific inhibitor Hirudin, to also abrogate this effect, suggested that thrombin could be the protease involved in mediating the LPS effect. LPS was also found to activate the proteolytic cleavage of a substrate by a thrombin-like protease, further supporting the involvement of the enzymes. However, free thrombin could not mimic, or potentiate activation of NF-κB in this system. It has been generally established in the past few years that the mammalian receptor complex for LPS detection and immune response signalling comprises

CD14, MD2 and TLR4. However, several recent studies have concluded that the complete LPS receptor complex is comprised of at least one additional protein. From the results presented here, I believe that the additional protein may comprise the proteolytically generated product of a thrombin-like serine protease.

Chapter Six

Final Conclusion and Comments

Chapter 6.

General Discussion and Future Perspectives

The recognition of infecting microbes followed by the induction of a pro-inflammatory response is essential for the survival of most multicellular organisms. NF-$\varkappa$B acts as the key modulatory transcription factor of the innate immune response, its activation a critical early indication of the presence of microbes within the host. Therefore, a greater understanding of the mechanisms and signal transduction pathways that lead to the activation of NF-$\varkappa$B are crucial for our understanding of host-pathogen interactions. The aim of this study was to investigate how two bacterial products, the invasive protein, InlB, from gram-positive *L. monocytogenes* and LPS from gram-negative bacteria, mediate activation of NF-$\varkappa$B.

This is the first study to report the activation of NF-$\varkappa$B by InlB and the investigation of the signal transduction pathway involved, increases our understanding of host-pathogen interactions of *L. monocytogenes* and its host. On the other hand, the signal transduction pathway induced by LPS that leads to NF-$\varkappa$B activation in mammals, is one of the most intensely studied transduction pathways of the immune system. However, it is only recently with the discovery of TLR4, in conjunction with MD2, as the signalling complex for LPS, has our attention once again focussed on the intracellular mechanisms by which the host recognises gram-negative bacteria.

An important feature of the InlB discovery relates to cell-type specificity. I have shown that InlB activates NF-$\varkappa$B in the macrophage cell lines P388D_1 and RAW 264, and the

epithelial cell line Hep2. However, InlB failed to induce NF-κB activation in a variety of other cell lines, in several of these InlB has been shown to activate PI-3 kinase. The understanding of signal transduction pathways is extremely complex, and it has been widely found that the same stimuli can induce varied effects upon a wide range of cell types. This problem is well illustrated by the example of Delhase et al (58) attempting to replicate the demonstration that in TNF signalling, Akt induces the phosphorylation of the IKK signalsome, mediating activation of NF-κB (199). Their attempts to replicate this finding in HeLa cells were unsuccessful, yet the original researchers were able to demonstrate that the effect was cell specific and depended on the amount of Akt present in the cells, and the extent of its activation. In chapter 3 I discussed the differences in effect of InlB-mediated NF-κB activation in different cell lines and related this to the possible differences in the cells types, and the abundance and expression of particular signal transducters (257). The discovery that Akt is acting as a downstream transducer of the effect of InlB suggests the levels of Akt could be responsible for the differences in cell-specific response that was observed here.

A second aspect concerns the downstream effects of InlB-mediated NF-κB activation. InlB-induces a pro-inflammatory response as evidenced by the expression of IL-6, IL-8 and TNF. NF-κB activation could also be part of an anti-apoptotic response, which involves Akt. NF-κB has been shown to be anti-apoptotic (25, 276), via induction of genes encoding anti-apoptotic proteins such as inhibitors of apoptosis (IAPs; IAP-1, IAP-2, and XIAP) (135) or the Bcl-2 family of anti-apoptotic proteins. Apart from NF-κB activation, the anti-apoptotic effect of Akt could be due to phosphorylation or

sequestering of proteins of the Forkhead and Bcl-2 families, causing their retention in the cytoplasm, thereby preventing pro-apoptotic events (55). Also, Akt-induced phosphorylation of pro-apoptotic caspase-9 decreases apoptosis by inhibiting its protease activity. Therefore, this single regulatory molecule could exert its anti-apoptotic effects in a variety of ways.

One possibility is that Akt activation is preventing Anoikis- a type of apoptosis triggered by inadequate or inappropriate cell-matrix contacts. Many mammalian cell types are dependent on adhesion to the extracellular matrix for their continued survival. Detachment from the matrix induces programmed cell death. The integrin family of surface proteins mediates interaction of cells with the extracellular matrix (reviewed in (82)). Upon binding to matrix proteins, integrins transmit an 'outside-in' signal to the cells, thus triggering a large array of intracellular signals and rearrangement of the cytoskeletal matrix. Engagement of integrins with extracellular matrix proteins activates PI-3 kinase (125), thus providing a protective signal through Akt that blocks apoptosis. InlB, through interaction with its receptor c-Met, has been shown to induce membrane ruffling, cell scattering and the disruption of cell-cell junctions. InlA interacts with its receptor E-cadherin, a member of the integrin family that mediates formation of adherens junctions between epithelial cells, to promote *L. monocytogenes* invasion (174, 175). It has been proposed that InlB may promote access for InlA to E-cadherin *in vivo*. Therefore, InlB-induced cell detachment from the matrix, or InlA binding of E-cadherin, may trigger programmed cell death, or Anoikis. It has previously been shown however, that detached cells can be rescued from Anoikis by the stimulation of Ras, PI-3 kinase

and Akt (127, 129, 224, 225). Thus PI-3 kinase and Akt play a critical role in sensing the adhesion status of the cell and promoting its survival. Therefore, InlB-mediated activation of Ras, PI-3 kinase and Akt, may act as a survival signal that compensates for InlB-induced cell disruption from the extracellular matrix, or InlA interaction with E-cadherin, which would lead to Anoikis. This would allow successful *L. monocytogenes* invasion of the host, and also ensure cellular survival to allow bacterial replication and further cell-cell invasion. This effect may explain the finding that *L. monocytogenes* does not induce apoptosis in bone marrow-derived macrophages, but promotes a delayed necrosis mediated by LLO (20).

The data presented in this study correlates well with the previously known signal transduction pathways initiated by ligand binding of c-Met. Therefore our data further supports the earlier finding of Shen et al (244), of c-Met acting as the mammalian receptor for InlB, a demonstration of a bacterial product utilising a mammalian receptor, the downstream function of which may be beneficial to the bacterium. It remains to be determined if the InlB-mediated activation of NF-$\varkappa$B via c-Met, is a 'by-product' of Akt activation in a cell specific manner, or an example of the host pro-inflammatory response in reaction to bacterial invasion, or possibly both. These findings in regard to InlB-mediating cytosolic signalling raise many interesting points regarding bacterial invasion of cells, the host response, bacterial survival and subsequent replication.

While InlB activation of NF-$\varkappa$B via the subjugation of a mammalian growth factor receptor is an excellent example of bacterial adaptability, the demonstration that LPS-

induced activation of NF-κB in monocytes is susceptible to serpin inhibition, further illustrates the evolutionary conservation of the innate immune response.

