INTERLEUKIN-1β SIGNALING IN INTESTINAL EPITHELIAL CELLS

by

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ABSTRACT

Although long regarded as providing a passive physical barrier to the contents of the lumen, a large body of evidence implicates intestinal epithelial cells (IECs) as playing a central role in the regulation of the gut immune response. One of the major immune functions of intestinal epithelial cells is the production of chemotactic cytokines, known as chemokines, which are responsible for the recruitment and activation of the underlying mononuclear cells, neutrophils, and dendritic cells. Several soluble agonists, including bacterial products as well as host-derived cytokines, are capable of driving this response. The prototypic proinflammatory agonist interleukin-1β (IL-1β), signals through the interleukin-1 receptor (IL-1R), the founding member of the IL-1R superfamily, and robustly activates this pro-inflammatory cascade. Synthesis of many downstream chemokines in response to IL-1β, requires the activation of the transcription factor NFκB. The objective of the work presented here is to characterize the regulatory pathways responsible for chemokine synthesis in IECs in response to IL-1β, and to examine their relationship with NFκB. Three important pathways in IL-1β signaling were examined. Initially the MAPK family of pathways was examined. IL-1β treatment led to activation of ERK, p38 MAPK, as well as JNK. Inhibition of ERK had no effect on either chemokine synthesis, or NFκB activation. Inhibition of p38 MAPK caused a 50% reduction in IL-1β induced chemokine release. This effect was due to regulation of the IL-8 promoter, and independent of NFκB and posttranscriptional regulation. JNK inhibition, using curcumin or a pharmacological inhibitor, attenuated

NFκB activation and IL-8 promoter activation; however, only curcumin was able to inhibit chemokine release. The next chapter focuses on regulation by protein kinase CK2. CK2 was found increased in tissues of ulcerative colitis patients when compared to normal uninflamed tissue. Its activity was required for the activation of NFκB, and this was through regulation of p65 transactivation in the nucleus of the cell, via serine 529. As a result, CK2 inhibition leads to attenuation of chemokine synthesis. Finally the phosphatidylinositol-3 kinase (PI3K) pathway was examined. Activation of PI3K, protein kinase B (PKB) and 3'-phosphoinositide dependant kinase (PDK1) were all required for chemokine synthesis as well as NFκB activation, although they had differing regulatory roles. PI3K and PKB regulated NFκB transactivation, through an undetermined mechanism. PDK1 regulated the IKK complex, potentially through a phosphorylation at serine residues 180/181 on IKKα and IKKβ. This resulted in PDK1 regulation of NFκB DNA binding, as well as p65 transactivation through phosphorylation of serine 536. The work presented here has examined the signaling pathways important in the regulation of IEC chemokine release. This may provide potential targets for *in vivo* studies of chronic inflammation, and thus have implications for chronic inflammatory conditions such as IBD.

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ABBREVIATIONS

Abbreviation	Definition
3' UTR	3' untranslated region
AP-1	activator protein 1
ARE	AU-rich cis regulatory elements
ATF-2	activating transcription factor-2
C/EBP	CAAT/ enhancer binding protein
CAMK IV	calmodulin activated kinase IV
CBP	CREB binding protein
CD	Crohn's disease
cFLIP	cellular inhibitor of FLICE
CHOP	C/EBP homologous protein
cIAP	cellular inhibitor of apoptosis
COX-2	cyclooxygenase-2
CTCF	CCCTC-binding factor
DRB	5,6 dichloro-ribifuranosylbenzimidazole
DSS	dextran sodium sulfate
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ENA-78	epithelial cell derived neutrophil attractant 78
ERK	extracellular signal regulated kinase
EV	empty vector
FBS	fetal bovine serum
GROα	growth related oncogene α
HAT	histone acetyl transferase
HSP	heat shock protein
IκB	inhibitor of κB
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
IKK	inhibitor of κB kinase
IL-1β	interleukin-1β
IL-18R	interleukin 18 receptor
IL-1R	interleukin-1 receptor
IL-1RA	interleukin-1 receptor antagonist
IL-1RI	interleukin-1 receptor, type 1
IL-1RII	interkeukin-1 receptor, type 2
ILK	integrin linked kinase
iNOS	inducible nitric oxide synthase
IP-10	interferon γ inducible protein
IRAK	interkeukin-1 receptor associated kinase
IRS	insulin receptor substrate
I-TAC	interferon inducible T cell attractant
JNK	c-jun NH2-terminal kinase

KD	kinase dead
LPMNC	lamina propria mononuclear cell
LPS	lipopolysaccharide
LTBR	leukotriene β receptor
MAPK	mitogen activated protein kinase
MAPKAPK2	mitogen activated protein kinase activated protein kinase-2
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MCP	monocyte chemoattracant protein
MDC	macrophage derived chemokine
MEKK	MEK kinase
MHC	major histocompatibility complex
MIG	monokine induced by interferon γ
MIP	macrophage inflammatory protein
MLK3	mixed linease kinase-3
MSK1	mitogen and stress activated kinase-1
NFκB	nuclear factor κB
NIK	NFκB inducing kinase
NRE	negative regulatory element
NRF	nuclear factor κB repressing factor
p90 RSK	p90 ribosomal S6 kinase
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PDK1	3' phosphoinositide dependant kinase-1
PI(3,4,5)P3	phosphoinositol(3,4,5)triphosphate
PI(4,5)P2	phosphoinositol(4,5)biphosphate
PI3K	phoshphatidylinositol-3 kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PRR	pathogen recognition receptor
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel eletrophoresis
SH2	src-homology 2
TAD	transactivation domain
TAK1	transforming growth factor β activated kinase
TGFβ	transforming growth factor β
TH_1	T helper cell, type 1
TH_2	T helper cell, type 2
tir	toll/IL-1R receptor
TNFα	tumor necrosis factor α
TNFR1	tumor necrosis factor receptor 1
TPCK	n-tosyl phenylalanyl chloromoethyl ketone
TRAF-6	tumor necrosis factor receptor associated factor-6
TWEAK	tumor necrosis factor weak inducer of apoptosis
UC	ulcerative colitis

WT	wildtype

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CHAPTER 1 – GENERAL INTRODUCTION

1.1 INTESTINAL EPITHELIAL CELLS

1.1.1 Biology

The intestinal epithelium is a rapidly renewing tissue for which homeostasis means striking not only a balance between proliferation and cell death, but also balancing communications and interactions between the myriad of cell types with which it interacts. Cells arise at the base of the crypt, where the stem cell compartment is located, and they become progressively more differentiated as they proceed along the villus to the top of the crypt, before being shed into the lumen(1). Due to their location intestinal epithelial cells (IECs) will interact with cells of myeloid, lymphoid, and as well as mesenchymal lineage, while they travel up the crypt. This crucial single layer of cells functions to provide an important physical barrier, separating the host from what is often deemed hostile contents of the gut lumen. The past few years have led to an ever increasing body of evidence that shows that in addition to providing a passive barrier, IECs are in fact important players in the activation of gut inflammatory response.

1.1.2 Innate Immune Response

As mentioned before, the role of IECs has evolved from a passive bystander to more of a sentinel of the intestinal immune system. IECs are able to respond to a milieu of adherent and invasive bacteria, as well as evolutionarily conserved soluble bacterial antigens that are known as pathogen associated molecular patterns (PAMPs). PAMPs include such things as CpG DNA, lipopolysacharide (LPS), flagellin, and cell wall

polymers(2). A number of recent studies have reported that IECs can express receptors that are capable of recognizing these PAMPs. These receptors are highly conserved throughout evolution, and known as pathogen recognition receptors (PRRs). They include extracellular receptors that belong to the toll-like receptor family (TLR)(3-9), as well as soluble internal receptors such as CARD4/Nod1 and CARD15/Nod2(10-14). In addition IECs have the ability to respond to proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα)(15). As a result of IEC activation, a powerful and robust pro-inflammatory cascade is set off, including the resultant expression of proinflammatory cytokines, chemoattractants (such as chemokines), as well as the induction of adhesion molecules(16-23). This results in the activation and recruitment of adjacent lamina propria mononuclear cells (LPMNCs), dendritic cells, mast cells, as well as the recruitment of peripheral blood mononuclear cells (PBMNCs), and polymorphonuclear cells (PMNs). Such a robust activation of the inflammatory cascade highlights the need for equally strong regulatory mechanisms that maintain a state of hyporesponsiveness between IECs and the commensal bacteria of the gut. These mechanisms are not completely understood, however may involve T-regulatory cells, as IECs have been shown to activate and expand T-regulatory cells(24, 25), and may also involve probiotic commensal bacteria of the GI flora, which have been shown to suppress intestinal inflammation(26). Breakdown of the homeostasis can result in chronic inflammatory conditions such as inflammatory bowel disease (IBD). It should be noted that although IECs have been reported to express major histocompatibility complex (MHC) class II, they are not capable of expressing the

appropriate co-stimulatory molecule and thus can not fully activate T cells, but instead annergize T cells to promote local tolerance to intestinal antigens(27, 28).

Inducers of NFκB in IECs

Cytokines and Growth Factors
Interleukin-1
Interleukin-2
Interleukin-17
Interleukin-18
Leukotriene B4
Tumor necrosis factor
Platelet-derived growth factor

Oxidative Stress
Hydrogen peroxide
Ozone Reactive oxygen intermediates

Viruses and Viral Products
Adenovirus

Bacteria and Bacteria Products
Salmonella
Shigella
Enteropathogenic *E. coli*
Listeria monocytogenes
LPS
Peptidoglycan-polysaccharide
Toxic shock syndrome toxin 1

Molecules regulated by NFκB in IECs

Cytokines and chemokines
Interleukin-1β
Interleukin-6
Interleukin-8
GROα/β
RANTES
Macrophage inflammatory protein-2

Cell surface receptor
Interleukin-2R
CD95/APO-1 (Fas)
Adhesion molecule
ICAM-1
Inflammatory enzyme
Inducible nitric oxide synthase
Cyclooxygenase-2

Stress proteins
Complement factors B, C3, C4

Immunoregulatory molecules
Major histocompatibility complex I and II

Table 1 – Activators and Inducers of NFκB in IECs.

1.2 CHEMOKINES

1.2.1 Structure, Expression, and Function

One of the hallmarks of the inflammatory response is the migration of leukocytes from the blood to sites of active inflammation. Chemotactic cytokines, also known as chemokines, are small polypeptides (8-14 kDa) that play an essential role in the homing of immune cells to areas of active inflammation. There are over 50 chemokines that can be subdivided into four families based upon their N-terminal motif of cysteines. The two main superfamilies are the C-C chemokines and C-X-C chemokines, along with two smaller families, the C and the CX_3C family(29, 30). Although many of these chemokines play a role in the recruiting immune cells during inflammation, chemokines are also responsible for homing during normal homeostasis, as well some chemokines can also control angiogenesis, cell growth, gastrointestinal (GI) and cardiac organogenesis(31, 32).

Under normal conditions the large and small bowel have large numbers of cellular infilitrates that include B and T lymphocytes as well as monocytes, dendritic cells, and a small number of mast cells and eosinophils. As a result of this, the intestine is considered to be physiologically inflamed, and this would be considered pathological inflammation in any other tissue setting. The expression of chemokines is highly regulated, being restricted to specific cell and tissue types. IECs are important in the activation of the gut inflammatory response, and as a result can synthesize and release numerous chemokines and cytokines, including members of both the C-C, and C-X-C group of chemokines, thus playing an important role in the recruitment of these

infiltrating immune cells. Macrophage and neutrophil chemoattractants that can be produced by IECs include IL-8/CXCL8, epithelial-cell derived neutrophil attractant (ENA-78/CXCL5), growth related oncogene α (Groα/CXCL1), monocyte chemoattractant protein-1 (MCP-1/CCL2), and macrophage inflammatory protein-1 α (MIP-1α/CCL3), however this list is not exhaustive(20-22). IECs also produce macrophage inflammatory protein-3α (MIP-3α) which is important for chemoattracting $CD45RO^+$ T cells as well as immature dendritic cells(18). IECs also produce a large body of chemoattractants for $CD4^+$ T cells. The large proportion of T cells in the lamina propria are of TH_1 phenotype (interferon γ (IFNγ) producing cells), and IECs are capable of producing interferon-gamma inducible protein (IP-10/CXCL10), monokine induced by interferon γ (MIG/CXCL9), as well as interferon inducible T cell attractant (I-TAC/CXCL11), which can attract T helper type 1 cells (TH_1) phenotype T cells expressing chemokine receptor CXCR3(19, 33). A small proportion of $CD4^+$ T cells in the bowel are T helper type 2 cells (TH_2) (which produce anti-inflammatory cytokines such as IL-4 and IL-10), and IECs are capable of producing macrophage derived chemoattractant (MDC/CCL20) that is important for attracting TH_2 phenotype T cells that express CCR4(17). The balance between a large proportion of proinflammatory TH_1 $CD4^+$ T cells, and small proportion of anti-inflammatory TH_2 $CD4^+$ T cells maintains this basal inflammation of the intestine in a controlled fashion. As a result chemokine regulation of this balance by IECs plays an essential role in intestinal homeostasis.

1.2.2 IL-8

IL-8 is a very well studied prototypic CXC chemokine involved in the recruitment of neutrophils, macrophages, and T lymphocytes(21). Sources of IL-8 have been reported to include CD14+ macrophages(34), neutrophils(34), endothelial cells(35), fibroblasts(36), T lymphocytes, and epithelial cells. In normal homeostatic tissue, IL-8 is often undetectable, however during inflammation it is robustly induced, resulting in a ten to 100 fold increase in concentrations(37).

The regulation of IL-8 production has been studied extensively, and often used as a paradigm for the regulation of other chemokine family members. The transcription of IL-8 relies on the activation of an essential core promoter region spanning (positions –1 to –133) within the 5' proximal promoter(38-40). Within this core promoter there are binding sites for a number or transcription factors, including NFκB, activator protein-1 (AP-1), and CAAT/enhancer binding protein β(C/EBP-β), which is also known as NF-IL-6. Promoter analysis studies have revealed that binding of NFκB is essential for transcriptional activation of IL-8 promoter, however AP-1 and C/EBP are dispensable. In order to get full gene activation however all three transcription factors are required(37, 39-43).

A number of other signaling pathways also regulate the transcription of IL-8, through a variety of transcriptional interactions. In some systems the transcription of IL-8 requires the activation the Jun NH2-terminal kinase (JNK) pathway. This activation is independent of the requirement of NFκB(44). There is also a role for the repressor, NFκB repressing factor (NRF) as a negative transcription regulator of the IL-8 promoter. NRF binds to a negative regulatory element (NRE), which overlaps the NFκB binding

site. Depletion of this factor using antisense RNA, leads to spontaneous IL-8 synthesis(45). NRF thus has a dual role in that it is required for the stimulus-induced activation of IL-8, and requires the activation of JNK in order to do this(45). Another transcriptional interaction is that of transcriptional co-activator creb binding protein (CBP)/p300. CBP/p300 has histone acetyl transferase (HAT) activity and binds many of these transcription factors to form an enhancesome that can mediate efficient transcription of the IL-8 gene(46, 47).

A further epigenetic level of regulation of IL-8 transcription is also present. Signaling pathways can modify histone protein complexes, which in turn regulate the availability of specific genes to the appropriate transcription factor. In the case of IL-8, the p38 mitogen activated protein kinase (p38 MAPK) pathway has been shown to regulate the phosphorylation and acetylation of histone H3, and thus allow the promoter of IL-8 to be available for access to the transcription factor NFκB(48).

Once transcribed, IL-8 also is privy to post transcriptional regulation. During a state of homeostasis, IL-8 is not present due to the lack of both transcription of the message, as well as the instability of the message. IL-8 contains AU-rich cis regulatory elements (AREs) in the 3' untranslated region (3' UTR). The 3' UTR can be regulated and stabilized by many signaling pathways. A signaling pathway that has been implicated to regulate this in some systems is the p38 MAPK pathway. Inhibition of p38 during stimulus-induced IL-8 activation results in a marked reduction in the half life of IL-8 message, and as a result the rapid destabilization and degradation of IL-8(49-52).

1.3 NFκB

1.3.1 Structure

NFκB is a family of inducible heterodimeric transcription factors (Fig 2), including p65/relA, relB, c-rel, NFκB1, and NFκB2(53-55). These proteins are structurally related through their evoluntionarily conserved Rel homology domain. p65/RelA, relB, and c-rel are all synthesized in their mature form, while NFκB1 and NFκB2 are processed from p100 to p52, and p105 to p50, respectively. p100 and p105 also share a region of homology in their ankyrin repeats, which are homologous to those found on IκB, the cytoplasmic binding partner of NFκB(53-55). Upon activation, p65 mainly associates with p50. In addition, c-rel also associates with p50, however much less efficiently. Interestingly p100, in its unprocessed form, associates with and sequesters relB. p50 is constitutively processed whereas p52 is inducibly processed(56, 57). Upon activation and processing, they form p52/relB heterodimers. p65/p50 heterodimers are the most common form found in IECs(58). In addition to being synthesized in their mature form, p65, relB, and c-rel are all related because they all contain at least one transactivation domain.

Another group of proteins, the inhibitor of kappa B (IκB) proteins, bind to NFκB family members and keep them sequestered in the cytoplasm. These are a group of 5 members including IκBα, IκBβ, IκBγ, IκBε, and BCL-3, in addition to the processed IκB portions of p100 and p105. They all contain 6-7 ankyrin repeats, which mediates their binding to rel homology domains(53-55).

1.3.2 Regulation

Activation of NFκB usually occurs through one of two pathways, the classical/ canonical pathway, or the recently defined alternate pathway; however in both cases the IκB kinase complex is paramount in the convergence of upstream activators of NFκB and the subsequent activation of the appropriate NFκB response(53, 54, 57).

The classical pathway is responsible for the activation p65:p50 heterodimers. These individual proteins are normally found sequestered in the cytoplasm, by the inhibitory protein IκB. Upon receiving a pro-inflammatory stimulus such as TNFα or IL-1β, IκB is quickly phosphorylated by the inhibitor of kappa-B kinase (IKK) complex at serine residues 32 and 36 (detailed upstream regulation of IKK will be discussed later). This is a process that requires IKKβ, and that does not require IKKα(59-63). Phosphorylated IκB is polyubiquitinated by the E3-$SCF^{\beta\text{-}TRCP}$ ubiquitin ligase, complex with the β-TRCP complex functioning as the IκB specific ubiquitin ligase(64). Polyubiquitinated IκB is targeted for subsequent 26S proteasomal mediated degradation. Loss of IκB binding reveals the p65 nuclear localization signal allows it to proceed to the nucleus.

Before p65 can become transcriptionally active it requires post-translational modifications including both phosphorylations and acetylations. These are crucial regulatory steps in p65 mediated transactivation. Many molecules have been shown to regulate these post translational modifications. CBP/p300, which contains HAT activity, provides a necessary acetylation of p65 on lysines 218, 221, and 310, with lysine 310 being an essential acetylation for full transcriptional activity of p65(65, 66). Similarly, protein phosphorylations at serines 276, 311, 529, 535 and 536 have all been shown to

be required for full transcriptional acitivity of p65. Several protein kinases have been implicated in the regulation of some of these sites including mitogen and stress activated protein kinase (MSK) and protein kinase A (PKA) for ser 276(67-69), protein kinase C zeta (PKCζ) for ser 311(70), protein kinase CK2 for ser 529(71-73), calmodulin-dependent kinase IV (CaMKIV) for ser 535(74), and IKKβ, protein kinase B (PKB), phosphatidylinositol-3 kinase (PI3K), and NFκB inducing kinase (NIK) for ser 536(75-84). These post-translation modifications can affect which co-activators are capable of associating with p65.

The alternative pathway requires a signal from ligands that are usually distinct from those that activate the classical pathway. These include TNF superfamily members leukotriene-β receptor (LTβR), CD40 ligand, as well as TNF like weak inducer of apoptosis (TWEAK)(85-88). These signals converge on the IKK signaling complex and activate it, so that the p100 subunit may get phosphorylated. In contrast to the classical pathway, this process requires IKKα, not IKKβ(57). Once phosphorylated p100 is processed into p52 and an IκB portion that is targeted for polyubiquitination and subsequent proteasome mediated degradation. p52 dimerizes with RelB and has been noted to be a slower NFκB response than the classical, however it is a far more sustained response(89).

1.3.3 Function

NFκB has typically been associated with providing a protective cellular response. This can take a variety of shapes and forms, however for the most part can be divided into two groups; a protective anti-apoptotic/proliferative response(53), or a protective

innate immune response(55). In this thesis we will be discussing the protective innate immune response, for the most part, however it is important to note that NFκB is equally a potent regulator of the anti-apoptotic response, through its ability to respond to pro-apoptotic signals such as fas ligand, TNFα, and tumor necrosis factor weak inducer of apoptosis (TWEAK), and its regulation of the expression of many anti-apoptotic molecules such as BCL_{XL}, cellular inhibitor of flice (cFLIP), and cellular inhibitors of apoptosis (cIAPs), as well as proliferative proteins such as cyclin D1(53).

NFκB can respond to a variety of immunogenic agents. These range from cytokines and growth factors (such as IL-1β, TNF, M-CSF) to bacteria (Salmonella, and enteropathogenic *E. coli*) or bacterial soluble antigens, some of which include LPS, CpG DNA, peptidoglycan. In addition viral products such as double stranded RNA can also activate NFκB. Oxidative stress including hydrogen peroxide and reactive oxygen intermediates can also activate NFκB(15, 55).

Likewise the NFκB family plays an integral role in the transcription of many important mediators of the innate immune response. These include the very cytokines and chemokines that are capable of activating NFκB itself, such as IL-1β and TNFα, as well as the majority of the chemokines that have been described in the previous section. In addition, the expression of a wide variety of cell surface receptors, adhesion molecules, and complement cascade proteins are also regulated by NFκB. Inflammatory enzymes that also potentiate inflammation such as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (cox-2) are also targets of NFκB transcription(15, 55).

Studies using genetic knockouts have provided much insight into the function of specific members of the NFκB family. Knockout mice lacking the IKK1 (encodes IKKα) are viable however have severe defects in keratinocyte differentiation, bone and limb development, and lack mature B cells(63, 90-92). These mice are incapable of p100 processing(57). Likewise knockout of the NFκB1 gene (encodes p100) results in mice that have no B cell development(93). In contrast, knockout of either NEMO (encodes IKKγ) or IKK2 (encodes IKKβ) is embryonic lethal at E11.5-E12.5 due to TNFα dependant liver apoptosis(59, 60, 94, 95). Mouse embryonic fibroblasts (MEFs) from these embryos lack the ability to activate p65. Similarily p65 knockout in mice is also embryonic lethal at E15.5-E16.5, due to TNFα dependant liver apoptosis(96). RelB knockout mice die postnatally from multiorgan inflammation(97), and c-rel knockout mice have no developmental defects, but instead have defective lymphocyte and macrophage function(98). Disruption of p50 results in viable mice however these mice have defects in lymphocyte function(99).

1.4 IEC SIGNALING

1.4.1 IL-1 signaling

IL-1 is a very important pro-inflammatory cytokine that is responsible for the synthesis of many inflammatory mediators, including enzymes, adhesion molecules, chemokines, tissue degrading molecules, and acute phase proteins. There are two types of IL-1: IL-1α, IL-1β, as well as a closely related protein IL-1 receptor antagonist (IL-1RA) that acts as an antagonist to the type 1 IL-1 receptor (IL-1RI). Both IL-1α and IL-1β signal through the type 1 IL-1 receptor(100).

The type I IL-1 receptor is a member of the IL-1 receptor/toll like receptor (IL-1R/TLR) superfamily. This family consists of over 33 members that are all related based on a homologous cytoplasmic domain named the toll IL-1R (tir) domain. The members of the IL-1R/TLR are divided into 3 families based upon their extracellular domains. The first group is based on those family members that have Ig domains in their extracellular domain(100). This group includes the IL-1RI and the interleukin-18 receptor (IL-18R)(100, 101). An interesting member of this subgroup is also the interleukin-1 receptor type II (IL-1RII) that acts as a decoy receptor to inhibit IL-1RI signaling(102). The IL-1RII does not contain a tir domain, and thus can not participate in downstream cellular signaling, even though it is capable of binding and internalizing IL-1(103). The second group has family members that contain leucine-rich repeats (LRRs) in the extracellular domains, as opposed to Ig domains, in addition to containing a cytoplasmic tir domain. These include a variety of members that act as PRRs, such as a large group of toll like receptors (TLRs 1-9)(2). A small third group contains family member that contain only cytoplasmic tir domains. These proteins act largely as adaptors to facilitate downstream signaling. Members of this family include MyD88, and mal(104-106). Due to the conserved nature of the tir domain between family members of this group, they evoke similar downstream responses.

Binding of IL-1 to the IL-1R activates numerous cell signaling pathways (many of which will be discussed below). This results in the formation of a complex including the adapter protein MyD88(104, 107, 108). As mentioned before MyD88 contains a tir domain, as well as a death domain, through which MyD88 can dimerize with other molecules. Attraction of MyD88 allows the recruitment of the serine threonine kinase

interleukin receptor associated kinase-1 (IRAK-1)(107, 109). Prior to associating with MyD88, IRAK-1 is phosphorylated and activated by IRAK-4 which releases it from the cytoplasmic inhibitor tollip(109, 110). Activated IRAK-1 recruits tumor necrosis factor associated factor-6 (TRAF-6)(111). TRAF-6 then signals down to transforming growth factor activated kinase-1 (TAK1). At this branch point, several signaling pathways are activated including the MAPK family, as well as the IKK pathway.

1.4.2 MAP Kinases

The MAP kinase signaling cascades are very evolutionarily conserved, and perhaps some of the most ancient signaling pathways. These pathways play a very important role in the activation of the innate immune response. There are three main families, including the extracellular signal regulated protein kinases (ERK)(112), the c-Jun NH2-terminal kinases (JNK)(113, 114), as well as the p38 MAPKs(115). The activation of these three members requires a dual phosphorylation on a Thr-X-Tyr motif present on all three of these members. This phosphorylation occurs via a MAPK kinase (MKK). These MKKs are in turn activated by MKK kinases (MKKK), which themselves are thought to be activated by small G proteins.

1.4.2.1 p38 MAPK

p38 MAPK family is activated by MAPKKs, MKK3 and MKK6. These in turn are activated by upstream Rho family of GTPases, through mixed lineage kinase-3 (MLK3) and TAK1 (115). Although the role of p38 MAPK in the gut inflammatory response is not well known, several reports show that is plays a very important role in other systems. Inflammatory mediators such as IFNγ, iNOS, COX-2, IL-6 and TNFα, have all

been shown to be subject to regulation via p38 MAPK(116-120). In addition, a few reports have also described p38 being an upstream regulator of the activation of NFκB, being able to modulate the phosphorylation and transactivation of NFκB. p38 also has the ability to regulate the transcription factor AP-1 by regulating activating transcription factor-2 (ATF-2) phosphorylation. A third line of evidence that implicates p38 MAPK in the regulation of the inflammatory response is its ability to regulate message stability of several cytokines/chemokines through their 3' UTR. In the gut inflammatory response, the role of p38 MAPK is largely unexplored.

1.4.2.2 JNK

JNKs consist of over 10 isoforms encoded as splice variants from 3 separate gene loci. Activation of JNKs occur via the MAPKKs, MKK4 and MKK7. These are activated through the upstream Rho family of GTPases through the MEK kinase 1 and 4 (MEKK1/4) and MLK3. The role of JNK in proinflammatory signaling has been controversial, however it is clear that JNK is able to activate and regulate the transcription factor AP-1, through its ability to regulate c-jun(113, 114). This is important as AP-1 has the ability to regulate numerous chemokines, such as IL-8, as previously discussed.

