STRUCTURE/FUNCTION ANALYSIS OF CD40; A KEY ACTIVATOR OF B LYMPHOCYTES

by

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ABSTRACT

B lymphocytes need two signals in order to differentiate into antibody-producing cells, one delivered by the B cell antigen receptor (BCR) and a second delivered by CD40. In the absence of the CD40 signal, B cells that receive only the BCR signal are rendered non-responsive or undergo apoptosis The ability of CD40 to rescue B cells from BCR-induced apoptosis can be demonstrated using the WEHI-231 B lymphoma cell line. I have used this cell line to investigate the role of mitogen-activated protein (MAP) kinases in integrating BCR and CD40 signaling. The three types of MAP kinases, the ERKs, the c-Jun N-terminal kinases (JNKs), and p38, each phosphorylate a distinct set of transcription factors. Thus, activating different combinations of MAP kinases could lead to distinct biological responses. I found that BCR engagement in WEHI-231 cells strongly activates ERK2 and weakly activates ERK1, JNK and p38. CD40 engagement did not activate either of these kinases, nor did it affect BCR-induced ERK activation. In contrast, CD40 engagement markedly stimulates JNK and p38 as well as MAPKAP kinase-2, a downstream target of p38. The BCR weakly activates JNK and p38 by itself, however, it potentiates CD40-induced JNK activation. Thus, activation of ERK2 alone correlates with apoptosis in WEHI-231 cells, whereas full activation of all three MAP kinase pathways correlates with cell survival. The role of MAP kinases in regulating these responses remains to be tested.

To identify signaling motifs in the CD40 cytoplasmic domain that are responsible for activation of the JNK and p38 MAP kinases, I created a set of 12 chimeric receptors consisting of the extracellular and transmembrane domains of CD8 fused to portions of the murine CD40 cytoplasmic domain. These chimeric receptors were expressed in WEHI-231 B lymphoma cells. I found that amino acids 35-45 of the CD40 cytoplasmic domain constitute an independent signaling motif that is sufficient for activation of the JNK and p38 MAP kinase pathways, as well as for induction of

IκBα phosphorylation and degradation. Amino acids 35-45 were also sufficient to protect WEHI-231 cells from anti-IgM-induced growth arrest. This is the same region of CD40 required for binding to the TRAF2, TRAF3 and TRAF5 adapter proteins. These data support the idea that one or more of these TRAF proteins couple CD40 to the kinase cascades that activate NF-κB, JNK and p38.

Another aim of this thesis was to test the hypothesis that ATAR, a recently discovered tumor necrosis factor (TNF) superfamily receptor, can mimic the effects of CD40 on B cells. Like CD40, ATAR is expressed on B cells and interacts with ligands expressed by activated T cells. To study ATAR signaling, two chimeric receptors consisting of the extracellular and transmembrane domains of CD8 fused to portions of the ATAR cytoplasmic domain were constructed and expressed in WEHI-231 cells. We found that the cytoplasmic tail of ATAR mediated phosphorylation of JNK and p38, phosphorylation and degradation of IκBα, as well as protection of WEHI-231 cells from anti-IgM-induced growth arrest. The C-terminal portion of the ATAR tail containing the TRAF-interaction domain was sufficient to mediate these signaling events. Our results support a model in which TRAF2 and/or TRAF5 link ATAR to the activation of JNK, p38 and NF-κB, as well as to B cell survival. The ability of ATAR to mimic some of the effects of CD40 on B cells suggests that this novel TNFR superfamily member may provide an alternative second signal to B cells.

In addition to B lymphocytes, CD40 is highly expressed on dendritic cells (DC). Both CD40 and lipopolysaccharide (LPS) have been shown to activate these antigen presenting cells. The final aim of this thesis was to determine whether the MAP kinases might be involved in CD40 or LPS-induced activation of DCs. I tested whether ERK, JNK or p38 are activated by CD40 and LPS in the murine D1 DC line. I found that both CD40 and LPS strongly activated ERK2 in D1 cells. In contrast, little or no activation of JNK and MAPKAP kinase-2 was induced by either of these stimuli. Our

collaborators extended these findings by showing that ERK activation is essential for the ability of LPS to protect DCs from growth factor withdrawal-induced apoptosis.

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ABBREVIATIONS

Ab	antibody
Ag	antigen
APC	antigen presenting cell
ASK-1	apoptosis signal-regulating kinase
ATAR	another TRAF-associated receptor
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCR	B cell antigen receptor
BSA	bovine serum albumin
CD	cell differentiation factor
cDNA	complimentary deoxyribonucleic acid
CD40L	CD40 ligand
CIP	calf intestinal alkaline phosphatase
CM	conditioned medium
CRAF	CD40 receptor-associated factor
CREB	cAMP-responsive element binding protein
dH_2O	distilled water
ddH_2O	double distilled water
DC	dendritic cell
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotidyl triphosphates
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethlylenediamine tetra-acetic acid

ERK	extracellular signal-regulated kinase
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSB	FACS sorter buffer
g	gram
GCK	germinal center kinase
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione-S-transferase
h	hour
HBS	Hepes buffered saline
HEPES	N-[2-hydroxyethyl]piperazine-N' -[2-ethanesulfonic acid]
HRP	horseradish peroxidase
Hsp25	heat shock protein 25
HVEM	Herpes virus entry mediator
ICAM-1	intercellular adhesion molecule-1
IDC	interdigitating cell
Ig	immunoglobulin
IgG	immunoglobulin G
IκB	inhibitor of NF-κB
IKK	IκBα kinase
IL	interleukin
IRF-1	interferon regulatory factor-1
JNK	c-jun amino-terminal kinase
kB	kilobase
kDa	kilodalton
LB	Luria Bertani

LPS	lipopolysaccharide
LTR	long terminal repeat
M	molar
mAb	monoclonal antibody
MAP kinase	mitogen-activated protein kinase
MAPKAP kinase-2	MAP kinase-activated protein kinase-2
MBP	myelin basic protein
MCP-1	macrophage chemoattractant protein-1
2-ME	2-mercaptoethanol
MEK	ERK kinase
MEKK	MEK kinase
MHC	major histocompatibility complex
min	minute
MIP	macrophage inflammatory protein
MKK	MAP kinase kinase
MKKK	MKK kinase
MOPS	3-[N-morpholino]-propanesulphonic acid
μCi	microCurie
μg	microgram
μL	microlitre
mL	millilitre
mRNA	messenger ribonucleic acid
NAPS	Nucleic acid and protein services
ng	nanogram
NGF	nerve growth factor
NGFR	nerve growth factor receptor
OD	optical density

PAKs	p21-activated kinases
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
pmol	picomolar
PMSF	phenlylmethylsulfonyl fluoride
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
RIP	receptor-interacting protein
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SAPK	stress-activated protein kinase
SB	sample buffer
sCD40L	soluble CD40 ligand
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SH2	Src homology 2
Sos	Son of sevenless
STAT	signal transducer and activator of transcription
Sulfo-NHS-biotin	sulfo-N-hydroxy succinimide biotin
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TBST	TBS with 0.05% Tween 20
TCR	T cell receptor

TLR2	Toll-like receptor 2
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TPA	12-*O*-tetradecaonylphorbol-13-acetate
TRAF	TNFR-associated factor
Tris	Tris (hydroxymethyl) amino methane
Txn	transcription
U	unit
V	volt
VCAM-1	vascular cell adhesion molecule-1

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I dedicate this thesis to my late grandmother,

Marie-Claire Robichaud Ramsay

CHAPTER 1

INTRODUCTION

1.1 Overview of T-dependent immune responses

The main function of B lymphocytes in the immune system is to produce antibodies (Abs) against invading microorganisms such as bacteria and viruses. Abs can prevent diseases caused by pathogens in several ways. First, Abs bind to pathogens and thereby block their access to cells. Abs also help to destroy pathogenic organisms by activating the complement cascade. Finally, Abs facilitate the removal of foreign antigens (Ags) by the process of opsonization (reviewed in (1)). Given the importance of Abs in fighting infection, it is not surprising that the immune system has developed a sophisticated method to regulate Ag-dependent B cell activation.

In the resting state, mature B lymphocytes circulate between the blood and secondary lymphoid organs such as the spleen and lymph nodes. In the secondary lymphoid organs, B cells check for the presence of trapped antigens that their antigen receptors recognize. The binding of antigen to the B cell antigen receptor initiates B cell activation, a process that culminates in clonal expansion and antibody secretion. However, most antigens are monovalent and do not cause the extensive BCR aggregation that is required to initiate BCR signaling. These antigens are termed T-dependent, since in addition to a signal through the B cell antigen receptor (BCR), the B cell must receive costimulatory signals from $CD4^+$ T cells in order to become activated by these antigens. However, before a T cell can provide costimulatory signals to B cells, the T cell must first be activated by processed antigen bound to MHC on the surface of specialized antigen presenting cells (APCs) termed dendritic cells

(DCs). Thus, for the generation of primary Ab responses to T-dependent Ags three types of cells must interact, the dendritic cell, the $CD4^+$ T cell and the B cell (reviewed in (1,2)).

Dendritic cells are antigen presenting cells that act as the initiators of primary, T-dependent Ab responses. When a foreign antigen enters the body, dendritic cells such as Langerhans cells in the skin capture and process the antigen. DCs then migrate into the T-cell rich areas of the secondary lymphoid organs where they are termed interdigitating DCs (IDCs). IDCs present processed antigen in the context of MHC to naive T cells and induce an Ag-specific primary T cell response (reviewed in (3,4)). Once primed by DCs, T cells promote B cell activation both by secreting T-cell derived cytokines such as IL-2, IL-4 and IL-5, and by direct cell to cell contact (reviewed in (2)).

Of the signals involved in T-dependent B cell activation, the interaction between CD40 on B lymphocytes and its ligand (CD40L) expressed on activated $CD4^+$ T cells delivers the most important activating signal to B cells. In mice, blocking this interaction with either a soluble CD40L fusion protein (5) or anti-CD40L mAbs (6), or by targeted disruption of the CD40 (7) or CD40L genes (8), leads to severe deficiencies in the generation of Ab responses to T-dependent Ags. Such studies have demonstrated that the CD40-CD40L interaction is essential for germinal center formation, for the induction of immunoglobulin (Ig) class switching and for the generation of B cell memory in response to T-dependent Ags. The finding that loss-of-function mutations in CD40L are responsible for X-linked hyper-IgM syndrome, a severe immunodeficiency disease in humans, further underscores the importance of CD40-CD40L interactions in T-dependent immune responses (9-11). Given the key role of CD40-CD40L interactions for the development of humoral immunity, the main goal of this thesis was to study CD40 signaling in B lymphocytes.

1.2 CD40L and CD40

1.2.1 CD40L

a). CD40L expression pattern, induction

CD40 signaling is initiated by the binding of CD40 to its ligand. The CD40 ligand (CD40L, gp39, CD154) is a type II transmembrane glycoprotein of approximately 39 kDa that is expressed by activated $CD4^+$ T cells, basophils, mast cells and eosinophils (reviewed in (12,13)). *In vitro*, activation of T cells through CD3 only weakly upregulates CD40L expression. However, high expression of CD40L is induced within hours of T cell activation by phorbol esters plus calcium ionophore (14). The natural activation signal that induces CD40L expression on T cells *in vivo* is not known, but is likely to be through the T cell Ag receptor plus a second signal such as CD28 interacting with B7 on the APC (15). Thus, a pre-requisite for B cell activation by T-dependent Ags is that an Ag-specific T cell be activated first.

Several members of the tumor necrosis factor (TNF) family including CD40L, TNFα and Fas ligand occur in both soluble and transmembrane forms (16-18). In response to T cell activation, CD40L is cleaved inside microsomes to create the soluble, secreted form of CD40 (sCD40L) (19). Both the soluble and membrane-bound forms of CD40L are biologically active (19). Thus, cleavage of CD40L to create sCD40L might not simply represent a mechanism to down-regulate CD40L expression.

b). CD40L structure

Structurally, CD40L belongs to the TNF superfamily of ligands. Members of this family are characterized by sequence homology in their C-terminal, extracellular receptor binding domains (reviewed in (20)). This sequence conservation appears to be responsible for the tendency of members of the TNF superfamily to trimerize.

CD40L, TNFα and lymphotoxin-α each exist as trimers (19,21). Signaling by CD40 appears to require aggregation of CD40 monomers which is presumably induced upon binding of CD40 to its trimeric ligand (reviewed in (20)). In support of this idea, anti-CD40 Abs can serve as a surrogate CD40L *in vitro* by clustering CD40. The aggregation of CD40 cytoplasmic domains may create composite sites that initiate CD40 signaling either by attracting signal transducing proteins or by activating constitutively-associated signal transducing proteins (161,165,166).