Studies have shown that TLR4 associates with MD2 to form an active signalling complex (122), where the interaction of CD14 and LPS induces the activation of NF-κB in response to gram-negative bacterial invasion. It has become increasingly evident though that there may be another component of this signalling complex that is yet to be elucidated. From the data presented in this study, I would suggest that other component might comprise a proteolytically generated product of a serine protease that is activated by LPS. I have shown that ATIII and Hirudin can inhibit LPS activation of NF-κB, and the serpin activity of ATIII was required to observe this effect. The identity of the serine protease involved is unknown. As Hirudin is a specific inhibitor of thrombin, it would be assumed that thrombin is the protease responsible for the effect. Thrombin has been shown induce a pro-inflammatory response in cells, and Fibrinogen, a thrombin substrate of the coagulation cascade, has recently been found to activate TLR4 (250). However, I was unable to show any thrombin involvement in LPS or thrombin-mediated activation of NF-κB. The experiments conducted here, however, may not mimic membrane-bound thrombin, which could be responsible for the effect.

The inhibitory effect of ATIII and Hirudin was further supported by the demonstration of LPS-induced activation of a thrombin-like serine protease. This response however was low, but consistent. It remains to be determined if a more sensitive assay could be used to enhance the increase of protease activity, or lower the concentration of LPS required, to

observe an effect. This may also allow the inhibitory effect of ATIII and Hirudin upon LPS-induced protease activity to be determined.

It has been twelve years since the introduction of the concept of pattern recognition receptors (116). The description and characterisation of the TLR family as the mammalian receptors for microorganisms and microbial products has greatly increased our understanding of the innate immune system. Much of this work was informed by the evolutionary conservation of the innate immune system found in plants, insects and mammals. Activation of TLRs induces an immune response involving expression of anti-microbial effector molecules (116) as found in *Drosophila*, but also cytokines and co-activator molecules that activate the adaptive immune system. Significant progress has been made in understanding the specific roles of the Rel proteins in the antifungal and antibacterial immune response in *Drosophila*. However, specific responses of the mammalian Rel family members are yet to be fully understood. The specific target genes induced by TLR signalling in response to different microbial antigens also remain largely unknown. Different TLRs are already known to elicit different responses, with the recent discovery of the TLR4 adapter protein MyD88-adapter like (Mal) (79, 104), further illustrating the complexities of specific TLRs in mediating specific responses to individual microbial products. These findings suggest that the innate immune response has conserved evolutionary aspects and a hitherto unsuspected specificity.

In conclusion, this study reveals intricacy and complexity in the interaction between bacterial products, host receptors and signalling pathways. These findings increase our

understanding of host-pathogen interactions and may provide targets for drug development against the virulent pathogenesis of listeriosis and septic shock.

Chapter Seven

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Chapter 7

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Chapter Eight

Record of Publications

CHAPTER 8

8. RECORD OF PUBLICATIONS

1. Mansell A, Khelef N, Cossart P, O'Neill LA. (2001), Internalin B activates NF-κB via Ras, PI-3 kinase and Akt. J Biol Chem. 2001 Sep 24 [epub ahead of print].

2. Mansell A, Reinicke A, Worrall D.M and O'Neill LAJ. (2001) The Serine Protease Inhibitor Antithrombin III Inhibits LPS-mediated NF-κB Activation by TLR4. FEBS Letters (Submitted September)

3. Mansell A, Braun L, Cossart P, O'Neill LA. (2000) A novel function of InlB from *Listeria monocytogenes*: activation of NF-κB in J774 macrophages. Cell Microbiol., **2**:127-36.

Chapter Nine

Appendices

CHAPTER 9

Appendices

APPENDIX I

Addresses of suppliers

Amersham International	Export Sales Division Lincoln Place, Green End Aylesbury Bucks HP20 2TP United Kingdom.
BDH Limited	P.O. Box 8 Dagenham Essex RM8 1RY United Kingdom
Boehringer Mannheim GmbH	Biochemica P.O. Box 31 01 20 D-6800 Mannheim 31 Germany
Calbiochem	Freepost Nottingham NG7 2BR United Kingdom
Clonetics Corporation	9620 Chesapeake Drive Suite 201 San Diego CA 92123 USA
European Collection of Animal Cell Cultures	PHLS Center for Applied Microbiology and Research Porton Down Salisbury SP4 OJG United Kingdom

Gibco BRL *Life Technologies Ltd.*	P.O. Box 35, Trident House Renfrew Road Paisley PA3 4EF United Kingdom
Genentech	Genentech, Inc. 1 DNA Way South San Francisco, CA 94080-4990 USA
Greiner GmbH	Maybachstrasse P.O. Box 1162 D-7443 Frichenhausen Germany
HyClone	1725 South HyClone Road Logan Utah 84321 USA
ICN Biomedicals Inc.	PO Box 26221, Birmingham, AL 35226, USA
Invitrogen	PO Box 2312, 9704 CH Groningen, Holland
National Diagnostics	305 Patton Drive Atlanta Georgia 30336 USA
New England Biolabs	32, Tozer Road, Beverly, MA01915-5599, USA
Nunc A/S	Kamstrupvej 90

	DK-4000 Roskilde Denmark
Pharmacia Biosystems Ltd	Pharmacia LKB Biotechnology AB Davy Avenue Knowhill Milton Keynes MK5 8PH United Kingdom
Promega Corporation	2800 Woods Hollow Road Madison WI 53711-5399 USA
QIAGEN Ltd.	Boundary Court Gatwick Road Crawley West Sussex RH10 2AX United Kingdom
R&D Systems Europe Ltd.	4-10 The Quadrant Barton Lane Abingdon OX14 3YS United Kingdom
Santa Cruz Biotechnology, Inc.	2161 Delaware Avenue Santa Cruz California 95060 USA
Sigma Chemical Company Ltd	Fancy Road Poole Dorset BH17 7BR United Kingdom
Southern Biotechnology	Birmingham, Alabama, USA

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A novel function of InlB from *Listeria monocytogenes*: activation of NF-κB in J774 macrophages

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Summary

***Listeria monocytogenes* causes a pro-inflammatory response on adhesion to macrophages. Upregulation of inflammation genes involves the transcription factor NF-κB. Several components of *L. monocytogenes*, including lipoteichoic acid (LTA), phospholipases and listeriolysin O (LLO), have since been shown to mediate NF-κB activation. Here, we report that purified recombinant InlB, but not internalin (InlA), is a potent activator of NF-κB in the mouse macrophage-like cell line J774. Expression of InlB in *Listeria innocua* enhances its ability to activate NF-κB, while deletion of InlB from *L. monocytogenes* marginally decreases its effect on NF-κB, possibly because of the presence of NF-κB activators such as LTA and LLO. The effect correlates with the rapid degradation of IκBα, a sustained degradation of IκBβ and increases in tumour necrosis factor alpha (TNF-α) and interleukin (IL) 6 production, two cytokines controlled by NF-κB. Using a series of anti-InlB monoclonal antibodies and domains of InlB, NF-κB activation was shown to be dependent upon the N-terminal 213-amino-acid leucine-rich repeat (LRR) domain of InlB, recently demonstrated to be responsible for InlB-mediated *L. monocytogenes* invasion and phosphoinositide-3 (PI-3) kinase activation. The effect of InlB was blocked by PI-3 kinase inhibitors, indicating the involvement of PI-3 kinase in this response. This report thus illustrates that InlB not only promotes invasion, but also contributes to the macrophage pro-inflammatory response.**

Introduction

Listeria monocytogenes is a Gram-positive, food-borne pathogen, which infects both phagocytic and non-phagocytic cell types (Dramsi *et al.*, 1996; Cossart and Lecuit, 1998). Once the bacterium has been internalized into cells, it rapidly replicates within the cytosol. Concomitantly, it becomes coated with actin filaments, starts moving intracellularly and spreads from cell to cell, thus avoiding circulating antibodies or other extracellular bactericidal components (Ireton and Cossart, 1997). Immunity to this bacterium is T-cell mediated and appears to require pro-inflammatory cytokines, such as interleukin (IL) 1 and tumour necrosis factor alpha (TNF-α) (Unanue, 1997).