1.4.2.3 ERK

ERKs are robustly activated by growth and differentiation factors, as well as cytokines. The activation of ERKs occurs via the MAPKKs, MKK1 and MKK2, which are activated by Ras through the Raf family of MKKKs(112). The two major isoforms of

ERK are ERK1/p44 and ERK2/p42. ERKs have previously been described to be responsible for growth and differentiation of IECs(121, 122), however their functional role in mediating the gut inflammatory response has been largely unexplored.

1.4.3 Protein Kinase CK2

Protein kinase CK2 (formerly known as casein kinase 2) is a ubiquitously expressed protein kinase that has been linked to over 160 cellular substrates(123). In spite of the numerous cellular targets that have been identified, its regulation and cellular function remain largely enigmatic. The CK2 holoenzyme is composed of 2 catalytic subunits (α or α') with two regulatory subunits (β)(124-126). CK2 is found to have basal activity even in the absence of stimulation, supporting the notion that it is a constitutively active kinase, however its specific activity can be modulated by growth and stress factors(127-129). CK2 has been co-localized in all cellular compartments including the nucleus, where its nuclear-matrix bound activity has previously been shown to be important for the regulation of apoptosis(130). It has also been linked to the regulation of many transcription factors, including C/EBP homologous protein (CHOP), CCCTC-binding factor (CTCF), c-myc, c-fos, β-catenin as well as NF-κB(131-136). Its role in activating NF-κB has been largely controversial, with different regulatory mechanisms in different cell systems. In mammary epithelial cells it has been linked to direct association with the IKK complex(135, 136), whereas other groups have shown it to regulate the transactivation of NFκB, through a direct phosphorylation of serine 529(71, 72). The role of protein kinase CK2 in the context of the gut inflammatory response is largely uncharacterized.

1.4.4 PI3K Pathway

PI3Ks compose a family of kinases that catalyse the 3' phosphorylation on inositol rings. These produce a variety of phosphoinositide products, including most notably the second messenger phosphatidylinositol(3,4,5)triphosphate ($PI(3,4,5)P_3$). $PI(3,4,5)P_3$ acts as a second messenger to regulate many cellular events including cell growth, cell survival, cytoskeletal remodeling, and intracellular organelle trafficking(137-143). The PI3K family contains four classes: I_A, I_B, II, and III, based on their structure and specificity for substrates. Class I_A PI3Ks are responsible for catalyzing the reaction from phosphatidylinositol(4,5)biphosphate $PI(4,5)P_2$ to $PI(3,4,5)P_3$ (140-143). Class I_A PI3K is most often composed of a regulatory subunit (p85) as well as a catalytic subunit (p110)(140-143). The p85 subunit contains 2 src-homology-2 (SH2) domains that facilitates the recruitment of tyrosine phosphorylated substrates at the membrane to the p110 subunit, which then can be activated, as recruitment of adaptor proteins (such as insulin receptor substrate (IRS)) relieves the constitutive inhibition in the p85-p110 complex(140-143). Other downstream effectors include PKB, some PKC isoforms, 3'-phosphoinositide dependant kinase 1 (PDK1), as well as others.

There is strong evidence for a role for PI3K in regulating the immune response. Cytokines such as IL-1β, IL-2, IL-3, IL-6, IL-7, IL-15, TNFα, and granulocyte/monocyte-colony stimulating factor (GM-CSF), have all been shown to activate PI3K(140-143). PI3Ks have been shown to act downstream of these cytokine receptors as well as T and B cell receptors and as a result regulate numerous immune functions including aspects of both the innate and adaptive immune response. In the IEC system the role of PI3K is largely unexplored however it has been shown to be a negative regulator of IEC

differentiation, a negative regulator of TNFα induced cox-2 synthesis, as well as being important for mediating cellular proliferation(144-147).

Although it is well accepted that PI3K can modulate NFκB signaling, its precise role, and that of one of its downsteam effector kinases, PKB, has been controversial. No work has yet been done to characterize these pathways in the IEC cell system.

1.4.4.1 PKB

PKB (also known as AKT or Rac) is known largely as a prosurvival kinase, regulating numerous proliferative and survival effectors including BAD, caspase-9, and the forkhead family of transcription factors(148). Its activation requires its membrane localization in a $PI(3,4,5)P_3$ dependant fashion, where it can undergo activating phosphorylations(148). The initial phosphorylation comes from as yet unidentified kinase termed phosphoinositide-dependant kinase 2 (PDK2) in its hydrophobic motif at serine 473. Some candidate kinases for this phosphorylation include PKB itself via autophosphorylation, integrin linked kinase (ILK), or an unidentified membrane associated kinase (149-152). Once phosphorylated at serine 473, this allows threonine 308 to be phosphorylated by PDK1, to induce its kinase activity fully(148). It is generally agreed that both phosphorylations are required for full activity, however examination of the phosphorylation status at serine 473 is not sufficient to assess kinase activity.

The role of PKB in the immune response is largely unexplored. PKB has been linked to the activation of NFκB, but the precise mechanism of activation has been controversial. There are reports that PKB is a direct regulator of IKK activity, through direct phosphorylation of IKKα, and thus being able to regulate the ability of p65 to

separate from IκB and translocate to the nucleus, in a PI3K dependant fashion(153, 154). More interestingly a number of reports have linked the PI3K/PKB pathway to the regulation of NFκB through regulation of p65 transactivation. This suggests that regulation of NFκB by PI3K, and PKB may be stimulus and system specific.

1.4.4.2 3'-Phosphoinositide dependant kinase 1

PDK1 is a nodal kinase regulating several downstream signaling pathways including several AGC kinases such as p70 S6 kinase, p90 ribosomal s6 kinase (p90 RSK), PKC, and PKB(155). PDK1 itself is an AGC kinase, and becomes activated through an essential phosphorylation of tyrosine 9 and trans-autophosphorylation of serine 241 (156, 157). PDK1 provides a critical phosphorylation on the activation loop of its downstream targets also(155). It is found to have high constitutive activity. Despite this several potential mechanisms exist to regulate its cellular activity, including regulation by $PI(3,4,5)P_3$, binding to heat shock protein 90 (HSP90), binding to 14-3-3, and subcellular distribution(155, 158, 159). Also there is some evidence that PDK1 can function in PI3K independent ways(160). Its role in the IEC cell system is largely unexplored, especially with respect to chemokine synthesis and NFκB activation.

1.4.4 IKK complex

Signal induced activation of IKK remains the rate limiting step in the activation of NFκB, thus its understanding is fundamental to understanding NFκB activation. The IKK signalsome is composed of a homodimer of the IKKγ/NEMO regulatory subunit with either a catalytic IKKα/IKKβ heterodimer or an IKKα homodimer(54, 161, 162). Some

specific functions have been ascribed for each of the subunits. IKKα has been shown to be essential for p100 processing (57), and regulating chromatin remodeling(163, 164). IKKβ has been shown to be essential for IκB phosphorylation on serines 32 and 36(94), as well as p65 phosphorylation on serine 536(81). Activation of IKK requires phosphorylation of the catalytic subunits on their activation loops. IKKα requires phosphorylation on serine residues 176 and 180, whereas IKKβ requires phosphorylations on serine residues 177 and 181(94). The mechanism through which upstream activation of these phosphorylations occurs is controversial. One possible mechanism is that a potential "IKK kinase" exists that phosphorylates these sites. Based on genetic knockout experiments, several candidate kinases have been implicated, depending on the cell system studied. In HeLa cells, using siRNA, TAK1 has been implicated(165). MEKK3 has been shown to be essential in MEFs(166). PKCς has been implicated only in mouse lungs cells but the same was not true in MEFs(167). A second possibility is that the IKK catalytic subunits trans-autophosphorylate themselves. This notion is supported by the observation that recombinantly expressed IKK α or β purified from insects or mammalian cells is constitutively active, due to phosphorylations on their active loops(168-173). In addition several reports have shown that homotypic oligomerizations results in trans-autophosphorylations(168). It is still unclear how these oligomerizations occur in the absence of overexpression, and where the initial phosphorylation comes from. It is hypothesized that there is a small pool of IKK that is active and capable of phosphorylating the remainder IKK molecules when there is ligand. Other factors that may play a role in the activation of IKK include HSP90 and cdc42(174).

1.5 INFLAMMATORY BOWEL DISEASE

1.5.1 Pathology

Inflammatory bowel disease (IBD) collectively refers to 2 conditions, Crohn's disease (CD), and ulcerative colitis (UC), which are characterized by a chronic idiopathic relapsing and remitting inflammation of the large or small bowel, and often leads to an irreversible impairment of gastrointestinal function. The North American prevalence of IBD is roughly 10-200 per 100,000 people. Although similar, CD and UC are defined based upon some markedly different characteristics. CD affects the terminal ileum, cecum, and large bowel, and often has patch lesions in between normal areas. UC on the other hand affects the rectum and continues proximal and is continuous. Inflammation in CD is transmural, affecting all muscle layers, and is characterized by lymphocyte infiltration as well submucosal fibrosis. UC inflammation is more superficial, however has a large lymphocytic and granulocytic infiltration, as well as a loss of goblet cells. In both cases this results in severe diarrhea, as well as blood loss. CD can lead to strictures, as well as bowel obstructions. UC can lead to loss of peristaltic function and rigidity of colon wall, as well as eventual toxic megacolon. Long term chronic inflammation as a result of IBD also increases the risk of colon carcinomas. Current treatments for IBD include 5-ASA compounds which target the activation of the transcription factor NFκB, corticosteroids, azathioprine/6-MP which target Rac and T cell apoptosis, surgery as well as monoclonal antibody therapy to TNFα for CD(175).

1.5.2 Pathogenesis

The pathogenesis of IBD still remains largely enigmatic, however it is generally well accepted that both CD and UC are complex genetic disorders. Despite this, studies using numerous murine models of IBD have brought forth a number of emerging concepts important to understanding human IBD. Currently there are several murine models of IBD; some of these are spontaneous, some require a haptenating agent, and while others require adoptive transfer of populations of T-cells. A role for genetics is highlighted by murine models that develop spontaneous colitis, and thus highlight a very important principle that there are several gene products, that once dysregulated, are sufficient to result in the development of very similar models of intestinal inflammation(176). Another important tenet is that the host background plays a paramount role in determining disease susceptibility. IL-10 knockout mice develop spontaneous colitis only on certain inbred backgrounds, whereas other inbred backgrounds are completely resistant(177). This underscores the notion of a complex interplay between a number of different gene products as a requirement for the pathogenesis of IBD.

A second and very important concept is the requirement of normal gut flora for the development and sustenance of colitis. Mice kept in germ-free conditions generally do not develop experimental colitis(178). Despite this there has not been one organism, or one group of organisms that has been found responsible for experimental colitis. In addition, a group of bacteria also seem to have protective effects have been termed probiotic bacteria(26).

A third emerging concept is that an imbalance between effector T-cell function and regulatory T-cell function can lead to IBD. Many models of murine colitis rely on an overproduction of pro-inflammatory cytokines to induce inflammation (eg tri-nitro benzene sulphonic acid (TNBS) induced colitis is dependant upon an IL-12 response to LPS in the gut). Likewise, murine models that are defective in the generation of regulatory T cells (Tgε26 mice), or defective in regulatory cytokine signaling (IL-10 -/-, transforming growth factor (TGFβ -/-) develop colitis as well(179, 180).

A fourth emerging concept is the imbalance of profile of T cells that occurs in IBD. Polarization to either TH_1 or to TH_2 leads to a major driving force for inflammation. Thus a critical balance between TH_1 and TH_2 CD4+ T cells must be maintained. CD is characterized by a TH_1 profile of cytokines, with IL-2, IL-12, IFNγ, and TNFα being very important players. In contrast, UC is often characterized by a TH_2 profile of cytokines, with increases in IL-4, IL-5, and IL-13. To further support this notion, experimental colitis (eg TNBS induced colitis) that is driven by a TH_1 response, histologically resembles CD. Likewise, experimental colitis driven by a TH_2 response (eg Oxazalone colitis), histologically resembles UC.

The role of the mucosal epithelium is an emerging concept in the pathogenesis and is the fifth concept. Maintenance of the barrier integrity of the mucosal epithelium is essential, due to the number of infiltrating lymphocytes that are basally present in the underlying lamina propria. Some of the best evidence for this comes from a transgenic model where a dominant negative N-cadherin was expressed. These mice had severe barrier dysfunction and as a result acquired colitis(181). In addition IECs are capable of expressing many PRRs such as TLRs and NOD receptors. These are important in

normal host defense and normally inert to the contents of the lumen. Dysregulation of this hyporesponsiveness may play an important role in the initiation of inflammation in IBD, as IECs are the first to encounter intestinal antigens, and damage. In addition defects in the innate immune response may be important as mutations in the intracellular signaling receptor NOD2 are responsible for between 5-15% of all people with CD(182, 183).

1.5.3 The role of IECs, chemokines, and NFκB

Although a large majority of work on IBD pathogenesis is looking at the breakdown of adaptive immune response, there is growing body of evidence that suggests that IECs, the transcription factor NFκB, and chemokines play critical roles. Breakdown in the regulation of these components may be instigating factors in the pathogenesis of IBD. Overproduction of the potent pro-inflammatory cytokines IL-1β and TNFα in the intestinal mucosa of patients with IBD is well documented(184-190). In cell culture systems, IL-1β and TNFα can robustly activate the transcription factor NFκB, thus it is not surprising that *in vivo* examination of IBD patients shows increased activation of NFκB in patients with active inflammation when compared to normal uninflamed control tissue(191, 192). In addition, a study examining localization of this NFκB activation reveals that it is limited to IECs and macrophages(191). One of the functional consequences of NFκB activation is that it can drive the expression of numerous inflammatory mediators, but most notably chemokines. There have been numerous studies examining levels of chemoattractants in tissues from patients with inflammatory bowel disease. These studies have shown that there are significant

increases in a variety of chemokines including IL-8, MCP-1, MCP-2, MCP-3, MIP-1α, MIP-1β, IP-10, RANTES, as well as GROα, in actively inflamed tissues when compared to normal uninflamed tissues(193-198). A recent study examining over 500 biopsy samples from patients with IBD, used immunohistochemistry to characterize the localization of these chemoattractants. Epithelial cells were determined to be one of the major producers of chemokines(198). Thus it can be hypothesized that a potential dysregulation of chemokine expression through activation of NFκB in IECs may be an important mechanism in the pathogenesis of IBD.

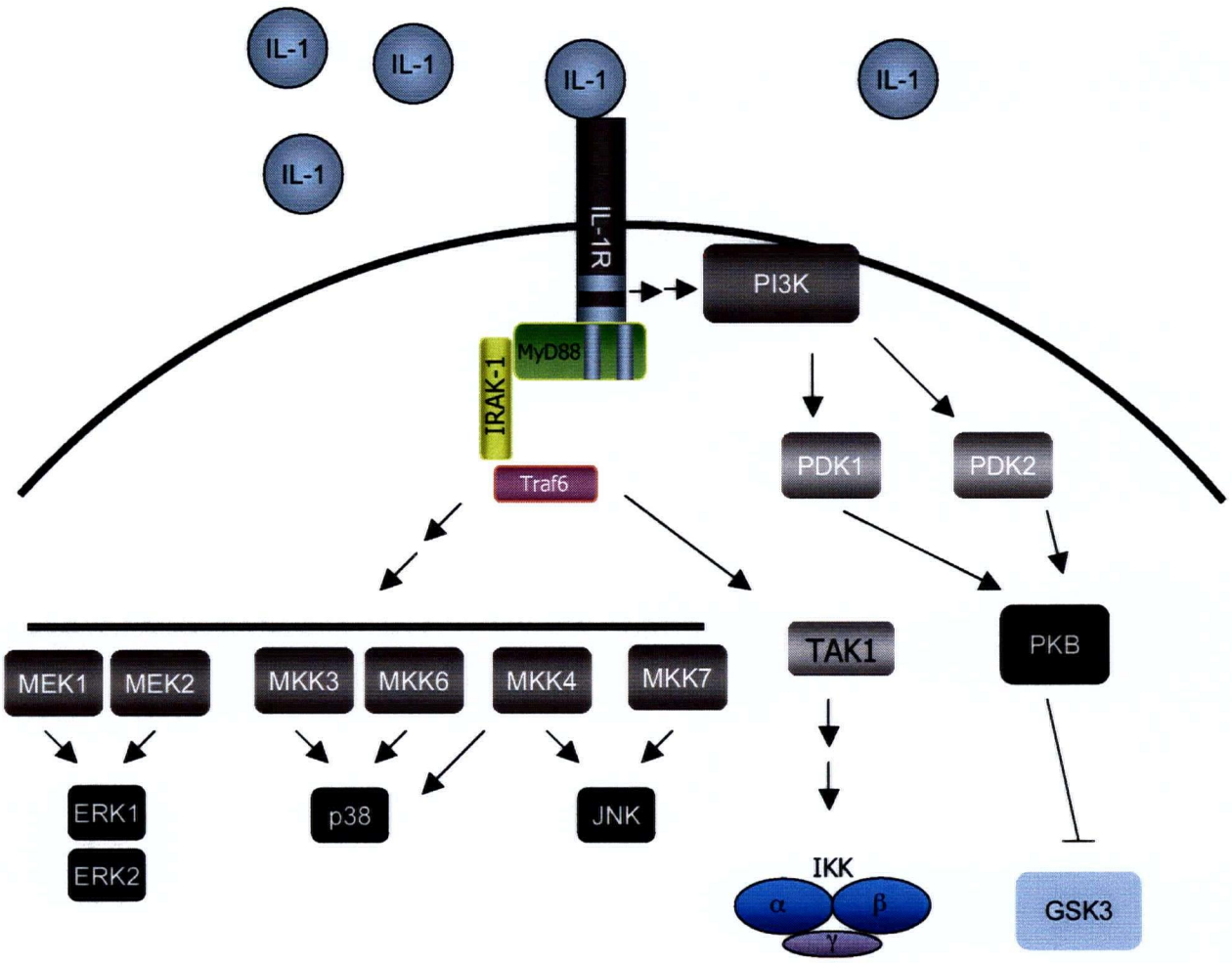


Figure 1. Signaling pathways activated by IL-1β

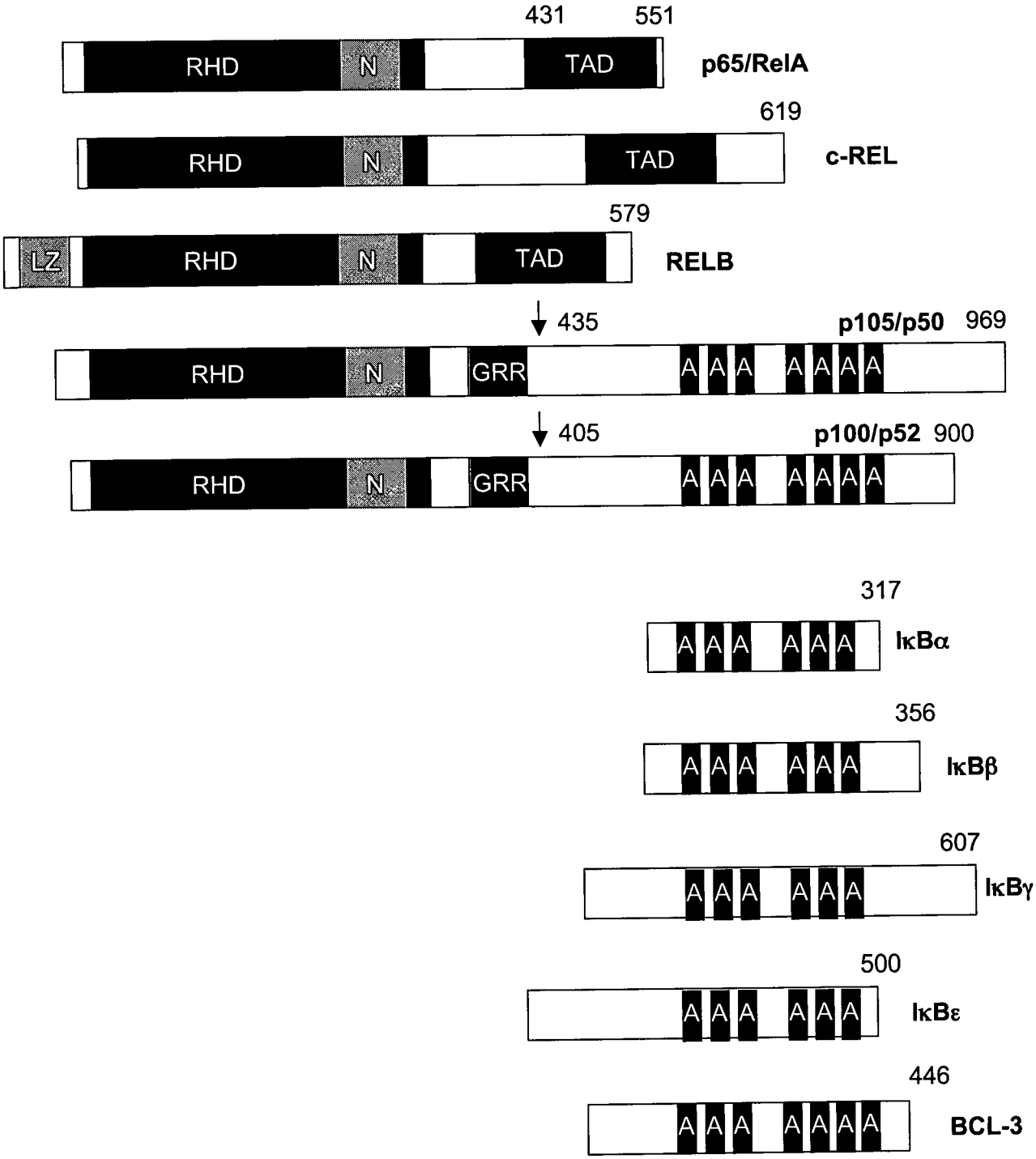


Figure 2. NFκB family members

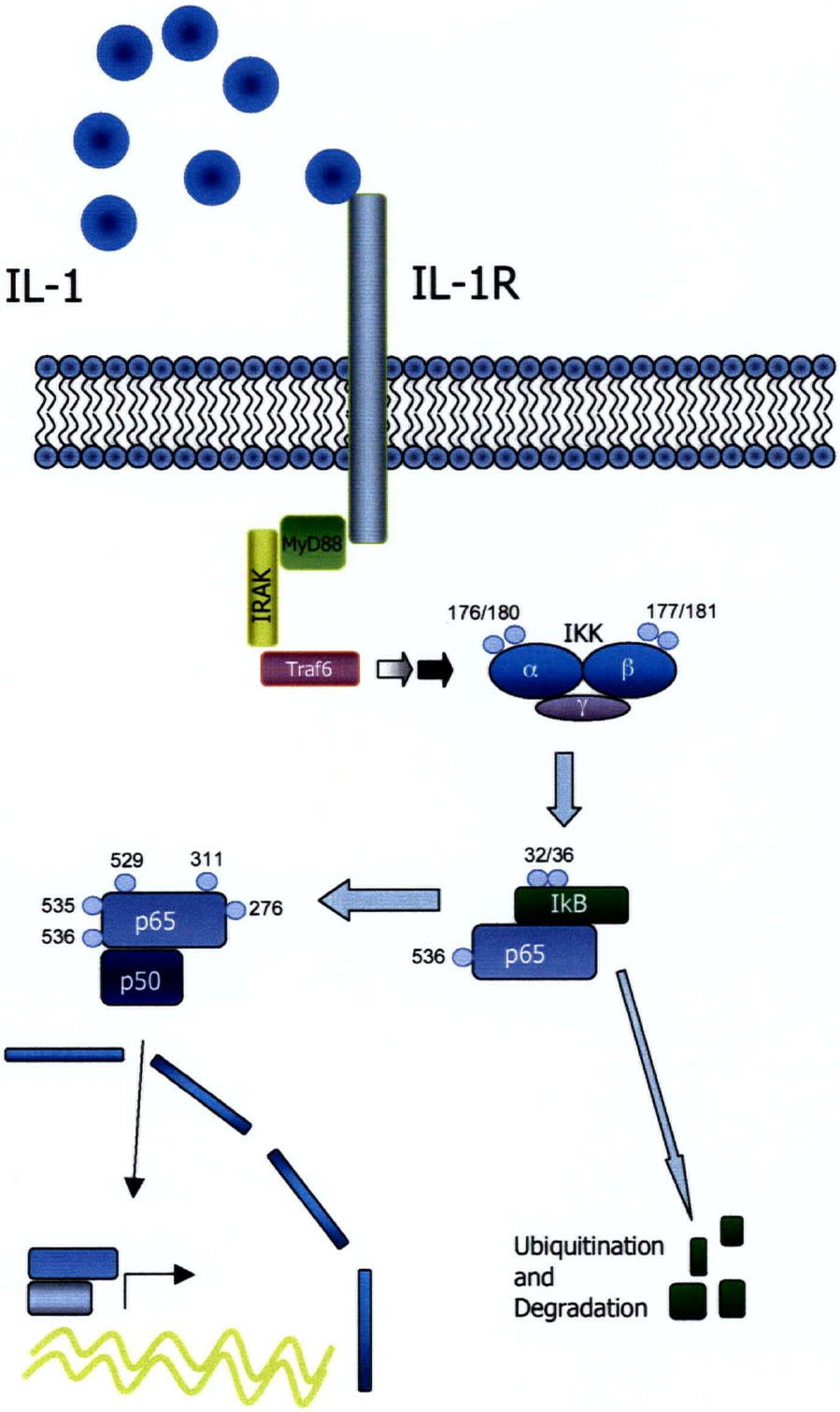


Figure 3. Detailed activation of NFκB, including important regulatory phosphorylations

CHAPTER 2 – MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines and Cell Culture

HCT-116, of IEC origin, were a kind gift of Bert Vogelstein (Johns Hopkins, Baltimore, Maryland). HCT-116 cells were cultured in McCoys 5A Medium (Gibco, Burlington, Ontario) containing 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah) with 100 U/mL of penicillan and 100 ug/mL of streptomycin (Gibco, Burlington, Ontario). Caco-2 cells and HT29 cells, of IEC origin, were acquired from the American Type Cell Culture (ATCC, Manassas, VA). Caco-2 and HT-29 cells were cultured in M199 Medium containing 10% FBS with 100 U/mL of penicillin and 100 ug/mL of streptomycin (Gibco, Burlington, Ontario). HEK 293T cells, of human embryonic kidney cell origin, were a kind gift of Alice Mui (University of British Columbia, Vancouver, BC). They were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat inactivated FBS with 100 U/mL of penicillan and 100 ug/mL of streptomycin. All cell lines were grown at 37°C with 5% CO_2 (v/v).