1.2.2 CD40

a). CD40 discovery

The CD40 Ag was independently identified in 1985 and 1986 as the target of mAbs that react with B lymphocytes (24) and which have costimulatory effects on B cells (25). At the 1989 International Workshop on leukocyte Ags, this Ag was designated as CD40. Prior to 1989, CD40 was denoted as p50, Bp50 or CDw40. Since its discovery, it has become clear that CD40 and its ligand play a central role in the regulation of immune responses (reviewed in (12,26,27)). The function of CD40 has been most extensively studied on mature B lymphocytes where it regulates B cell activation, proliferation, Ig class switching, survival, and memory formation (reviewed in (12,26,27)). However, more recent studies have examined the function of CD40 on other cell types including endothelial cells, fibroblasts, monocytes/macrophages and dendritic cells. Such studies have revealed that in addition to its well recognized role in regulating B cell functions, CD40-CD40L interactions influence many aspects of T-cell mediated inflammatory responses.

b). CD40 structure

CD40 is a 48-kDa type I transmembrane glycoprotein belonging to the TNF receptor (TNFR) superfamily (reviewed in (12,20)). Members of this superfamily are

characterized by the presence of 2 to 6 repeats of a cysteine-rich motif in their extracellular domains. In addition to CD40, the TNFR superfamily currently includes CD30, the nerve growth factor receptor (NGFR), TNFR1, TNFR2, the poxvirus proteins PV-T2 and PV-A53R, 4-1BB, OX-40, Fas, CD27 and a predicted family member, TNFR-RP (Fig. 1.1). Recent additions to the superfamily include death receptor-3 (DR-3), the receptor for lymphotoxin-β and another tumor necrosis factor-associated receptor (ATAR).

Human and murine CD40 share 62% identity in their extracellular domains, including 22 cysteines that help form the ligand binding site. These cysteines are dispersed over four homologous extracellular domains (Fig. 1.2). Human CD40 is 277 amino acids long while murine CD40 is 289 amino acids. The cytoplasmic domains of the two proteins share 68% identity and contain a "homology box" region with 98% amino acid identity (Fig. 1.2) that mediates many CD40 signaling events (see below). The murine CD40 cytoplasmic domain continues an additional 12 amino acids, resulting in a cytoplasmic tail of 74 amino acids instead of 62 amino acids as found in human CD40 (Fig. 1.2). This unique murine cytoplasmic tail region appears to be dispensable for CD40 signaling since human CD40 can substitute for murine CD40 (28).

c). Cells expressing CD40

In murine bone marrow, CD40 is expressed at low levels on about 25% of pre-B cells, at intermediate levels on about 75% of immature B cells and at relatively high levels on essentially all mature B cells (29). CD40 is also expressed on mature B cells in the follicles of peripheral lymphoid organs such as the spleen, tonsils and lymph nodes, (12,24,25). Thus, CD40 is expressed relatively late during B cell differentiation. The level of CD40 expression remains essentially constant on naive mature B cells, activated germinal center B cells and memory B cells (12).

Figure 1.1: The TNF Receptor Superfamily

Homologous extracellular domains are depicted as open ovals and cysteine residues by horizontal lines. Stippled boxes in the cytoplasmic regions represent death domains. Adapted from (20).

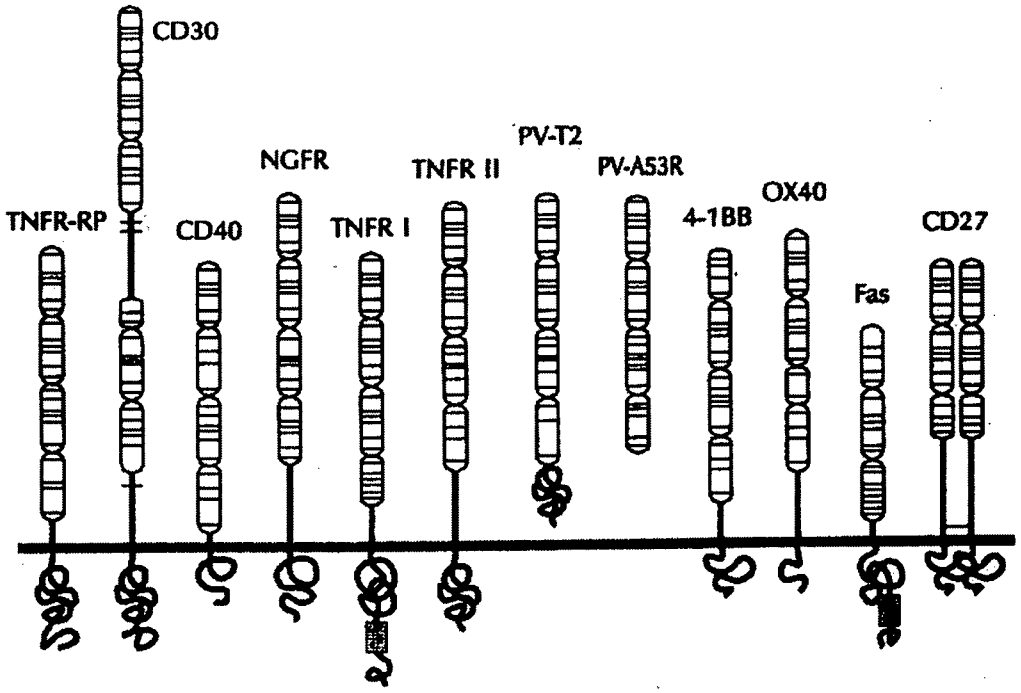
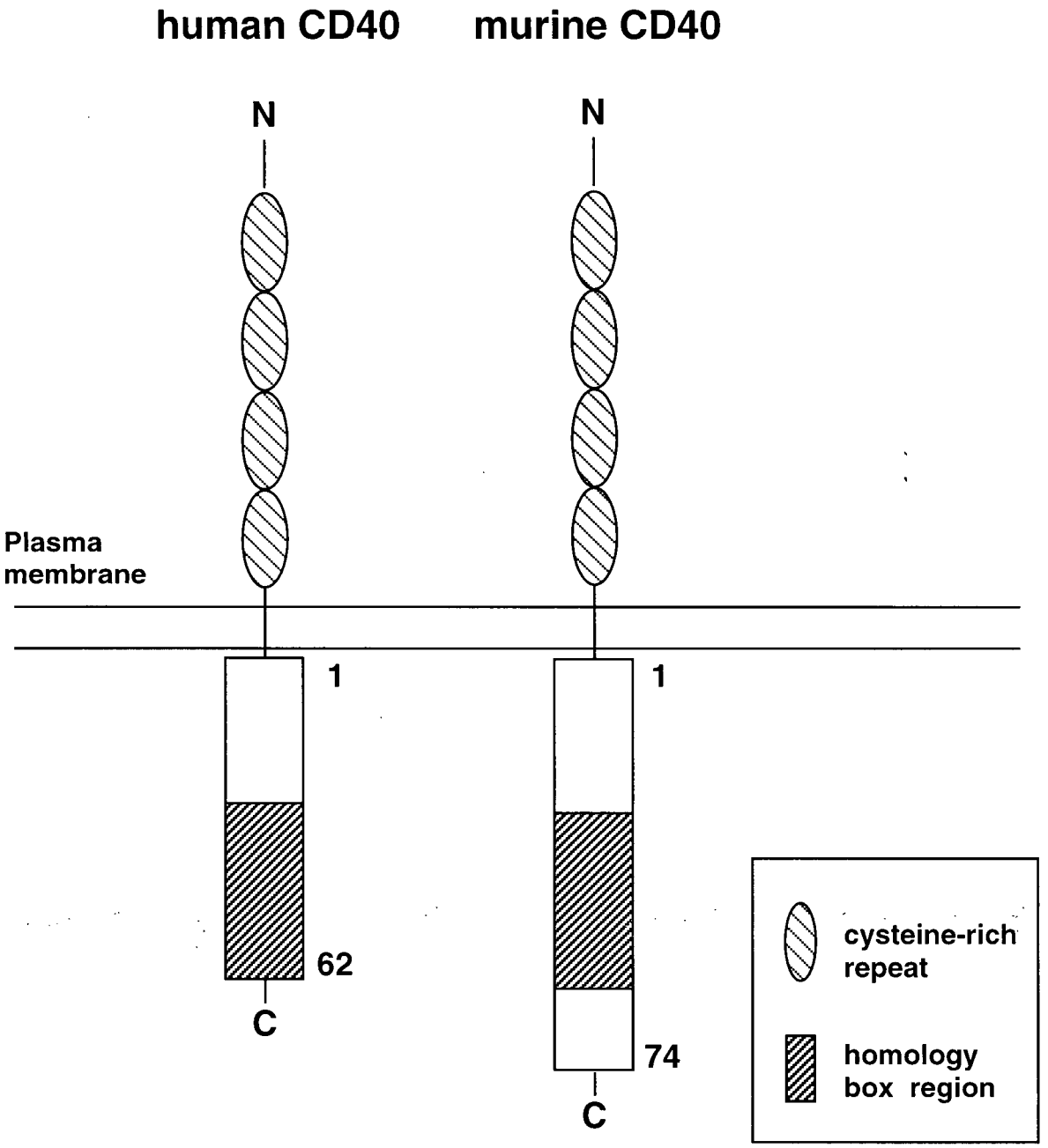

CD30
TNFR-RP
CD40
NGFR
TNFR I
TNFR II
PV-T2
PV-A53R
4-1BB
OX40
Fas
CD27

Figure 1.2: Schematic diagram of the human and murine CD40 proteins

Human CD40 is 277 amino acids long while murine CD40 is 289 amino acids. The cysteine-rich extracellular domains are indicated. The cytoplasmic domain of human CD40 is 62 amino acids long, whereas the cytoplasmic domain of murine CD40 is 74 amino acids. Human and murine CD40 share a homology box region with 98% amino acid identity. This region of CD40 mediates many CD40 signaling events.


human CD40
murine CD40
N
N
Plasma membrane
1
1
62
74
C
C
cysteine-rich repeat
homology box region

Although initially thought of as a B cell-specific receptor, CD40 is now known to be widely expressed. In addition to B cells, CD40 is expressed on epithelial cells (30,31), endothelial cells (27), fibroblasts (27), keratinocytes (27), T lymphocytes (32), monocytes (33), macrophages (34), dendritic cells (35), follicular dendritic cells (27), and some carcinomas (36,37). This expression pattern indicates that in addition to being a key regulator of humoral immunity, CD40 regulates various aspects of cell-mediated immunity.

1.3 Effects of CD40-CD40L interaction on B lymphocytes

1.3.1 Activation and proliferation of B lymphocytes

CD40 regulates multiple steps in B cell differentiation and activation. Anti-CD40 mAbs, acting as surrogate CD40L, induce homotypic aggregation (38,39), and an increase in cell size (39). Anti-CD40 mAbs also induce the expression of B7.1 (CD80) and B7.2 (CD86) on B cells (29). B7.1 and B7.2 bind to CD28 on T cells and deliver the second signal that prevents T cell anergy and promotes T cell proliferation (2). Thus, CD40 enhances the ability of B cells to act as APCs for T cells.

Anti-CD40 mAbs produce a stimulatory signal that synergizes with signals delivered by Abs to the BCR or to CD20 or by exposure of B cells to the cytokine IL-4 (12). These signals synergize to promote B cell activation and proliferation (40-43). Anti-CD40 mAbs induce Ig class switching, leading to the production of various Ig isotypes in the presence of different cytokines (44-46).

CD40L is a 39 kDa TNF-related protein expressed on activated $CD4^+$ T cells which plays an essential role in T cell-dependent B cell activation (47,48). Soluble CD40-Ig fusion proteins block the ability of $CD4^+$ T cells to activate resting B cells (47). Consistent with an essential role for CD40L in B cell activation, soluble recombinant CD40L (sCD40L) synergizes with known B cell stimuli such as anti-CD20 Abs and the

phorbol ester PMA to induce B cell proliferation and Ab production (49,50). By itself, sCD40L stimulates only a modest degree of B cell proliferation (45,50).

1.3.2 CD40 protects B cells from BCR-induced anergy or cell death

In addition to its roles in B cell activation and proliferation, CD40 also generates survival signals for B lymphocytes. In a properly functioning immune system, only B cells specific for foreign Ag are activated while self-reactive B cells are rendered anergic or deleted. For this to occur, B cells are subjected to a series of positive and negative selection events during their development, activation and differentiation into plasma cells (51-54). These selection events are primarily conveyed by signals transduced through the BCR and CD40. For most types of Ags, BCR signaling alone induces anergy or deletion, rather than activation, unless the B cell receives a second signal through CD40 (28,55,56). This two signal requirement may be a mechanism by which self-reactive B cells are deleted due to the absence of Ag-specific activated T cells expressing CD40L. For example, CD40 ligation rescues both immature (WEHI-231) and mature (Ramos) B cell lines from anti-IgM induced apoptosis (28,56). CD40 also prevents germinal center B cells from undergoing spontaneous apoptosis during the process of affinity maturation (57-59). This rescue of germinal center B cells from apoptosis is essential for the generation of memory B cells (7) that are capable of producing high avidity Abs.

Although CD40 is often thought of as a viability factor for B cells, engagement of CD40 on primary B cells induces Fas expression and sensitizes them to Fas-dependent apoptosis (60,61). Fas ligation has been shown to inhibit the later stages of CD40-dependent B cell proliferation and eventually induce apoptosis of CD40-activated B cells (60,61). The delayed response to Fas after CD40 engagement may represent a mechanism to control the expansion of antigen-specific B cell clones (61). Fas-dependent apoptosis is averted if B cells are stimulated by both CD40L and anti-

IgM (60). This two signal requirement for B cell survival may provide a mechanism to eliminate bystander B cells that are activated by CD40L-expressing T cells while ensuring the survival of antigen-selected B cells that have bound antigen (60).

1.3.3 The WEHI-231 B cell lymphoma as a model system for studying CD40

The WEHI-231 immature B cell line (62) provides a model system for studying CD40 signaling in B cells. Engagement of the BCR on these cells with anti-IgM Abs results in growth arrest followed by apoptosis (63-65). The BCR-induced growth arrest and apoptosis can be abrogated by anti-CD40 Abs, by transfected fibroblasts expressing the CD40L, or by a soluble form of CD40L (28,66,67). BCR-induced apoptosis of WEHI-231 cells is often thought of as a model for central tolerance that would occur in the bone marrow. Similar to WEHI-231 cells, the immature B lymphoma CH31 and CH33 cell lines are also susceptible to BCR-induced growth arrest (68,69). In this thesis I have used WEHI-231 cells to identify signaling pathways activated by CD40 and to identify signaling motifs in the CD40 cytoplasmic tail.