Two surface proteins of *Listeria*, internalin (InlA) and InlB, are crucial in mediating *L. monocytogenes* cell invasion (Ireton and Cossart, 1997; Cossart and Lecuit, 1998). InlA is an 800-amino-acid protein consisting of 15 highly conserved 22-amino-acid leucine-rich repeats (LRRs), an inter-repeat region (IR) and a second repeat region consisting of two repeats of 70 amino acids and a third of 49 amino acids (B repeats). The carboxyl-terminal part contains the LPXTG motif, which is found in more that 50% of Gram-positive surface proteins, and allows a covalent linkage to peptidoglycan (Navarre and Schneewind, 1994). The receptor for InlA is E-cadherin, a transmembrane protein normally involved in cell–cell adhesion (Mengaud *et al.*, 1996a; Lecuit *et al.*, 1998).

InlB is a 630-amino-acid protein consisting of eight LRRs, each comprising 22 amino acids with a high degree of homology to InlA LRRs, an inter-repeat region (IR) similar to that of InlA, one B-repeat containing 80 amino acid repeats and a 232-amino-acid carboxyl-terminal region beginning with the sequence GW. InlB is loosely attached to the bacterial surface via the so-called GW repeats and is found in culture supernatants of wild-type *L. monocytogenes* (Dramsi *et al.*, 1995; Braun *et al.*, 1997). Purified, recombinant InlB confers invasiveness upon inert latex beads or non-invasive bacteria (Braun *et al.*, 1998). InlB has also been shown to activate phosphoinositide-3 kinase (PI-3 kinase) in Vero cells causing a rapid and transient increase in the lipid products of the PI 3-kinase p85-p110, tyrosine phosphorylation of the mammalian adaptor proteins Gab1, Cbl and Shc and association of these proteins with p85 (Ireton *et al.*, 1996; 1999). Recently, it has been reported that the 213-amino-acid LRR region of InlB is sufficient to promote InlB-mediated invasion, stimulation of PI-3 kinase and membrane ruffling (Braun *et al.*, 1999).

A diverse range of bacterial species and products has been shown to be capable of triggering an inflammatory

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response in mammalian cells (reviewed by Henderson *et al.*, 1996). Macrophage, neutrophils, natural killer cells and γδ T lymphocytes are the host's primary means of early defence against bacterial infection. Upon contact with bacteria, macrophages secrete cytokines and chemokines that stimulate natural killer cells and T lymphocytes, which in turn activate macrophages to become killers of bacterial invasion.

L. monocytogenes has been shown to initiate a pro-inflammatory response in macrophages (Kuhn and Goebel, 1994; Demuth *et al.*, 1996). Further research has indicated that this pro-inflammatory response was initiated by the activation of the transcription factor NF-κB after adhesion of *L. monocytogenes* (Hauf *et al.*, 1994; 1997). The eukaryotic transcription factor NF-κB plays an important role in the regulation of a number of genes involved in inflammation and immunity. Target genes include genes for cytokines, adhesion molecules, acute-phase proteins and cell surface receptors. In the cytoplasm of unstimulated cells, NF-κB is sequestered as a dimer bound to the inhibitory protein IκB (May and Ghosh, 1997; 1999; Baeuerle, 1998). There are several types of IκB, including IκBα, IκBβ and IκBϵ (Ghosh *et al.*, 1998). The currently known subunit members of the NF-κB family in mammals are p50, p65 (RelA), c-Rel, p52 and RelB (Liou and Baltimore, 1993). The major form of NF-κB found in resting cells is the heterodimer p50/p65 complexed to IκBα. Upon stimulation with agents such as the cytokines IL-1 or TNF, IκBα is quickly phosphorylated by the IκB–kinase complex (IKK), which targets the inhibitor protein for ubiquitination and subsequent degradation (Baeuerle and Henkel, 1994). There are two known IKKs, IKK-1 and 2 (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997; Zandi *et al.*, 1997), with IKK-2 being important for NF-κB activation in immunity and inflammation. The degradation of IκB allows NF-κB to translocate to the nucleus and bind to promoter regions, allowing the transcription of genes under its control (O'Neill and Greene, 1998).

L. monocytogenes has already been demonstrated to activate NF-κB (Hauf *et al.*, 1997). As both TNF-α and IL-1 expression are NF-κB dependent, the ability of *L. monocytogenes* to activate NF-κB could be part of the host response to the bacterium (Baeuerle and Henkel, 1994). Other bacteria, such as *Shigella flexneri*, heat-killed *Staphylococcus aureus* and pneumococcal cell walls, have also been shown to activate NF-κB (Busam *et al.*, 1992; Dyer *et al.*, 1993; Spellerberg *et al.*, 1996). Bacterial products such as lipopolysaccaride (LPS), a surface component of Gram-negative bacteria, and lipoteichoic acid (LTA) from Gram-positive bacteria and, in particular, that of *Listeria* are potent activators of NF-κB (Baeuerle and Baichwal, 1997: Hauf *et al.*, 1997). Listeriolysin O (LLO) from *L. monocytogenes* has also recently been shown to activate NF-κB potently (Kayal *et al.*, 1999).

Given that PI-3 kinase has recently been shown to have a role in NF-κB activation (Reddy *et al.*, 1997; Ozes *et al.*, 1999; Sizemore *et al.*, 1999) and as InlB activates PI-3 kinase (Ireton *et al.*, 1996; 1999; Braun *et al.*, 1999), we tested whether InlB activates NF-κB.

We found that InlB activates NF-κB in the murine macrophage-like cell line J774. The effect is rapid and sustained, involving both IκBα and IκBβ degradation. Activation is mediated by the LRR region of InlB and involves PI-3 kinase.

Results

InlB contributes to Listeria*-induced NFκB activation*

As InlB has been shown to be involved in PI-3 kinase activation (Ireton *et al.*, 1996; 1999; Braun *et al.*, 1999), when deleted from or transformed into bacterial species, and given that PI-3 kinase has been shown to activate NF-κB (Reddy *et al.*, 1997; Ozes *et al.*, 1999; Sizemore *et al.*, 1999), we wished to examine whether InlB could activate NF-κB.