2.1.2 Reagents, Enzymes, and Chemicals

PD 98059, SB 203580, SP 600125, 5,6-dichloro-ribifuranosylbenzimidazole (DRB), apigenin, LY294002, wortmannin, actinomycin-D, and N-tosyl phenylalanyl chloromethyl ketone were purchased from Calbiochem (San Diego, California). All chemical stock solutions were made up in dimethylsulfoxide (DMSO) except for DRB in 100% ethanol,

and TPCK in methanol. All restriction enzymes were purchased from New England Biolabs (Missisauga, Ontario, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

2.1.3 Plasmids and cDNAs

Numerous plasmids and cDNAs were generously provided for use in this work:

WT and KD FLAG-p38	J. Han	Scripps Institute (San Diego, California)
WT and KD HA-CK2 α	D. Litchfield	University of W. Ontario (London, Ontario)
WT and KD HA-CK2 α'	D. Litchfield	University of W. Ontario (London, Ontario)
WT and KD MYC-PDK1	JS. Park	Friedrich Miescher Instit (Basel, Switzerland)
WT AND KD HA-PKB	Kinetek Pharmaceuticals (Vancouver, BC)	
4xκB-Luc	Kinetek Pharmaceuticals (Vancouver, BC)	
IL8-Luc	G. Wu	University of Pensylvania (Philadelpha, PA)
pfr-Luc	Stratagene	(La Jolla, California)
pTADI-III	BR Cullen	Duke University, (Durham, North Carolina)
pTADIII	BR Cullen	Duke University, (Durham, North Carolina)
pM-p65 (521-551)	T. Okamoto	Nagoya University (Nagoya, Japan)
pM-p65 (521-551, S529A)	T. Okamoto	Nagoya University (Nagoya, Japan
pM-p65 (521-551, S536A)	T. Okamoto	Nagoya University (Nagoya, Japan)

2.1.4 Primers

Primers used in RT-PCR are as follows:

IL-8 Forward: 5'-TCTGCAGCTCTGBTGTGAAGGTGCAGTT-3'
IL-8 Reverse: 5'-TTCCTTTGACCCACGTCTCCCAA-3'

MCP-1 Forward: 5'-TCTGTGCCTGCTGCTCATAGC-3'

MCP-1 Reverse:	5'-GGGTAGAACTGTGGTTCAAGAGG-3'
Actin Forward:	5'-CCAACCGCGAGAAGATGACC-3'
Actin Reverse:	5'-GATCTTCATGAGGTAGTCAGT-3'
IκB Forward	5'-TACACCTTGCCTGTGAGCAG-3'
IκB Reverse	5'-AGGATTTTGCAGGTCCACTG-3'
GROα Forward	5'-ACTCAAGAATGGGCGGAAAG-3'
GROα Reverse	5'-TGGCATGTTGCAGGCTCCT-3'
TNFα Forward	5'-CGGGACGTGGAGCTGGCCGAGGAG-3'
TNFα Reverse	5'-CACCAGCTGGTTATCTCTCAGCTC-3'
iNOS Forward	5'-CGGTGCTGTATTTCCTTACGAGGCGAAGAAGG-3'
iNOS Reverse	5'-GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC-3'
RANTES Forward	5'-CCAACCCAGCAGTCGTCTTTG-3'
RANTES Reverse	5'-CTCCCAAGCTAGGACAAGAGC-3'

2.2 METHODS

2.2.1 Nuclear Preps

Cells were seeded onto 60 mm plates and grown to confluence, and then pretreated with the appropriate inhibitor for 1 h, before being stimulated with IL-1β for 30 min. Cells were washed once with ice cold phosphate buffered saline (PBS), scraped into 1 ml of PBS before being centrifuged at 14,000 RPM. The pellet was resuspended in 200 uL Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 min, before adding 13 uL of 10 % Nonidet NP-40. The cell suspension was vortexed for 10 s before being centrifuged again at 14,000 RPM for 30 s. The supernatant was removed and the nuclear pellet resuspended in 30 uL of Buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM

DTT, 1 mM PMSF). The sample was vigorously rocked for 15 min and subsequently centrifuged for 5 min at 14,000 RPM. The supernatant was retained and the protein concentration determined by the Bradford assay (Bio-Rad, Mississuagua, Ont). Samples were stored at -80°C until use.

2.2.2 Electrophoretic Mobility Shift Assay (EMSA)

A synthetic κb oligonucleotide was cloned into the cloning vector, pBS (Stratagene, La Jolla, CA), using the *Eco*RI and *Hin*dIII sites, to create pBS-EMSAκb. To radiolabel the probe, it was excised from pBS-EMSAκb using *Eco*RI and *Hin*dIII, and labelled using [γ-^{32}P]dCTP (Amersham, Montreal, Québec, Canada) and the Klenow fragment of DNA polymerase (New England Biolabs, Missisauga, Ont., Canada). The probe was then purified by running on a 5% non-denaturing gel, cutting out the fragment, and incubating the gel slice in elution buffer [(0.6 M ammonium acetate, 1 mM EDTA, 0·1% sodium dodecyl sulphate (SDS)] overnight.

Briefly, 10 ug of nuclear extract were preincubated in binding buffer (20 mM Hepes pH 7.9, 100 mM KCl, 10% glycerol, 1 mM DTT) and 1 ug of poly dIdC (Amersham, Montreal, Quebec), for 15 min. 20,000 CPM of hot probe was then added, and the reaction mixture incubated at room temperature for 30 min, and subsequently resolved on a 5% non-denaturing polyacrylamide gel in 0.25 x TBE at 200V for 1.5 h. The gel was then dried for 45 min before phosphoimaging analysis using a Bio-Rad molecular imager FX (or alternatively exposed to film overnight at -80°C and then developed). For supershift or cold competitor reactions, the nuclear extract was preincubated with 1 ug of anti-p65 antibody (Calbiochem, San Diego, California), or

100-fold excess of unlabelled probe with binding buffer and poly dIdC for 30 min before adding the radiolabelled probe. Alternatively, AP-1 oligonucleotides were labeled using polynucleotide kinase, and [^{32}P]ATP. Labelled probe was cleaned as described above. The oligonucleotide sequence used were:

CGCTTGATGACTCAGCCGGAA, Human AP-1 EMSA Forward

TTCCGGCTGAGTCATCAAGCG, Human AP-1 EMSA Reverse

2.2.3 Immunoblotting

Cells were washed once with ice cold PBS, resuspended in homogenization buffer (20 mM MOPS, 50 mM b-glycerophosphate, 5 mM EGTA, 50 mM NaF, 1 mM DTT, 1 mM sodium vanadate, 1% NP-40 and 1 mM PMSF) for 30 min, sonicated for three 5 s intervals on ice at 30% output, before being centrifuged at 14,000 RPM for 15 min. The protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad, Mississauga, Ont). 50 mg of protein from each sample was resolved using 10% SDS-PAGE before transferring to nitrocellulose membranes (Bio-Rad). The blots were blocked in 5% skim milk in TBST (20 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.05% Tween-20) for 1 h before probing for 2-4 h using the appropriate primary antibody. The blots were washed with TBST for 10 min three times, before being incubated with the appropriate secondary antibody for 1.5 h. Following 3 further washes in TBS-T, they were developed using the enhanced chemiluminescence detection system (ECL, Amersham, Montreal, Quebec). Phospho-IκB, IκBα, phospho-IKK, phospho-p38, phospho-JNK, JNK, phospho-ERK, ERK, phospho-PKB, phospho-PDK1, phospho-p65 antibodies were purchased from Cell Signaling (Missisaugua, Ont). Antibodies to PKB

kinase (PDK1), p65, IKKγ, IKKβ, CK2 α and α' were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), or were a kind gift of Steve Pellech (UBC, Vancouver, BC, Canada). Antibody to p38 MAPK was purchased from Stressgen Biotechnologies (Victoria, BC).

2.2.4 CK2 Phosphotransferase Activity Assays

CK2 assays were carried out as previously described (129). Briefly 5 ug of protein lysate was incubated in a final volume of 25 uL with 5 ug of partially phosphorylated casein or the specific CK2 substrate RRADDSDDDDD and 100 □M [^{32}P]GTP (2.5 □Ci/assay in Buffer C (12 mM MOPS (pH 7.2), and 15 mM $MgCl_2$) for 15 min at 30°C. The phosphorylation of casein or the specific substrate was quantitated by spotting 20 ml on to a 1.5 cm^2 piece of Whatman P-81 phosphocellulose paper. The papers were washed extensively in 1% (w/v) phosphoric acid, transferred into 6-ml plastic vials containing 0.5 ml of Ecolume (ICN) scintillation fluid, and the incorporated radioactivity was measured in a Wallace (LKB) scintillation counter. CK2 immunocomplex assays were also carried out as previously described. Immunoprecipitations were performed by incubating 500 ug of whole lysate with 4 ug of CK2α polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) for overnight and collected with 20 ul of protein A-Sepharose (preblocked with 1% BSA) for 3-4 h at 4°C. The beads were washed four times with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40. The immunoprecipitated beads were washed one additional time with buffer A (12 mM MOPS (pH 7.2) and 15 mM MgCl2), and incubated with either 5 mg of casein of the specific CK2 substrate RRADDSDDDDD. Reactions were stopped by the addition of SDS-PAGE sample

buffer. After boiling, the samples were subjected to sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) for autoradiography with X-ray film or by immunoblotting analysis.

2.2.5 Transient Transfections

Cells were seeded at a confluency of 75%. 24 hours later cells were transfected using Lipfectamine 2000, as per the manufacturers instructions, at a ratio of 2:1 (liposome uL:DNA ug). 1 ug of plasmid DNA per 60 mm plate was used. Cells were incubated overnight, and cell medium was aspirated and fresh medium was added. Cells were allowed to express the proteins for over 24 hours, before the experiment was carried out.

2.2.6 Luciferase Assay

0.5 ug of an NFκB-dependant reporter containing 4 repeats of the κb consensus sequence from the IL-1β gene (4xκb-Luc), was cotransfected with a LacZ plasmid that constitutively encodes a β-galactosidase gene (kindly donated by William Jia, UBC). Cells were pretreated for 1 h with the appropriate inhibitor, stimulated with IL-1β for 6 h, before being harvested. Luciferase and β-galactosidase activities were measured according to the manufacturer's instructions (Promega, Madison, WI). Light emission was measured using a luminometer, and the luciferase readings were normalized using β-galactosidase activity.

2.2.7 Isolation of RNA and RT-PCR

RNA was isolated using the TRIZOL method (Life Technologies, Burlingtion, Ontario). The purity of the RNA was determined by running 1 mg of RNA on a 1 % agarose gel for 1.5 h at 75 volts. 1 mg of RNA was reverse transcribed using 0.5 mg of oligo (dT)12-18 (Amersham, Montreal, Quebec), 1 ml of 10 mM dNTPs, 2 ml of 0.1 M DTT, 40 units of RNA-guard (Amersham, Montreal, Quebec) in 1x first strand buffer (Life Technologies, Burlington, Ontario) using 200 units of M-MLV reverse transcriptase, by incubating the reaction mixture for 50 min at 37°C. 2 ml of cDNA was used in each subsequent polymerase chain reaction (PCR) reaction. For each 50 uL PCR reaction, 2 U of TAQ (PE Biosystems, Branchburg, New Jersey), 1 x PCR Buffer (PE Biosystems, Branchburg, New Jersey), 10 pmol of each primer, 1 ml of 10 mM dNTPs, and 3 ml of 25 mM MgCl2 were used. The PCR temperatures used were: 94°C denaturing for 45 s, 56°C annealing for 45 s, and 72°C extension for 1 min. 10 uL aliquots of the reaction were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Negative controls for cDNA synthesis were run without template, and also without RT. Linearity of PCR reactions was determined in the range between 20-40 cycles. Linearity of the reaction was further determined by template dilutions (1 in 10 and 1 in 100). Densitometry was performed using Bio-Rad Quantity-One software.

2.2.8 Tissue Procurement and Immunohistochemistry

A total of 18 cases of normal or active IBD were obtained through Dr. D. Owen from the Division of Anatomical Pathology at Vancouver Hospital and Health Sciences Centre (VH&HSC). Paraffin-embedded colonic tissue samples were de-waxed in xylene twice for 5 min, rehydrated in a series of ethanol (100%-70%)for 3 min each

followed by rehydration in PBS for 30 min. After rehydration the endogenous peroxidase was blocked with 0.3 % hydrogen peroxide followed by antigen retrieval by microwaving sections in citrate buffer pH 6.0 (10 mM Na-citrate). Following antigen retrieval, the sections were stained using the above mentioned kit according to manufacturer 's recommendations but with the following modifications. Sections were incubated with the primary antibody at 4° C overnight at the indicated dilutions: CK2α (1:100). Sections were stained with Vectastain ABC elite kit and DAB secondary detection kit (Vector Laboratories, CA, USA). Each section had its own control using the secondary antibody only. Pre-immune serum was initially used to ensure specificity of the signal with each of the antibodies.

2.2.9 ELISA assays for IL-8 and MCP-1

Cells were pretreated with the appropriate inhibitors and stimulated for 36 hr with IL-1β. The supernatants were sampled and the chemokine concentration was determined, in triplicate, by using enzyme-linked immunosorbent assay (ELISA) (BD Pharmingen, Missisaugua, Ont., Canada), as per the manufacturer's instructions. If required, supernatants were stored at -80°C prior to use. The sensitivity of the MCP-1 and IL-8 ELISA assays was 1.0 pg/mL and .8 pg/mL, respectively. This was determined b y the manufacturer and defined as two standard deviations above the mean optical density of 20 replicates of the zero standard.

2.2.10 MAPKAPK2 assay

This was carried out as an *in vitro* assay using crude lysate. Briefly, Caco-2 cells were preincubated with SB 203580 for 2 h, stimulated with IL-1β (2 ng/ml) for 30 min and

then harvested in homogenization buffer (described under section 2.2.3 Immunoblotting). Ten uL (equal to 10 µg of protein) of cell lysate was then used in a kinase assay, using heat shock protein 27 (HSP 27) (1 µg; Stressgen Biotechnology) as the substrate and 0.5 µg of ATP (250 µM ATP, 1 µCi [γ-^{32}P]-labeled), for 20 min at 30°C. Samples were then boiled in 5x sample buffer and resolved on SDS-PAGE. After transfer to nitrocellulose membranes, the substrate bands were visualized using autoradiography. Ponceau staining was carried out to demonstrate equal loading.

2.2.11 Immunoprecipitation

Lysates were homogenized and normalized for protein using Bradford assay as described under *immunoblotting*. 400 ug of soluble protein in a final volume of 500 uL of homogenization buffer was used. 5 uL of antibody was added and the tubes placed on a rotator overnight at 4°C. The following morning, 30 uL of 1:1 slurry of protein A/G (Sigma, Oakville, Ontario) beads were added and then rotated for 1 h at 4°C. Following this the beads were washed twice with homogenization buffer and twice with KII buffer (12.5 mM -glycerol phosphate, 20 mM MOPS pH 7.2, 5 mM EGTA, 7.5 mM MgCl 2, 50 mM NaF and 0.25 mM DTT). The beads were then resuspended in 15 uL of KII and subjected to an immune complex kinase assay as described below.

2.2.12 Immune complex assays for PKB and IKK signalsome

10 uL of the substrate cocktail, containing either histone H2B (1 ug), GST-IκB (1 ug), or GST-p65 (1 ug) in assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM -glycerophosphate, 20 mM MgCl 2, 5 mM EGTA, 2 mM EDTA, 1 mM DTT and 1 mM sodium vanadate), were added to the washed beads. and the reaction carried out as

above for 20 min using 5 uL of the ATP cocktail (250 mM ATP, 1 Ci [γ32 P] ATP). 10 uL of 5X sample buffer were added to the beads and after boiling for 5 min. The samples were resolved by 10% SDS-PAGE, and stained with coomasie blue. The gel was then dried for 45 min before phosphoimaging analysis using a Bio-Rad molecular imager FX (or alternatively exposed to film overnight at -80°C and then developed).

CHAPTER 3 – MAPK REGULATION OF IL-1β SIGNALING

3.1 RATIONALE AND HYPOTHESIS

As mentioned previously, there is strong evidence for MAPK signaling downstream of IL-1β. The functional role of each of the MAPK family members in IECs is not known, especially with respect to activation of NFκB, and subsequent synthesis of chemokines. In other systems, recent work has shown a role for p38 in the synthesis of many other inflammatory mediators. These observations include regulation of IFNγ in lymphocytes, iNOS in astrocytes and glial cells, and cox-2, IL-6, and TNFα in monocytes(116-120). Also the food agent curcumin has been shown to be an inhibitor of JNK(199). Preliminary reports have shown that curcumin can in fact modulate both NFκB signaling and chemokine production(200). Thus there is strong evidence to support the notion that MAPK family will regulate IEC chemokine production.

3.2 RESULTS

3.2.1 IL-1β activates protein tyrosine phosphorylation and MAPKs in IECs

The effect of IL-1β stimulation on the temporal characteristics of the activation of the MAPK family members was first examined. The data indicate that all three MAPKs were activated within 10-15 min, with p38 MAPK exhibiting the earliest activation (Fig. 4A). In addition it is apparent that the activation was sustained for at least 60 min. The lowest panel shows that proteins corresponding to MAPKs (with a molecular mass of 40-44 kDa) become tyrosine phosphorylated at similar times.

To address the role of p38 MAPK the selective inhibitor SB 203580 was used(201). To confirm that the SB 203580 inhibited signaling through to downstream mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), *in vitro* phosphorylation reactions were performed using its substrate, heat shock protein (HSP 27). The data indicates that preincubation with the p38 inhibitor attenuated activation of this downstream target (Fig. 4B).

3.2.2 SB 203580 attenuates IL-8 and MCP-1 production by IL-1β stimulated IECs

Next the effect of the p38 MAPK inhibitor, SB 203580, on the production of an α-chemokine (IL-8) and a β-chemokine (MCP-1) by IECs was examined. This inhibitor acts by binding to the ATP-binding pocket of p38(201). Pretreatment of both Caco-2 cells and HT-29 with SB 203580 led to a significant reduction in IL-8 production (Fig. 5A). A similar inhibitory effect of the p38 MAPK inhibitor was observed on MCP-1 production in Caco-2 cells (Fig. 5B). HT-29 cells do not produce any MCP-1 in response to IL-1β alone, as previously reported(202). PD 98059 (an inhibitor of ERK via upstream activator-dependent phosphorylation) had no effect upon the production of either chemokine in the Caco-2 cell line (lane 4). The data indicates that p38 MAPK is activated in IECs in response to IL-1β and is involved in the production of important immunoregulatory chemokines.

3.2.3 IκB phosphorylation and degradation are independent of p38 MAPK

As previously mentioned NFκB has been reported to be a major regulator of IL-8 and MCP-1 transcription. The phosphorylation and subsequent degradation of IκB is an integral step in the activation of NFκB. Previously, protein kinase CK2 has been shown to be downstream of p38 MAPK in response to stress(129). Therefore the possibility that p38 MAPK or downstream CK2 were involved in the phosphorylation and degradation of IκB was explored. However, as the data indicates, pretreatment of cells with either SB 203580 or DRB, a specific inhibitor of protein kinase CK2 at the concentrations used, did not prevent IL-1β induced phosphorylation and degradation of IκB (Fig. 6A).

3.2.4 NFκB DNA binding and transactivation are independent of p38 MAPK activation

NFκB is regulated by its ability to translocate to the nucleus and bind to its consensus sequence. Therefore, the potential role of p38 MAPK on this aspect of NFκB activation was investigated. Electrophoretic mobility shift assays (EMSAs) were performed on nuclei isolated from Caco-2 cells. Pretreatment of cells with SB 203580 or DRB did not prevent nuclear translocation or the ability of NFκB to bind to its consensus sequence (Fig. 7A). It can be seen that there was no effect upon the bound probe signal with either of the two inhibitors. In order to confirm that in fact p65 was the major NFκB family member binding to the NFκB consensus sequence, a supershift assay was performed, by preincubating nuclear extracts with the anti-p65 antibody before addition

of the probe. As seen, the p65/RelA band was almost entirely shifted up with the addition of the antibody (Fig. 7B). DNA binding alone does not confer transcriptional activation, as NFκB requires multiple transactivating phosphorylations in order to be functionally active once bound to DNA(203). To investigate the possibility that p38 MAPK may be phosphorylating and regulating the transactivation of p65, we performed luciferase reporter assays with four synthetics repeats of a consensus κB site fused to a luciferase gene(4xκB-Luc). The data indicates that there is a threefold increase in NFκB activation upon exposure of Caco-2 cells to IL-1β (Fig. 7C). Pretreatment with SB 203580 did not attenuate the activation of NFκB. Curcumin was found to attenuate NFκB activation, in accordance with a previous report(200). This data excludes an effect of p38 MAPK upon NFκB activation in this cell system.

To confirm that there was no involvement of p38 MAPK upon NFκB DNA binding, we transiently transfected Caco-2 cells with both the wild-type and kinase-dead versions of p38 MAPK, and investigated their influence upon DNA binding in response to stimulation with IL-1β. These interventions were found to have only a marginal effect upon this parameter (Fig. 8A). In addition the effects of transient transfections of the p38 MAPK constructs upon NFκB reporter activation were examined. The data indicated (Fig. 8B) that despite the small changes observed in the DNA-binding study (Fig. 8A), this is not translated into a reduction in transactivation. We believe that this complements the data obtained with the SB 203580 inhibitor, and establishes that p38 MAPK is not involved at any step of NFκB activation within the Caco-2 cell system.

The control western immunoblot (Fig. 8C) shows that the p38 MAPK protein was overexpressed. In contrast to the data obtained for NFκB, we were able to show a

reduction in IL-1β induced AP-1 DNA binding using SB 203580 (Fig. 9). This reduction was limited to IL-1β induced AP-1 binding and had no effect on basal constitutive AP-1 DNA binding.

3.2.5 IL-8 and MCP-1 messages are regulated by p38 in IECs

Next the role of the p38 MAPK inhibitor on the relative amounts of both IL-8 and MCP-1 message was investigated. A time-course investigation using semiquantitative reverse transcriptase PCR (RT-PCR) was carried out, and after resolving the PCR products the bands were quantified by densitometry and corrected for using actin expression. The data indicate (Fig. 10A and 10B) that there was a reduction in the message for both of the chemokines investigated. The magnitude of this reduction was 40% for IL-8 and 50% for MCP-1. In order to verify or exclude a role for message stability being the mechanism for reduction of chemokine expression, RT-PCR assays were repeated using actinomycin-D to stop active transcription, as previously reported(204). The possibility of message stability being the mechanism for reduction of chemokine expression was excluded by our findings (Fig. 10C). More specifically, whereas Wang et al. were able to show an almost 80% reduction in LPS stimulated messages for both TNFα and IL-6 in human monocytes within 20 min, our findings showed negligible changes, even as late as 120 min into the assay.

3.2.6 p38 is involved in activation of the IL-8 promoter

Our findings did not confirm an effect on chemokine message stability, and thus further studies were performed with the previously characterized IL-8 luciferase (IL8-

Luc) promoter construct to determine whether p38 was involved at this level(41). The data clearly indicate that using SB 203580 resulted in a significant attenuation of promoter activation (Fig 11A). The data also indicate that the p38 kinase dead (p38KD) construct reduces IL-8 promoter activation when compared to cells transfected with the empty vector (EV). Unexpectedly there was also a smaller reduction of activation using the wildtype (p38WT) construct when compared with the empty vector (Fig. 11B). The explanation for this is not clear and may be a result of the activation of counter-regulatory pathways by a constitutively active p38 signal.

3.2.7 Pharmacological inhibitor SP 600125 inhibits IL-1 induced JNK activity however has no effect on p65 DNA binding

To expand on the previous reports using curcumin, as well as our own work that have successfully reproduced the curcumin induced inhibition of chemokine production (data not shown), the selective JNK inhibitor SP 600125 was used(205). Using curcumin we have observed inhibition of IL-8 promoter activation, as well as inhibition of NFκB activation (Fig 7C AND 8A). Thus this work was repeated using SP 600125. Treatment of cells with 20 uM SP 600125, prevented the IL-1β induced activation of JNK (Fig 12A). When the phosphorylation status of JNK was examined, it clear that it inhibits JNK phosphorylation (Fig 12B). Interestingly, p38 phosphorylation was also inhibited, suggesting a degree of nonspecificity. When ERK was examined, there was no effect on its IL-1β induced phosphorylation (data not shown). When the DNA binding of p65 was examined, SP 600125 pretreatment had no effect (Fig 12C). Similarily there was no effect on IL-1β induced IκB degradation (data not shown).

3.2.8 SP 600125 inhibits IL-1β induced NFκB activation and IL-8 promoter activation

Since SP 600125 had no effect on NFκB nuclear translocation and DNA binding, we tested its ability to regulate the complete activation of NFκB, using the 4xκB-luc construct. SP 600125 inhibited NFκB activation by 50% (FIG 13A). When IL-8 promoter activation was examined using SP 600125, it also attenuated IL-8 promoter activation by 50% (FIG 13B). Interestingly when RT-PCR was performed and the mRNA of several chemokines was examined, including IL-8, there was no effect (data not shown).

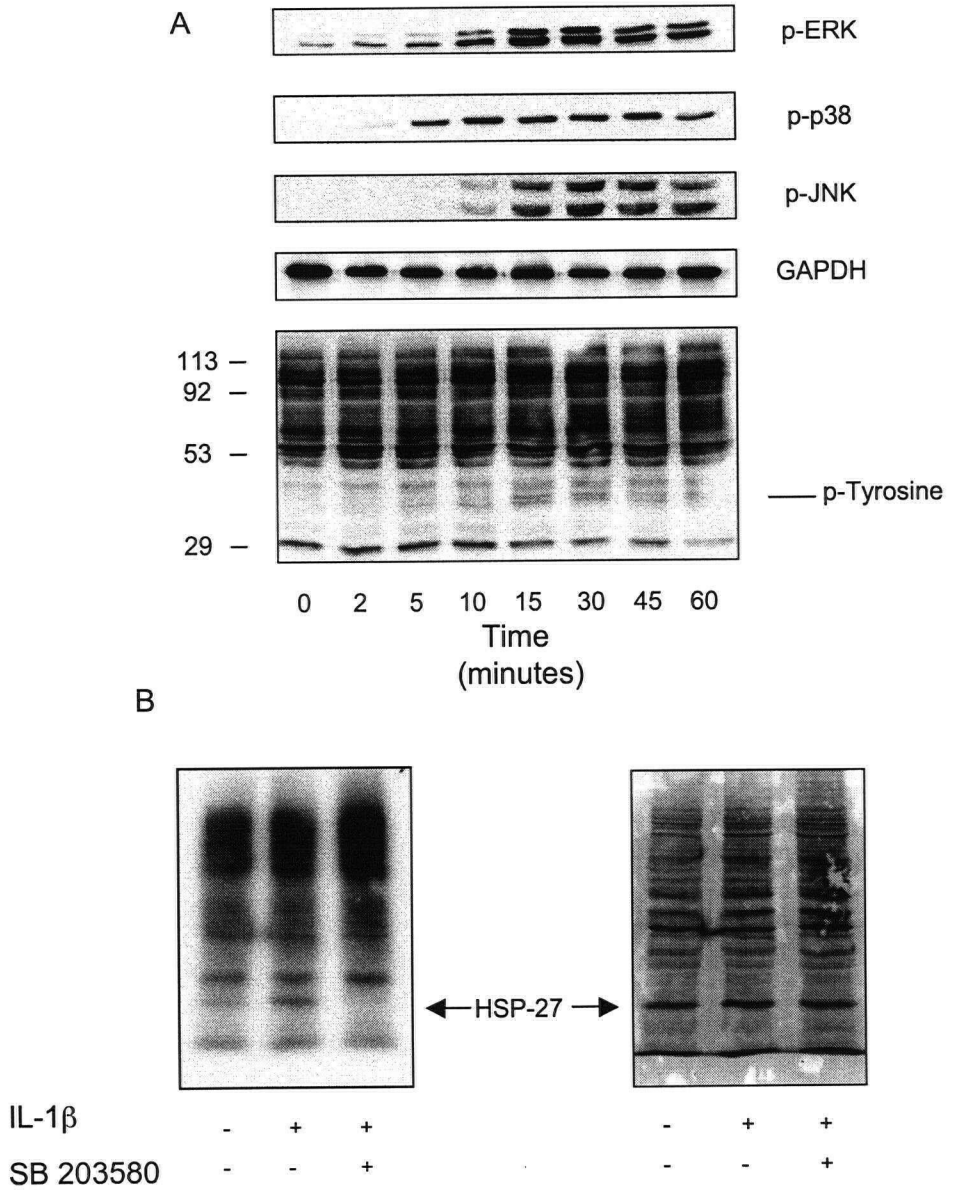


Figure 4. IL-1β stimulation of IECs results in the activation of ERK, JNK and p38. (A) Caco-2 cells were stimulated with IL-1β (2 ng/ml) for the indicated times, before being harvested. Western immunoblotting was carried out. (B) Caco-2 cells were preincubated with SB 203580 for 2 h before being stimulated with IL-1 (2 ng/ml) for 30 min and harvested. Kinase assays were performed, with HSP27 as substrate. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and visualized using autoradiography. The panel on the left is an autoradiogram and the one on the right is a Ponceau stain of the same membrane, showing co-localization of the HSP27 band. The results shown are representative of at least three independent experiments.