1.4 Effects of CD40-CD40L interactions on other cell types

The importance of CD40-CD40L interactions for non B cells was first indicated by the finding that, in addition to defective humoral immunity, X-linked hyper IgM syndrome patients display an increased susceptibility to infections that would normally be controlled by the cell-mediated branch of the immune system. Consistent with this finding, recent studies have demonstrated that CD40 is expressed on a variety of cells where it regulates various aspects of cell-mediated immunity. I will summarize these briefly in the following sections.

1.4.1 Endothelial cells, epithelial cells and fibroblasts

CD40 is expressed on several types of nonhematopoietic cells including endothelial cells, basal and thymic epithelial cells as well as fibroblasts (12,27). Engagement of CD40 on these cells induces a variety of responses, most of which contribute to inflammation. For example, engagement of CD40 on vascular endothelial cells induces expression of selectins and adhesion molecules such as CD54 (intercellular adhesion molecule [ICAM]-1), CD62E (E-selectin), and CD106 (vascular cell adhesion molecule [VCAM]-1) (34). These molecules promote leukocyte extravasation at sites of inflamed tissue (2). CD40 triggering on kidney epithelial cells induces secretion of IL-8, macrophage chemoattractant protein-1 (MCP-1) and RANTES (27). These chemotaxins work to attract extravasated granulocytes, monocytes and lymphocytes to sites of tissue injury (2). Finally ligation of CD40 on fibroblasts induces proliferation (27) and IL-6 production (70). Once produced, IL-6 acts together with TNFα to perpetuate an inflammatory response (71).

1.4.2 Monocytes/macrophages

Peripheral blood monocytes express high levels of CD40 following exposure to cytokines such as IFNγ, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (33,72). CD40 ligation induces monocytes to secrete the cytokines TNFα, IL-1α, IL-1β, IL-8, and MIP-1α (72,73). TNFα and IL-1 contribute to vasodilation, a key feature of inflammation, whereas IL-8 and MIP-1α attract leukocytes to sites of tissue injury (2,34). CD40 cross-linking also prevents the apoptosis of circulating monocytes (74). Thus, in addition to augmenting inflammatory responses by inducing monocytes to secrete cytokines, CD40 could prolong inflammation by contributing to monocyte survival at sites of inflammation.

Following recruitment to a site of inflammation, monocytes can become tissue macrophages. Interactions between CD40L on activated T cells and CD40 on

macrophages are important for the induction of macrophage effector functions. For example, CD40 induces macrophages to secrete metalloproteinases (75). These collagenases are thought to be important for the restructuring of damaged tissue and may also cause joint degradation in rheumatoid arthritis. CD40 ligation also induces antigen-presenting macrophages to secrete IL-12 (76), a cytokine that promotes maturation of Th1 cells and the development of cell-mediated immunity rather than Th2-mediated humoral immunity (77). Finally, studies with CD40L-deficient mice have shown that T cells require CD40L in order to induce macrophages to generate the antimicrobial agent nitric oxide (78,79).

1.4.3 Dendritic cells

Dendritic cells (DCs) play a central role in the initiation of primary immune responses as they are the major APCs that activate naive T cells (80). CD40 is expressed at high levels on peripheral blood DCs, on follicular DCs and on interdigitating DCs in the T cell rich areas of secondary lymphoid organs (12). Interaction between CD40 on the DC and CD40L on the T cell induces the expression of T cell costimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) on the DC (73,80). CD40 also upregulates the expression of the adhesion molecules CD54 (ICAM-1) and CD58 (lymphocyte function-associated antigen [LFA]-3) on the DC (27,74). These costimulatory and adhesion molecules make DCs better antigen presenting cells for T cells.

Stimulation via CD40 has a number of other important effects on DCs. CD40 prevents DCs from undergoing apoptosis in response to growth factor withdrawal (18). CD40 engagement also induces DCs to secrete cytokines such as IL-12 and TNFα as well as chemokines such as IL-8, MIP-1α and MIP-1β (reviewed in (73)).

1.5 CD40-CD40L interactions: role in infection and immunity

Numerous recent studies indicate that blocking CD40-CD40L interactions would have serious consequences on human health. These findings are briefly summarized below.

1.5.1 Immunodeficiency disease

The first evidence that CD40-CD40L interactions are critical for B function *in vivo* came from the discovery that hyper-IgM syndrome, an X-linked immunodeficiency disease, is due to genetic defects in CD40L (15,81). These mutations either prevent CD40L expression or prevent its binding to CD40 (10,11,81). Hyper-IgM syndrome is characterized by defective B cell responses including an inability of B cells to undergo Ig class switching and to generate memory cells in response to T-dependent Ags. B cells from these patients are capable of producing normal antibody responses *in vitro* when cultured with $CD4^+$ T cells from normal individuals, indicating that the hyper-IgM syndrome defect is due to the inability of the patient's T cells to activate their B cells (13). Presumably, mutations in the CD40 gene that affect its ability to bind CD40L or its ability to signal would cause similar immunodeficiencies as seen in patients with hyper-IgM syndrome.

1.5.2 Autoimmunity

Anti-CD40L Abs block the development of autoimmune symptoms in several murine models including those for human rheumatoid arthritis (82), systemic lupus erythematosus (83) and multiple sclerosis (84). In addition, CD40L-deficient mice carrying a transgene for a myelin basic protein-specific T cell receptor fail to develop experimental allergic encephalomyelitis (34). It remains to be determined whether these reduced T cell-mediated inflammatory responses are due to decreased induction of inflammatory cytokines, decreased leukocyte extravasation and/or

decreased T cell activation. However, these findings demonstrate that CD40-CD40L interactions are important for the establishment of several T cell-mediated autoimmune diseases.

1.5.3 Transplantation tolerance

Several studies indicate that CD40-CD40L interactions are important for the rejection of transplanted cells and organs. For example, anti-CD40L Abs block the onset of graft-versus-host disease during allogeneic bone marrow transplants (85,86). Anti-CD40L Abs also promote long-term survival of skin and cardiac allografts (87,88).

1.5.4 Infection

Given that CD40 is involved in regulating many types of cells in the immune system, it is not surprising that blocking CD40 signaling decreases the ability of the immune system to fight off pathogens. Patients with X-linked hyper-IgM syndrome are prone to *Histoplasma*, *Cryptococcus* and *Pneumocystis* infections (34). Similarly, CD40-deficient mice display an increased susceptibility to *Leishmania* infections (89) as well as impaired antiviral Ab responses (34).

1.6 CD40 signaling

Understanding how CD40 signals is important because mutations that block CD40 signaling could cause immunodeficiency diseases. Moreover, mutations that mimic continual CD40 signaling could cause excessive B cell activation and lead to leukemia or autoimmune diseases such as rheumatoid arthritis, as discussed above. By elucidating the signal transduction pathways used by CD40 in various cell types, pharmacological agents could be developed that block or augment intracellular signaling pathways activated by CD40. These agents might be useful in treating diseases that are due to deregulated CD40 signaling.

At the start of this thesis, almost nothing was known about CD40 signaling. However, over the last five years several CD40 signaling pathways have been identified. Most of these studies were performed with B lymphocytes and it is possible that CD40 may activate different signaling pathways in other cell types. Presented below is a brief summary on CD40 signaling in B cells.

1.6.1 Tyrosine kinase-based signaling by CD40

a). Tyrosine phosphorylation and protein tyrosine kinase (PTK) activation

Phosphorylation of proteins on tyrosine residues is an important mechanism used by many growth factor receptors to transmit signals into cells (reviewed in (1)). The tyrosine phosphorylation state of proteins in the cell is controlled by the action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Tyrosine phosphorylation can regulate the function of proteins by initiating protein-protein interactions that are important for signal transduction. For example, many signaling proteins contain structural domains known as Src homology 2 (SH2) domains that bind phosphotyrosine-containing regions of proteins with high affinity. In addition, several key signaling enzymes such as the Src family kinases, are regulated by phosphorylation of critical tyrosine residues (1).

At the start of this thesis, several observations suggested that PTK activation may play an important role in mediating the biological effects of CD40. For example, tyrosine phosphorylation of various signaling proteins, including phospholipase C-γ2 (PLC-γ2) and the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (PI3K), was shown by one group to be induced within minutes of CD40 engagement on Daudi B cells (90). However, this data has not been reproduced by other groups. Other studies showed that PTK inhibitors blocked CD40-mediated B cell aggregation (91), Ig class switching (92) and rescue of germinal center B cells from spontaneous apoptosis (59). However, they did not demonstrate that these inhibitors blocked CD40-induced

protein tyrosine phosphorylation. Finally, engagement of CD45, a transmembrane PTP, appears to inhibit CD40-mediated B cell proliferation (93). Thus, there is circumstantial evidence that CD40 may signal, at least in part, through PTKs.

If PTK activation plays a role in CD40 signaling, it remains to be determined how CD40 might activate PTKs. The CD40 cytoplasmic region does not contain a kinase domain (36), indicating that CD40-mediated tyrosine phosphorylation is mediated by a separate tyrosine kinase. During the course of this study, there have been several reports that members of several different PTK families are activated in response to CD40 engagement. For example, Ren et al. (90) and Faris et al. (94) showed that CD40 induces tyrosine phosphorylation and activation of the Src family PTK, Lyn in the Daudi and Raji B cell lines. In addition, one group has found that engagement of CD40 on the BJAB human B cell line and on tonsillar B cells induces tyrosine phosphorylation and activation of the Janus kinase 3 (Jak3) tyrosine kinase (95). Once activated by CD40, JAK3 was found to phosphorylate and activate the signal transducer and activator of transcription factor-3 (STAT3). Jak3 binding to CD40 was found to require a proline-rich sequence in the membrane-proximal region of human CD40 (95). These results suggest that JAK3 activation may play an important role in mediating CD40 functions. However, CD40 activation of B cells was recently found to be normal in JAK3-deficient mice (unpublished data, 1998 MidWinter Conference of Immunologists).

It remains to be determined whether CD40 activates members of other PTK families such as Zap70 or Btk. In preliminary studies, we did not detect significant activation of Btk by CD40 in the WEHI-231 B cell line (C. Sutherland and T. Roth, unpublished observations). In summary, more work needs to be done to evaluate the importance of PTKs in CD40 signaling. The observation that CD40 engagement fails to induce tyrosine phosphorylation in B cell lines such as WEHI-231 which respond

vigorously to CD40 engagement, indicates that PTK activation may not be a major mode of CD40 signaling.

b). Phospholipase C (PLC)

There has been some suggestion that CD40 activates PLC-γ2 (90), an enzyme that breaks down the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate to produce the second messengers diacylglycerol and inositol 1,4,5-trisphosphate. These second messengers activate members of the protein kinase C (PKC) family of serine/threonine kinases and elevate intracellular Ca^{2+} concentration, respectively (1). Activation of PLC-γ2 and the associated increase in intracellular Ca^{2+} by CD40 would be consistent with reports that CD40 activates NF-AT (96), a transcription factor that is induced to translocate from the cytosol to the nucleus in response to increased intracellular Ca^{2+} (97).

c). The phosphatidylinositol 3-kinase (PI3K) pathway

CD40 engagement on the Daudi human B cell line has been shown to activate phosphatidylinositol 3-kinase (PI3K) (90). PI3K catalyzes the phosphorylation of inositol phospholipids on the 3' position of the inositol ring, generating lipid second messengers that activate several serine/threonine protein kinases (1). Among the targets of these second messengers is PKB/Akt, a serine/threonine kinase that mediates a survival signal from various cell surface receptors (98). PKB phosphorylates the pro-apoptotic factor Bad, resulting in the dissociation of Bad from Bcl-x_L. Once released, Bcl-x_L suppresses apoptotic cell death pathways involving cytochrome c and the caspase protease cascade. It remains to be determined whether CD40 activates PKB and whether the PI3K/PKB pathway plays a role in CD40-mediated protection of WEHI-231 cells from anti-IgM-induced apoptosis.

1.6.2 CD40 signaling via cAMP

Many receptors, primarily seven transmembrane G protein-coupled receptors, use the cAMP pathway as a mechanism of signal transduction. These receptors activate the enzyme adenylate cyclase which produces the second messenger cAMP. cAMP, in turn activates the cAMP-dependent protein kinase (PKA). PKA phosphorylates and activates the CREB transcription factor (99,100). There is some suggestion that the cAMP pathway may be involved in CD40-induced B cell proliferation. Treatment of splenic B cells with CD40L expressed on activated T cell membranes induces increases in intracellular cAMP levels (101). This increase in cAMP levels is blocked by soluble CD40-Fc fusion protein. Furthermore, a PKA inhibitor, H-89, suppressed the B cell proliferation induced by these membranes (101). However, it has not been directly demonstrated whether CD40 engagement activates adenylate cyclase in B cells.

1.6.3 Mitogen-activated protein (MAP) kinase activation

In addition to tyrosine phosphorylation, there was some suggestion at the start of this study that CD40 uses serine/threonine phosphorylation as a signal transduction mechanism. In particular, engagement of CD40 on various human B cell lines and on tonsillar B cells was shown by in-gel kinase assays to activate several serine/threonine protein kinases of 120 kDa, 93 kDa, 76 kDa, 55 kDa and 48 kDa (102). However, the identity of these kinases was not known.