We first tested two species of *Listeria* to determine whether InlB was contributing to the activation of NF-κB. A mutant strain of *L. monocytogenes,* EGDΔInlB (Dramsi *et al.*, 1995), which does not express the InlB protein, was used in conjunction with wild-type *L. monocytogenes* EGD (Mackaness, 1962). J774 cells were stimulated for 60 min with a multiplicity of infection (MOI) of 20:1 bacteria to eukaryotic cells. NF-κB activation was analysed using the electrophoretic mobility shift assay (EMSA; see *Experimental procedures*). As shown in Fig. 1, both strains were able to potently activate NF-κB (compare lanes 3 and 4 with lane 1). However, it was possible to discern a difference in the intensity of activation between EGD and mutant EGDΔInlB (compare lanes 3 and 4). Densitometric analysis indicated a 20% reduction in activation by EGD-ΔInlB over wild-type EGD.

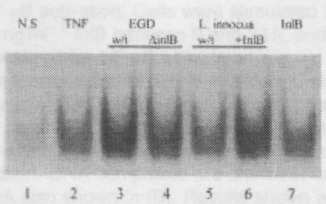

Fig. 1. Effects of *Listeria* species on NF-κB activation. J774 cells were seeded at 5×10^4 cells ml^{-1} in six-well plates 48 h before stimulation with bacteria. Cells were stimulated with *L. monocytogenes* strains (lanes 3 and 4) at a multiplicity of infection (MOI) of 20:1, bacteria to eukaryotic cell. *L. innocua* (lanes 5 and 6) strains were used at an MOI of 50:1. Positive control consists of stimulation with 20 ng ml^{-1} TNF-α (lane 2) compared with non-stimulated (NS) cells (lane 1). Lane 7 shows the effect of 500 ng ml^{-1} InlB. NF-κB activation was assayed by EMSA as described in *Experimental procedures*. Results are representative of four independent experiments.

To illustrate further the ability of InlB to activate NF-κB, the avirulent species *Listeria innocua*, which do not express either InlB or LLO, was used in conjunction with the *L. innocua* strain transformed with a plasmid expressing InlB. J774 cells stimulated for 60 min at an MOI of 50:1 clearly illustrate *L. innocua* (InlB) (Fig. 1, lane 6) stimulating NF-κB at a higher intensity than that of *L. innocua* alone (lane 5). Densitometric analysis confirmed that *L. innocua* (InlB) was able to stimulate NF-κB activation 2.5-fold over that of *L. innocua* alone. Treatment of cells for 60 min with purified recombinant InlB (lane 7) also activated NF-κB.

These results therefore clearly indicate that InlB can activate NF-κB, either alone or when expressed in *L. monocytogenes* or *L. innocua*.

Characterization of NF-κB activation by InlB

We next investigated the effects of recombinant InlB and a series of InlB domains on NF-κB activation. A schematic diagram illustrates the different domains of InlB used in this study (Fig. 2A). Their purity was assessed by SDS–PAGE (Fig. 2A).

We first investigated whether purified recombinant InlB was capable of activating NF-κB. As shown in Fig. 2B, InlB-mediated NF-κB activation occurred over a prolonged time range, initially being observed at 5 min after stimulation, peaking at 1–2 h after stimulation, with the activation persisting for at least 4 h after stimulation.

We next stimulated cells with a range of InlB concentrations. Activation was initially observed at 5 ng ml^{-1} InlB, reaching a maximal response at 50 ng ml^{-1} (Fig. 2C). A concentration of 100 ng ml^{-1} InlB, together with a 60 min stimulation, was used for further experiments.

Using these parameters, the ability of InlB to stimulate NF-κB activation was tested on other cell lines. We found that the murine macrophage cell line P388D$_1$ and the human epithelial cell line Hep2 were both responsive to InlB stimulation (Fig. 3). P388D$_1$ cells were less sensitive than J774 cells, however, requiring 1 μg ml^{-1} for an effect to be evident. The human epithelial cell lines, Hela, Hep G2, Caco-2 and LoVo, were all found to be unresponsive to InlB, as was found with the monkey fibroblast-like cells Vero and human endothelial cells ECV 304 (data not shown). The basis for these differences is being investigated. Subsequently, J774 cells were used for all further stimulation studies with InlB.

We next examined the subunit composition of the activated complex. The activated complex consisted of p50 and p65, as both subunits were supershifted upon incubation of nuclear extracts with the relevant anti-p50 and anti-p65 antibodies, while the anti-c-Rel antibody had no effect (Fig. 4A). The antibodies used were not directly comparable in terms of titre or avidity, and the result is therefore qualitative.

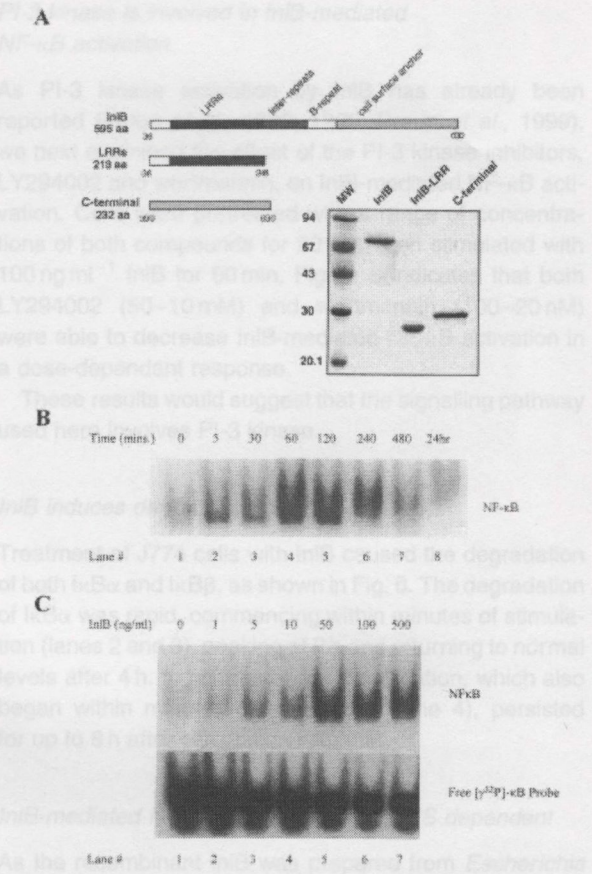

Fig. 2. A. Schematic diagram representing the different constructs of InlB and domains used for stimulation of J774. Wild-type InlB and analogues (InlB-LRR and C-terminal) of InlB are indicated by numbers corresponding to amino acid positions relative to wild-type InlB.
B. Purity of analogues as determined by SDS–PAGE of recombinant proteins and stained with Coomassie blue. Sample concentrations were 2 μg of protein per lane.
B. Effects of recombinant InlB on NF-κB activation in J774 cells. Cells were seeded in six-well plates at a concentration of 1×10^5 cells ml^{-1} 2 days before stimulation, such that final concentrations of cells were $\approx 5\times10^6$ cells ml^{-1}. Upon stimulation, cells were incubated at 37°C and then tested for NF-κB. Time course of InlB-mediated NF-κB activation. Cells were stimulated for a range of times with 100 ng ml^{-1} InlB between 0 and 24 h, and samples were taken and assayed for NF-κB activation.
C. The dose-dependent effects of InlB stimulation. Cells were stimulated for 60 min at 37°C with a range of concentrations of InlB, increasing from 1 ng ml^{-1} to 500 ng ml^{-1} (lanes 2–7), and assayed for their ability to stimulate NF-κB in comparison with unstimulated cells (lane 1). Protein–DNA complexes are shown in each case, with unbound DNA also shown in (B). Results shown are representative of three separate experiments.