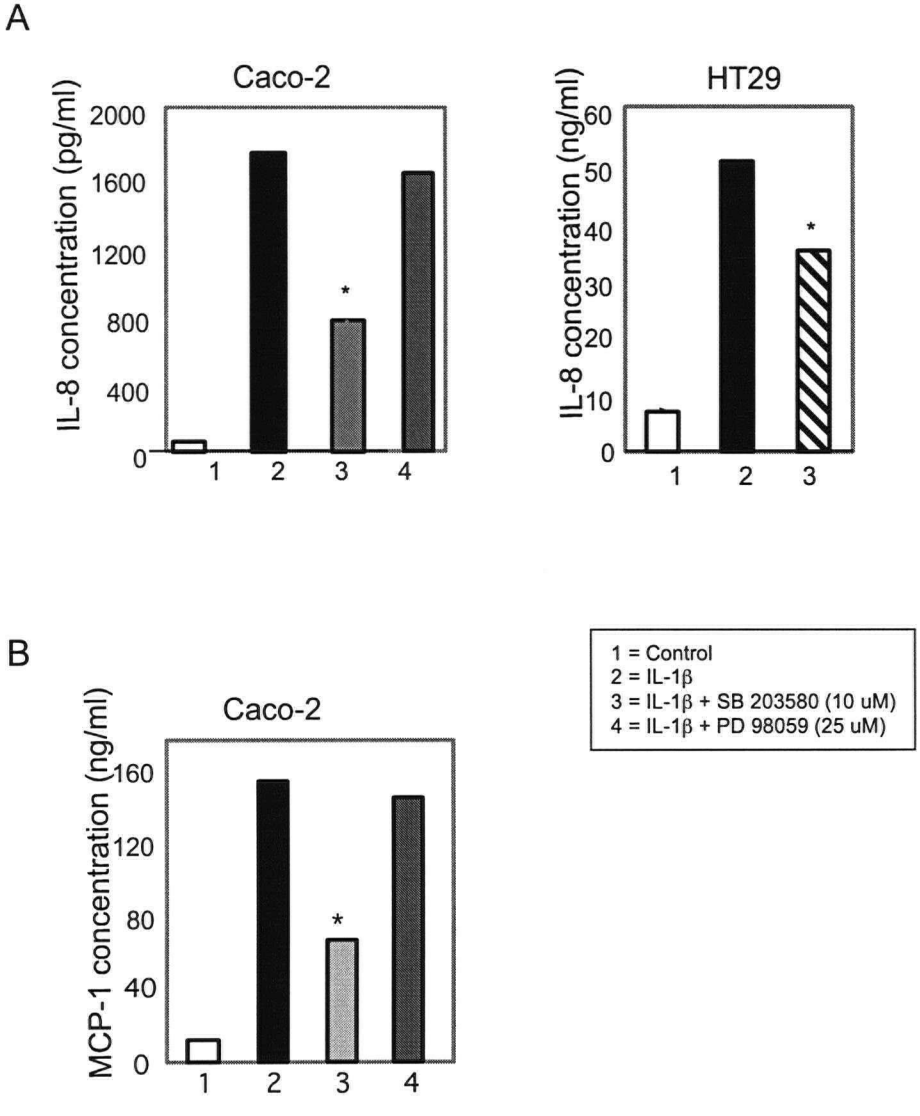


Figure 5. Inhibition of p38 MAPK attenuates IL-1β induced IL-8 production. Caco-2 were pretreated with SB 203580 (10 uM) or PD 98059 (25 uM) for 2 h, stimulation with IL-1β (2 ng/ml) for 36 h. Supernatants were collected and analysed for the presence of IL-8 (A) or monocyte chemotactic protein-1 (MCP-1) (B) by ELISA. (Lane 1, control; lane 2, IL-1β; lane 3, IL-1β with SB 203580; lane 4, IL-1 with PD 98059.) The results shown are representative of at least three independent experiments. The results are expressed as mean ± standard error of the mean (SEM) (* P <0·01).

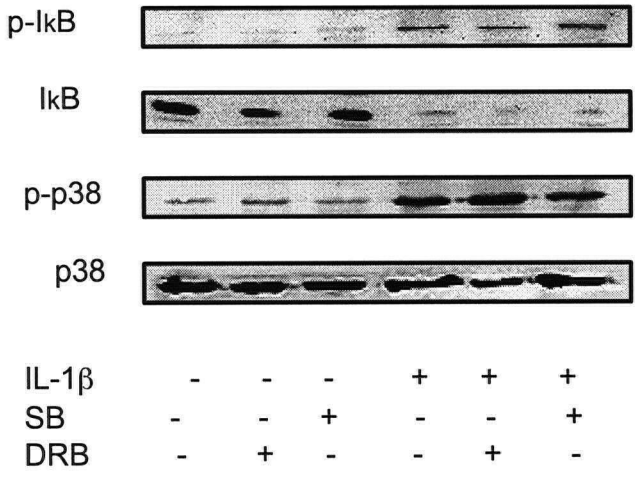


Figure 6. IL-1β induced degradation of IκB is independent of p38. Caco-2 cells were preincubated with DRB (10 uM) or SB 203580 (10 uM) for 2 h. Cells were stimulated with IL-1β (2 ng/ml) for 30 min, then harvested. Cells were lysed, resolved by SDS-PAGE and immunoblotted with the appropriate antibody. The results are representative of at least four independent experiments.

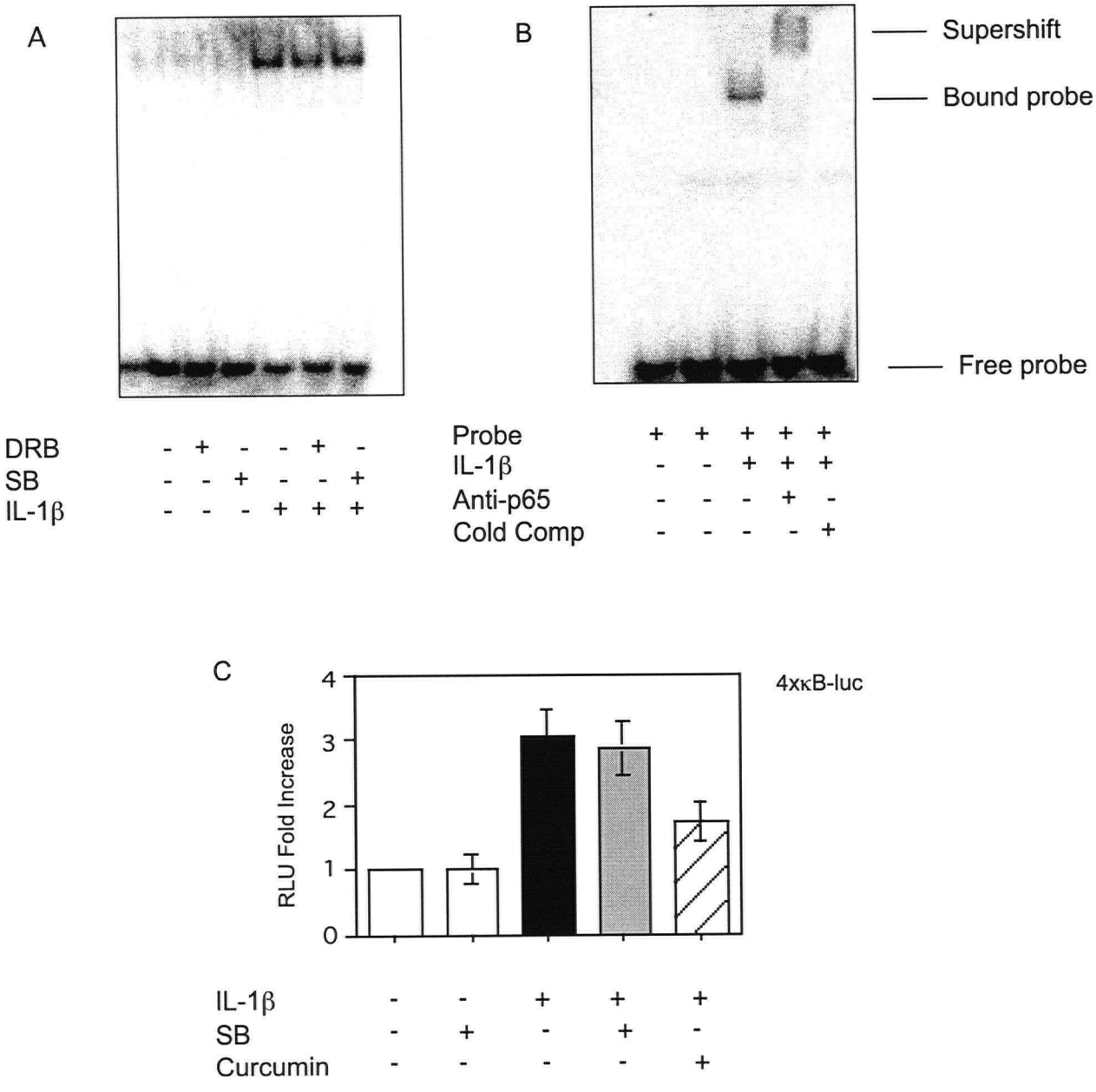


Figure 7. IL-1β induced activation of NFκB is independent of p38. Caco-2 cells were preincubated overnight with DRB (10 uM) or SB 203580 (10 uM) for 2 h before being stimulated with IL-1β (2 ng/ml) for 30 min. (A) Nuclear extracts were prepared and assayed for NFκB binding by using electromobility shift assays (EMSAs). (B) Specificity of NFκB binding was determined by antibody supershifting or competition with cold probe. Nuclear extracts were preincubated with an anti-Rel A antibody or 100-fold excess of cold unlabelled probe, before adding radiolabelled probe. (C) Cells were co-transfected with the NFκB reporter 4xκB-luc and LacZ. 24 hours later cells were preincubated with the appropriate inhibitor for 2 h, before being stimulated with IL-1β (2 ng/ml) for 6 h. Cells were harvested and the luciferase activity was determined. Results were normalized for transfection efficiency using LacZ activity. Results are representative of at least five independent experiments.

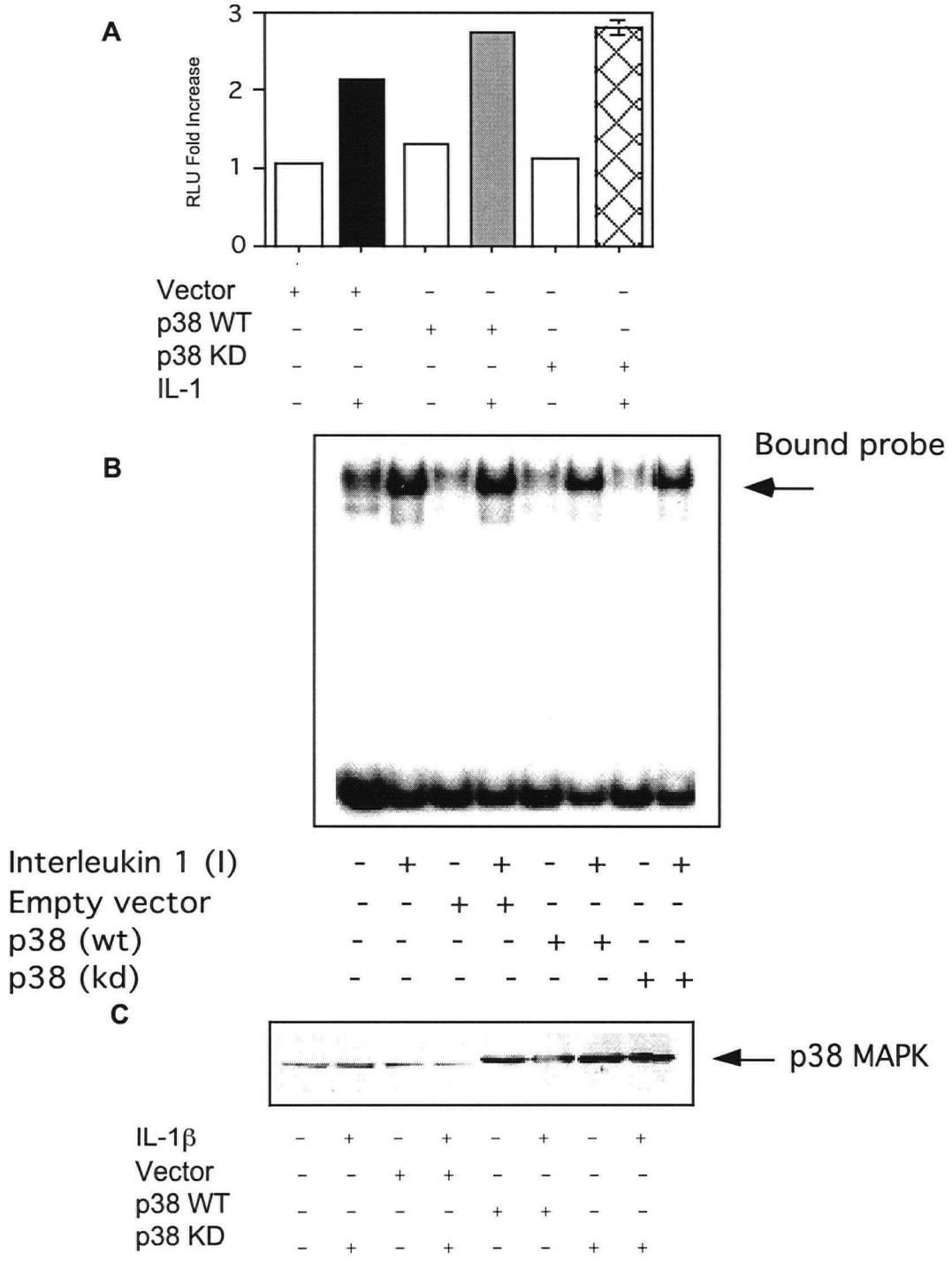


Figure 8. IL-1β induced activation of NFκB is not modulated by overexpression of kinase inactive p38. Caco-2 cells were transfected with the indicated plasmids (p38wt = p38 wild-type, p38kd = p38 kinase-dead) and then stimulated with IL-1β for 30 min (A) or 6 h (B). (A) DNA binding was carried out using EMSA, as previously described. (B) In addition to p38wt and p38kd, cells were also transfected with the NFκB reporter 4xκB-luc. Cells were harvested and the luciferase activity was determined. Results were normalized for transfection efficiency using LacZ activity for p38 expression. (C) Expression of both native and transfected p38 MAPK is shown by immunoblotting. Results are representative of at least five independent experiments.

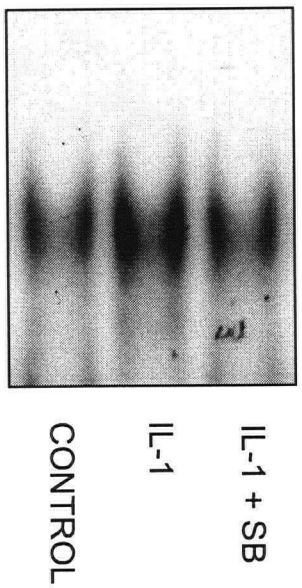


Figure 9. Inhibition of p38 attenuates IL-1β induced AP-1 DNA binding. Caco-2 cells were pretreated with SB 203580 (10uM) for 2 h before being DNA binding was assessed using EMSA, as previously described. Results are representative of at least two independent experiments.

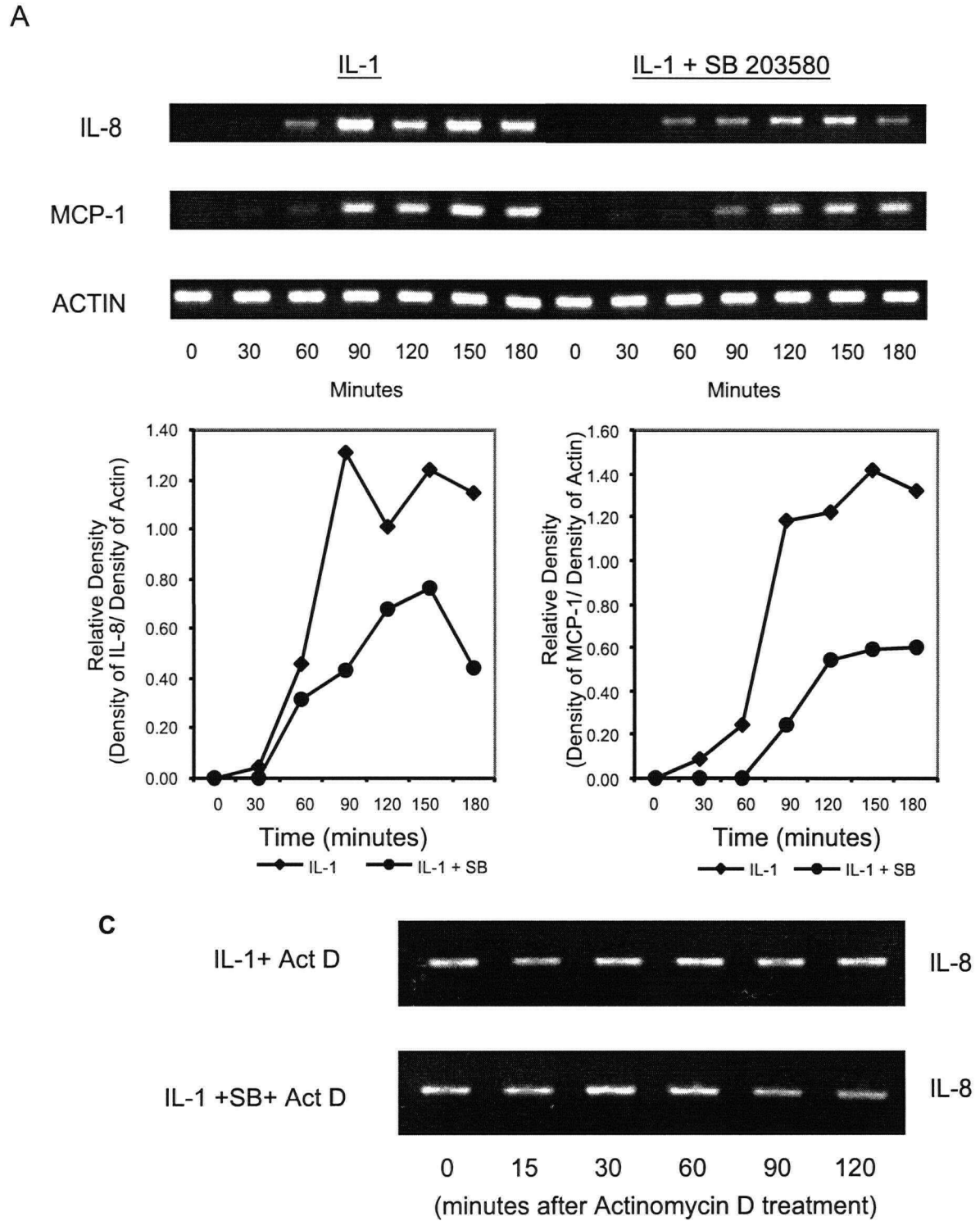


Figure 10. p38 MAPK regulates IL-1β induced IL-8 and MCP-1 mRNA expression. (A) Caco-2 cells were preincubated with the appropriate inhibitor for 2 h, before being stimulated with IL-1β for 3 h. Total RNA was isolated, and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed and PCR products were resolved using agarose gel electrophoresis. (B) Densitometry was performed using Bio-Rad Quantity One Software. (C) Cells were stimulated with IL-1 for 3 h and then cultures were adjusted so that they contained 10 μg/ml of actinomycin D (Act D), either alone or together with 10 μm SB 203580 (SB). The samples were then harvested at the indicated time-points, total RNA extracted and semiquantitative PCR for IL-8 was performed. The data shown are representative of three independent experiments.

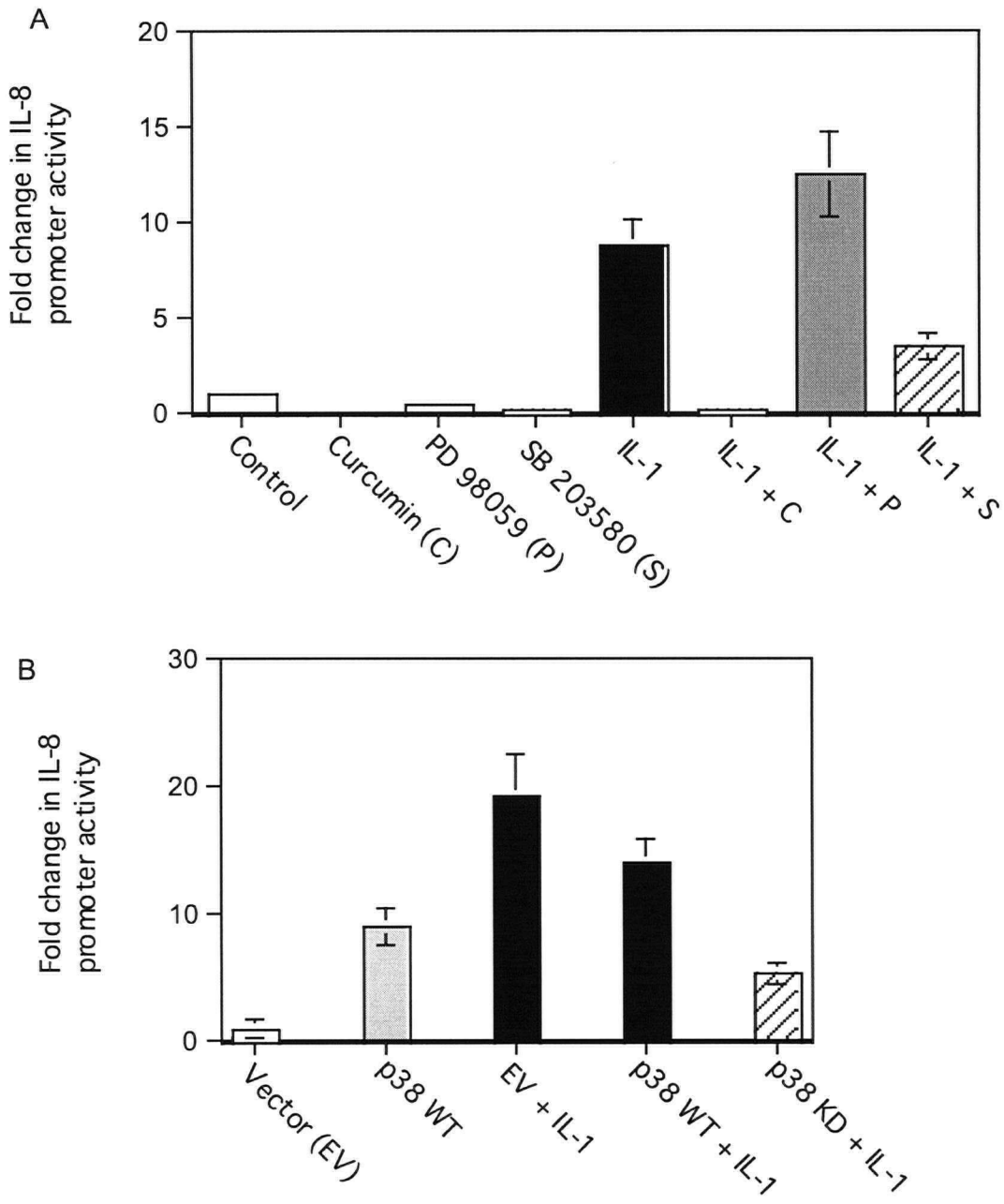


Figure 11. IL-1β induced activation of the IL-8 promoter is dependent on p38 MAPK regulation. (A) Caco-2 cells were co-transfected with the IL-8 promoter and LacZ for 24 h. Cells were preincubated with the appropriate inhibitor for 2 hr, before being stimulated with IL-1β (2 ng/ml) for 6 h. (B) Alternatively, the IL-8 promoter was co-transfected with the p38 MAPK constructs (p38WT = p38 wild-type, p38KD = p38 kinase-dead) and then stimulated with IL-1β. Cells were harvested and the luciferase activity determined. Results were normalized for transfection efficiency using LacZ activity. Results are representative of at least three independent experiments.

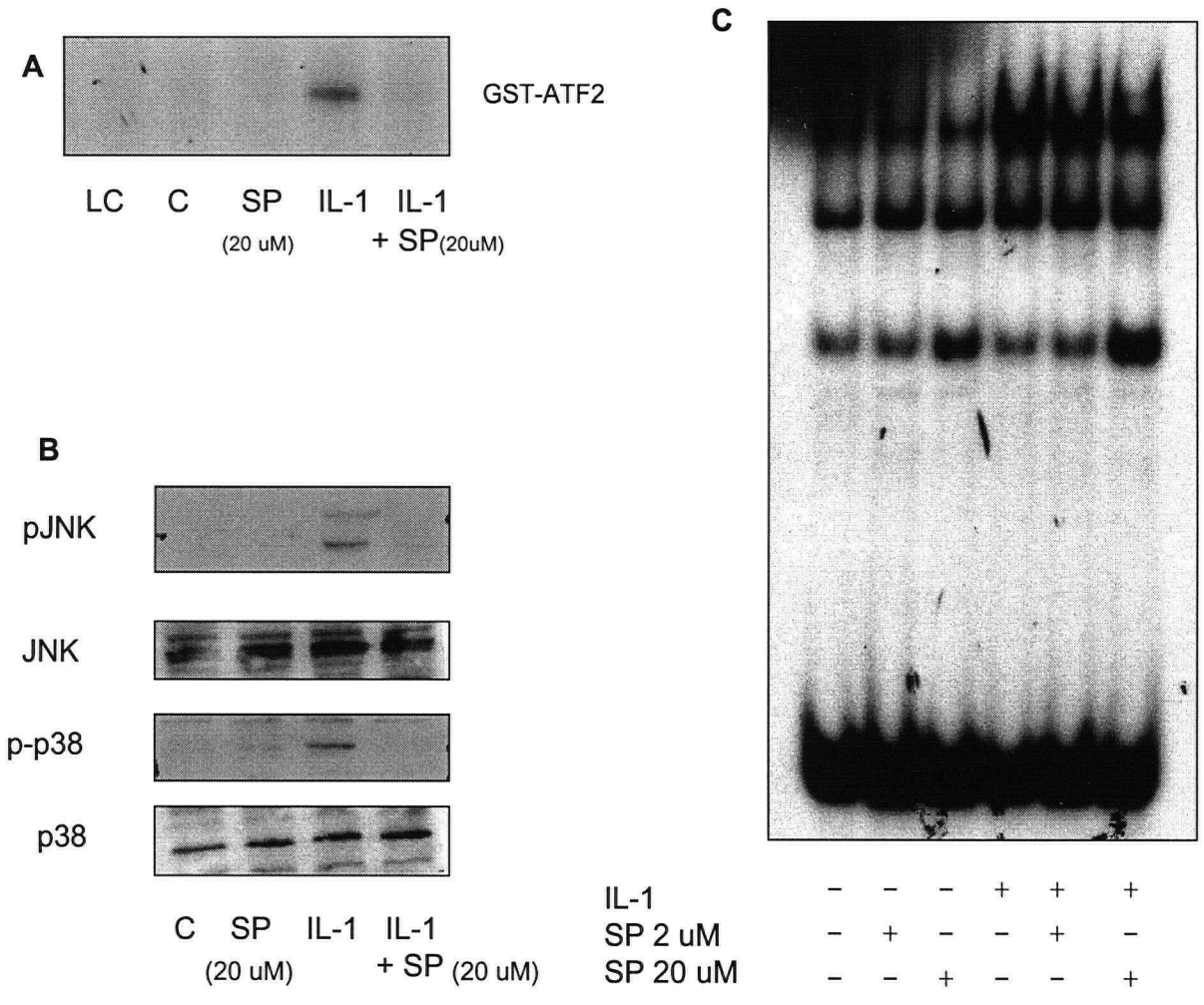


Figure 12. Selective JNK inhibitor SP 600125 inhibits JNK activity, however does not regulate NFκB DNA binding. HCT-116 cells were pretreated with SP 600125 at the indicated concentration for 2 h before being stimulated with IL-1β for 30 min. (A) JNK activity was determined by immunoprecipitating JNK and using GST-ATF2 as a substrate. Phosphorylated substrate was resolved using SDS-PAGE, and visualized using autoradiography. LC-Lysate and non specific IgG control, C-control, SP-SP 600125 at 20 uM (B) Immunoblotting was performed to examine phosphoryaltion status of p38 and JNK. (C) EMSA was performed to assess NFκB DNA binding. Results are representative of at least 3 independent experiments.