MAP kinases are serine/threonine kinases that are activated by many receptors and have been implicated in both mitogenic and apoptotic responses to receptor signaling (103-106). For example, multiple hematopoietic growth factors had been shown to stimulate activation of ERK MAP kinase family members (104). Our lab had previously shown that BCR-induced apoptosis in murine B lymphoma lines correlates with activation of ERK2 (107). In addition, genetic and biochemical studies have

demonstrated that the JNK signaling pathway regulates cellular proliferation and apoptosis (reviewed in (108)). Moreover, as described below, activated MAP kinases can migrate to the nucleus where they phosphorylate and activate transcription factors. Given the key role of MAP kinases in signaling by many receptors, I tested the hypothesis that CD40 activates MAP kinases.

At the onset of this work, three MAP kinase families had been identified, the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 kinases (Fig. 1.3). The MAP kinases are activated by dual-specificity kinases called MAP kinase kinases (MKKs) which phosphorylate both the threonine residue and the tyrosine residue in a threonine-X-tyrosine activation motif (109,110). This phosphorylation has recently been shown to promote MAP kinase homodimerization and subsequent nuclear translocation (111). As discussed below, the ERK, JNK, and p38 kinases have different threonine-X-tyrosine motifs and are regulated by different MKKs (109,110), allowing for their independent regulation (Fig. 1.3). Although MAP kinases have numerous substrates, many of their effects on growth regulation are likely due to their ability to phosphorylate and activate nuclear transcription factors (110,112).

a). The ERK MAP kinase cascade

The best characterized MAP kinase family consists of the ERK1 and ERK2 kinases (reviewed in (113,114)). These kinases are most strongly activated by tyrosine kinase-linked growth factor receptors. ERK1 and ERK2 are activated by the MKKs, MEK1 and MEK2. MEK1 and MEK2 are, in turn, activated through phosphorylation by one of three different MKK kinases (MKKKs); Raf, MEKK1 or c-Mos (Fig. 1.3). The small G protein, Ras has been shown to link growth factor receptors to Raf by recruiting Raf to the plasma membrane (115). Transcription factors targeted by

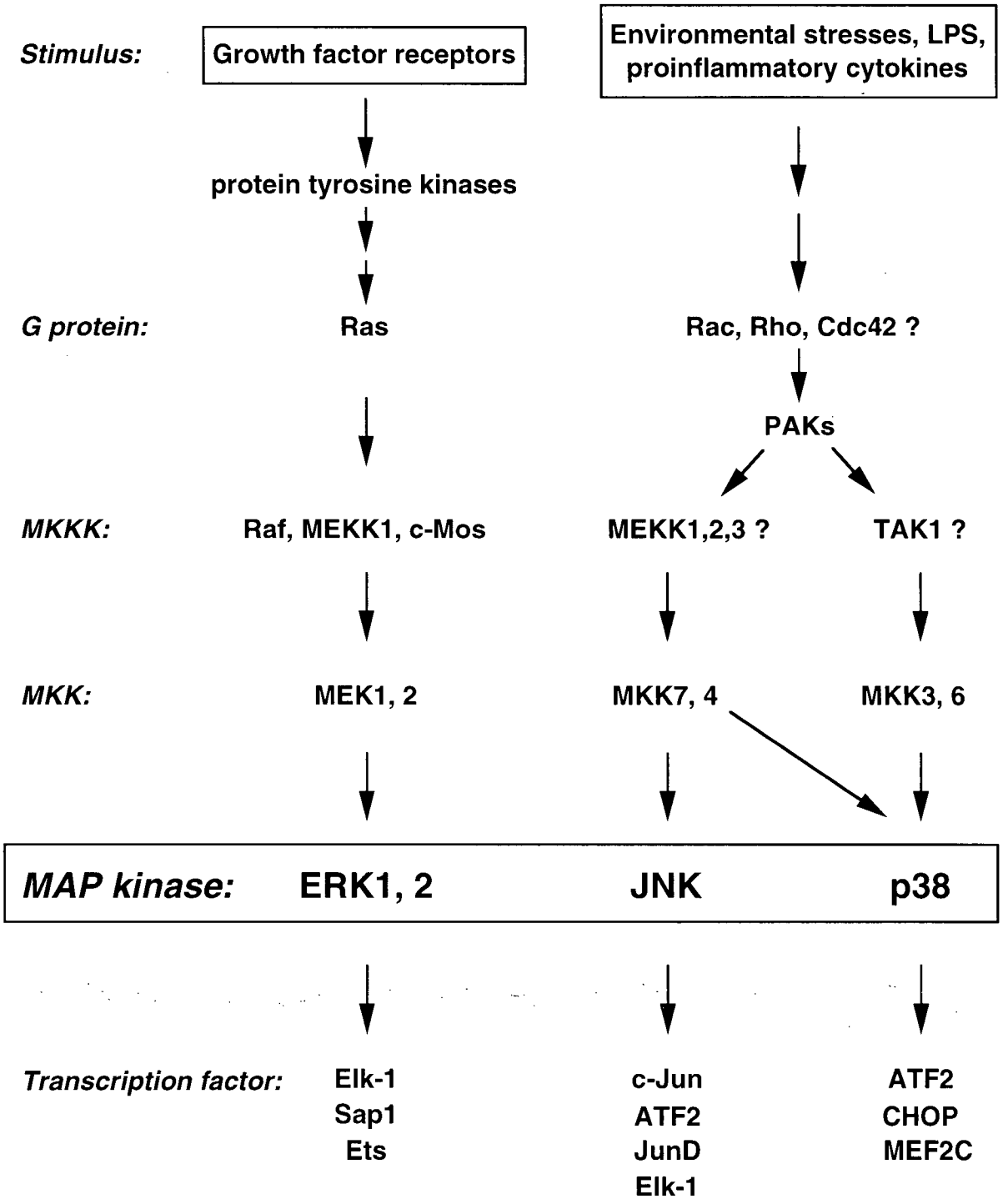


Figure 1.3: The mitogen activated protein kinase pathways in mammalian cells (see text for details).

the ERKs include Ets domain-containing transcription factors such as Elk-1 (116), Sap1 (117) and Ets-1 (114) (Fig. 1.3).

b). The JNK MAP kinase cascade

The JNKs are activated by environmental stresses such as ultraviolet light, as well as by inflammatory cytokines and LPS (reviewed in (108)). Ten members of this family have been identified in humans by molecular cloning. These kinases correspond to alternatively spliced isoforms that are derived from three different genes: *jnk1*, *jnk2* and *jnk3*. All three of these genes are expressed as 46 kDa and 54 kDa forms due to alternative splicing of the 3' coding region of the gene transcripts. Functional differences caused by this form of alternative splicing of the mRNA transcripts have not been reported. However, a second form of alternative splicing that involves the mutually exclusive utilization of two exons within the kinase domain of transcripts of the *jnk1* and *jnk2* genes does have functional consequences. The resulting forms of JNK1 and JNK2, termed JNK1α1 (p46 JNK1α), JNK1β1 (p46 JNK1β), JNK1α2 (p54 JNK1α), JNK1β2 (p54 JNK1β), JNK2α1, JNK2β1, JNK2α2, JNK2β2, differ in their substrate binding abilities *in vitro* (118). Thus, individual members of the JNK family may selectively activate specific transcription factors *in vivo*. JNK1, JNK2 and JNK3 are homologous to three protein kinases that were independently identified in rat cells and were called the stress-activated protein kinases (SAPKs) (119). Thus, JNK1 is the human homologue of rat SAPKγ, JNK2 is the homologue of SAPKα and JNK3 is the homologue of SAPKβ.

The JNKs are phosphorylated and activated by several MKKs including MKK4 and MKK7 (Fig. 1.3). MKK4, in turn, is phosphorylated and activated by the MKKK, MEKK1. Although there is currently much confusion concerning the upstream elements of the JNK signaling pathway that lead from receptors to the activation of MKKKs, small G proteins seem to be involved. Unlike the ERKs, however, activated

Ras only weakly activates JNK (120). Rather, the small GTP-binding proteins, Rac, Rho and Cdc42 appear to be more effective activators of JNK (Fig. 1.3) (121-123).

The JNKs were initially characterized by their ability to phosphorylate and activate the transcription factor c-Jun (124), a component of the AP-1 transcription factor. However, several other transcription factors including ATF2, JunD and Elk-1 are now known to be phosphorylated by the JNKs (Fig. 1.3) (reviewed in (113)).

c). The p38 MAP kinase cascade

p38 MAP kinase was identified as a protein kinase that regulates the production of inflammatory cytokines such as IL-1 and TNFα in monocytes stimulated with LPS (125). In addition to the original isoform of p38, now referred to as p38α, three additional members of the mammalian p38 family (p38β (126), p38δ (127), and p38γ) (128) have been cloned. These four p38 MAP kinases are encoded by separate genes but display significant sequence homology to each other.

Many stimuli including environmental stresses, inflammatory cytokines and LPS activate both JNK and p38. This similarity in the regulation of JNK and p38 activities is not surprising given that MKK4 is a direct activator of both JNK and p38 (Fig. 1.3) (113). However, a few stimuli that activate p38 but not JNK have been reported (129,130). Consistent with these findings, MKK3 and MKK6 activate p38 but not JNK (131). A candidate MKKK for p38 is TAK1 (132), which activates both MKK3 and MKK6 (133). By analogy to the JNK cascade, MKKKs that activate p38 are likely to be regulated by a GTPase such as Rac1 (134) or Cdc42 (122). A group of serine/threonine kinases called PAKs (p21-activated kinases) are direct targets of Rac1 and Cdc42 (135,136) and appear to link the small G proteins to the p38 and JNK MAP kinase cascades (134,135) (Fig. 1.3).

p38 phosphorylates and activates the transcription factors ATF2 (105,137) CHOP (138) and Sap1 (139). p38 also activates MAPKAP kinase-2 (140), a

serine/threonine kinase that phosphorylates the small heat shock protein Hsp25 and the transcription factor CREB (141).

d). MAP kinase activation by CD40

As mentioned previously, it was known at the start of this thesis that signals transmitted through CD40 and the BCR are involved in regulating B cell survival, proliferation, Ab production and apoptosis. Given the implicated roles of MAP kinases in cell survival, proliferation and apoptosis, in this thesis I examined whether the ERK, JNK and p38 MAP kinases are activated in response to CD40 or BCR engagement on B cells. Since integration of CD40 and BCR signals has unique effects on B cells, I also investigated whether there was any dual regulation of these kinases by CD40 and the BCR.

I found that BCR engagement in the WEHI-231 cells strongly activated ERK2 and weakly activated ERK1. CD40 did not activate either of these kinases, nor did it affect BCR-induced ERK activation. In contrast, CD40 engagement strongly activated JNK. BCR ligation cause a small increase in JNK activity by itself and also potentiated CD40-induced JNK activation. Finally, CD40 caused strong activation of p38, as well MAPKAP kinase-2, a downstream target of p38. BCR ligation induced only a modest activation of the p38 pathway. The significance of these findings are discussed in Chapter 3.

1.7 Activation of transcription factors by CD40

Several transcription factors are activated in response to CD40 engagement on B cells. Treatment of splenic B cells with sCD40L induces activation of the NF-AT, AP-1, NF-κB and STAT6 transcription factors (96,142). In addition, treatment of the BJAB human B cell line with anti-CD40 mAb activates the STAT3 transcription factor (95). Each of these transcription factors has been implicated in the expression of several

genes, some of which presumably are important for CD40 effects on B cells. Although the importance of NF-AT in CD40 signaling has yet to be determined, recent work has shown that NF-AT mediates anti-IgM induction of CD5 expression in splenic B cells (143). AP-1, a family of dimeric transcription factors composed of Jun, Fos or ATF subunits, regulates the expression of genes containing an AP-1 binding site in their 5' regulatory regions (144). Although the target genes for different AP-1 complexes have yet to be identified, AP-1 factors appear to be involved in regulating cell proliferation and survival (144). Finally, STAT6 and STAT3 are thought to mediate CD40-induced expression of interferon regulatory factor-1 (*IRF-1*) (95), a gene whose product has been implicated in the regulation of cellular proliferation and differentiation (145).

1.7.1 NF-κB

By far the transcription factor that has received the most attention lately with regard to its role in CD40 signaling is NF-κB. NF-κB has been shown to mediate CD40 protection from BCR-induced apoptosis in the WEHI-231 B cell line (146). CD40 engagement on these cells was found to prevent the decrease in the level of nuclear NF-κB/Rel complexes that occurs following BCR engagement. CD40-induced maintenance of NF-κB activity was, in turn, shown to prevent the catastrophic drop in c-Myc levels that follows BCR engagement and which is responsible for these cells undergoing growth arrest and apoptosis (146-149).

In most cell types, NF-κB is present as a heterodimer of p50 and p65 subunits that is sequestered in the cytoplasm by the IκB inhibitor proteins, IκBα and IκBβ (150,151). IκB proteins mask the nuclear localization signal of NF-κB, thereby preventing its nuclear translocation. A wide variety of stimuli including viruses, LPS and UV light activate NF-κB by stimulating phosphorylation of IκB proteins on specific serine residues. The kinases that phosphorylate IκB have recently been identified (152) and are discussed further in Section 4.8.4. This phosphorylation targets IκB

proteins for ubiquitination and subsequent proteasome-mediated degradation, thereby freeing NF-κB to translocate to the nucleus (reviewed in (153)). In this thesis I have shown that CD40 also induces phosphorylation and degradation of IκB proteins.