The specificity of the binding of NF-κB to its radiolabelled κB consensus sequence was verified by preincubation of extracts with an oligonucleotide containing unlabelled wild-type κB sequence. Binding was inhibited (compare lanes 2 and 1 in Fig. 4B), but remained unaffected by preincubation with an oligonucleotide containing

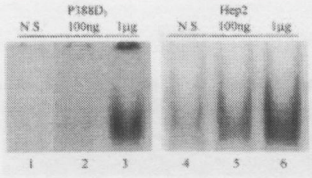

Fig. 3. Activation of $P388D_1$ cells and Hep2 with InlB. Both cell lines were seeded at 1×10^5 cells ml^{-1} 48 h before stimulation with 100 ng ml^{-1} and 1 μg ml^{-1} InlB respectively. Lanes 1 and 4 correspond to non-stimulated (NS) cells for both cell lines.

a mutant κB sequence (compare lane 4 with lane 1 in Fig. 4B).

Taken together, these results indicate that InlB is a potent activator of NF-κB and that the activated complex contains p50 and p65 subunits. There is some variation in the strength of the response as a result of differences in the basal level of NF-κB, which varies somewhat between experiments. However, InlB gave a consistently strong activation of NF-κB.

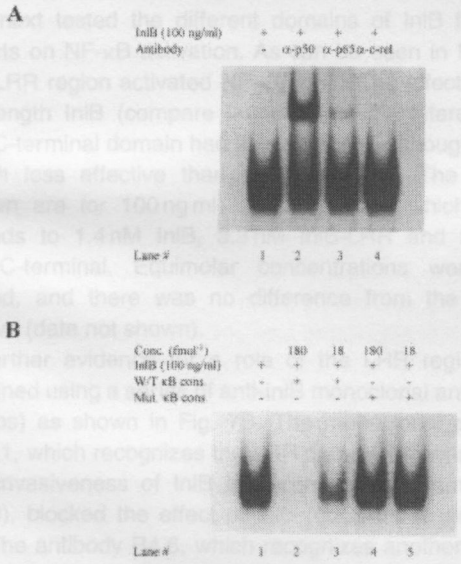

Fig. 4. Specificity of NF-κB activation using supershift and competition assays (A and B).
A. The composition of the NF-κB complex was determined by incubating prestimulated samples (2 μg $sample^{-1}$) for 30 min at 4°C with antibodies against p50 (lane 2), p65 (lane 3) and c-Rel (lane 4) compared with a control (lane 1), which had not been incubated with antibody. Samples were then assayed for NF-κB using EMSA. Supershifted complexes can be seen in lanes 2 and 3, indicating the presence of p50 and p65 subunits respectively.
B. Nuclear extracts from InlB-treated cells (2 μg $sample^{-1}$) were preincubated for 30 min with wild-type or mutant non-radiolabelled κB-consensus oligonucleotide, as indicated, at the following concentrations: 180 $fmol^{-1}$ (lane 2) and 18 $fmol^{-1}$ (lane 3). Samples were then assayed for NF-κB by EMSA.

PI-3 kinase is involved in InlB-mediated NF-κB activation

As PI-3 kinase activation by InlB has already been reported (Ireton *et al.*, 1996; 1999; Braun *et al.*, 1999), we next examined the effect of the PI-3 kinase inhibitors, LY294002 and wortmannin, on InlB-mediated NF-κB activation. Cells were pretreated with a range of concentrations of both compounds for 20 min, then stimulated with 100 ng ml^{-1} InlB for 60 min. Figure 5 indicates that both LY294002 (50–10 mM) and wortmannin (100–20 nM) were able to decrease InlB-mediated NF-κB activation in a dose-dependant response.

These results would suggest that the signalling pathway used here involves PI-3 kinase.

InlB induces degradation of IκBα and IκBβ

Treatment of J774 cells with InlB caused the degradation of both IκBα and IκBβ, as shown in Fig. 6. The degradation of IκBα was rapid, commencing within minutes of stimulation (lanes 2 and 3), peaking at 2 h and returning to normal levels after 4 h. In contrast, IκBβ degradation, which also began within minutes of stimulation (lane 4), persisted for up to 8 h after stimulation with InlB.

InlB-mediated NF-κB activation is not LPS dependent

As the recombinant InlB was prepared from *Escherichia coli*, it was possible, although unlikely given the purification protocol used, that the effect of InlB activation was caused by contamination of samples with LPS. To verify that this was not the case, InlB was incubated with the LPS-neutralizing agent polymyxin B (Srimal *et al.*, 1996). Polymyxin B at concentrations of up to 10 μg ml^{-1} was found to have no effect on the InlB response. Furthermore, LPS at concentrations of up to 1 μg ml^{-1} were required to observe an activation of NF-κB in J774 cells similar to that of InlB. Polymyxin B at 1 μg ml^{-1} inhibited this effect (data not shown). From these results, it can be concluded that LPS was not a contaminant causing NF-κB activation.

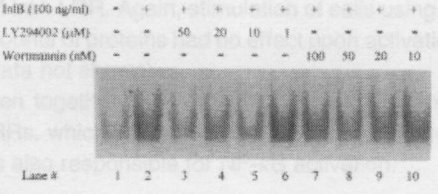

Fig. 5. Effect of PI-3 kinase inhibitors on InlB-mediated NF-κB –activation. Cells were seeded at 1×10^5 cells 48 h before –treatment. J774 cells were pretreated for 20 min with a –concentration range, 50–1 μM, of LY294002 (lanes 3–6) and 100–10 nM wortmannin (lanes 7–10). InlB (100 ng ml^{-1}) was then used to stimulate the cells for 60 min at 37°C, after which they were assayed for NF-κB activation by EMSA.

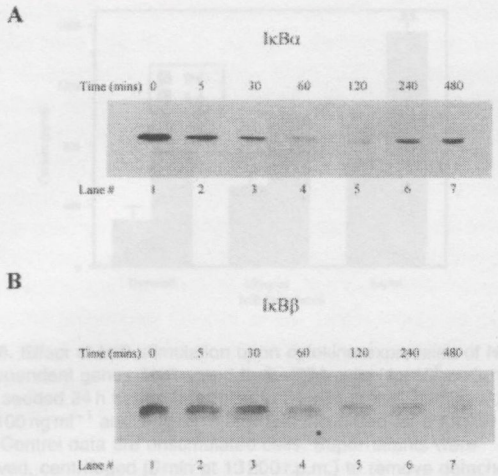

Fig. 6. The effect of InlB on the degradation of IκBα and IκBβ. J774 cells were seeded at 1×10^5 cells ml^{-1} in six-well plates 2 days before treatment, stimulated with 100 ng ml^{-1} InlB for corresponding time periods, then assayed for IκBs in cytosolic fractions by Western blot analysis (A and B). Only one band of 37 kDa, corresponding to each IκB, was detected. The results shown are representative of two experiments.