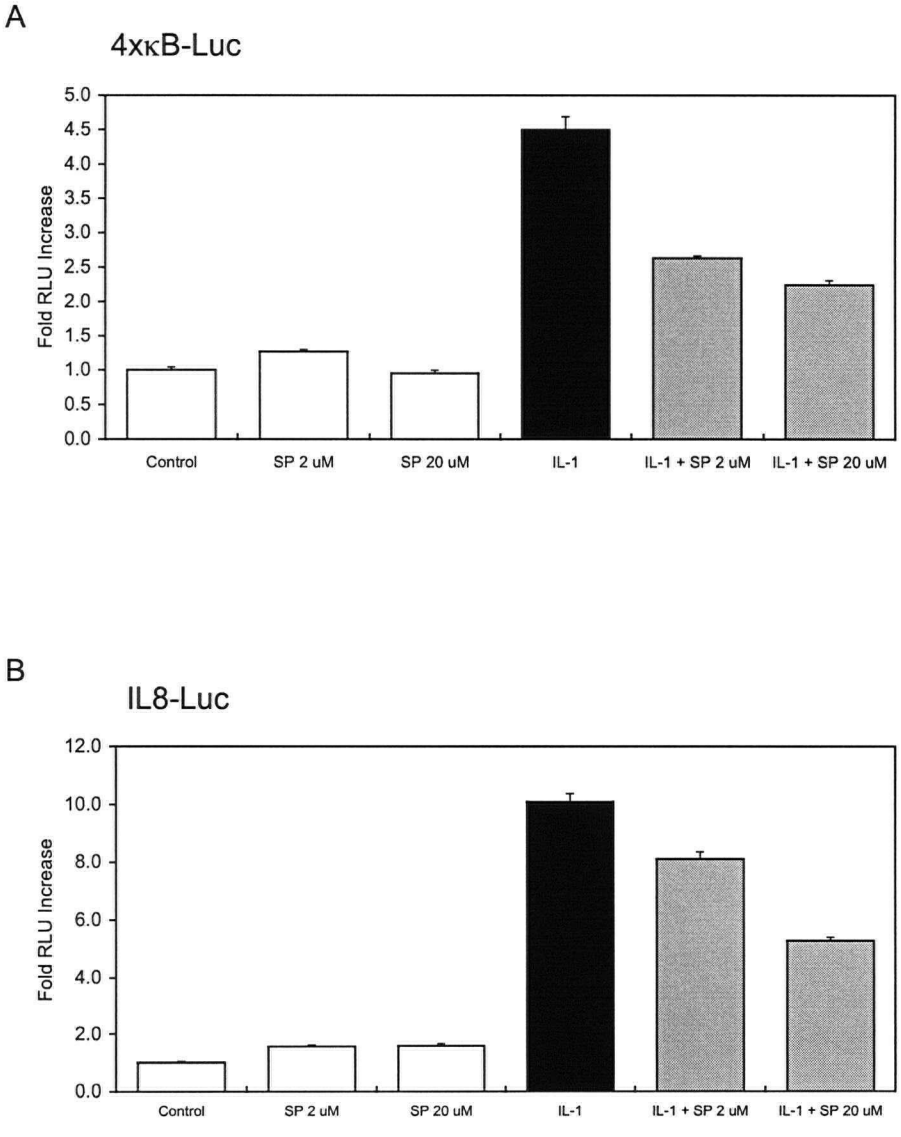


Figure 13. Selective JNK inhibitor SP600125 inhibits NFκB activation and IL-8 promoter activation. HCT-116 cells were transfected with the κB-Luc (A) or IL8-Luc (B) for 24 h. Cells were then pretreated with the SP600125 at the indicated concentration for 2 hours before being stimulated with IL-1β for 6 h. Results are representative of at least three independent experiments.

3.3 DISCUSSION

This section of work was devoted to the investigation of the role of MAPK family members in the regulation of IL-1β induced IEC chemokine release. Inhibition of ERK had no effect on the activation of NFκB, and subsequently no effect on IL-8 or MCP-1 production. Inhibition of p38 had significant effects on IL-8 and MCP-1 production; however, this effect was not through a p38 dependant regulation of either message stability or NFκB. The effect was determined to be at the proximal IL-8 promoter, potentially through disruption of AP-1 activation. Inhibition of JNK activity using curcumin or selective JNK inhibitor SP 600125 reduced IL-8 promoter activation as well as NFκB activation. Curcumin prevented chemokine expression; however, using SP 600125, enigmatically, there was no effect on message production of a number of chemokines including IL-8, MCP-1, Groα, and RANTES (data not shown).

ERK activation in response to IL-1β is well documented(206-208). Many reports in several different systems have dissociated ERK activation from both NFκB activation, and chemokine synthesis, so the lack of ERK regulation on chemokine synthesis in the IEC system was not surprising. Interestingly at the same time these studies were completed, a report was published looking at TNF induced IL-8 production in IECs(209). This report showed that ERKs did in fact play a role in the IL-8 synthesis. This is possible because due to the difference in downstream signaling pathways induced by TNF and IL-1β. Although both the IL-1R and tumor necrosis factor receptor-1 (TNFR1) are capable of activating NFκB as well as the MAPK family members, the intermediates,

such as the adaptor proteins that are recruited immediately following receptor activation, can be completely different. As a result, differences in the cytoplasmic portion of different families of receptors, such as those seen here between the IL-1R superfamily and the TNFR superfamily are important. The question of the functional role of ERK activation in response to IL-1β remains, and it may be responsible for things such as IEC migration, and epithelial cell restitution, which are important processes during the inflammatory response.

A role for p38 MAPK in functional responses to IL-1β in human IECs has never been investigated, although the production of IL-8 in response to invasion of IECs with Salmonella typhimurium has been previously demonstrated(207). IL-1β regulation of MCP-1 has been investigated, and these reports show a regulatory effect of p38 MAPK on MCP-1 production(210-212). In many other systems p38 MAPK has been shown to regulate chemokine production. Several mechanisms occur to explain this regulation, and these appear to be system and stimulus specific.

Several reports have indicated that p38 MAPK is involved in the transactivation of NFκB as one potential mechanism(213-215). This may occur via indirect mechanisms, including modulation of p65 transactivation and phosphorylation of the associated TATA-binding protein, required for transcriptional activation. Additionally, a hypothesized (but unproven) second signaling pathway involving MAPKs, which may allow fine-tuning of transcriptional responses within the nucleus, has been invoked to explain this effect. Perhaps the most compelling evidence for cross-talk between these two integral signalling pathways is derived from data in cardiac myocytes, where there is evidence of an interaction between MKK6 and IKK(216). With particular reference to

the IEC system, our study clearly contrasts these observations by showing that p38 MAPK plays a role distinct from that of regulating NFκB activation. p38 MAPK did not significantly influence the ability of NFκB to bind to DNA, or to phosphorylate IκB, nor did it affect the ability of translocated p65 to activate a target gene.

Regulation of mRNA transcript levels is probably one of the major determinants of cytosolic levels of protein, and this is a potential second mechanism. The transcripts of many short-lived cytokines and proto-oncogenes are often characterized by AREs found in their 3' UTR, and it is these AREs that mediate their subsequent destabilization and decay(217, 218). IL-8 and MCP-1 both feature AREs, which provide stimulus-specific regulation of their decay through the MKK6/p38 pathway(51, 219). Knockout mice that have AREs deleted from the TNFα gene exhibit increased levels of TNFα and develop chronic inflammatory arthritis and Crohn's-like IBD(220). Predictably, peritoneal macrophages from these animals were found to be unresponsive to modulation by the p38 pathway using SB 203580 at concentrations similar to those used in our study (10 uM). The studies presented here preclude this mechanism, despite the strong evidence of this regulation in other systems.

A final mechanism may be the regulation of the chemokine promoters as we have presented here. Another candidate transcription factor that is regulated by p38 is AP-1. Although it is not required for chemokine synthesis, it can play an important role in modulating the maximum production of chemokines. As such it has been previously reported that mutation of the AP-1 binding site results in an approximate 50% decline in promoter activity(41), similar to what was seen here. This does not preclude a role for

p38 in other aspects of promoter regulation. The coactivator p300/CBP is essential for the formation of the transcriptional activation complex. Although no work has looked at p38 regulation of this coactivator, a recent study looking at IL-2 promoter assembly showed that inhibition of p38 led to an inability to form a functional AP-1, NFκB, CREB transcriptional complex(221). Inhibition of p38 prevented the ability of CREB to associate with CBP/p300 and rendered the complex inactive. This also presents the final aspect of promoter assembly, p38 MAPK may regulate chromatin remodeling and access to chemokine promoters, as previously reported(48). Our initial studies looking at histone proteins shown to be under p38 regulation, showed a lack of increased phosphorylation of histone H3 at serine 9, as well as a lack of histone H3 dual phosphorylation and acetylation in response to IL-1β (data not shown). Pretreatment of SB 203580 was not able to modulate histone H3 modification both basally and in response to IL-1β.

The role of JNK in chemokine synthesis is well explored in some systems, however still remains largely enigmatic in the IEC system. Two approaches were taken in this work to examine the potential role of JNK in IEC chemokine release. Curcumin which has been shown to inhibit both signals upstream of IKK activation(200) as well as JNK(199, 222), was used as well as the selective JNK inhibitor SP 600125. Both the inhibitors attenuated NFκB activation as well as IL-8 promoter activation. Many reports have shown a role for JNK in regulating the IL-8 promoter by directly binding to it and being an important part of the transcriptional assemble complex. Alternatively both of these inhibitors may have additional effects on other kinases and regulatory pathways. Recently both PD 98059 and SB 203580 were shown to have a non-selective inhibitory

effect on the related MSK1 and this led to inhibition of both NFκB activation as well as chemokine release(69, 223). The same may be happening with the JNK inhibitor, due to the high degree of homology between MAPK family members. Further investigation using molecular and genetic approaches is required to fully delineate the mechanism of JNK control on promoter activation.

CHAPTER 4 – CK2 regulation of IEC NFκB Activation and Chemokine Synthesis

4.1 RATIONALE AND HYPOTHESIS

Protein kinase CK2 is highly conserved throughout evolution, thus underscoring its important biological role. Enigmatically, however, the precise role of this kinase has yet to be defined. An area in which CK2 may play a significant role, but yet to be explored, is its role in IEC inflammatory signaling. Although there has been no formal connection between CK2 and IEC chemokine production, some interesting reports in other cell systems do set a very interesting precedence. CK2 has been linked to the regulation of NFκB, as previously discussed. This regulation however has been controversial, with different mechanisms in different cell systems. Some describe regulation of the basal stability of IκB(224-227), some describe regulation of IKK activity(135, 136), whereas some describe regulation of p65 phosphorylation(71-73). In addition, a recent report has also linked CK2 to IFNγ mediated signaling(228). Thus it can be hypothesized that CK2 will play a role in chemokine expression in IECs, through its regulation of NFκB.

4.2 RESULTS

4.2.1 CK2 activity is increased in patients with active IBD

Patient samples were acquired from both normal uninflamed tissue as well as inflamed tissue from people suffering from IBD. These were immunohistochemically stained for CK2α protein expression. Staining of normal tissue revealed a relatively ubiquitous expression of CK2α (Fig 14A). It was present in both epithelial cells and infiltrating lymphocytes, however absent from mucous containing vacuoles of goblet cells. Staining of actively inflamed tissues showed an increase in CK2α staining. There was increased CK2α staining in each epithelial cell cytoplasm. Infiltrating lymphocytes also stained with a higher intensity.

4.2.2 CK2 activity is increased in a murine model of colitis

In order to assess CK2 kinase activity, normal and inflamed tissue was examined in mice induced with colitis by treating with 2% dextran sodium sulphate (DSS). Tissue homogenates were subjected to CK2 kinase assays, using GTP and a CK2 specific peptide substrate. There was a significant increase in the activity of CK2 in tissue from inflamed mice, as compared to their normal control counterparts (Fig 15). Similar results were also seen in an alternate murine model of colitis, the TNBS model (data not shown). As with the human samples, immunohistochemistry analysis revealed that there was a concomitant increase in the protein expression of CK2α (data not shown).

4.2.3 Inhibition of CK2 prevents IL-1β induced NFκB activation

NFκB plays a very important role in the activation of the IEC inflammatory response, thus the role of CK2 in regulating NFκB was next investigated. The IEC cell lines Caco-2, and HCT-116 were used as they have been well characterized, can form tight junctions, as well as release numerous cytokines and chemokines. CK2 activity was inhibited using the selective CK2 inhibitor, apigenin, as previously reported(229). Cells were transfected with a κB dependant reporter (4xκB-luc) for 24 h before being pretreated with the inhibitor at the appropriate concentration for 1 h, and stimulated with IL-1β (2ng/mL). Pretreatment with apigenin led to a dose dependant decrease in the IL-1β induced activation of NFκB (Fig 16). Data from Caco-2 is presented, and is representative of that from HCT-116 (data not shown).

4.2.4 Overexpression of a kinase inactive CK2 inhibits IL-1β induced NFκB activation

The catalytic dimer in CK2 holoenzyme can be made up of either α or α', so the effect of overexpression of CK2 α or α' on NFκB luciferase reporter activity was examined next. Overexpression of CK2α was sufficient to activate the NFκB reporter (4xκB-luc) (Fig 17). Interestingly this was not repeated by overexpression of the CK2α'. Upon treatment with IL-1β, both α and α' overexpression led to a mild hyperactivation of the NFκB dependant reporter above empty vector stimulated, however this effect was additive, and not synergistic. Overexpression of either kinase inactive α or α' led to both an inhibition of basal NFκB activity, as well as IL-1β induced activity.

4.2.5 CK2 regulates the transactivation of p65

Next the mechanism of regulation of CK2 was investigated in more depth. The phosphorylation and subsequent degradation of IκB, the first regulatory step in activation of NFκB, was examined by immunoblotting. Pretreatment of cells with apigenin(229) or a second CK2 inhibitor, 5,6-dichloro-ribifuranosylbenzimidazole (DRB)(230), had no effect on IL-1β induced phosphorylation and degradation of IκB (Fig 18A). Subsequently, when the IL-1β induced nuclear translocation and DNA binding of NFκB was examined (by EMSA), pretreatment of cells with either CK2 inhibitor had no effect (Fig 18B). The ability of p65 itself to undergo post-translational modifications provides an important mechanism to regulate its transactivation. We examined the ability of CK2 to regulate the IL-1β induced transactivation of the p65 subunit. We employed the one-hybrid system whereby a portion of the C-terminal transactivation domain (amino acids 501-551, or 521-551) was fused to the yeast Gal4 DNA binding domain. This construct was co-expressed with Gal4 responsive luciferase reporter to assess signal specific activation of the p65 transactivation domain-1 (TAD1). IL-1β was able to activate the C-term transactivation domain, and the pretreatment of CK2 inhibitors prevented this activation (Fig 18C). Previously it has been shown that CK2 can regulate the C terminus transactivation domain via serine 529. When CK2 was overexpressed with a WT construct, it was able to induce its activation, however when overexpressed with a serine 529 to alanine mutant construct, it was unable to

transactivate this construct (Fig 18D). Thus CK2 regulates IL-1β induced activation of NFκB through the regulation of transactivation domain 1 (TAD1) via serine 529.

4.2.6 CK2α associates with p65, exclusively in the nucleus

The possibility that there may be an association of CK2α with p65 was next examined. HCT-116 cells were stimulated with IL-1β for 15 minutes, and subsequently immunoprecipitated for p65 and CK2α, and immunoblotted for the other protein. CK2α was found to associate with p65 (Fig 19A). Interestingly the amount of association did not change with IL-1β stimulation, even when time points as long as one h were examined. The cellular localization of this association was examined, by fractionating the cellular components into cytoplasm and nucleus, before immunoprecipitating them for p65 and CK2α. CK2α and p65 were found to associate exclusively in the nucleus, suggesting that this association was found to be after IκB has released p65 and revealed its nuclear localization signal (Fig 19B). Also when p65 immunoprecipitates were immunoblotted for CK2 α', it was found to associate with p65 as well. To confirm that the nuclear and cytoplasmic separations were clean, whole cell lysate was immunoblotted for histone H3, a protein marker found only in the nucleus (Fig 19C).

4.2.7 p65 associated CK2 activity is increased with IL-1β stimulation

Since a pool of CK2 is able to associate with p65, and the amount of this association remained constant, the specific activity of this p65 bound pool of CK2 was examined. HCT-116 cells were stimulated with IL-1β for varying lengths of time, before

being immunoprecipitated for p65. p65 immunocomplexes were then subjected to CK2 assays, using casein as a substrate and GTP as the phosphate donor. p65 bound CK2 activity was increased at 30 minutes of stimulation (Fig 20). When compared to the kinetics of degradation of IκB, the p65 bound CK2 was activated most likely at the point when it entered the nucleus. Similar results were also achieved using a specific substrate instead of casein. When total cellular CK2 activity was examined, there was no significant change with IL-1β stimulation (data not shown).

4.2.8 Inhibition of CK2 prevents IL-1β induced activation of the IL-8 proximal promoter

Next we examined if CK2 could regulate the activation of the downstream NFκB target, IL-8, through its regulation of NFκB transactivation. NFκB has been shown to essential for the activation of the proximal IL-8 promoter(41). Cells were transfected with a luciferase reporter gene construct under the control of the IL-8 promoter. IL-1β robustly activated this construct, and pretreatment with the CK2 inhibitors dose dependently attenuated this activation (Fig 21).

4.2.9 Inhibition of CK2 prevents the message synthesis of NFκB downstream targets

To further confirm CK2 was able to regulate downstream NFκB targets we examined the IL-1β induced message synthesis of NFκB downstream targets. IκB

synthesis one of the simplest NFκB target promoters. Pretreatment with apigenin prevented the resynthesis of IκB message that is required for the resynthesis of IκB following its degradation (Fig 22A). In addition, we examined several chemokines and cytokines. Many of these had been attenuated by pretreatment with apigenin (Fig 22).

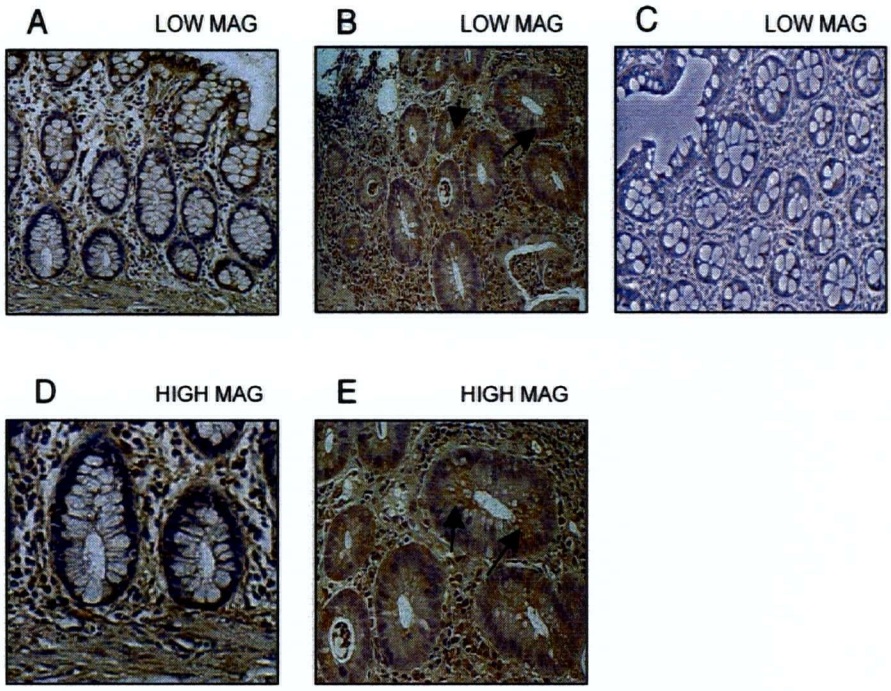


Figure 14. CK2α is overexpressed in patients with active UC. Immunohistochemical staining of CK2α in a patient with normal uninflamed colon (A/D) or in actively inflamed tissue from a patient diagnosed with UC (B/E). Immunohistochemical staining of uninflamed with non-specific rabbit IgG (C).

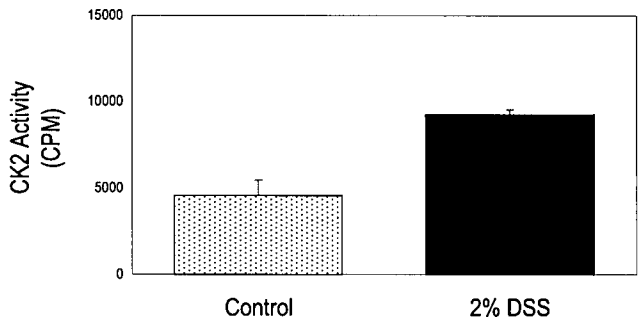


Figure 15. DSS induced colitis in mice increases CK2 activity in actively inflamed tissue. DSS colitis was induced in mice using 2.0% DSS (3 animals per group). 48 h later, mice were sacrificed, and inflamed or normal tissue from the large bowel was collected and homogenized for protein. Samples were normalized for protein concentration before performing CK2 kinase assays using CK2 specific substrate and GTP.

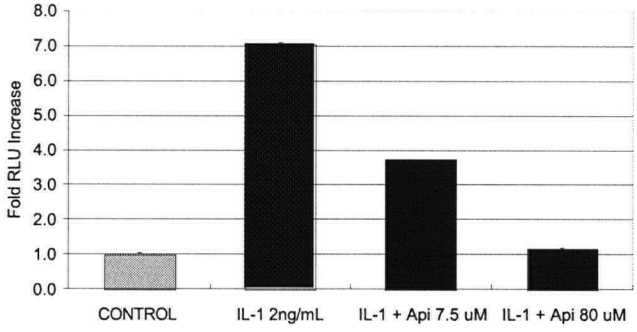


Figure 16. Inhibition of CK2 using a selective inhibitor prevents IL-1β induced activation of NFκB in intestinal epithelial cells. Caco-2 cells were transfected with an NFκB responsive luciferase reporter (wxκb-Luc). 36 h later cells were pretreated with the selective CK2 inhibitor apigenin at various concentrations before being stimulated with IL-1β (2 ng/mL) for 6 h. Cells were lysed and measured for luminescence. Results are representative of at least four independent experiments.

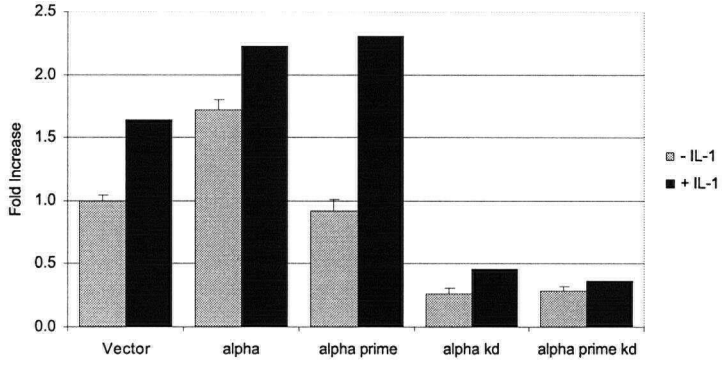


Figure 17. Overexpression of CK2 α or α' modulates both basal and IL-1β induced NFκB activation. HCT-116 colonic epithelial cells were transfected with an NFκB responsive luciferase reporter (4xκb-Luc) and with 1 ug of either empty PCDNA3 vector (EV), CK2α wt (a WT), CK2α' wt (a' WT), CK2α kinase dead (α KD), or CK2 (α' KD). 36 h later cells were stimulated with IL-1β (2 ng/mL) for 6 h. Cells were lysed and measured for luminescence. Results are representative of at least three independent experiments.