NF-κB regulates the expression of a large number of genes including those that encode cytokines, cytokine receptors, leukocyte adhesion molecules and other regulatory molecules that are involved in immune responses (154). In addition to *c-myc*, several other genes that are activated by CD40 such as *A20*, *IL-6* and *ICAM-1* (155) have NF-κB consensus sites in their 5' regulatory regions. Thus, it is likely that NF-κB also mediates CD40 induction of these genes.

At the onset of this study, it was not known how CD40 activated NF-κB. To gain insights into this process, in this thesis I sought to identify signaling motif(s) in the CD40 cytoplasmic region that activated NF-κB. Once this region(s) was identified, I determined whether it was the same region that mediated the biological effects of CD40 on B cells.

1.8 Activation of signaling pathways by CD40: The role of TRAF proteins

Like other members of the TNFR superfamily, the cytoplasmic domain of CD40 has no kinase domain and no tyrosine residues with which to recruit SH2-domain containing signaling proteins. Furthermore, CD40 has no known consensus sequence for binding kinases. Rather, during the course of this study, it was discovered that members of the TRAF (TNFR-associated factors) family of adapter proteins bind to the cytoplasmic domains of CD40 and other TNFR superfamily members and link these receptors to signaling pathways.

To date, the TRAF family includes TRAF1, TRAF2, TRAF3 (also known as CRAF1, CD40bp or LAP-1), TRAF4, TRAF5 and TRAF6. The TRAF molecules share homology in their C-terminal TRAF domains, which are responsible for receptor

binding (156) and for homo- or heterodimerization (156). Using the yeast two-hybrid system, TRAF2 (157), TRAF3 (158), TRAF5 (159) and TRAF6 (160) have been shown to directly associate with the cytoplasmic domain of CD40. Aggregation of CD40 by the trimeric CD40L initiates CD40 signaling, presumably by recruitment of these TRAF proteins to the CD40 cytoplasmic domain (161).

Several recent studies indicate that the TRAF proteins may link CD40 to the MAP kinase pathways as well as to the transcription factor NF-κB. For example, TRAF2, TRAF5 and TRAF6 can activate JNK and NF-κB, when overexpressed in the 293 cell line (157,159,160,162). Furthermore, amino-terminally truncated forms of these TRAFs act as dominant negatives by blocking CD40-mediated activation of NF-κB (157,159,160). TRAF6 also mediates activation of ERK when overexpressed in 293 cells, and amino-terminally truncated TRAF6 suppresses ERK activation by CD40 (163). Finally, TRAF3 may mediate CD40-induced activation of p38 since dominant negative TRAF3 suppresses p38 activation by CD40 (164). Thus, TRAF2, TRAF3, TRAF5 and TRAF6 are likely to be key adapter molecules that couple CD40 to important signaling pathways.

1.9 Signaling motifs in the cytoplasmic domain of CD40

Although it was known at the start of this project that CD40 plays a critical role in regulating B cell function, the mechanism by which CD40 activates signaling pathways was completely unknown. The TRAF adapter proteins had not been identified and JAK3 had not been shown to bind to human CD40. Although the sequence of the extracellular domain of CD40 had led to its classification as a member of the TNFR superfamily, the cytoplasmic domain of CD40 bears little homology to other known proteins. Thus, few clues were available at the start of this project as to how CD40 is linked to its signaling pathway(s) and whether these pathways were independent of each other. To better understand CD40 proximal signaling events, one goal of this

thesis was to perform a structure/function analysis on the cytoplasmic domain of CD40. My hypothesis was that there would be short linear amino acid sequence(s) or "signaling motif(s)" that mediate CD40 signaling.

The cytoplasmic domain of human CD40 is 62 amino acids long while that of murine CD40 is 74 amino acids. During the course of this thesis investigation, data from other groups suggested that the cytoplasmic domain of CD40 contains at least two potential signaling regions. Two CD40 cytoplasmic regions that are important for binding the TRAF proteins have been identified by coprecipitation experiments in which glutathione *S*-transferase (GST)-CD40 fusion proteins containing the entire cytoplasmic tail of CD40 or various deletion mutants were incubated with cell extracts from fibroblast cell lines overexpressing TRAF2, TRAF3, TRAF5 or TRAF6. These studies have showed that TRAF2 and TRAF3 can bind to peptides corresponding to amino acids 31-50 of human CD40 or amino acids 36-52 of murine CD40 (165,166). These regions are completely conserved between human and murine CD40 (167). TRAF5 may bind to an identical or overlapping site since residues 15-54 of murine CD40 are required for TRAF5 binding (159).

In addition to the TRAF binding sites, reports published during the course of this study indicated that the membrane-proximal region of CD40 may also contain signaling motifs. Deletion analysis showed that a membrane-proximal proline-rich region corresponding to amino acids 7-14 of the cytoplasmic domain of human CD40 is required for the association of the JAK3 tyrosine kinase with human CD40 (95). In addition, residues 15-30 of murine CD40 are required for TRAF6 binding (160).

1.10 The role of phosphorylation in CD40 signaling

In human B cells CD40 has been shown to be constitutively phosphorylated. (168-170). Furthermore, treatment with interleukin 6 increases CD40 phosphorylation within minutes (170). Presumably this phosphorylation is on serine/threonine residues

since human CD40 contains no tyrosines. It is not known whether phosphorylation of CD40 contributes to its signaling function. At the onset of this thesis, it was known that the threonine at position 39, a potential site of phosphorylation, was important for CD40 signaling since mutation of this residue to an alanine disrupts the growth inhibitory effects of CD40 on M12 cells whereas mutation of the other threonine residues in the human CD40 cytoplasmic tail had no effect (168). In this thesis, I further examined the importance of this threonine residue in CD40 signaling. While our structure function analysis on the CD40 cytoplasmic tail was underway, it was shown that mutating this threonine in human CD40, or the corresponding threonine at position 40 in murine CD40, to an alanine disrupts the ability of CD40 to associate with TRAF2 (95,160), TRAF3 (95,158) and TRAF5 (159).

1.11 Activation of B cells by Another TRAF-Associated Receptor (ATAR)

Since other TNFR superfamily members bind TRAFs, it is expected that these receptors are coupled to many of the same signaling pathways as CD40. We hypothesized that other TNFRs expressed in B cells could activate the same signaling pathways as CD40 and, thus, mimic CD40. These receptors may substitute for CD40 and activate B cells under some circumstances. Another TRAF-associated receptor (ATAR) is a recently discovered member of the TNFR superfamily (171). Human ATAR shares over 99% sequence identity with the Herpes virus entry mediator (HVEM) that is expressed on human T cells (172) and is probably the same protein. ATAR/HVEM has recently been shown to be expressed on human B cells and, similar to T cells, mediate Herpes simplex virus (HSV) entry into B cells (172). The consequences of B cell infection in the etiology of Herpes virus infection remains to be determined.

In addition to the HSV envelope glycoprotein (gD), ATAR binds two cellular ligands, LTα and LIGHT, a new member of the TNF family (173). Similar to CD40L,

both LTα and LIGHT are produced by activated T cells (173,174). Thus, ATAR could participate in T cell-dependent B cell activation like CD40.

Very little is known about the signal transduction pathways utilized by this new TNFR superfamily member. An initial study in which ATAR and the TRAF protein were coexpressed in 293 cells indicates that, like CD40, ATAR initiates signaling events by binding the TRAF2 and TRAF5 adapter proteins (171). In addition, ATAR activates NF-κB, a target of CD40 signaling, in transiently transfected 293 cells (171). It remains to be determined, however, whether ATAR is capable of activating these pathways in B cells and under physiological conditions. In this thesis, we tested whether ATAR can activate B lymphocytes. We also tested the hypothesis that signaling by ATAR can induce many of the same responses in B cells as signaling by CD40.

1.12 Summary of objectives

B lymphocytes need two signals in order to differentiate into antibody-producing cells, one delivered by the B cell antigen receptor (BCR) and a second delivered by CD40. In the absence of the CD40 signal, B cells that receive only the BCR signal undergo apoptosis. In this thesis, the WEHI-231 B lymphoma cell line was used as a model system to study BCR and CD40 signaling and in particular to investigate how signals from these two receptors are integrated.

The first aim of this research was to test the hypothesis that CD40 and the BCR activate MAP kinases. These serine/threonine kinases have been implicated in both apoptotic and mitogenic responses to receptor signaling. Each of the three types of MAP kinases, the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and p38, phosphorylates a distinct set of transcription factors. Thus, activating different combinations of MAP kinases could lead to distinct biological outcomes.

A second aim of this research was to test the hypothesis that the BCR and CD40 signals are integrated by mitogen activated protein (MAP) kinases. I tested whether any of the MAP kinases are subject to dual regulation by both the BCR and CD40 since, in T cells, JNK is involved in signal integration during costimulation of T lymphocytes through the TCR and CD28 (175). In T cells, the TCR and CD28 are often thought of as an analogous receptor pair to the BCR and CD40 in B cells.

The third aim of this thesis was to identify signaling motif(s) in the CD40 cytoplasmic domain that mediate MAP kinase activation. Identification of these motifs would improve our understanding of how CD40 is linked up to the MAP kinase pathways. To identify these motifs, eleven chimeric receptors consisting of the extracellular and transmembrane domains of human CD8α fused to progressively smaller portions of the CD40 cytoplasmic domain were constructed. The chimeric receptors, as well as the parental CD8α molecule which contains only the extracellular and transmembrane portions of CD8α, were stably expressed in WEHI-231 cells. After identifying the regions in the CD40 tail that mediate MAP kinase activation, I determined if they were the same residues that confer protection from BCR-induced apoptosis, as well as activation of NF-κB, a transcription factor essential for CD40-mediated rescue of WEHI-231 cells from BCR-induced death. By mapping the region in the CD40 cytoplasmic tail that is responsible for activating these signaling pathways, I have determined which CD40-associated adapter proteins might be relevant for CD40-induced activation of MAP kinase, NF-κB and survival pathways in B cells.

The fourth aim of this thesis was to test the hypothesis that ATAR/HVEM a recently discovered tumor necrosis factor (TNF) superfamily receptor, can mimic the effects of CD40 on B cells. Although the functions of ATAR are completely unknown, it is expressed on B cells. Furthermore, initial studies suggest that ATAR uses similar mechanisms as CD40 to initiate intracellular signaling. Since the ligand for ATAR is

unknown and antibodies to ATAR are unavailable, we produced chimeric receptors containing the ATAR cytoplasmic domain to study ATAR signaling. Two chimeric receptors consisting of the extracellular and transmembrane domains of CD8α fused to portions of the ATAR cytoplasmic domain were constructed and expressed in WEHI-231 cells. We tested whether the cytoplasmic region of ATAR could mediate activation of the MAP kinases and NF-κB, as well as confer protection of WEHI-231 cells from anti-IgM-induced growth arrest.

In addition to B lymphocytes, CD40 is highly expressed on dendritic cells (DC). Both CD40 and lipopolysaccharide (LPS) have been shown to activate these professional antigen presenting cells. However, DCs have traditionally been difficult to study due to the absence of immortalized DC lines. Ricciardi-Castagnoli and colleagues have recently established the murine D1 DC line (176,177). To evaluate whether MAP kinases might be involved in CD40 or LPS-induced activation of DCs, the final aim of this thesis was to test which MAP kinases are activated by CD40 and LPS in D1 cells. Our collaborators, M. Rescigno and M. Martino, under the direction of Dr. P. Ricciardi-Castagnoli (Milano, Italy) extended these findings by testing the role of LPS-induced ERK activation in DC survival and maturation.

CHAPTER 2

MATERIALS AND METHODS

Materials

2.1 Antibodies

Goat anti-mouse IgM (μ-chain specific) and goat anti-mouse IgG-FITC were obtained from Bio-Can (Mississauga, ON, Canada). The rat hybridoma producing the 1C10 anti-murine CD40 mAb (178) was obtained from Dr. M. Howard (DNAX Research Institute, Palo Alto, CA). The 1C10 mAb was purified using a protein G-Sepharose column. The murine hybridoma cell line producing the 4G10 anti-phosphotyrosine mAb was obtained from Dr. D. Morrison (National Cancer Inst., Frederick, MD), while the murine hybridoma cell lines producing the OKT8 and 51.1 anti-human CD8α mAbs were obtained from the American Type Culture Collection (ATCC). All 3 of these mAbs were purified from tissue culture supernatant using protein A-Sepharose columns. Agarose-conjugated rabbit anti-ERK2 (Ab C-14) and rabbit anti-ERK1 (Ab C-16) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), as were rabbit Abs against JNK1 (Ab C-17), Crk II and IκBα (Ab FL). The rabbit anti-p38 Ab, provided by Dr. P. Young (SmithKline Beecham Pharmaceuticals, King of Prussia, PA), was raised against full length CSBP2 (human p38α) (125,179). The rabbit anti-p38 Ab used for Western immunoblotting was from Santa Cruz (Ab C-20). The sheep anti-MAPKAP kinase-2 Ab was from Upstate Biotechnology Inc. (Lake Placid, NY). Abs specific for phosphorylated IκBα (Ser-32) were from New England Biolabs Inc. (Beverly, MA). Horseradish peroxidase (HRP)-conjugated protein A and HRP-conjugated sheep anti-mouse IgG were from Amersham Corporation (Oakville, Ontario, Canada). Goat anti-rabbit IgG-HRP was purchased from BioRad (Mississauga, ON, Canada).