NF-κB activation is caused by the N-terminal LRR region of InlB

We next tested the different domains of InlB for their effects on NF-κB activation. As can be seen in Fig. 7A, the LRR region activated NF-κB almost as effectively as full-length InlB (compare lanes 3 and 2). Interestingly, the C-terminal domain had some activity, although it was much less effective than full-length InlB. The results shown are for 100 ng ml^{-1} each protein, which corresponds to 1.4 nM InlB, 3.3 nM InlB-LRR and 3.75 nM InlB-C-terminal. Equimolar concentrations were also tested, and there was no difference from the results shown (data not shown).

Further evidence for a role of the LRR region was obtained using a series of anti-InlB monoclonal antibodies (mAbs) as shown in Fig. 7B. The monoclonal antibody D23.1, which recognizes the LRR domain responsible for the invasiveness of InlB into Vero cells (Braun *et al.*, 1999), blocked the effect of InlB (compare lanes 3 and 2). The antibody B4.6, which recognizes another part of the LRR domain of InlB, but is unable to inhibit invasion of Vero cells, had no effect on stimulation, as did antibodies to the LRR inter-repeat region and the C-terminal domain of InlB (lanes 4–6). Of note here, the antibody directed against the LRR and/or inter-repeat region of InlB, mAb 3.3, which has been found to inhibit InlB-mediated invasion of Vero cells (Braun *et al.*, 1999), had no effect upon NF-κB activation (lane 3).

As the LRR region of InlB is highly similar to that of InlA, we tested both the native InlA and the shorter analogue InlA-LRR proteins for NF-κB activation. As shown in

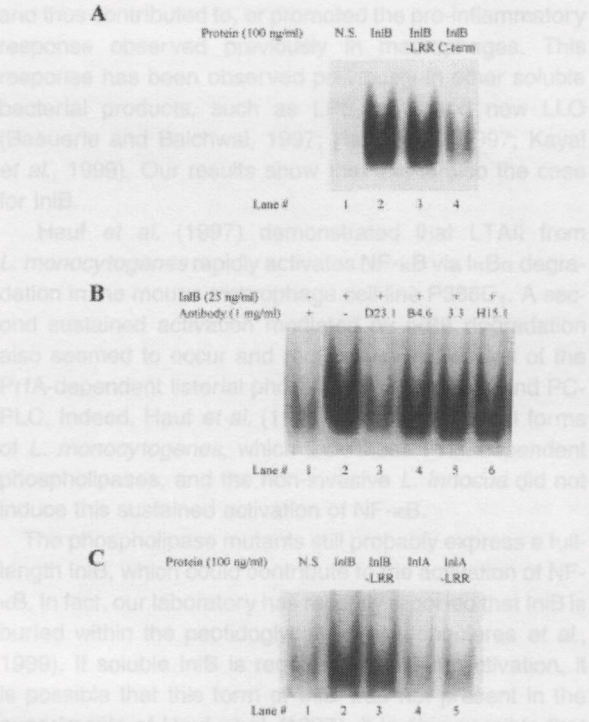

Fig. 7. Domains of InlB responsible for NF-κB activation.
A. J774 cells were stimulated with 100 ng ml^{-1} InlB (lane 2), InlB-LRR (lane 3) and the InlB C-terminal region (lane 4) for 60 min as described in *Experimental procedures* (refer to Fig. 1 for schematic diagram of domains) NS, non-stimulated cells (lane 1).
B. InlB (25 ng ml^{-1}) was preincubated for 60 min with 1 mg ml^{-1} antibodies directed against specific regions of InlB and then used to stimulate J774 cells. Antibodies correspond to D23.1, anti-InlB-LRR (lane 3); B4.6, anti-InlB-LRR (lane 4); 3.3, anti-InlB-LRR-IR (lane 5); and H15.1, anti-C-terminal (lane 6). Control cells (lane 1) were incubated with a corresponding amount of 1 mg ml^{-1} D23.1 antibody.
C. Proteins (100 ng ml^{-1}) corresponding to InlB (lane 2), InlB-LRR (lane 3), InlA (lane 4) and InlA-LRR (lane 5) were assayed by EMSA as described previously. Control cells (lane 1) were unstimulated. Protein–DNA complexes corresponding to NF-κB are shown. These results are representative of three independent experiments.

Fig. 7C, both the InlA and the InlA-LRR were inactive in NF-κB activation, compared with full-length InlB and InlB-LRR.

The molar concentrations tested were 1.16 nM InlA and 2.1 nM InlA-LRR. Again, stimulation of cells using equimolar amounts of proteins had no effect upon activation intensity (data not shown).

Taken together, these results indicate that a region of the LRRs, which is required for InlB-mediated internalization, is also responsible for NF-κB activation.

InlB regulates NF-κB-dependent gene expression of TNF-α and IL-6 in J774 cells

Finally we examined whether InlB could induce the expression of NF-κB-regulated genes in J774 cells. We

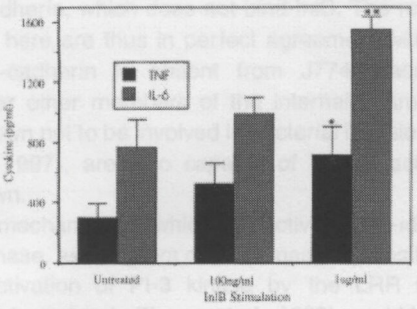

Fig. 8. Effect of InlB stimulation upon cytokine expression of NF-κB-dependent genes TNF-α and IL-6. J774 cells (1×10^6 cells ml^{-1}) were seeded 24 h before treatment in 24-well plates, stimulated with 100 ng ml^{-1} and 1 μg ml^{-1} InlB and incubated for a further 24 h. Control data are unstimulated cells. Supernatants were removed, centrifuged (5 min at 13 200 r.p.m.) to remove detached cells and assayed as described in *Experimental procedures*. Results are shown as cytokines (pg ml^{-1}) and are expressed as means ± standard deviations ($n = 3$). Data indicate significant differences ($^*P = 0.05$, $^{**}P = 0.007$) when compared with control values.

tested the expression of TNF-α and IL-6, as the genes for these two proteins are controlled by NF-κB and their promoters containing NF-κB binding sites (Baeuerle and Henkel, 1994). As shown in Fig. 8, TNF-α and IL-6 protein levels increased in a dose-responsive manner after 24 h stimulation with InlB.