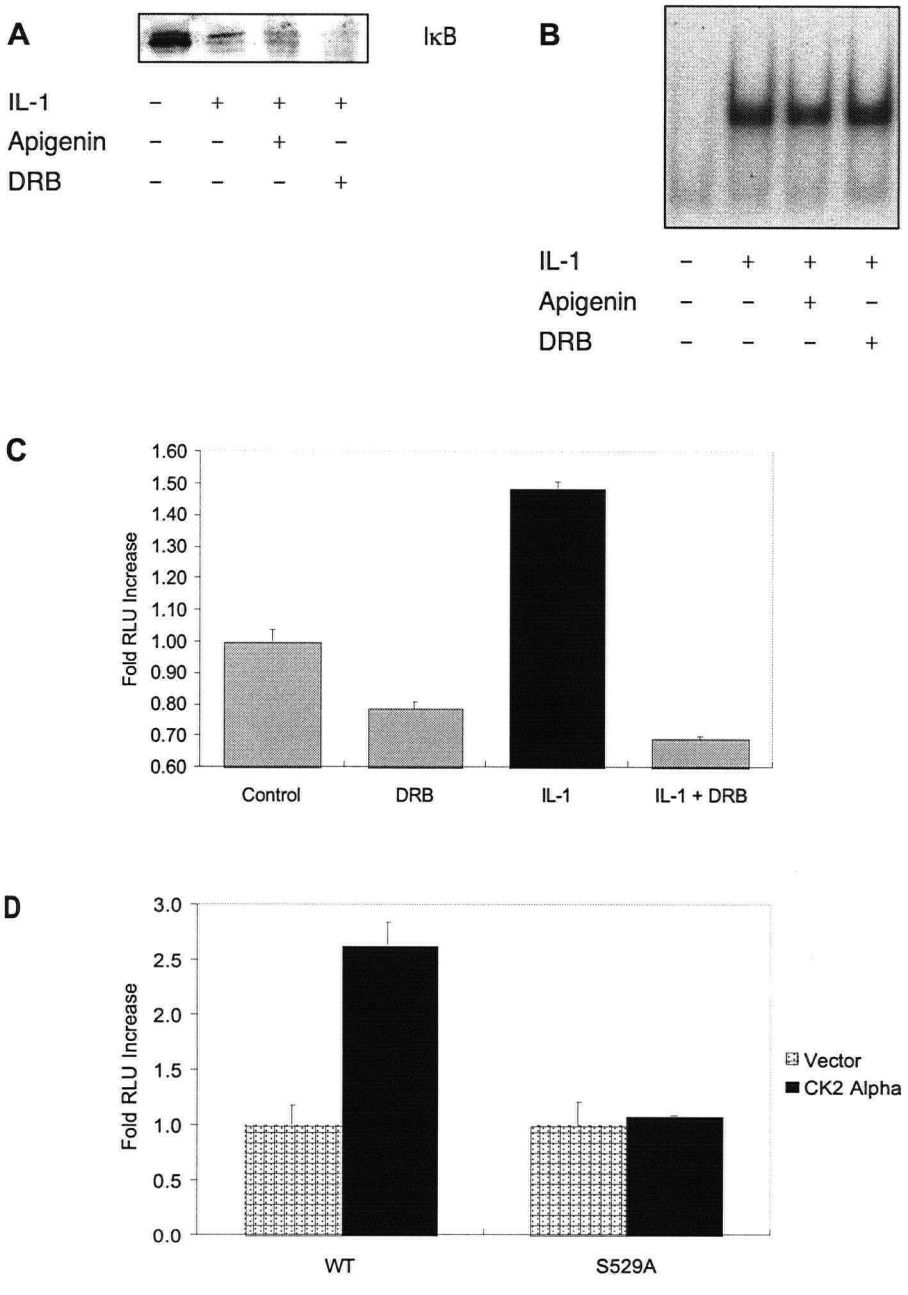


Figure 18. CK2 regulation of IL-1β induced NFκB activation is at the level of transactivation through p65 serine 529. Caco-2 cells were pretreated for 1 h with the selective CK2 inhibitors apigenin (80 uM) and DRB (20 uM), before being stimulated with IL-1β (2ng/mL) for 30 min. Cells were lysed for total proteins and subsequently resolved by SDS-PAGE and probed for IκB (A) or lysed for nuclear proteins and subjected to electrophoretic mobility shift assay (EMSA), using a probe containing a consensus κB binding site (B). (C) To assess transactivation, cells were transfected with a Gal4 responsive luciferase plasmid (pfr-Luc) along with p65 transactivating domain 1 - Gal4 fusion (amino acids 501-550) together. 36 h later cells were preincubated with DRB and stimulated with IL-1β (2 ng/mL) for 6 h. Cells were lysed and measured for luminescence. (D) Transactivation was assessed by transfecting cells with a Gal4 responsive luciferase plasmid (pfr-Luc) along with p65 transactivating domain 1 - Gal4 fusion (amino acids 501-550, either wildtype or ser529ala mutant) together along with empty vector or wildtype CK2 for 36 h. Cells were lysed and measured for luminescence. Results are representative of at least 3 independent experiments. (D) CK2

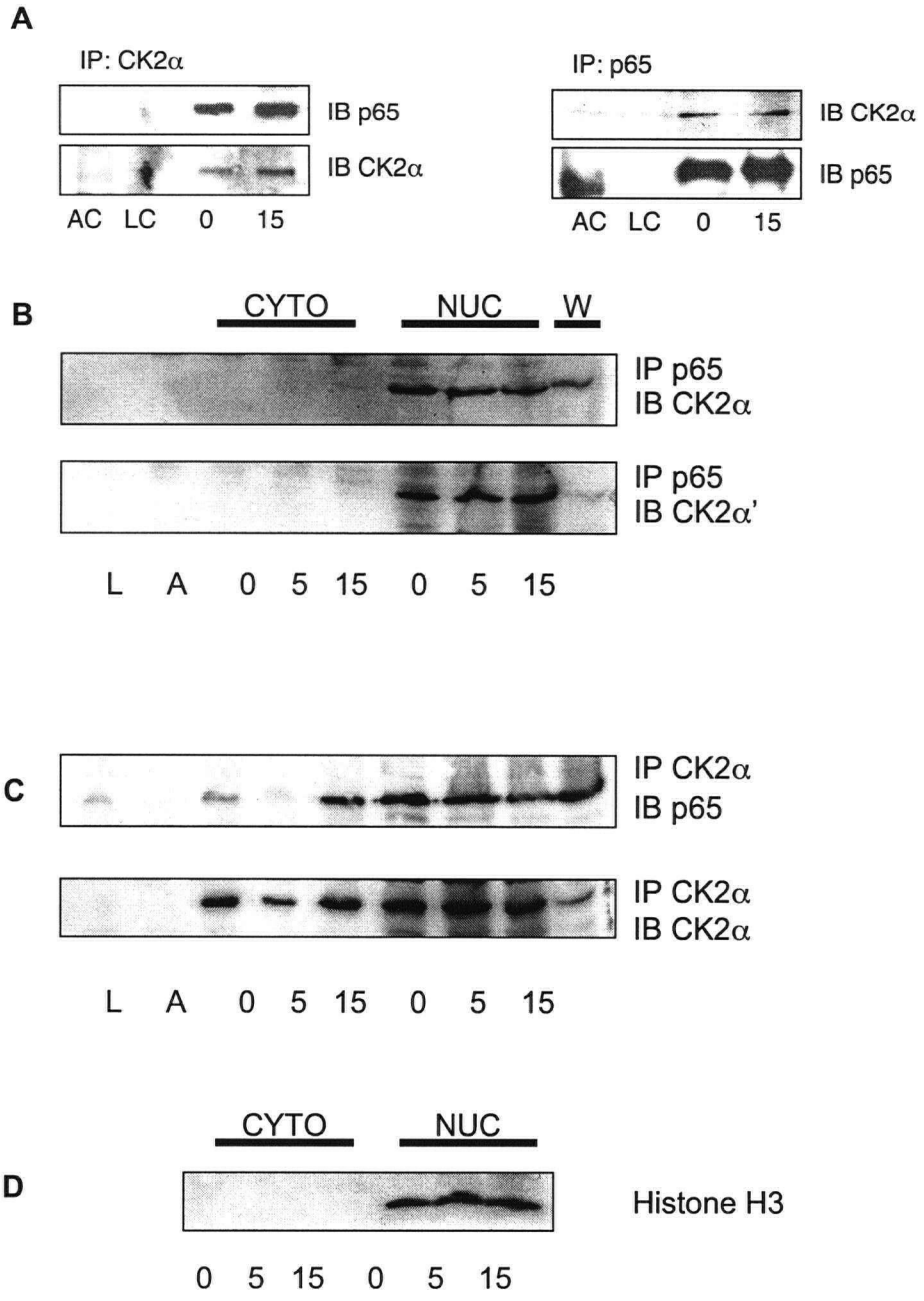


Figure 19. CK2α associates with p65, and this association occurs exclusively in the nucleus. (A) HCT-116 colonic epithelial cells were serum starved for 6 h before being stimulated with IL-1β (10 ng/mL) for the indicated lengths of time. p65 or CK2α was immunoprecipitated overnight before immunocomplexes were collected with protein A/G, subjected to SDS-PAGE, and immunoblotted for CK2α, C2α', or p65. (B/C) HCT-116 colonic epithelial cells were serum starved for 6 h before being stimulated with IL-1β (10 ng/mL) for the indicated lengths of time. Cells were fractionated into nuclear and cytoplasmic fractions. p65 was immunoprecipitated overnight before immunocomplexes were collected with protein A/G, subjected to SDS-PAGE, and immunoblotted for CK2α, CK2α', or p65. (D) Fractions from the nuclear and cytoplasm extracts were immunoblotted for histone H3. AC= Ab and beads control, LC=lysate and non specific IgG control.

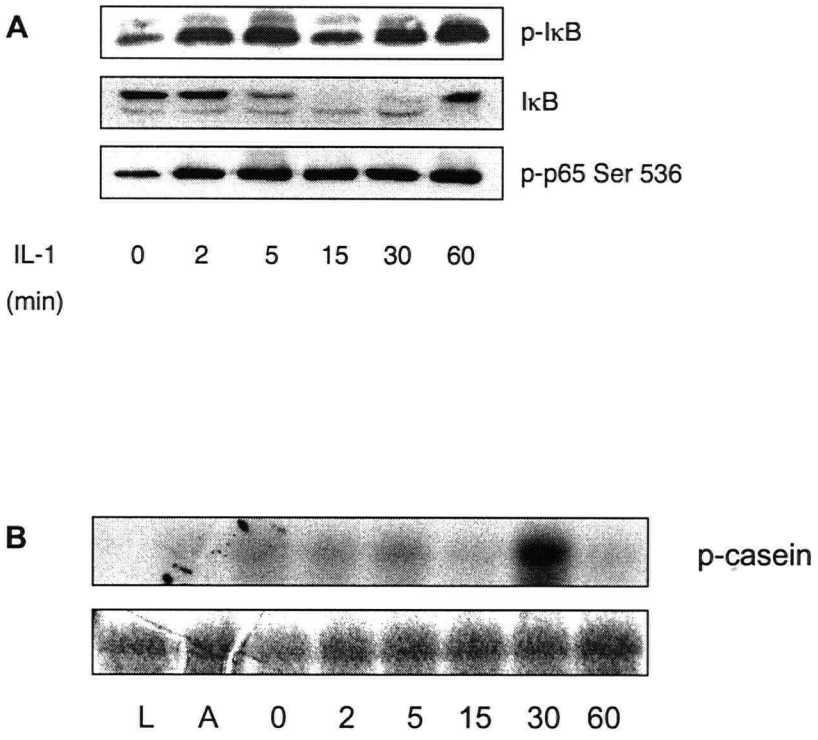


Figure 20. p65 bound CK2α activity increase with IL-1β treatment. HCT-116 colonic epithelial cells were serum starved for 6 h before being stimulated with IL-1β (10 ng/mL) for the indicated lengths of time. (A) Cells were immunoblotted for phospho-p65, pIκB, or IκB (B) p65 was immunoprecipitated overnight before immunocomplexes were collected with protein A/G, CK2 kinase assays were performed using GTP and casein as a substrate. The reactions were then resolved by SDS-PAGE, coomasie stained, followed by exposure to film for 6h at -80 C.

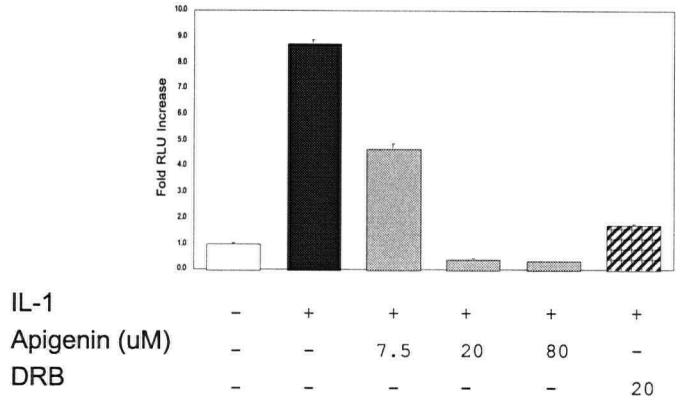


Figure 21. Inhibition of CK2 prevents IL-1β induced activation of the IL-8 proximal promoter. HCT-116 cells were transfected with the IL-8 promoter luciferase reporter (IL8-Luc). 36 h post-transfection cells were pretreated for 1 h with the selective CK2 inhibitors apigenin and DRB at the appropriate concentrations, before being stimulated with IL-1β (2ng/mL) for 6 h. Cells were lysed and measured for luminescence. Results are representative of at least 4 independent experiments.

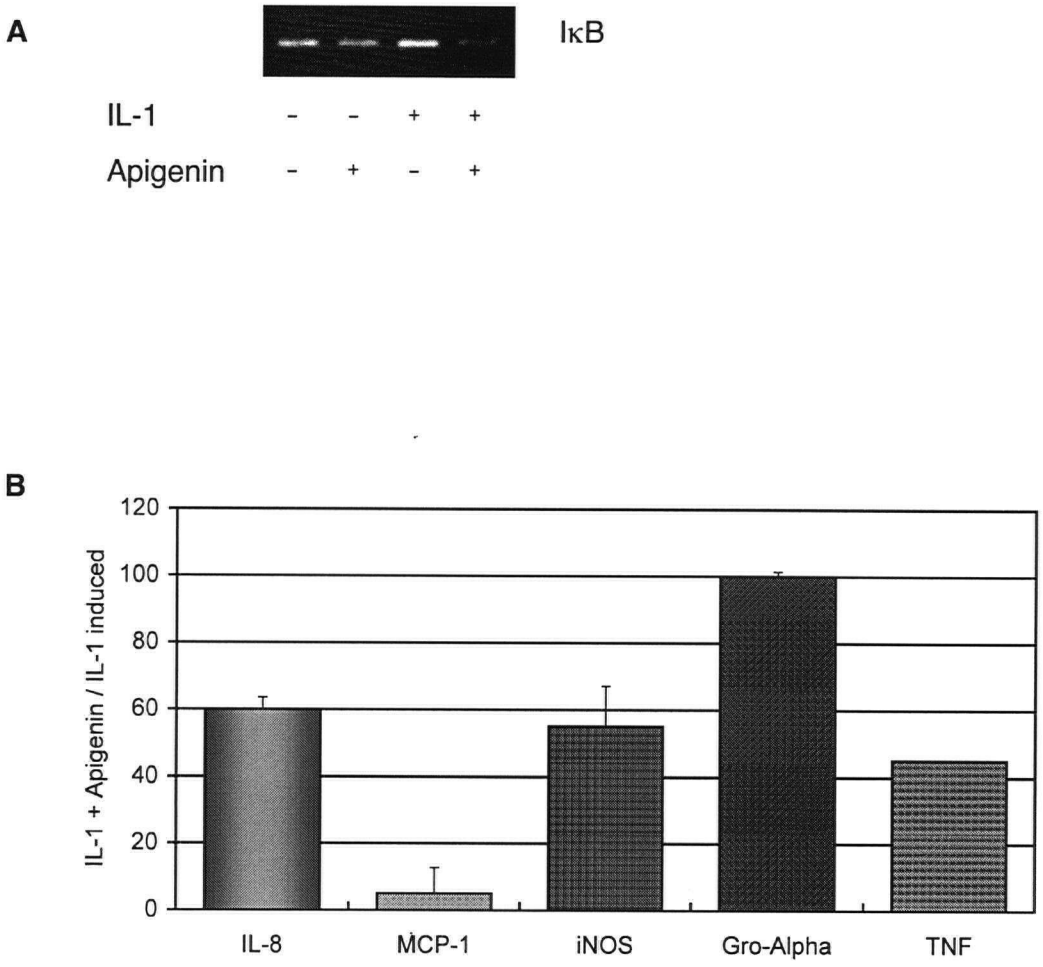


Figure 22. Inhibition of CK2 prevents the synthesis of IL-1β induced IκB and chemokines. Caco-2 cells were pretreated with apigenin for 1 h before being stimulated with IL-1β for 3 h. RNA was isolated and subsequent RT-PCR was performed for IκB (A), as well as IL-8, MCP-1, iNOS, and GROα (B). Results are representative of three independent experiments.

4.2 DISCUSSION

This section of work presents a number of novel observations about inflammatory signaling in IECs. In actively inflamed tissue there seems to be an increase in CK2 expression and also activity. The functional role of CK2 in IECs was then examined and a regulatory role for CK2 in the regulation and activation of the p65 subunit of NFκB was observed. This activation is required for the subsequent downstream activation of several NFκB dependant promoters, including most notably IL-8. The activation of p65 is due to the phosphorylation of p65 at serine 529 by CK2 that appears to occur by a pool of CK2 that is constitutively bound to p65. Upon the addition of IL-1β this pool increases in specific activity.

Although CK2 has been described to be elevated in many solid tumors and rapidly proliferating tissues, the precise functional consequence of CK2 overexpression has not yet been determined(126, 231). The data presented here provides some evidence for the potential functional consequences of overexpression of CK2. In an inflammatory setting, this may lead to elevated basal NFκB activity, or a hyperactive NFκB response, especially in response to proinflammatory cytokines.

Although normally considered a constitutively active kinase, CK2 modulation by growth factors and stress factors has been described(129, 232). Activation of its activity by cytokines has also been described in response to TNFα, and IFNγ(129, 228). This is the first report of its activity being modulated by IL-1β. In addition due to the large number of substrates for protein kinase CK2, the mechanism through which it can

regulate these substrates is not known. A possible mechanism through which it may accomplish this is by distinct pools of CK2 that are capable of acting independently of one another. In the situation presented here a nuclear pool, bound specifically to p65, regulates p65 transactivation potential, and its ability to become activated. When compared to total CK2 activity, there is only a negligible change in total cellular activity (data not shown). This phenomenon of pools has been described before, as nuclear matrix bound pools of CK2 in cancer cells can regulate apoptosis in response to anticancer agents(130).

Although CK2 previously has been reported to regulate NFκB activation, its method of regulation has been controversial. The c-terminus PEST sequence is a major point of regulation of IκB, as proteins that have regions of sequence with a high proportion of proline (P), glutamic acid (E), serine (S), and threonine (T) residues are degraded much quicker (233). Regulation here is mediated by the phosphorylation of the PEST sequence of IκB by CK2 at serine 283, threonine 288, serine 291, and serine 293, however preferentially at serine 293(224-227). This phosphorylation does not affect the cytokine mediated degradation of IκB, however it regulates the steady state turn over of IκB by decreasing its stability. An important point to consider is that in the absence of stimulation, there is constitutive translocation of NFκB to the nucleus, due to the short basal half-life of IκB. Loss of function experiments involving the depletion of CK2 and site directed mutation of the CK2 phosphorylation sites results in increased IκB stability and decreased constitutive NFκB translocation (225, 227, 234-236). An independent study has also cited that CK2 is able to phosphorylate IκB *in vitro*, however this report failed to show the activation of CK2 in response to TNFα, a potent activator

of the NFκB cascade (237). The data presented here in this report provides some preliminary evidence that overexpression of CK2 may be able to modulate basal NFκB activation (Fig 17). Interestingly, the functional differences between CK2 α and α' are not well characterized or reported, however, in this situation only α overexpression, not α' overexpression, was capable of inducing basal NFκB activation. This is one the few functional differences that has ever been observed thus far, between CK2α and α'.

A more direct role has recently been elucidated for CK2 in regulating NFκB as it has been shown to directly phosphorylate the p65 subunit on serine 529 and regulate its transactivation potential(71, 73). This site is exclusively phosphorylated in response to TNFα and it was shown to be due to CK2. Phosphorylation at this site did not modulate the ability of p65 to translocate to the nucleus, only its transactivation potential. Interestingly this is located in transactivation domain-1 of the C-terminal of p65, the area that interacts with other transcriptional coactivators such as CBP, and p300 as well as TBP, TFIIB. As a result phosphorylation at this site may be responsible for interaction with other transcription factors, or with the ability of NFκB to disrupt chromatin, suggesting a specific role for each promoter. This report now shows for the first time that this site is also important for mediating IL-1β induced p65 transactivation.

CHAPTER 5 – PI3K and PDK1 regulation of IL-1β induced NFκB and chemkokine production

5.1 RATIONALE

The PI3K pathway, including PDK1 and PKB, is perhaps one of the most intriguing cell signaling pathways. It has been implicated in the regulation of numerous cell functions including viability, apoptosis, cell metabolism, migration, and endocytosis, just to name a few(140). PI3K and PKB have been strongly implicated in the inflammatory response, by regulating NFκB(80, 83, 153, 154). The mechanism of regulation is different in different systems. No work to this date has examined the role of PDK1 regulation of NFκB, or chemokine production, especially in IECs. Thus in this section the precise regulation of chemokines and NFκB by PI3K, PKB, and PDK1 was examined. Due to their previous roles in regulating NFκB, it can be hypothesized that PI3K and PKB will regulate NFκB activation, as well as chemokine synthesis. Also it can be hypothesized that PDK1 can regulate NFκB, although its role is yet completely undefined.

5.2 RESULTS

5.2.1 Inhibition of both PI3K and PDK1 results in attenuation of IL-1β induced NFκB activation.

Initially using the NFκB reporter (4xκB-luc), cells were pretreated with inhibitors to PI3K (LY 294002 (LY) or wortmannin)(238) or a recently identified inhibitor to PDK1 (N-tosyl-L-phenylalanyl chloromethyl ketone/TPCK)(239), and stimulated with IL-1β for

6 h. The results show that inhibition of either PI3K (Fig 23A) or PDK1 (Fig 24A) attenuates IL-1β induced NFκB activation. To confirm that LY and wortmannin were inhibiting PI3K, the phosphorylation status of PKB (serine 473) was examined, and its IL-1β induced activation was in fact attenuated by the inhibitors (Fig 23B). Similarly TPCK inhibited both basal and IL-1β induced PDK1 trans-autophosphorylation on serine 241, as well as phosphorylation of downstream target S6 ribosomal protein (Fig 24B). As previously reported inhibition of PDK1 led to a mild activation in ERK phosphorylation(239). These data thus confirmed the selectivity of the PDK1 inhibitor, TPCK.

5.2.2 Overexpression of PDK1 or PKB is sufficient to drive NFκB activation.

To further confirm the role of PDK1 and PKB in the activation of NFκB, empty vector, PDK1, or PKB were overexpressed with the 4xκB-luc construct. Compelling evidence from three different cell lines was acquired (Fig 25 A/B/C). In all cases the overexpression of PKB and PDK1 was sufficient to drive both basal and IL-1β induced activation of NFκB. Most importantly this data was reproducible in the non-tumorigenic cell line HEK 293T (Fig 25C).

5.2.3 IL-1β induces PKB serine 473 phosphorylation and also its specific activity.

Since serine 473 phosphorylation is not sufficient to assess activation of PKB in response to IL-1β, the kinetics of serine 473 phosphorylation were compared with PKB activity. IL-1β activated PKB in a biphasic manner, with the initial activity coming very quickly at 2 min, and a second increase in activity at 30 min (Fig 26B). Maximal activity coincided with serine 473 phosphorylation, although serine 473 phosphorylation was not

detected until 30 minutes (Fig 26A). PDK1 activity is absolutely required for PKB activation(240), thus it can be inferred that PDK1 is also activated very early in this system. This data was reproducible in both HCT-116 cells and Caco-2 cells.

5.2.4 Overexpression of PDK1 but not PKB activates the IKK complex

In order to examine the mechanism through which PDK1 and PKB were regulating NFκB, the activity of the IKK complex was first examined. 293T cells were transfected with vector, PDK1, or PKB and 24 h later immunoprecipitated for IKKγ, which pulls down a complete IKK complex including IKKγ, IKKα, and IKKβ (data not shown). Immunocomplexes were then used in a radioactive kinase assay with either the GST-p65 or GST-IκB substrate, because both of these molecules are endogenous substrates of the IKK signalsome. Although both PKB and PDK1 were capable of activating NFκB (Fig 25), surprisingly only overexpression of PDK1, but not PKB, was sufficient to activate the IKK complex (Fig 27A). To further confirm this, the effects of overexpressing PDK1 and PKB on IκB phosphorylation were examined. Overexpression of PDK1, but not PKB, induced endogenous IκB phosphorylation (Fig 27B). In addition, pretreatment with TPCK, but not LY and wortmannin, led to prevention of IL-1β induced IκB degradation (Fig 27C). This meant that PDK1 and PKB were activating NFκB in distinct ways, and that PKB was acting at a more distal regulatory location.

5.2.5 Pharmacological inhibition of PDK1 but not PI3K results in the inhibition of p65 DNA binding

Since PDK1 regulates IKK activation, it should therefore also regulate NFκB DNA binding. Using EMSA, the effects of TPCK, LY, and wortmannin on p65 DNA binding were thus examined. Not surprisingly, when cells were pretreated with TPCK, there was attenuation of DNA binding (Fig 28A/B). Pretreatment of cells with LY and wortmannin had no effect on DNA binding (Fig 28A/B), also confirming the previous data that PDK1 was regulating the IKK complex, whereas PKB was regulating some other distal regulatory point. Overexpression of PDK1 but not PKB was sufficient to induced κB DNA binding (Fig 28C). This data was reproducible in both 293T and HCT-116 cells.

5.2.6 Pharmacological inhibition of PKB results in the attenuation of p65 transactivation

A potential mechanism for PKB regulation of NFκB may be through regulation of p65 transactivation. To assess this we used the one-hybrid system (p65 TADI-III fused to Gal4 DNA binding, transfected along with a Gal4 responsive luciferase reporter). Inhibition of p65 transactivation was observed with pretreatment of both LY and wortmannin (Fig 29A). Thus PKB was regulating NFκB activation through regulation of p65 transactivation. When p65 phosphorylation at serine 536 was examined, there was no effect of LY or wortmannin on IL-1β induced p65 phosphorylation at serine 536 (Fig 29B).

5.2.7 Pharmacological inhibition of PDK1 results in the attenuation of p65 transactivation

The IKK signalsome has two main regulatory functions. The first is that it regulates the phosphorylation of IκB. The second function is that it phosphorylates p65 and regulates its transactivation. Due to the fact that PDK1 was regulating the IKK complex, this would mean that PDK1 would also regulate the transactivation of p65. We tested this by using the Gal4 one-hybrid system. Pretreatment of cells with TPCK resulted in attenuation of IL-1β induced p65 transactivation (Fig 30A). Thus PDK1 can also regulate p65 transactivation. When p65 serine 536 was examined, TPCK did inhibit IL-1β mediated serine 536 phosphorylation on p65 (Fig 30B).

5.2.8 Overexpression of PKB or PDK1 is sufficient to induce p65 transactivation

To confirm the abilities of PKB and PDK1 to regulate p65 transactivation, they were overexpressed with the Gal4 one-hybrid system. Both PKB and PDK1 were sufficient to activate the Gal4-dependant luciferase reporter (Fig 31). This confirmed the data presented with the pharmacological inhibitors, that both PKB and PDK1 were able to regulate p65 transactivation; however PDK1 was regulating transactivation via IKK, and PKB was not.

5.2.9 PDK1 and PKB regulate p65 transactivation through unique sites

The IKK signalsome regulates p65 transactivation by phosphorylating p65 at serine 536, in an IKKβ dependant fashion(81). To confirm this in our system, mutant GST-constructs that were mutated at serine 529 or serine 536 and changed to alanines were generated. Serine 529 is the CK2 regulated site and this mutant was used as a

control (Chapter 4). The IKK complex was assayed by immunoprecipitating IKKγ, and performing radioactive IKK assays using either GST-p65 WT, GST-p65 S529A, or GST-p65 S536A. As is clearly shown (Fig 32A), mutation of serine 536, but not serine 529 attenuates p65 phosphorylation. Thus only the serine 536 can be phosphorylated by the IKK signalsome in our system. Since PDK1 regulates the IKK signalsome complex, we hypothesized that PDK1 should regulate p65 transactivation through serine 536. Similarly PKB should not regulate serine 536 because it does not regulate IKK. Thus using the one-hybrid system with a mutant version of p65 TAD that contains a serine 536 to alanine mutation, both PKB and PDK1 were overexpressed. Overexpression of PDK1 and PKB both resulted in transactivation of the WT p65 construct. Mutation of serine 536 to alanine completely attenuated the ability of PDK1 to transactivate the p65 construct, whereas PKB was still able to transactivate the p65 construct (Fig 32B). This further substantiates the notion that PDK1 is regulating p65 via IKK and serine 536, whereas PKB is regulating p65 through an as of yet unknown site. To further confirm this, endogenous phospho-p65 (serine 536) was examined by immunoblotting. This shows that overexpression of PDK1 but not PKB is sufficient to induce serine 536 phosphorylation (Fig 32C).