2.2 Other reagents

Cells producing sCD40L (50) were provided by Dr. P. Lane (Basel Institute for Immunology). Tissue culture supernatants containing sCD40L were concentrated 10-fold using a Centricon 30 concentrator (Amicon Inc., Beverly, MA) and then diluted to the original volume with fresh tissue culture medium. LPS (*Escherichia coli* serotype 026:B6) was purchased from Sigma Chemical Corp. The p38 inhibitor, SB 203580, was obtained from Dr. P. Young (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). The MEK1/MEK2 inhibitor PD98059 was from BioMol (Plymouth Meeting, PA).

Protein A-Sepharose, protein G-Sepharose, glutathione-Sepharose, avidin and myelin basic protein (MBP) were purchased from Sigma (St. Louis, MO). Glutathione S-transferase (GST) fusion proteins containing either amino acids 1-79 or 1-169 of c-Jun were expressed in *Escherichia coli* and purified by glutathione-Sepharose affinity chromatography. Bacteria containing the plasmid encoding GST-c-Jun (1-79) were a gift from Dr. J. Hambleton (University of California, San Francisco). Bacteria containing the plasmid encoding GST-c-Jun (1-169) were obtained from Dr. S. Pelech (University of British Columbia, Canada). The GST-ATF2 (1-96) fusion protein was purchased from Santa Cruz Biotechnology. Recombinant murine Hsp25 was obtained from StressGen Biotechnologies Corp. (Victoria, BC, Canada). The enhanced chemiluminescence (ECL) detection system was from Amersham Corporation (Oakville, ON, Canada).

Methods

2.3 Affinity purification of monoclonal Abs

The 1C10, 4G10, OKT8 and 51.1 mAbs were affinity purified from tissue culture supernatants. Briefly, the hybridomas that produce these mAbs were grown in complete RPMI 1640 medium (see Section 2.6.1). When the cell concentration

reached approximately 0.8 X 10^6/mL, the medium was supplemented with glucose to a final concentration of 1%. In addition, 1/20th volume of 1 M sodium HEPES, pH 7.2 was added to neutralize the pH. When all of the cells had died (approximately one month from the onset of culture) they were removed by centrifugation at 3,000 rpm for 10 min. The pH of the tissue culture supernatant was adjusted to 8.0 by adding 1/10th volume of 1 M Tris HCl, pH 8.0 and sodium azide was added to 0.02%.

The 1C10 mAb was purified from the tissue culture supernatant using a protein G Sepharose column (Sigma), whereas the 4G10, 51.1 and OKT8 mAbs were purified using a Protein A-Sepharose column (Sigma). Column washes were performed with borate buffer, pH 8 (25 mM boric acid, 11.4 mM sodium borate, 0.5 M NaCl, 2.5 mM EDTA). The mAbs were eluted from the columns with 0.1 M glycine HCl, pH 2.5. One mL fractions were collected and each fraction was neutralized by the addition of 37.5 μL of saturated Tris base. Fractions containing significant amounts of Ab (as determined by OD_{280}) were pooled and dialyzed at 4°C over 2 days against 4 to 5 changes of phosphate-buffered saline (PBS). The final Ab concentration was then determined using an extinction coefficient of 1.46 for a 1 mg/mL solution of IgG and the solution was filter sterilized. Purified mAbs were stored in aliquots at -20°C.

2.4 Antibody biotinylation

The 51.1 anti-human CD8α mAb was biotinylated with Sulfo-NHS-Biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, 4 mg of Ab dissolved in 2 mL of 50 mM sodium bicarbonate buffer, pH 8.5, was mixed in a glass test tube with 80 μg of Sulfo-NHS-Biotin. The reaction was incubated for 30 min at room temperature in the dark. To remove unreacted biotin, the sample was centrifuged for 30 min at 3,000 rpm using a Centricon-30 Microconcentrator (Amicon). After centrifuging, the sample was diluted to 1.8 mL with 0.1 M sodium phosphate, pH 7.0. The sample was concentrated and then diluted with 0.1 M sodium phosphate, pH

7.0, a total of four times. The Ab solution was concentrated one more time and then diluted to 6 mL with phosphate buffered saline (PBS; 150 mM NaCl, 1.86 mM $NaH_2PO_4 \cdot H_2O$, 8.39 mM $Na_2HPO_4 \cdot 7H_2O$). The final concentration of the biotinylated 51.1 was determined by measuring its absorbance at 280 nm. Biotinylation was verified by performing a streptavidin-HRP Western blot on 0.5 μg of Ab. Biotinylated 51.1 was stored as sterile-filtered aliquots at -20°C.

2.5 Molecular biology methods

2.5.1 Cloning of the truncated CD8α cDNA into the pLXSN retroviral expression vector

The puc12/CD8 plasmid containing cDNA encoding human CD8α in which a *Bgl II* site had been inserted after the fourth codon of the cytoplasmic domain (180) was a gift from Dr. A. Weiss (Univ. of California, San Francisco). The CD8 cDNA was excised from the pUC12/CD8 plasmid by digesting with *XbaI* and *BamH1*. The ends of the CD8-containing cDNA fragment were then blunt-ended with Klenow. To subclone the CD8 fragment into the pLXSN retroviral expression vector (181), the vector was linearized with *BamH1* and blunt-ended with Klenow. The blunt-ended CD8 fragment was then ligated into the linearized pLXSN vector (Fig. 2.1). Insertion of the CD8 fragment into the pLXSN vector was performed with Danielle Krebs.

2.5.2 mRNA purification

mRNA was purified from WEHI-231 cells using oligo dT cellulose as described below. All solutions were made with RNase-free sterile milli-Q double distilled H_2O (ddH_2O).

a) Cell lysis: 1 X 10^8 WEHI-231 cells were centrifuged at 1,500 rpm in a Beckman GS-6R centrifuge for 5 min. Following centrifugation, the medium was aspirated and

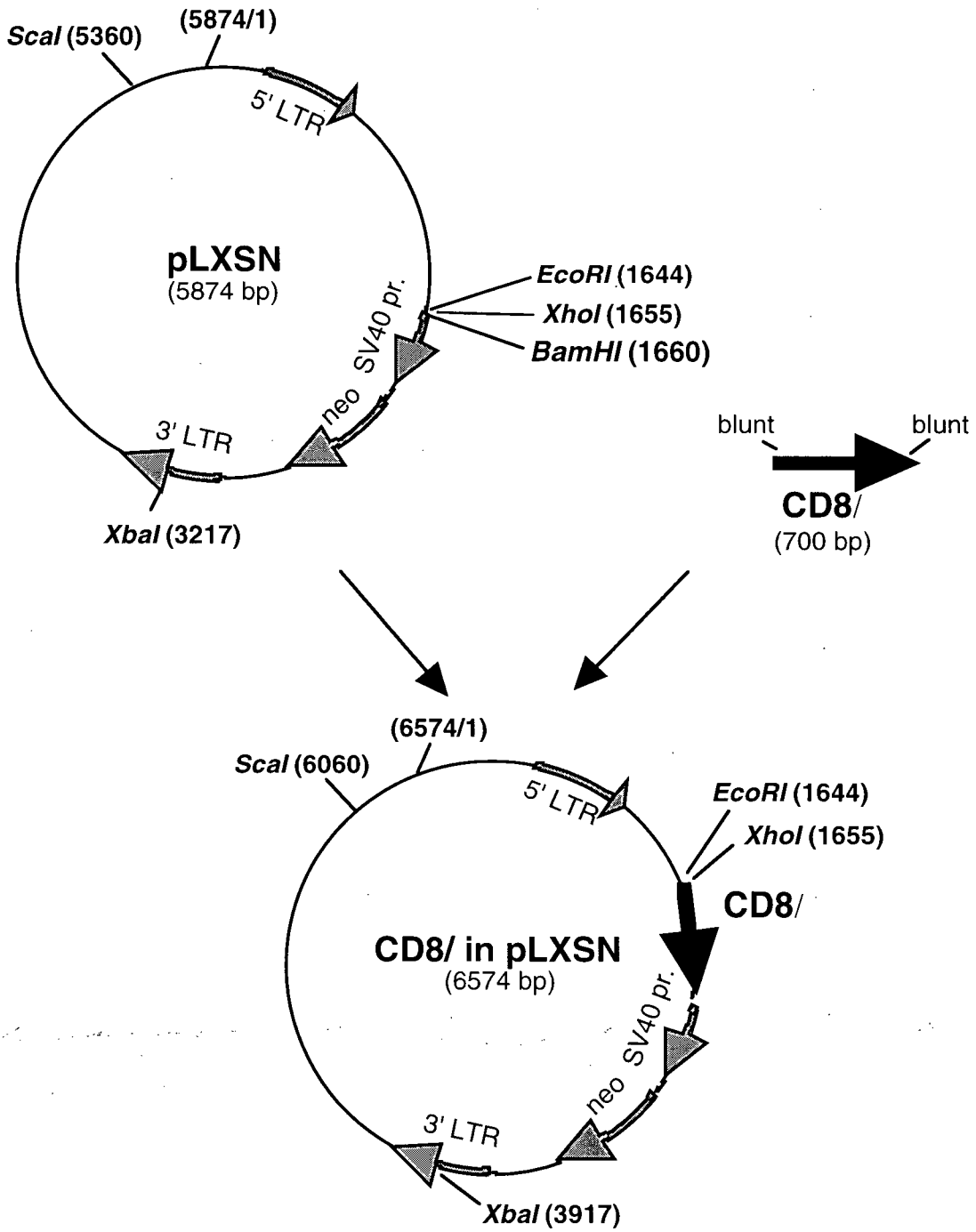


Fig. 2.1 Construction of the pLXSN/CD8 retroviral expression vector (see text for details).

the cell pellet was resuspended in 30 mL of ice-cold PBS and centrifuged again. The cells were washed a second time with PBS and then resuspended in 1 mL of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA containing 2 mg of proteinase K. The cells were lysed by adding 4 mL of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% SDS to the cell suspension. The DNA was then sheared by passing the cell lysate 7 to 8 times each through an 18 gauge needle followed by a 22 gauge needle. Following this, the lysate was incubated for 1 h at 37°C.

b) mRNA binding: 1 mL of Type 7 oligo(dT)-cellulose beads (Pharmacia) was treated for 5 min with 0.1 N NaOH to destroy RNAses. The beads were then washed 2 times with 5 mL of binding buffer (0.5 M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA and 0.1% SDS) and then resuspended in 4 mL of binding buffer plus 1 mL of 2.5 M NaCl. The cell lysate was added to the bead suspension and the mixture was rocked overnight at room temperature.

c) mRNA elution: The beads were poured into an autoclaved Poly-Prep column (BioRad) and then washed once with binding buffer and 3 times with wash buffer (0.1 M NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.1% SDS). The mRNA was eluted from the oligo(dT) beads with 2 mL of 10 mM Tris HCl, pH 7.4. The mRNA was ethanol precipitated by adding sodium acetate, pH 5.2, to a final concentration of 0.3 M, and then adding 2 volumes of ice-cold 100% ethanol. After incubation for 30 min at -20°C, the precipitated mRNA was recovered by centrifugation in an Eppendorf microfuge at 12,000 rpm for 20 min at 4°C. The precipitated mRNA was dissolved in 100 µL sterile ddH_2O with 0.3 U/mL RNAsin (Promega Corporation) and then quantitated by measuring its absorbance at 260 nm using a conversion of 1 OD unit = 40 µg/mL. The mRNA was stored at -80°C.

2.5.3 cDNA synthesis

First strand cDNA was synthesized using the Promega reverse transcription system (Promega Corporation, Madison, WI). A 20 μL reaction mixture containing 5 mM $MgCl_2$, 1X reverse transcription buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 1 mM of each dNTP, 20 units RNAsin, 15 units AMV reverse transcriptase (Promega Corporation, 0.5 μg oligo$(dT)_{15}$ primer, and 0.1 μg mRNA was incubated at 42°C for 15 min. The reaction was terminated by boiling for 5 min and then stored at -20°C.

2.5.4 n-Butanol purification of PCR primers

Oligonucleotide primers were synthesized by the Nucleic Acid and Protein Service (NAPS) Unit (University of British Columbia) and purified by the n-butanol method. Primers were dissolved in 100 μL of 30% ammonium hydroxide (NH_4OH) and 1 mL of n-butanol (Fisher Scientific). After vigorous vortexing, the sample was centrifuged at 12,000 rpm for 3 min in an Eppendorf microfuge. The liquid was aspirated and the oligonucleotide pellet was suspended in 100 μL of ddH_2O plus 1 mL of n-butanol and then centrifuged again. The pellet was dried under vacuum and resuspended in 500 μL of ddH_2O. The ratio of absorbancies at 260, 280, and 320 nm was used to calculate the concentration of primer recovered using the following equation:

μmol/mL of primer = (OD 260 nm/280 nm/320 nm ratio)/(10X length of primer)

2.5.5 Polymerase chain reaction (PCR)

The first strand WEHI-231 cDNA was used as a template in the PCR reactions to generate double stranded cDNA molecules encoding (1) the full length murine CD40 cytoplasmic domain (amino acids 1-74; where the amino acid residues are numbered starting at the beginning of the CD40 cytoplasmic domain), (2) the homology box

region of CD40 (amino acids 26-63) or (3) a truncated version of the homology box region of CD40 (amino acids 26-53). The primers used in these PCR reactions (Table 4.1) added a *Bgl II* site at the 5' end of the amplified cDNAs and a stop codon followed by a *Bgl II* site at the 3' end.