Discussion

In this study, we have demonstrated that the invasion protein InlB from *L. monocytogenes* activates NF-κB in the murine macrophage-like cell line J774, causing the expression of two cytokines, TNF-α and IL-6, which are part of a pro-inflammatory response. It has also been demonstrated that the PI-3 kinase pathway is involved in this InlB-mediated NF-κB activation. The effect is sustained and involves the transient degradation of IκBα and the prolonged degradation of IκBβ. This activation is mediated by the LRR domain of InlB, a region recently shown to be required for internalization in epithelial cells, and activation of PI-3 kinase (Braun *et al.*, 1999).

Infection of bone marrow-derived macrophages with *L. monocytogenes* has been shown previously to upregulate cytokines IL-1α, IL-1β and TNF-α after initial infection (Demuth *et al.*, 1996). This classical pro-inflammatory response was found to be unaffected by cytoskeletal inhibitors that prevent bacterial invasion, but not adhesion. These results suggested that secreted or surface molecules of mammalian cells are able to interact with a bacterial ligand and initiate a pro-inflammatory response. Given that InlB is a cell surface protein, also found in culture supernatants, and an activator of PI-3 kinase, we felt that it was an excellent candidate for activation of NF-κB, and thus contributed to, or promoted the pro-inflammatory response observed previously in macrophages. This response has been observed previously in other soluble bacterial products, such as LPS, LTA and now LLO (Baeuerle and Baichwal, 1997; Hauf *et al.*, 1997; Kayal *et al.*, 1999). Our results show that this is also the case for InlB.

Hauf *et al.* (1997) demonstrated that LTAII from *L. monocytogenes* rapidly activates NF-κB via IκBα degradation in the mouse macrophage cell line $P388D_1$. A second sustained activation mediated by IκBβ degradation also seemed to occur and require the expression of the PrfA-dependent listerial phospholipases PI-PLC and PC-PLC. Indeed, Hauf *et al.* (1997) found that mutant forms of *L. monocytogenes*, which lack these PrfA-dependent phospholipases, and the non-invasive *L. innocua* did not induce this sustained activation of NF-κB.

The phospholipase mutants still probably express a full-length InlB, which could contribute to the activation of NF-κB. In fact, our laboratory has recently reported that InlB is buried within the peptidoglycan layer (Jonquières *et al.*, 1999). If soluble InlB is required for NF-κB activation, it is possible that this form of InlB was not present in the experiments of Hauf *et al.* (1997). It is also possible that InlB expression or activity was very low in their bacterial cultures. A final explanation for the discrepancy could be that Hauf *et al.* (1997) used $P388D_1$ cells, which we have found to be less responsive to InlB than J774 cells.

Using a combination of shorter domains of InlB and monoclonal antibodies directed against some of these regions, we have been able to demonstrate that activation of NF-κB is mediated by the LRR domain. This region activated NF-κB at concentrations similar to native InlB, and activation was inhibited by an antibody (D23.1) raised against a region of the LRR involved in InlB-mediated invasion of Vero cells, but was unaffected by an antibody raised against the LRR motif of InlB that is unable to inhibit invasion (B4.6). Finally, another antibody, 3.3, raised against a protein comprising the LRR-IR region, was also unable to inhibit NF-κB activation. All antibodies were raised against recombinant proteins and have not been epitope mapped. Therefore, the exact regions of the LRR involved cannot be elucidated. Taken together, however, these results indicate that the LRR motif of InlB involved in invasion (Braun *et al.*, 1999) is critical for NF-κB activation.

InlA shares with InlB a similar LRR region (Dramsi *et al.*, 1996), as do the other members of the internalin family (Dramsi *et al.*, 1997). Yet our investigation has shown that native InlA and its LRR region are unable to activate NF-κB in J774 macrophages. Thus, the activation is specific and not caused by the presence of LRR motifs. The difference in the LRR domains of InlA and B is sufficient to allow both proteins to bind different receptors (L. Braun and P. Cossart, unpublished data). The receptor for InlA

is E-cadherin, which does not bind InlB. The results presented here are thus in perfect agreement with the fact that E-cadherin is absent from J774 macrophages. Whether other members of the internalin family, which are known not to be involved in bacterial invasion (Dramsi *et al.*, 1997), are also capable of NF-κB activation is unknown.

The mechanism by which InlB activates NF-κB involves PI-3 kinase, as inhibitors of PI-3 kinase repress the effect. The activation of PI-3 kinase by the LRR from InlB reported previously (Braun *et al.*, 1999) would be consistent with a role for PI-3 kinase in NF-κB activation. How InlB activates PI-3 kinase is not known. Akt/protein kinase B (PKB), which is downstream of PI-3 kinase, has been shown to activate NF-κB (Kane *et al.*, 1999; Ozes *et al.*, 1999) by phosphorylation of the IκB kinases and may mediate the effect of InlB here.

What are the downstream consequences of NF-κB activation in infected cells? As NF-κB has been implicated in many aspects of the pro-inflammatory response to bacterial invasion (Baeuerle and Baltimore, 1996), it is possible that the InlB-mediated NF-κB activation observed mainly represents a host defence response to the bacterium. Another possibility, however, is that NF-κB activation may be beneficial to the bacterium. NF-κB has recently been shown to be antiapoptotic in TNF-induced cell death (Beg and Baltimore, 1996; Wang *et al.*, 1996) and also via the induction of genes encoding antiapoptotic proteins such as IAPs (LaCasse *et al.*, 1998). Given that *L. monocytogenes* is an intracellular pathogen, it is possible that, via NF-κB, the bacterium prevents the killing of infected cells. Interestingly, it has been reported that another potent NF-κB activator, LPS, which regulates the production of pro-inflammatory cytokines produced by macrophages, can actually inhibit TNF-induced apoptosis in U-937 cells (Manna and Aggarwal, 1999). PI-3 kinase can also generate an antiapoptotic signal via Akt/(PKB) (Marte and Downward, 1997). As stated above, Akt/PKB has recently been shown to activate NF-κB (Kane *et al.*, 1999; Ozes *et al.*, 1999). If PI-3 kinase and NF-κB activation are linked via this signalling pathway, NF-κB activation could be another antiapoptotic signal activated by PI-3 kinase in response to InlB.

The finding that LLO, among others, is also a potent NF-κB activator demonstrates that *L. monocytogenes*, in many checkpoints of the infection process, may use different components to trigger similar signalling events.

In conclusion, our results indicate that InlB is not only involved in *L. monocytogenes* invasion. It also activates NF-κB in macrophages. Thus, NF-κB activation during *Listeria* infection seems to be mediated by a series of components, including LTA, phospholipids, LLO and InlB. We anticipate that these outcomes will have important consequences upon both bacterial survival and host defence.