5.2.10 PDK1 and IKK coassociate

To see if there was a physical association between IKK and PDK1, they were co-immunoprecipitated and immunoblotted for each other. As the data shows they are constitutively bound, and their association does not change with IL-1β stimulation (Fig 33). When we examined overall cellular PDK1 activity there was no change, nor was there a change in IKKγ bound PDK1 specific activity (data not shown). To confirm that

IKKγ (and the IKK signalsome) was successfully immunoprecipitated, the same membrane was stripped and reprobed for IKKβ. Thus there is constitutive association between PDK1 and IKK.

5.2.11 IKK α/β ser 177/181 is sensitive to TPCK, and is a potential PDK1 phosphorylation site

In order to explore the mechanism through which PDK1 regulates IKK, we looked at the activating phosphorylations of IKKα and β (serine 180/181). When cells were pretreated with TPCK, this activating phosphorylation was inhibited (Fig 34A). Close examination of the IKK α and β activation loop phosphorylation site reveals that it is a potential PDK1 phosphorylation site, as it matches those sites in other PDK1 downstream targets quite closely (Fig 34B).

5.2.12 Both PI3K and PDK1 are required for the activation of the IL-8 promoter

Now that a clear role for PDK1 and PKB regulation in the activation of the NFκB has been established, the activation of downstream NFκB target, IL-8, was examined. We used an IL-8 promoter attached to a luciferase reporter (IL8-luc) to examine IL-8 promoter activation. Cells pretreated with either LY or wortmannin, were unable to activate the reporter in response to IL-1β (Fig 35A). Likewise cells pretreated with TPCK were also unable to activate this reporter (Fig 35B).

5.2.13 Overexpression of PDK1 is sufficient to activate the IL-8 promoter

To further confirm the pharmacological data in Fig 35, PKB and PDK1 were overexpressed with the IL8-luc reporter. PDK1 was sufficient to activate the IL-8

proximal promoter. PDK1 robustly activated this promoter construct with a 20 fold activation basally, and almost a 200 fold activation with IL-1β stimulation (Fig 36). Overexpression of PKB was not sufficient to activate the IL-8 promoter construct; however, the addition of IL-1β led to a synergistic activation.

5.2.14 Overexpression of PDK1 is sufficient to IL-8 message synthesis

The expression of the chemokine IL-8 was examined by RT-PCR in order to see if RNA was transcribed as a result of promoter activation. Overexpression of PKB led to a very mild increase in IL-8 message, however PDK1 overexpression resulted in a very significant increase in IL-8 message (Fig 37). This increase in IL-8 message from PDK1 overexpression was almost equivalent to that of IL-1β induced IL-8 message

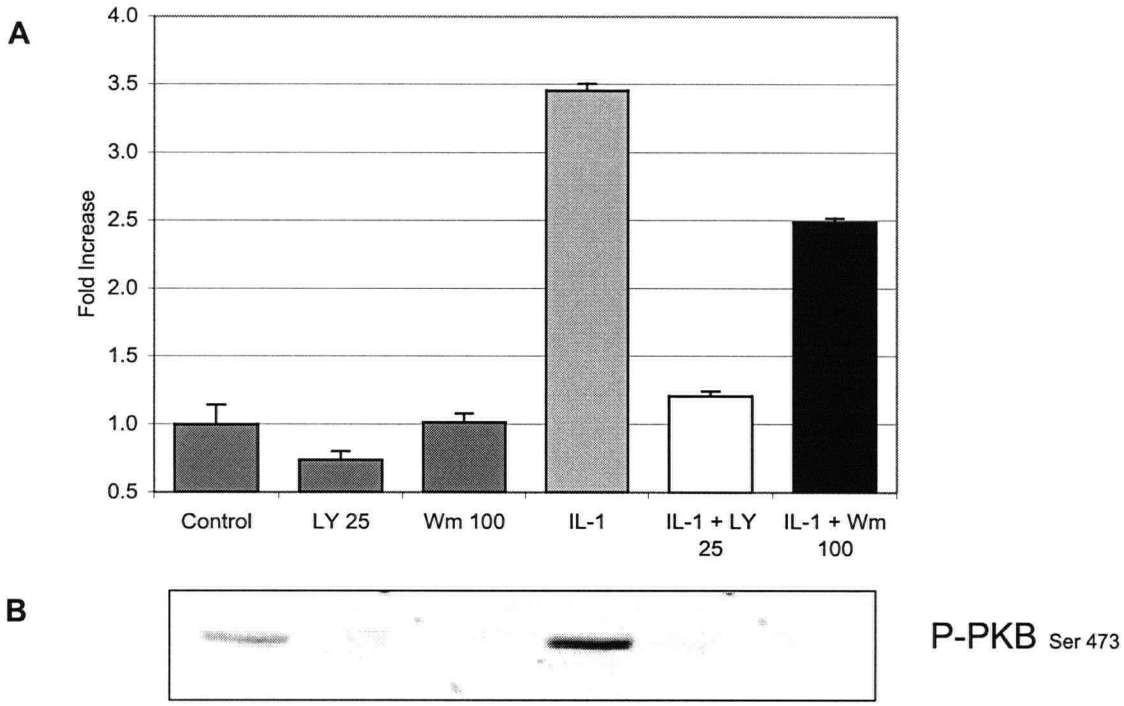


Figure 23. Inhibition of PI3K inhibits IL-1β induced NFκB activation. (A) HCT-116 colonic epithelial cells, transfected with an NFκB responsive luciferase reporter (4xκB-Luc), were pretreated with LY (25 uM), wortmannin (100 nM), for 1 h before being stimulated with IL-1β (2 ng/mL) for 6 h. (B) HCT-116 colonic epithelial cells were pretreated with LY (25 uM), wortmannin (100 nM), stimulated with IL-1β (2 ng/mL) for 30 min. Proteins were subsequently resolved by SDS-PAGE, and probed for phospho-PKB (serine 473).

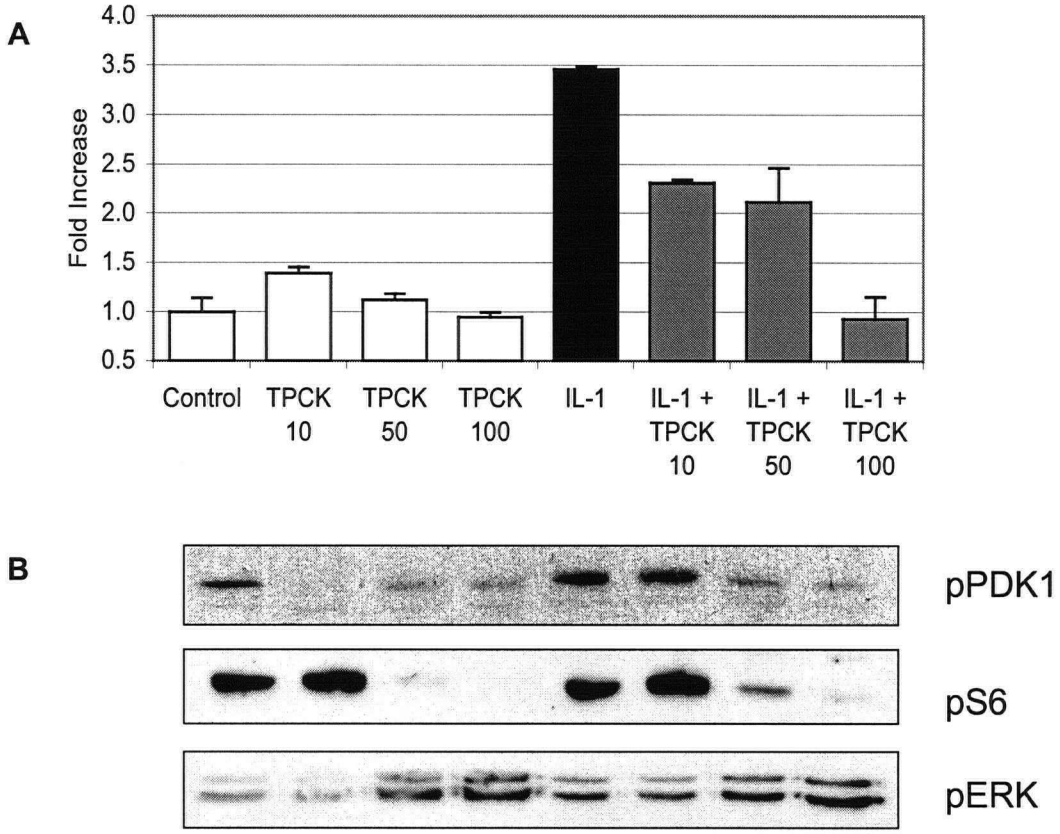


Figure 24. Inhibition of PDK1 inhibits IL-1β induced NFκB activation. (A) HCT-116 colonic epithelial cells, transfected with an NFκB responsive luciferase reporter (4xκB-Luc), were pretreated with TPCK (10-100 uM) for 1 h before being stimulated with IL-1β (2 ng/mL) for 6 h. (B) HCT-116 colonic epithelial cells were pretreated with TPCK (50-100 uM) for 1 h, stimulated with IL-1β (2 ng/mL) for 30 min. Proteins were subsequently resolved by SDS-PAGE, and probed for phospho-PDK1, phospho-S6, or phospho-ERK.

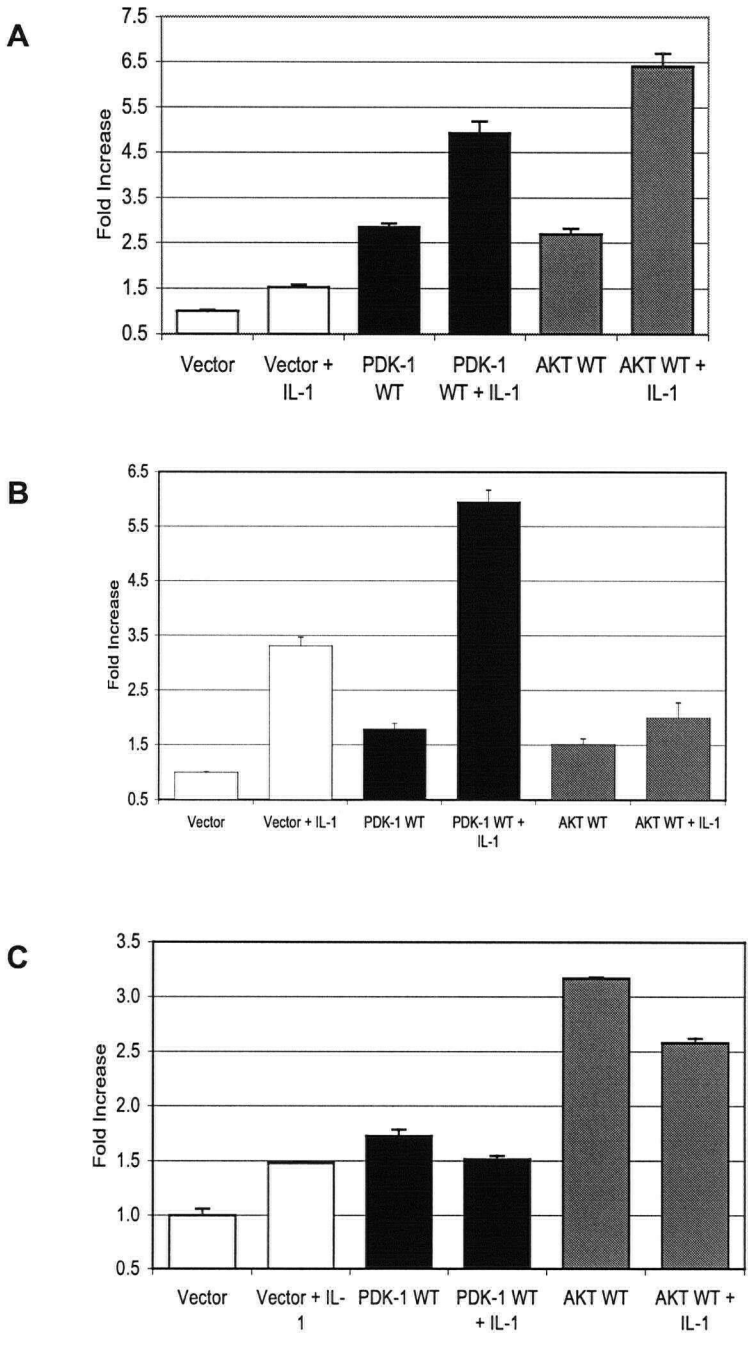


Figure 25. Overexpression of PDK1 or PKB is sufficient for the activation of NFκB. HCT-116 colonic epithelial cells (A) Caco-2 colonic epithelial cells (B) or 293T human embryonic kidney cells (C) were transfected with an NFκB responsive luciferase reporter (4xκB-Luc) as well as either empty PCDNA3 vector, myc-PDK1, or HA-PKB. 24 h post transfection cells were stimulated with IL-1β for 6 h.

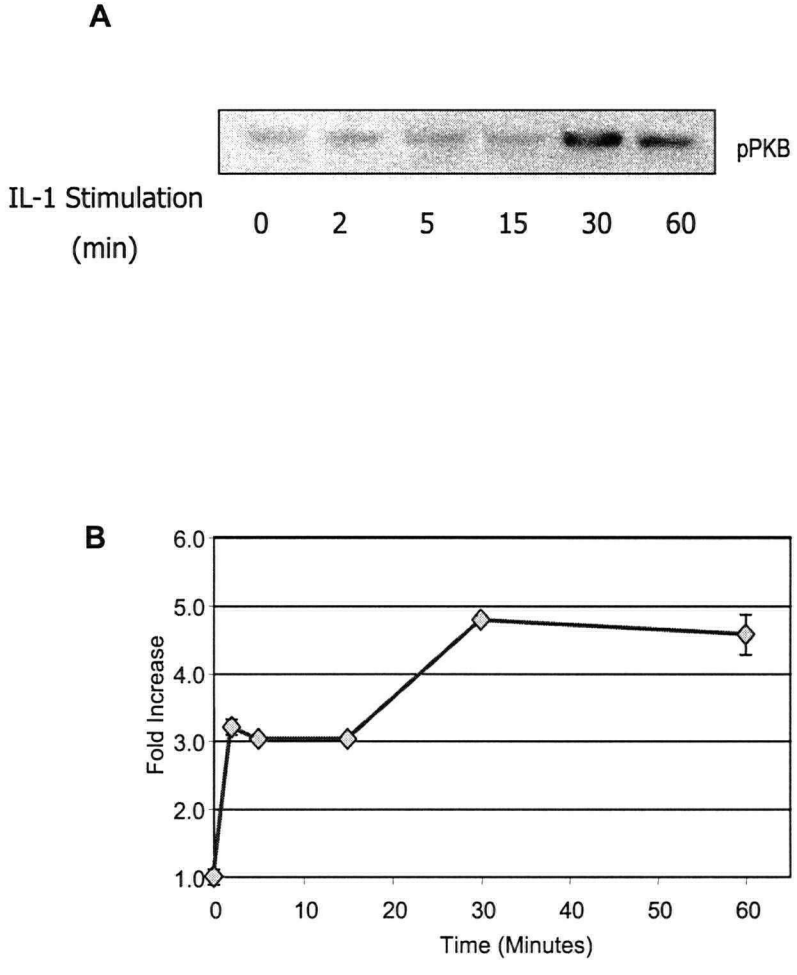


Figure 26. IL-1β activates PKB and the phosphorylation of IκB and p65. HCT-116 colonic epithelial cells were stimulated with IL-1β (10 ng/mL) for varying lengths of time. Proteins were subsequently resolved by SDS-PAGE, and probed for phospho-PKB (A) or were immunoprecipitated with an antibody against PKB, and a PKB immune complex kinase assay was performed (B).

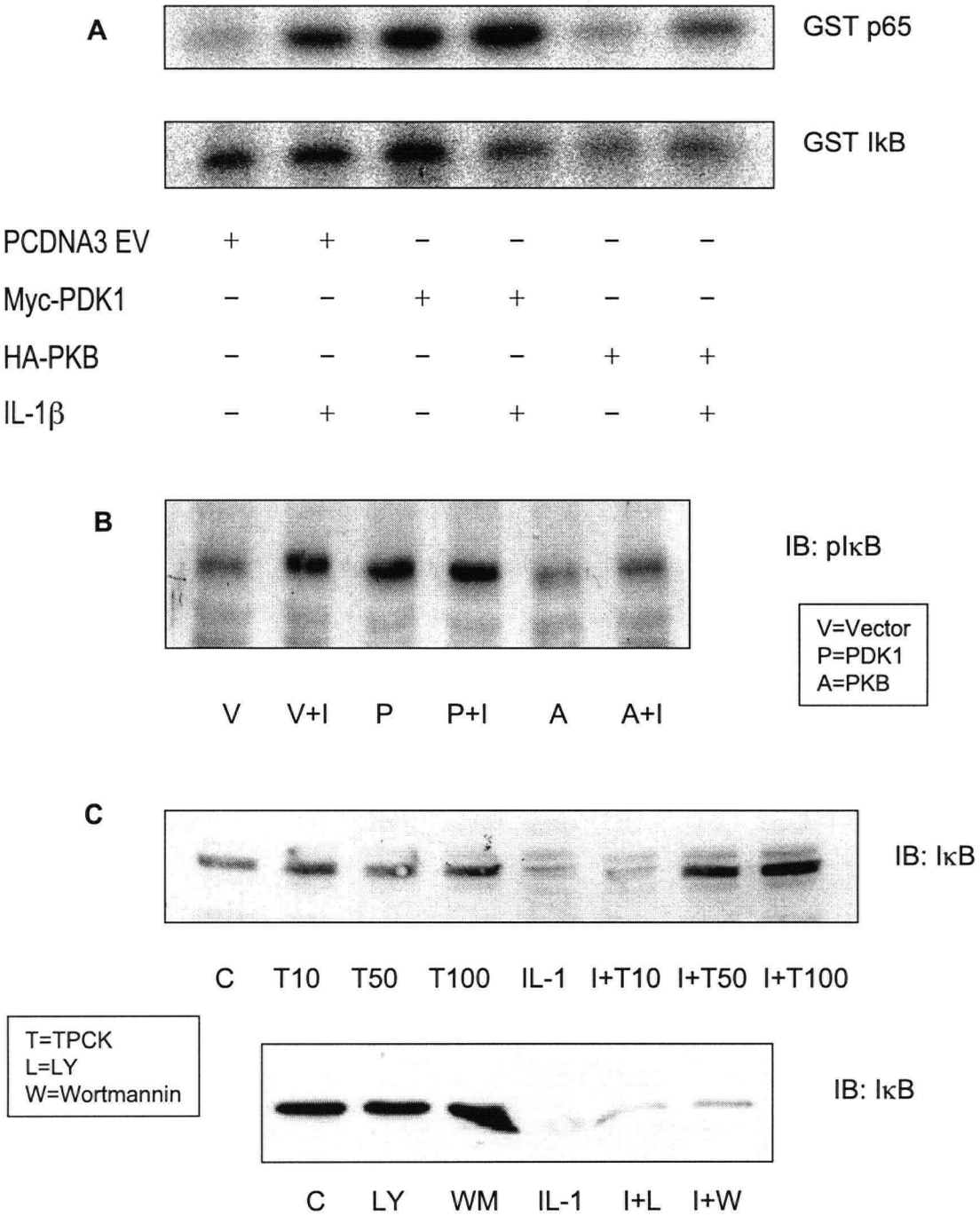


Figure 27. Overexpression of PDK1, but not PKB, results in the activation of IKK activity. (A) 293T cells were transfected with either empty PCDNA3 vector, myc-PDK1, or HA-PKB. 24 h post transfection cells were stimulated with IL-1β for 30 min. (A) IKK was subsequently immunoprecipitated and kinase assays were performed. (B) Lysates were immunoblotted for phospho-IκB. (C) Cells were pretreated with TPCK (T) or 25 uM LY294002 (L) or 100 nM wortmannin (W). Cell lysates were immunoblotted for protein IκB.

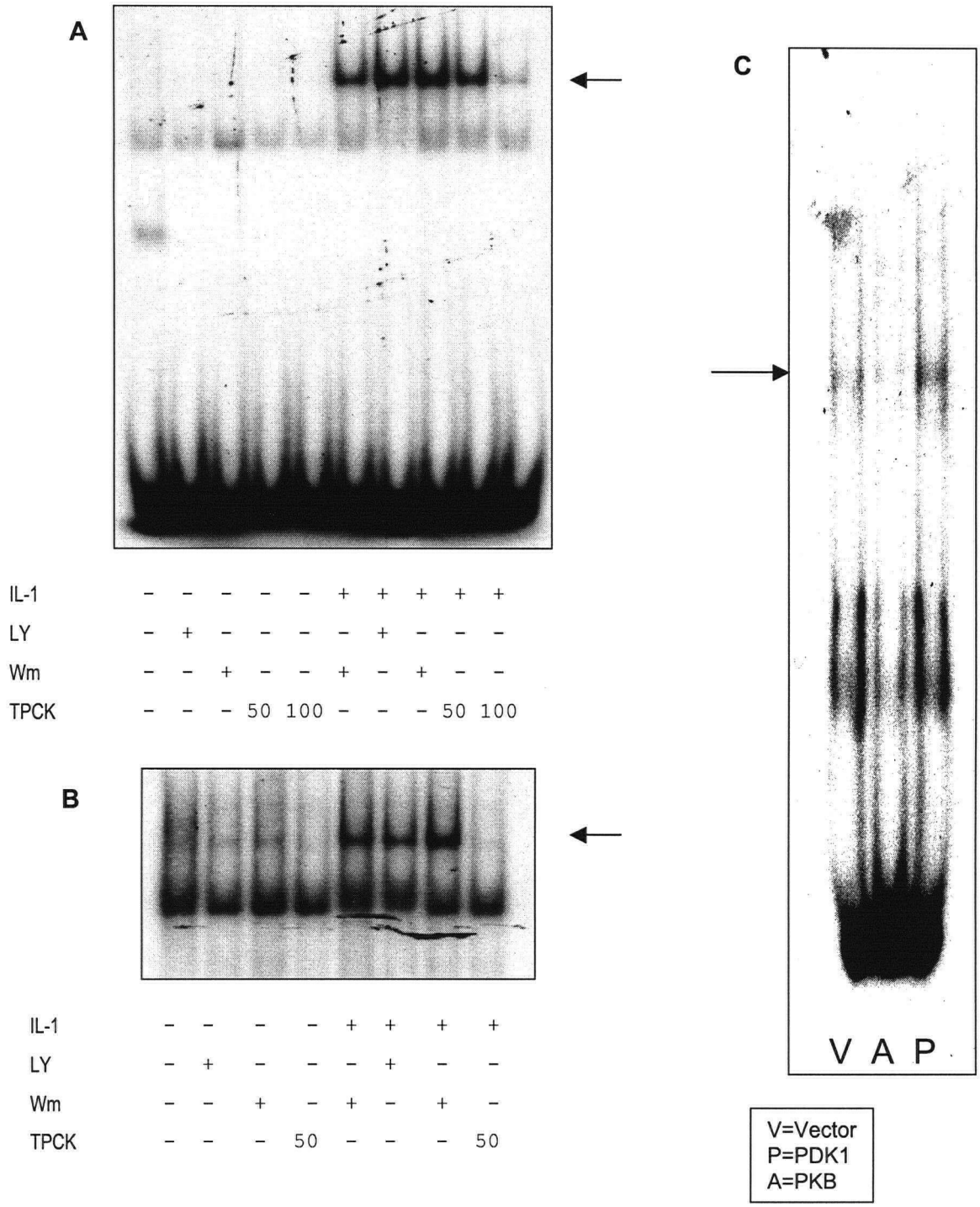


Figure 28. Inhibition of PDK1 but not PI3K results in the inhibition of NFκB nuclear translocation and subsequent DNA binding. HCT-116 colonic epithelial cells (A) or 293T human embryonic kidney cells (B) were pretreated with LY (25 uM), wortmannin (100 nM), or TPCK (50-100 uM) , for 1 h before being stimulated with IL-1β (2 ng/mL) for 30 min. Nuclear proteins were isolated and electrophoretic mobility shift assay (EMSA) was performed. (C) HCT-116 cells were transfected with empty PCDNA3 vector, myc-PDK1, or HA-PKB. 24 h post transfection cells were harvested for nuclear proteins and analyzed for p65 DNA binding by EMSA.

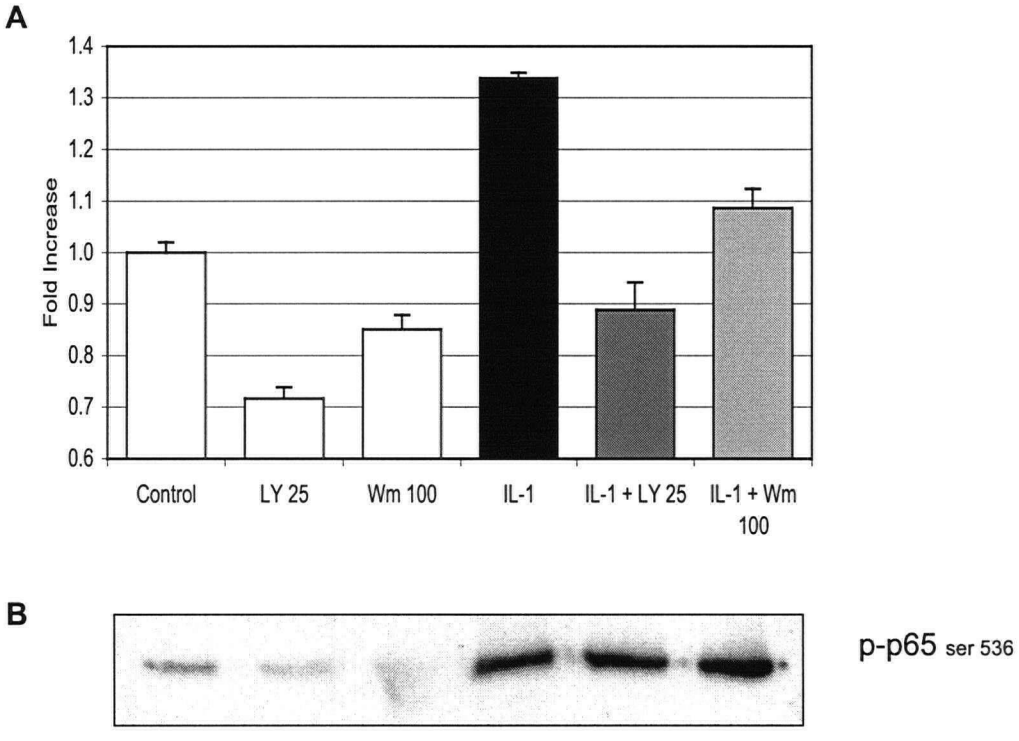


Figure 29. Inhibition of PI3K inhibits IL-1β induced NFκB transactivation. (A) HCT-116 colonic epithelial cells, transfected with a GAL4 responsive luciferase reporter (Gal4-Luc), along with pTADI-III (transactivation domains I-III fused to a Gal4 DNA binding domain), were pretreated with LY (25 uM), wortmannin (100 nM), for 1 h before being stimulated with IL-1β (2 ng/mL) for 6 h. (B) HCT-116 colonic epithelial cells were pretreated with LY (25 uM), wortmannin (100 nM), stimulated with IL-1β (2 ng/mL) for 30 min. Proteins were subsequently resolved by SDS-PAGE, and probed for phospho-p65.