PCR reactions were performed using 1 μL of a 1:5 dilution of WEHI-231 cDNA, 5 μL of 10X ThermoPol reaction buffer (New England Biolabs), 2 μL of 10 mM dNTPs (Pharmacia Biotech Inc., Piscataway, NJ), 50 pmol of each primer and 1 unit of Vent DNA polymerase (New England Biolabs) in a final volume of 50 μL. The PCR reactions were carried out in a PTC-100 Programmable Thermal Cycler (MJ Research, Inc., Watertown, Mass). Following an initial 2 min incubation at 94°C, samples were cycled 30 times with a denaturation step of 1 min at 94°C, an annealing step of 2 min at 55°C, and an extension time of 2 min at 72°C.

PCR products were analyzed on 2% agarose gels. A 100 bp DNA ladder (Gibco BRL, Burlington, ON) was used to determine the sizes of the PCR products. PCR products of the expected sizes for the full length CD40 tail DNA, the homology box DNA or the truncated homology box DNA were cut from the gel with a scalpel and purified using the QIAEX II Extraction Kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's protocol.

2.5.6 Oligonucleotides encoding CD40 and ATAR cytoplasmic domain fragments

Oligonucleotides were synthesized by the NAPS Unit at the University of British Columbia. The sequences of the oligonucleotides used to generate the murine CD40 tail segments are listed in Table 4.2. The sequences of the oligonucleotides used to generate the murine ATAR tail segments are listed in Table 5.1. Complementary synthetic oligonucleotides were annealed together to generate cDNAs encoding amino acid residues 1-25, 15-30, 35-53, 35-53 T_{40}-A, 26-44, 35-45, 45-63 and 43-53

of the murine CD40 cytoplasmic domain and the C-terminal half of the murine ATAR cytoplasmic domain (amino acid residues 27-46).

2.5.7 Annealing of oligonucleotides encoding CD40 cytoplasmic tail or ATAR inserts

Each oligonucleotide (40 nanomoles) was dissolved in 100 μL of TE, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA). Complimentary oligonucleotides were mixed together and annealed by boiling for 5 min in a H_2O bath and allowing them to slowly cool to room temperature. Once formed, the double stranded CD40 and ATAR cDNA fragments were cut with *Bgl II* and ligated into the *Bgl II* site of CD8 in pLXSN (Fig. 2.2).

2.5.8 Restriction endonuclease reactions

Restriction endonucleases were purchased from New England Biolabs or Gibco BRL. To digest plasmid DNA with restriction endonucleases, 5 μg of the DNA were mixed with 2 μL of the appropriate 10X restriction enzyme digestion buffer. The reaction volume was brought up to 18 μL with autoclaved dH_2O and then 2 μL of the appropriate restriction enzyme was added. The reaction was allowed to proceed for 2 to 4 h at 37°C.

2.5.9 Filling in 5' DNA overhangs with Klenow

To create blunt ends on DNA containing a 5' overhang after restriction enzyme digestion, the reaction mixture was first heated at 75°C for 10 min to denature the restriction enzyme. After cooling the reaction mixture, 5 units of Klenow DNA polymerase (New England Biolabs) were added and dNTPs were added to a final concentration of 33 μM. The reaction was allowed to proceed at 25°C for 15 min. Finally, the Klenow was denatured by heating at 75°C for 10 min.

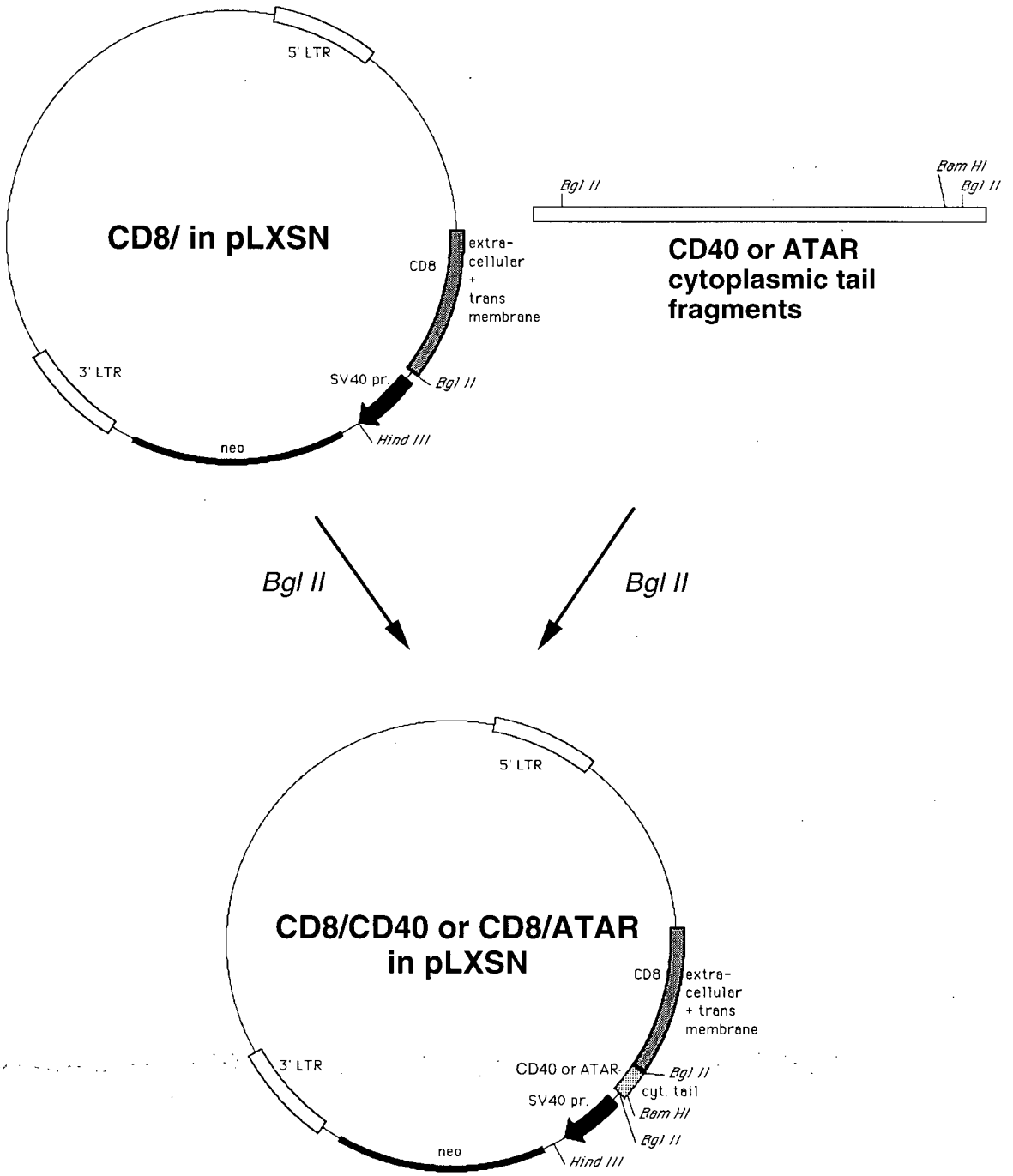


Figure 2.2: Cloning of CD40 and ATAR cytoplasmic tail inserts into pLXSN/CD8

The 6.6 kb pLXSN-CD8 plasmid (see Materials and Methods) and the CD40 or ATAR cytoplasmic tail inserts (see Materials and Methods) were digested overnight with *Bgl II*. After phosphatase treating pLXSN-CD8, the vector and inserts were purified by Geneclean. The CD40 and ATAR inserts were then ligated into the *Bgl II* site of pLXSN-CD8 which is located 4 codons into the CD8 cytoplasmic domain.

2.5.10 Phosphatase treatment of vectors

To prevent religation of linearized plasmid vectors, 5' phosphates were removed by treating the DNA with calf intestinal alkaline phosphatase (CIP, New England Biolabs). Dephosphorylation reactions were carried out by adding 0.5 units of CIP per pmol of DNA ends directly to a completed restriction enzyme digest and incubating the tube at 37°C for 60 min. The phosphatase reaction was terminated by adding EDTA to 5 mM and heating at 75°C for 10 min. Before use in ligation reactions, phosphatase-treated DNA was purified using the Geneclean Kit and then dissolved in dH_20.

2.5.11 Agarose gel electrophoresis

DNA samples were checked for correct size and purity on 0.8% or 1.5% (w/v) agarose gels. Gels were prepared by dissolving the agarose (Gibco BRL) in Tris-Borate-EDTA buffer (TBE) (0.045 M Tris-borate, 0.001 M EDTA) as described in (182). Agarose gels were run in TBE containing 0.1 μg/mL ethidium bromide (Sigma) at 100 V for 1 h. DNA in the agarose gels was visualized by illumination with ultraviolet light.

2.5.12 Purification of DNA

DNA was separated from various contaminants after reactions involving restriction endonucleases, alkaline phosphatase or Klenow polymerase. To purify DNA from an agarose gel, the DNA fragment of interest was first cut from the gel using a scalpel. DNA fragments less than 400 bp in size were purified using the QIAEX II Gel Extraction Kit (Qiagen Inc., Mississauga, ON). Larger DNA fragments were purified using the Geneclean Kit (Bio 101 Inc., La Jolla, CA). All fragments were purified according to the manufacturer's protocols.

2.5.13 Ligation reactions

One hundred ng of *Bgl II*-digested, phosphatase-treated vector was mixed with *Bgl II*-digested CD40 tail or ATAR tail inserts at a 1:3 molar ratio. The ligation reaction contained vector and insert DNA, 2 μL of 10X ligase buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$ 10 mM DTT, 1 mM ATP, 50 μg/mL BSA) and 400 U of T4 DNA ligase (New England Biolabs) in a final volume of 20 μL. Ligation reactions were allowed to proceed for 1 h or overnight at 16°C.

2.5.14 Preparation of competent bacteria for transformation

HB101 *Escherichia coli* (Gibco BRL) were rendered competent for transformation by pelleting 500 mL of a log phase culture (OD_{550} = 0.4) and resuspending the bacteria in 200 mL of 30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, pH 5.8. The cell suspension was incubated for 5 min on ice. The bacteria were then pelleted by centrifuging at 8000 rpm for 10 min at 4°C, resuspended in 20 mL of 10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% glycerol pH 6.5, and incubated on ice for 15 min. Small aliquots of the cells were made and frozen on dry ice. The competent bacteria were stored at -80°C until use.

2.5.15 Transformation of bacteria

To transform HB101 bacteria, 40 ng of plasmid DNA or 8 μL of a ligation reaction were added to 100 μL of competent HB101 bacteria and incubated on ice for 30 min. The cells were heat shocked for 45 sec at 42°C and then placed on ice for 2 min. At this point, 0.7 mL of SOC medium (2 g bactotryptone, 0.5 g yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgCl_2$, 20 mM glucose in a final volume of 100 mL) were added and the culture was incubated at 180 rpm, 37°C for 1 h. Either 50 μL or

250 μL of the transformed bacteria were then plated onto LB plates containing 50 μg/mL ampicillin and incubated overnight at 37°C.

2.5.16 Plasmid DNA preparations

Small quantities of purified plasmid DNA (minipreps) were prepared using the alkaline lysis method (182). Briefly, a single isolated colony was transferred to 3 mL of Luria-Bertani (LB) medium containing 50 μg/mL ampicillin and incubated overnight at 37°C,180 rpm. Approximately 1.5 mL of the culture were centrifuged and the pellet was resuspended in 100 μL of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8). At this point, 200 μL of freshly-prepared Solution II (0.2 N NaOH, 1% SDS) were added, the tube was mixed by inversion, and incubated on ice for 5 min. Following this, 150 μL of ice-cold Solution III (5 M potassium acetate, 11.5% glacial acetic acid) was added. The mixture was vortexed for 10 sec and then placed on ice for 5 min to allow precipitation of bacterial chromosomal DNA. After centrifugation at 12,000 rpm for 5 min at 4°C, the supernatant was transferred to a fresh tube and extracted with an equal volume of (1:1) phenol:chloroform to remove excess protein. The upper aqueous phase was recovered and the plasmid DNA was precipitated by adding 2 volumes of ethanol. The solution was then mixed by vortexing and incubated for 5 min at room temperature. The plasmid DNA was pelleted by centrifugation at 12,000 rpm for 5 min. The pellet was washed once with 70% ethanol and dried briefly in a Speed Vac (Savant Instruments Inc., Farmingdale, NY). After drying, the pellet was redissolved in 20 μL of dH_20 containing 5 μg of RNAse (Sigma). Plasmids were analyzed by digesting 10 μL of miniprep DNA with the appropriate restriction enzymes. Plasmid DNA was stored at -20°C.

To obtain large amounts of pure DNA for sequencing and subsequent transfection into eukaryotic cells, Nucleobond AX 100 column preps (Clontech, Palo Alto, CA) were performed according to the manufacturer's instructions.

2.5.17 Preparation of frozen stocks of bacteria

To prepare frozen stocks of bacteria, 3 mL of an overnight culture of bacteria were mixed with 1 mL of sterile glycerol. One mL aliquots of this mixture were stored at -80°C.

2.5.18 DNA sequencing

The sequence of each CD8α/CD40 and CD8α/ATAR chimeric cDNA was confirmed by DNA sequencing. Sequencing was carried out using the Sequenase Version 2.0 Kit (Amersham) according to the manufacturer's instructions. Alternatively, sequencing was performed by the NAPS Unit at the University of British Columbia. The primer used for sequencing was synthesized by the NAPS unit. This primer corresponds to codons 177-183 of the CD8 sense strand and consists of the following sequence: 5' CTG GAC TTC GCC TGT GAT ATC 3'.