Experimental procedures

Cells, media and chemicals

The murine macrophage-like cell line J774 was kindly provided by Kingston Mills (National University of Ireland, Maynooth) and was grown in 10% heat-inactivated fetal calf serum (FCS) in RPMI 1640 (both Euroclone), which was supplemented with 2 mM L-glutamine (Hyclone) and 2 mM penicillin/streptomycin (Hyclone). Wortmannin and LY294002 (Sigma) were both dissolved in dimethyl sulphoxide (DMSO). The 22 bp oligonucleotide, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, containing the NF-κB consensus sequence (underlined), and the T4 polynucleotide kinase kit were from Promega Corporation. The 22 bp oligonucleotide, 5′-AGT TGA GGC GAC TTT CCC AGC C-3′, containing the mutated NF-κB consensus sequence (underlined), and the rabbit polyclonal antibody to IκBβ were from Santa Cruz. Rabbit polyclonal antibodies to p50, p65 and c-Rel were a kind gift from Jean Imbert (INSERM, Marseilles, France), as was the mouse monoclonal antibody to IκBα from Ron Haye (St Andrews, UK). [γ-^{32}P]-ATP (3000 Ci mol^{-1}) was from Amersham, and the enhanced chemiluminescence (ECL) reagent was from Biolabs. Poly-(dI–dC) was supplied by Pharmacia. Secondary antibodies anti-mouse IgG peroxidase and horseradish peroxidase (type II), polymyxin B and other chemicals were purchased from Sigma. Internalin purification was performed as described previously (Mengaud *et al.*, 1996b).

Preparation of InlB6xHis and InlB analogues

All recombinant proteins were grown and purified as described previously (Braun *et al.*, 1998; 1999). Purified proteins were dialysed against 50 mM HEPES (pH 7.4), 500 mM NaCl, concentrated to the desired volume using Centriprep 10 (Amicon) and stored at −20°C. Purity of samples was determined by SDS–PAGE.

Bacterial strains, growth conditions and bacterial stimulation

L. monocytogenes and *L. innocua* were both grown in brain–heart infusion (BHI) at 37°C. For *L. innocua* strains containing pAT28 derivatives, erythromycin was added to a final concentration of 5 μg ml^{-1}. The strains used are described in Table 1.

For the bacterial stimulation of J774 cells described in Fig. 1, 1 ml of exponential-phase cultures (OD_{600} = 0.8–1.0) of strains *L. monocytogenes* and *L. innocua* was washed three times with phosphate-buffered saline (PBS), pelleted and resuspended in 1 ml of PBS. Bacteria were added to mammalian cells (≈ 1 × 10^6 cells ml^{-1}) to give an MOI of either 20:1 (*L. monocytogenes*) or 50:1 (*L. innocua*) and incubated for 60 min, after which cells underwent nuclear extract preparation for NF-κB analysis.

Activation assays and nuclear extract preparation

Murine macrophage-like cells J774 were seeded at 1 × 10^5 cells ml^{-1} in six-well plates (3 ml volume) 48 h before stimulation and incubated at 37°C, 5% CO_2. InlB was diluted in

Table 1. Bacterial strains used in this study.

Strain	Genotype/properties	Reference
BUG600	Wild type, serotype 1/2a	Mackaness (1962)
BUG1047	EGD:ΔinlB	Dramsi *et al.* (1995)
BUG994	*L. innocua*: pAT28 (vect)	Dramsi *et al.* (1995)
BUG1533	*L. innocua*: (InlB)	Jonquières *et al.* (1999)

35 mM Tris, 75 mM NaCl, 1 mM EDTA, pH 7.5. Relevant concentrations of proteins were added to cells and incubated for 1 h at 37°C.

Nuclear extracts were prepared using a modified version of the method of Osborn *et al.* (1989). Stimulation was terminated by aspiration of media from cells and replacement with 1 ml of ice-cold hypotonic buffer [10 mM HEPES buffer, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)], after which the cells were scraped and centrifuged (5 min, 13 200 r.p.m., 4°C). The subsequent cell pellet was lysed in 0.1% Igepal CA-630 in hypotonic buffer and held on ice for 10 min. Centrifugation (10 min, 13 200 r.p.m., 4°C) led to a cell debris pellet from which nuclear-associated proteins were extracted by the addition of 20 mM HEPES, pH 7.9, containing 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol and 0.5 mM PMSF, and this was maintained on ice for 20 min. After centrifugation (10 min, 13 200 r.p.m., 4°C), the supernatant was removed and mixed with 10 mM HEPES, pH 7.9, containing 50 mM KCl, 0.2 M EDTA, 20% glycerol, 0.5 mM DTT and 0.5 mM PMSF. Protein concentrations were determined using the method of Bradford (1970) and extracts stored at −20°C.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (4 μg) were incubated with 10 000 c.p.m. of a 22 bp DNA fragment oligonucleotide containing the NF-κB consensus sequence that had previously been labelled with [γ-^{32}P]-ATP (10 mCi mmol^{-1}) by T4 polynucleotide kinase. Incubations were performed for 30 min at room temperature in the presence of 2 μg of poly-(dI–dC) as non-specific competitor and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 4% glycerol and 100 μg ml^{-1} nuclease-free BSA. For competition studies, unlabelled wild-type or mutant NF-κB oligonucleotides were added to the binding reaction 30 min before the addition of the radiolabelled probe. In experiments involving antisera to NF-κB subunits, 0.5 μl of specific antibodies to p50, p65 and c-Rel were incubated with nuclear extracts for 20 min on ice before the binding reaction. All incubation mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels, which were subsequently dried and autoradiographed.

IκB immunoblot analysis

Murine macrophage-like cells J774 were seeded at 1×10^5 cells ml^{-1} in six-well plates (3 ml volume) 48 h before stimulation and incubated at 37°C, 5% CO_2. InlB (100 ng ml^{-1}) was added, and stimulation was terminated at relevant time points by aspiration of reaction media and the subsequent addition of 1 ml of ice-cold PBS. After aspiration of PBS from the cells, 200 μl of ice-cold RIPA buffer (1% Igepal CA-630, 0.5% deoxycholate, 0.1% SDS in PBS, containing 10 mg of PMSF, 7 μg of aprotinin and 1 mM sodium vanadate) was added. Plates were shaken on ice for 15 min and cells scraped to ensure lysis. After further disruption of cells by passage through a 21-gauge needle (10 strokes), a further 0.1 mg ml^{-1} PMSF was added to the samples, which were incubated for 45 min. Samples were then centrifuged for 20 min at 13 200 r.p.m. at 4°C, and the supernatant was removed from the cell debris and assayed for protein by the Bradford (1970) method.

Equal amounts of protein (4 μg) were resolved by SDS–PAGE and transferred to nitrocellulose, in which an IκB immunoblot was carried out as described previously (Mahon and O'Neill, 1995). After immunoblotting was completed, membranes were stained with Ponceau S to confirm equal protein loading.

TNF-α and IL-6 gene expression analysis

J774 cells were seeded at 1×10^6 cells ml^{-1} in 24-well plates (1 ml volume) 24 h before stimulation and incubated at 37°C, 5% CO_2. Cells were stimulated with 100 ng ml^{-1} and 1 μg ml^{-1} InlB in triplicate and incubated for a further 24 h. Supernatant was aspirated and centrifuged (1600 r.p.m. for 5 min) to remove any detached cells, and samples were stored (−70°C) or analysed. Protein concentrations were determined as described by Loscher *et al.* (1998).

Statistical analysis

Significance was evaluated using the Student's *t*-test for unpaired data.

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