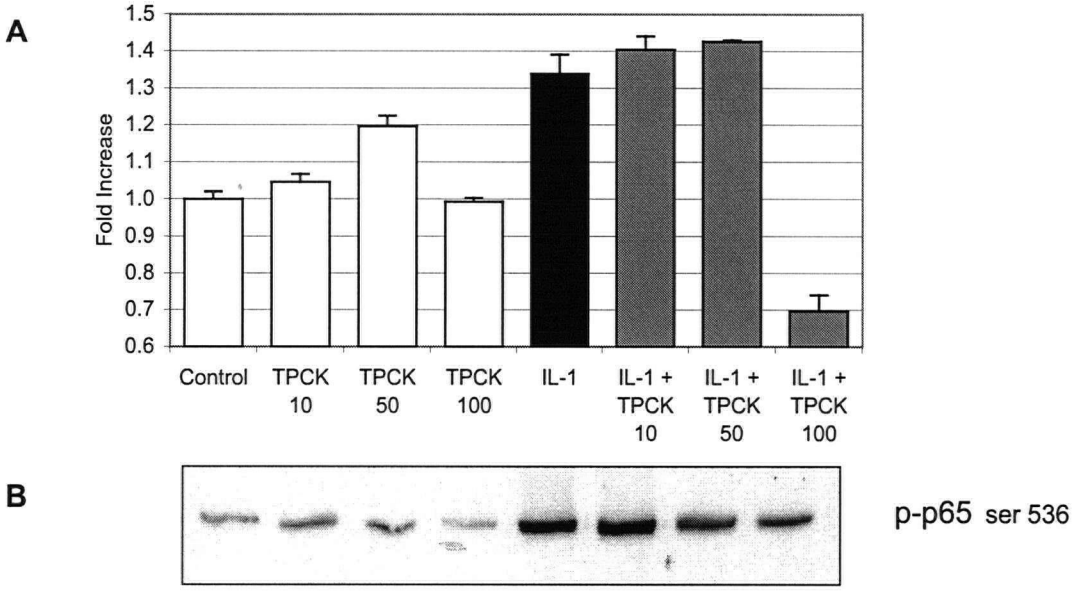


Figure 30. Inhibition of PDK1 inhibits IL-1β induced NFκB transactivation. (A) HCT-116 colonic epithelial cells, transfected with a GAL4 responsive luciferase reporter (Gal4-Luc), along with pTADI-III (transactivation domains I-III fused to a Gal4 DNA binding domain), were pretreated with TPCK (50-100 uM) for 1 h before being stimulated with IL-1β (2 ng/mL) for 6 h. (B) HCT-116 colonic epithelial cells were pretreated with TPCK (50-100 uM), stimulated with IL-1β (2ng/mL) for 30 min. Proteins were subsequently resolved by SDS-PAGE, and probed for phospho-p65.

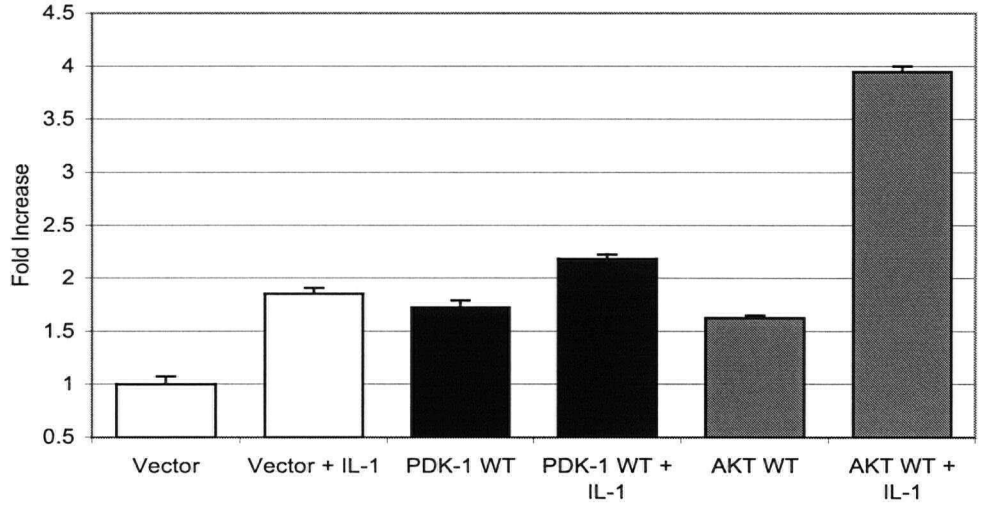


Figure 31. Overexpression of PDK1 and PKB results in increased transactivation. (A) HCT-116 colonic epithelial cells, transfected with a GAL4 responsive luciferase reporter (Gal4-Luc), along with pTADI-III (transactivation domains I-III fused to a Gal4 DNA binding domain) and with either empty PCDNA3 vector, myc-PDK1, or HA-PKB. 24 h post transfection cells were stimulated with IL-1β for 6 h.

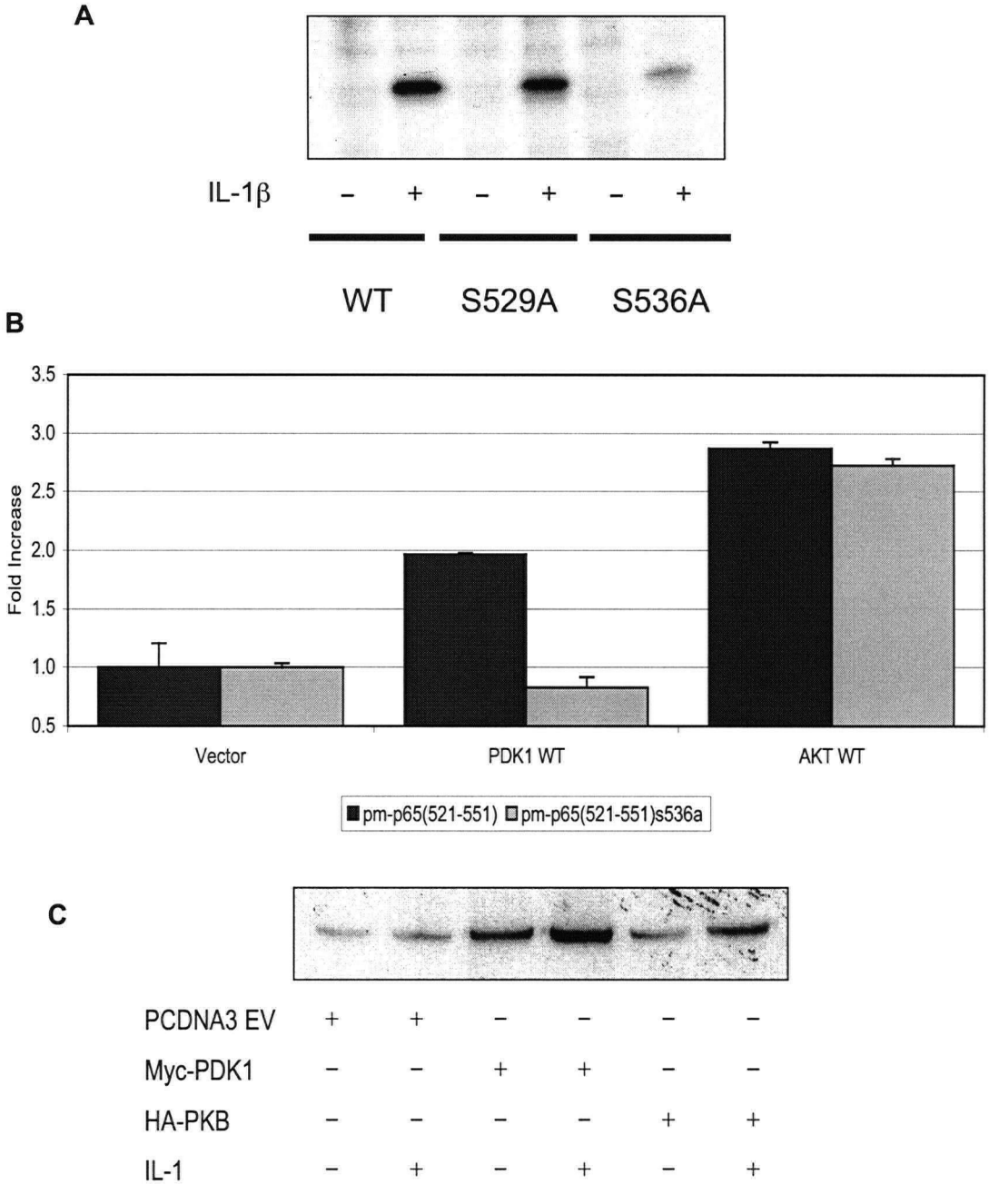


Figure 32. PDK1 mediated increased transactivation is dependant on p65 serine 536. (A) HCT-116 cells were stimulated with IL-1β for 15 min. IKK was subsequently immunoprecipitated and kinase assays were performed, using WT GST-p65, serine 529 to alanine GST-p65, or serine 536 to alanine GST-p65. (B) HCT-116 colonic epithelial cells, transfected with a GAL4 responsive luciferase reporter (Gal4-Luc), along with pTADIII (transactivation domain III fused to a Gal4 DNA binding domain – WT or S536A) and with either empty PCDNA3 vector, myc-PDK1, or HA-PKB. 24 h post transfection cells were stimulated with IL-1β for 6 h. (C) 293T cells were transfected with either empty PCDNA3 vector, myc-PDK1, or HA-PKB. 24 h post transfection cells were stimulated with IL-1β for 30 min. Proteins were subsequently resolved by SDS-PAGE, and probed for phospho-p65.

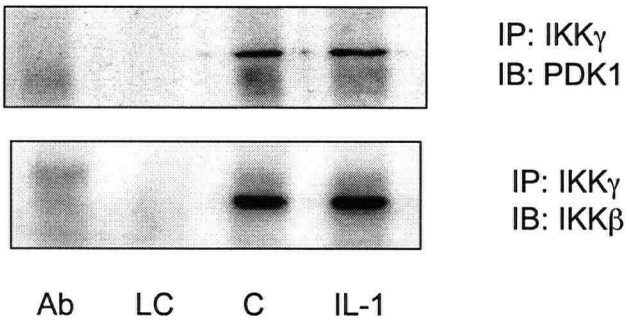
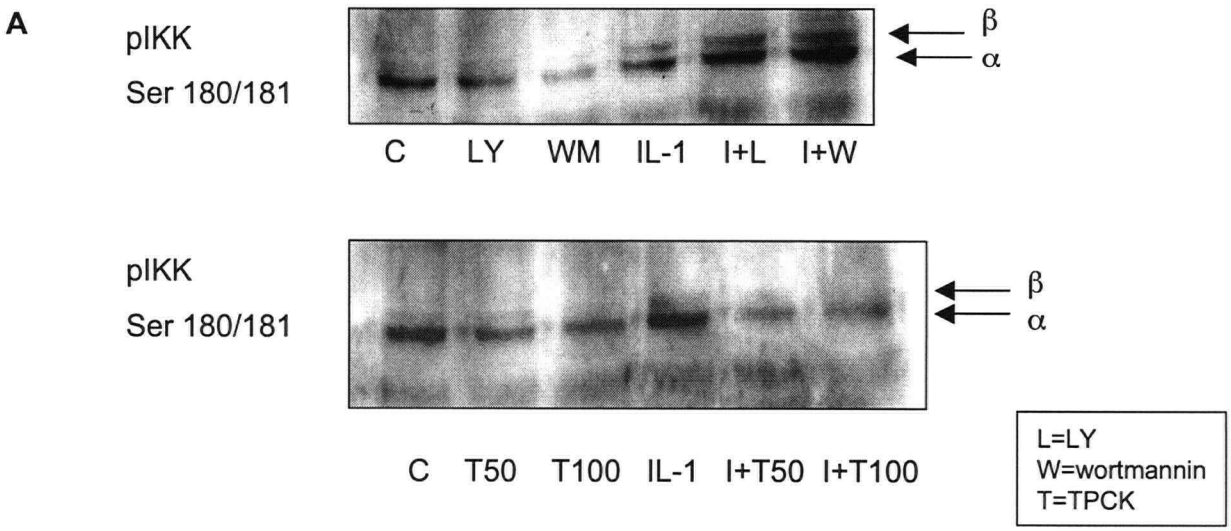


Figure 33. PDK1 coassociates with IKK signalsome. HCT-116 cells were stimulated with IL-1β for 15 min. IKK was subsequently immunoprecipitated and immunoblotted for either PDK1 or IKKβ. Ab = antibody and beads control, LC = lysate and non-specific IgG control

A



B

PKB	K	D	G	A	T	M	K	**T**	F	C	G	T	P	E	Y	L	A	P	E
SGK	E	H	N	S	T	T	S	**T**	F	C	G	T	V	E	Y	M	A	P	E
p70SGK	H	D	G	T	V	T	H	**T**	F	C	G	T	I	E	Y	M	A	P	E
p90RSK	D	H	E	K	K	A	Y	**S**	F	C	G	T	V	E	Y	M	A	P	E
PKA	R	V	K	G	R	T	W	**T**	L	C	G	T	P	E	Y	L	A	P	E
PRK	G	Y	G	D	R	T	S	**T**	F	C	G	T	P	E	F	L	A	P	E
PKC	M	D	G	V	T	T	R	**T**	F	C	G	T	P	D	Y	I	A	P	E
PDK1	S	K	Q	A	R	A	N	**S**	F	V	G	T	A	Q	Y	V	S	P	E
IKK ALPHA	D	Q	G	S	L	C	T	**S**	F	V	G	T	L	Q	Y	L	A	P	E
IKK BETA	D	Q	G	S	L	C	T	**S**	F	V	G	T	L	Q	Y	L	A	P	E

Figure 34. Ser 180/181 on IKKα/β are potential PDK1 phosphorylation sites and are sensitive to TPCK. (A) Alignment of consensus PDK1 phosphorylation sites. (B) HCT-116 colonic epithelial cells were pretreated with TPCK (50-100 uM) for 1 h, stimulated with IL-1β (2 ng/mL) for 30 min. Proteins were subsequently resolved by SDS-PAGE, and probed for phospho-IKK (serine 180/181).

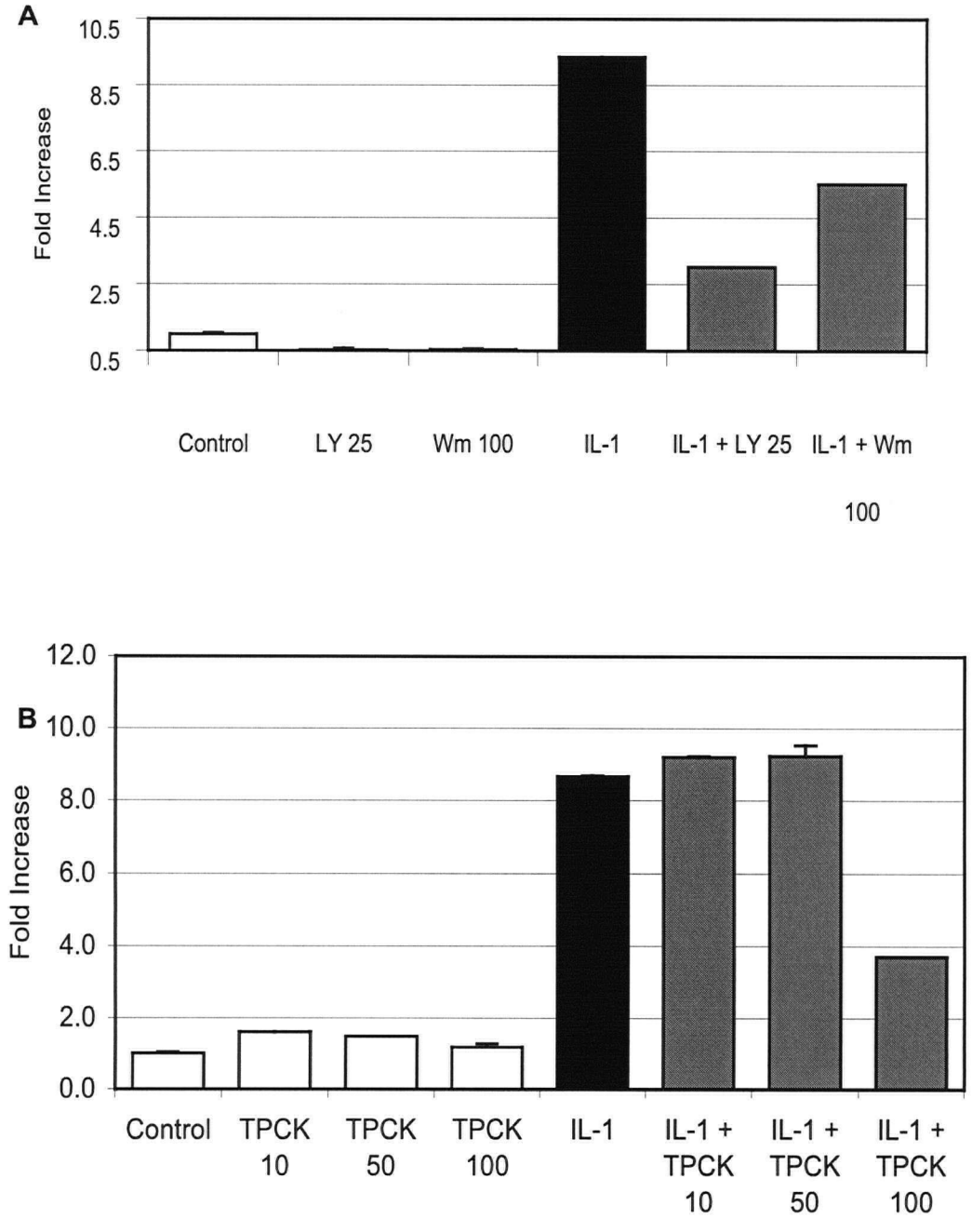


Figure 35. Inhibition of PI3K or PDK1 inhibits IL-1β induced proximal IL-8 promoter activation. (A) HCT-116 colonic epithelial cells, transfected with an IL-8 luciferase reporter (IL8-Luc), were pretreated with LY (25uM), wortmannin (100 nM) (A), or TPCK (50-100 uM) (B) for 1 h before being stimulated with IL-1β (2 ng/mL) for 6 h.

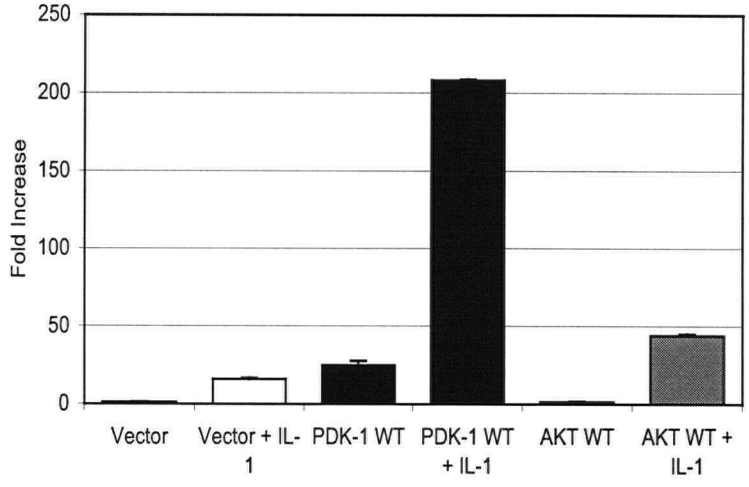


Figure 36. Overexpression of PDK1 or PKB results in the activation of the IL-8 proximal promoter. HCT-116 colonic epithelial cells, transfected with an IL-8 luciferase reporter (IL8-Luc), along with either empty PCDNA3 vector, myc-PDK1, or HA-PKB. 24 h post transfection cells were stimulated with IL-1β for 6 h.

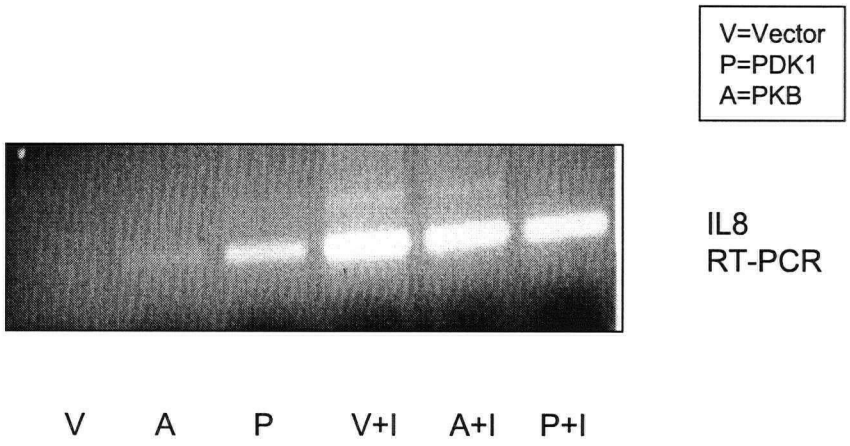


Figure 37. Overexpression of PDK1 but not PKB results in increased IL-8 message synthesis. HCT-116 cells were transfected with either empty PCDNA3 vector, myc-PDK1, or HA-PKB. 24 h post transfection cells were harvested for RNA and subjected to RT-PCR to examine IL-8 message.

5.3 DISCUSSION

In this section the PI3K, PKB axis was examined and compared with PDK1 with respect to its ability to regulate NFκB and chemokine synthesis. Both PKB and PDK1 were capable of regulating chemokine synthesis. In addition, both PKB and PDK1 were able to regulate NFκB. Interestingly however, although PKB and PDK1 are considered to be in the same pathway, they had distinct mechanisms of regulating NFκB, perhaps suggesting in the context of IL-1β signaling they work independently. PDK1 was shown to regulate the IKK complex, and as a result could modulate IκB phosphorylation at serine 32/36 as well as p65 transactivation through serine 536. Alternatively through the PI3K and PKB pathway, NFκB transactivation was being regulated, however in a serine 536 independent fashion. Interestingly, on closer examination, IKK was capable of co-associating with PDK1. The regulatory activating phosphorylation of IKKα and IKKβ were potential PDK1 phosphorylation sites, and were sensitive to TPCK.

The mechanism of regulation of NFκB by the PI3K pathway has been very contentious, and disputed. Some reports observe that PI3K and PKB can regulate the IKK complex, and thus regulate IκB phosphorylation and degradation(153, 154). Some reports instead observe that PI3K and PKB instead play a very important role in regulating the transactivation of p65(80, 83). Other reports argue any role at all for PI3K and PKB(241). Many of these studies are confounded by the diversity of cell systems used, along with the variety of stimuli used in the studies. The data presented here

shows in the IEC system, that PI3K regulates the transactivation potential of p65, and not IκB phosphorylation and degradation.

TPCK has only been recently identified as a PDK1 inhibitor. It was discovered as an *in vitro* inhibitor, designed to allow the study of the serine-protease chymotrypsin(242). TPCK was initially observed to inhibit tumorigenesis, reduce the rate of proliferation, despite the lack of knowledge as to its *in vivo* cellular target(243, 244). A study then reported that TPCK can inhibit RSK, S6K, and PKB(245). Many of these observations were not reproducible with the use of other chymotrypsin inhibitors, thus suggesting that the cellular target of TPCK was not a chymotrypsin but some other pathway. The same group then reported that the cellular target of TPCK was PDK1(239). TPCK has also been long regarded as an inhibitor of NFκB(173, 246). Inhibition of NFκB has been attributed to an attenuation of IκB phosphorylation, thus regulating a signal upstream of IKK. This observation is similar to our observations of the IKK complex being sensitive to TPCK signaling. Thus these reports provided a link between the previously inhibition of PDK1 by TPCK, and NFκB.

Much evidence supports the notion that PDK1 may be regulating IKK. Numerous studies have described the ability of IKK α and β to trans-autophosphorylate each other(168-173). At the same time these reports all leave the same question as to how the initial pool of IKK become phosphorylated and activated. The studies presented here show a constitutively bound PDK1 to IKK. Due to the constitutive activity of PDK1, this would allow IKK to become initially phosphorylated, allowing the IKK kinase subunits to trans-autophosphorylate each other. This may be mediated by a signal induced change in the IKK signalsome, either conformational, or with the addition of

loss of another protein. A candidate protein for this may be the cellular chaperone HSP90 that has been shown to associate and regulate both IKK as well as associate and regulate PDK1(159, 174). The data presented thus suggests a small pool of PDK1 may exist in the cell, that is bound to IKK, and that is not regulated by PI3K. PI3K independent activation of PDK1 has been previously reported(160).

Previously, PKB has been linked to regulation of serine 536 phosphorylation(80). Our data clearly shows that PDK1, not PKB, regulates this site in an IKK dependant fashion. This then leaves the question as to how PKB may be regulating p65 transactivation between residues 501-551. One potential mechanism may be that PKB directly phosphorylates p65. Another mechanism may be that PKB is working through another downstream effector. A candidate molecule for this may be GSK3β. Gene knockout of this molecule results in embryonic lethality due to TNF induced liver apoptosis(247). When examined, GSK3β was found to regulate NFκB in a method independent of IκB phosphorylation and degradation, similar to the mechanism through which PKB regulates NFκB in our system.

CHAPTER 6 – GENERAL CONCLUSIONS

The body of work presented here has examined the signaling pathways that are activated in IECs leading to the synthesis of chemokines, in response to IL-1β. Three important pathways were examined (MAPK, protein kinase CK2, and PI3K/PDK1). Their relationship with respect to activation of NFκB activation was also examined.

IL-1β, upon binding to its receptor, activated all three MAPK family member, as well as protein kinase CK2 and PKB. NFκB, which is required for the synthesis of many chemokines, was found to be a point of convergence of many of these pathways. Activation of the IKK complex is required for the signal induced phosphorylation and degradation of IκB. PDK1 was found to regulate the IKK complex, in a PI3K independent fashion, potentially by phosphorylating IKKα and β at serines 180/181. IKK also phosphorylates the p65 subunit at serine 536 to allow it to become functionally active. PDK1 was found to regulate this phosphorylation at serine 536, in a process dependant upon IKKβ.

In addition, further phosphorylations of p65 are required for full transactivational activity. PKB regulated the transactivation of p65 in a serine 536 independent fashion. This may occur by a direct phosphorylation of p65 or indirectly through a downstream effector, a potential candidate being GSK3β. Upon entering the nucleus p65 is further phosphorylated by protein kinase CK2 at serine 529. p65, now being fully active, binds its consensus DNA sequence and forms part of the transcriptional complex. For promoters such as IL-8, it will synergize with other trans-acting regulatory factors such

as other transcription factors and coactivators. p38 MAPK was found to regulate the IL-8 promoter, however independently of NFκB. Another important transcription factor, AP-1 is required for maximal transcriptional activation. Its DNA binding ability appears to be regulated by p38 MAPK, and this may be one the ways that p38 MAPK regulates chemokines. At this point JNK may play a role in interacting with functional p65 and thus regulating both p65 activity and the IL-8 promoter. This allows the promoter to be regulated by multiple inputs, all downstream of the IL-1R.

Overall this work maps the signaling pathways, relevant to IL-1β signaling, and describes their potential modes of action. Insight gained from this work may provide potential *in vivo* targets that can be modulated to relieve chronic gut inflammatory conditions such as IBD.

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