2.6. Cell culture

2.6.1 Culturing WEHI-231 cells

The WEHI-231 murine B lymphoma cell line (183) was grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) , 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol. FCS was heat-inactivated by incubation at 56°C for 30 min. WEHI-231 clones expressing CD8/CD40 or CD8/ATAR chimeric receptors were maintained for up to 3 months in complete medium supplemented with 1.8 mg/mL geneticin (G418, Gibco, BRL).

2.6.2 Culturing BOSC 23 cells

The BOSC 23 cells were a gift from Dr. W. Pear (Massachusetts Institute of Technology, Cambridge, MA). BOSC 23 cells were grown in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM

2-ME. To split these adherent cells, they were first washed with PBS and then removed from the tissue culture dishes by a 5 min treatment with 0.25% trypsin/1 mM EDTA. The cells were then pelleted by centrifugation at 1500 rpm for 5 min and resuspended in complete DMEM medium. Selection and maintenance of BOSC 23 cells was performed by Danielle Krebs and has been described previously (184). Briefly, BOSC 23 cells were grown in gpt selection medium (DMEM supplemented with 10% dialyzed FCS, 0.25 mg/mL xanthine, 14 μg/mL hypoxanthine, 25 μg/mL mycophenolic acid, 10 μg/mL thymidine and 20 μg/mL aminopterin) for two weeks. This selection ensured that the BOSC 23 cells expressed all of the proteins required for virus assembly. Multiple frozen aliquots were made. Each aliquot could then be thawed and grown without selection for up to two months, after which production of viral packaging proteins declined.

2.6.3 Culturing D1 dendritic cells (DCs)

The D1 cells were derived from murine splenic DCs and maintained *in vitro* as growth factor-dependent immature DCs (177,185). D1 cells were grown by M. Rescigno and M. Martino in IMDM (Sigma Chemical Corp., St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah) 2 mM L-glutamine, 50 μM 2-ME, 30% NIH/3T3 fibroblast supernatant and 10 ng/mL mouse recombinant GM-CSF (Genzyme, Cambridge, MA). Clusters of adherent cells with DC morphology were detached using PBS/3mM EDTA as described in (177).

2.6.4 Long term storage of eukaryotic cells

For long term storage, eukaryotic cells were frozen in 90% FCS/10% DMSO. After 2 days at -80°C, the cells were moved to liquid nitrogen for storage.

2.7 Retrovirus infection of WEHI-231 cells

2.7.1 Generation of retroviruses using the BOSC 23 packaging cell line:

The protocol used to produce retroviruses using the BOSC 23 cell line has been described previously (184). Briefly, on the day prior to transfection, BOSC 23 cells were plated in 6-well dishes at 2.0 X 10^6 cells per well. The cells were approximately 85% confluent when transfected. Immediately before transfection, the medium was aspirated from the cells and 1 mL of fresh medium containing 25 μM chloroquine was added. For each well, a transfection cocktail was prepared by adding 2 μg plasmid DNA to 0.2 mL of 250 mM $CaCl_2$ in a 12 X 75 mm polystyrene tube and then adding 0.2 mL HEPES-buffered saline (50 mM sodium HEPES, pH 7.05, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4) dropwise over 5 sec while vortexing at moderate speed. The mixture was vortexed for an additional 10 sec and the transfection cocktail was added to the BOSC 23 cells. At 10 h and 36 h post-transfection, the medium was aspirated from the cells and replaced with 2 mL of fresh medium. At 42-44 h post-transfection, the medium containing the retroviruses was collected and used for infection of WEHI-231 cells. In general, virus-containing cell supernatants were filtered and used immediately. Occasionally, the supernatants were frozen at -80°C. However, freeze-thawing was found to decrease the viral titer.

2.7.2 Infection of WEHI-231 cells:

On the day of infection, 5 X 10^5 WEHI-231 cells in 0.5 mL medium were added to each well of a 6-well dish. The virus-containing supernatant from one well of a 6-well dish of BOSC 23 cells (2 mL) was passed through a 0.22 μM filter (Millipore) into a tube containing 2.5 μL of 10 mg/mL polybrene (hexadimethrine bromide, Sigma). The filtered supernatant was then added to the cells. Twelve to 18 h post-infection, the cells were pelleted and resuspended in fresh medium without polybrene.

2.7.3 Obtaining drug-resistant clones:

Drug-resistant WEHI-231 clones were selected by culturing the cells in medium containing 1.8 mg/mL G418 (Gibco) starting 44 h post-infection. To obtain isolated clones, cells were diluted to 5 X 10^3/mL and plated by serial dilution into 96-well plates. Twenty-four serial 0.25-fold dilutions were performed by successively transferring 150 μL of cells into wells containing 50 μL of media. Approximately two weeks later isolated clones were tested for CD8α expression by flow cytometric analysis.

2.8 Flow cytometry

To test G418-resistant WEHI-231 cell clones for expression of CD8 chimeric receptors, cells were stained with the OKT8 anti-human CD8 mAb according to the following protocol. All steps were done in the cold. Briefly, 2 X 10^5 cells were placed in one well of a U-bottom non-sterile 96 well plate. The plate was centrifuged at 2,000 rpm at 4°C in a GS-6R centrifuge (Beckman) to pellet the cells. The supernatant was removed by blotting the inverted plate onto paper towels. The cells were washed by first resuspending the cell pellet in 200 μL of flow cytometry sorter buffer (FSB; PBS, 1% FCS, 0.1% sodium azide) and then repelleting the cells by centrifuging the plate as described above. After removing the supernatant, the cell pellet was resuspended in 50 μL of FSB containing 1.5 μg of the OKT8 anti-human CD8α mAb and incubated for 20 min. The cells were washed and incubated for an additional 20 min in 50 μL FSB containing 1.5 μg of goat anti-mouse IgG-FITC Ab. After a final wash step, the cells were resuspended in FSB and analyzed for CD8 expression using a Becton Dickinson FACSCAN. Data analysis was performed using Lysis II software.

2.9 WEHI-231 cell stimulation and preparation of cell lysates

For signaling experiments, transfected WEHI-231 clones were expanded in complete medium without G418. Cells were pelleted, washed once with modified HEPES-buffered saline (HBS; 25 mM sodium HEPES, pH 7.2, 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 mg/mL glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-ME) and then resuspended to 5 x 10^6 per mL in HBS. The cells were warmed to 37°C and stimulated with various concentrations of either anti-IgM Abs, the 1C10 anti-CD40 mAb, sCD40L, LPS or with biotinylated 51.1 anti-human CD8α mAb (51.1-biotin) and avidin for various times. Where indicated, the cells were pre-treated with 50 μM SB 203580 (p38 inhibitor) for 30 min at 37°C prior to being stimulated with 1C10. Reactions were stopped by adding ice-cold PBS containing 1 mM Na_3VO_4 and then centrifuging the cells for 3 min at 3,000 rpm in the cold. Cell pellets were washed once with ice-cold PBS/Na_3VO_4 and then solubilized in one of the following buffers: buffer A (20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/mL leupeptin, 1 μg/mL aprotinin); buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM MoO_4, 0.2 mM Na_3VO_4, 1 mM DTT, 10 μg/mL aprotinin, 2 μg/mL leupeptin, 0.7 μg/mL pepstatin, 40 μg/mL PMSF, 10 μg/mL soybean trypsin inhibitor); buffer C (20 mM Tris-HCl, pH 8, 137 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 10 mM β-glycerophosphate); buffer D (25 mM sodium HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 μg/mL leupeptin, 20 μg/mL aprotinin, 1 mM PMSF); buffer E (20 mM Tris-HCl, pH 7.4, 1% Triton-X 100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, 1 mM PMSF, 10 μg/mL leupeptin). After 10 min on ice (unless otherwise indicated), detergent-insoluble material was removed by

centrifugation. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

2.10 D1 dendritic cell stimulation and preparation of cell lysates

Five million D1 cells were resuspended in HBS and stimulated with 10 μg/mL of LPS for various times. Where indicated, the cells were pre-treated with 50 or 100 μM MEK inhibitor PD98059 for 30 min at 37°C prior to being stimulated with LPS. Reactions were terminated with ice-cold PBS. The cells were pelleted by centrifugation and lysed in buffer B as described in section 2.9. After removing detergent-insoluble material by centrifugation, ERK activity in the lysates was measured by ERK *in vitro* kinase assay. D1 cell lysates were prepared by our collaborator, M. Rescigno.

2.11 SDS-PAGE and Western immunoblot analysis

Protein samples were prepared for electrophoresis by adding SDS-PAGE sample buffer to a final concentration of 62.5 mM Tris, pH 6.8, 2.3% SDS, 100 mM DTT, 10% glycerol and boiling for 5 min. Samples were separated by SDS-PAGE on 1.5 mM thick (unless indicated otherwise) mini-gels according to standard protocols (186). After electrophoresis, gels were stained with Coomassie blue to visualize the proteins and then dried using a gel dryer (BioRad, model 583), or were electrophoretically transferred (75 min at 75 V) to nitrocellulose (BA85, Schleicher and Schuell, Keene, NH). Transferred proteins were visualized by Ponceau S (Sigma) staining.

For immunoblot analysis, filters were blocked overnight at 4°C with either 5% bovine serum albumin (BSA; ICN Biomedicals Inc., Aurora, OH), 0.02% sodium azide in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) or with 5% (w/v) nonfat dry milk (Carnation), 0.02% sodium azide in TBS. Primary antibodies were diluted in TBS and

incubated with the filter for 3 h or overnight at 4°C. After washing for 15 min with several changes of TBS/0.05% Tween 20 (TBST), filters were incubated with either horseradish-peroxidase-(HRP) conjugated goat anti-rabbit IgG (1:20,000 in TBST), HRP conjugated sheep anti-mouse IgG (1:10,000 in TBST) or protein A-HRP (1:10,000 in TBST) for 1 h. Filters were then washed for 2 h with several changes of TBST followed by a final 5 min wash in TBS. Immunoreactive bands were visualized by enhanced chemiluminescence detection (ECL, Amersham).

To re-probe filters with other Abs, the filters were first stripped of bound Ab by incubating in TBS, pH 2 for 15 min. Stripped filters were washed twice in TBS, reblocked and then probed as described above.

2.12 p38 tyrosine phosphorylation

Cell lysates (300 μg protein in buffer A) were precleared by mixing with 15 μL of protein A-Sepharose for 1 h at 4°C. p38 was immunoprecipitated by incubating the precleared lysate overnight at 4°C with 5 μg of rabbit-anti-p38 Ab (P. Young). Immune complexes were collected by mixing the lysate with 15 μL of protein A-sepharose for 1 h at 4°C. After washing the beads 3 times in buffer A, the beads were boiled 5 min in 1X SDS-PAGE sample buffer. Samples were separated on 11% polyacrylamide gels and transferred to nitrocellulose. Filters were blocked with BSA as described above.

Tyrosine phosphorylation of p38 was detected by probing the filter with the 4G10 anti-phosphotyrosine mAb, followed by sheep anti-mouse IgG-HRP as a secondary Ab. Immunoreactive bands were visualized by ECL. To ensure equal recovery of p38 protein in each lane, the filter was stripped and then re-probed for 1 h with a 1:5000 dilution of rabbit anti-p38 Ab (Santa Cruz, C-20), followed by goat anti-rabbit IgG-HRP.

2.13 ERK bandshift assays

To detect p42 and p44 ERK bandshifts which are indicative of phosphorylation and correlate with ERK activation, immunoblots using an Ab that recognizes both ERK1 and ERK2 were performed. Samples (1.5 μg of cell lysate in buffer A) were electrophoresed on 12.5% low bis gels (12.36% acrylamide, 0.14% bis-acrylamide, final concentrations) and transferred to nitrocellulose. The filters were blocked with BSA and incubated for 2 h with a 1:1000 dilution of rabbit anti-MAP kinase (Ab Sc-94, Santa Cruz Biotech). Bound Ab was detected with goat anti-rabbit IgG HRP and the p42 and p44 ERK bands were visualized using the ECL detection system.

2.14 In vitro kinase assays

2.14.1 ERK in vitro kinase assays

Cell lysates (150-250 μg protein in buffer B) were incubated with 15 μL of agarose-conjugated anti-ERK1 or anti-ERK2. After mixing in the cold for 1 h, the beads were washed three times with buffer B and once with kinase assay buffer (20 mM sodium HEPES, pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-ME, 2 mM Na_3VO_4, 10 μg/mL aprotinin, 1 mM PMSF). Reactions were initiated by adding 30 μL of kinase assay buffer containing 1 mg/mL myelin basic protein (MBP) and 5 μCi ^{32}P-γ-ATP. After 15 min at 30°C, reactions were terminated by adding 30 μL 2X SDS-PAGE sample buffer. Samples were loaded onto 15% mini-gels and transferred to nitrocellulose. The MBP bands were detected by Ponceau S staining. After autoradiography, the MBP bands were excised and ^{32}P incorporation was determined by liquid scintillation counting.

2.14.2 JNK in vitro kinase assays

Following stimulation, 10^7 cells were lysed in 350 μL buffer C. Cell lysates were precleared for 1 h at 4°C with 10 μL of protein A-Sepharose, then mixed with 0.5 μg of

rabbit anti-JNK1 Ab for 14 h at 4°C. Immune complexes were collected by adding 10 µL of protein A-Sepharose and mixing for an additional hour. The beads were washed twice with buffer C and once with kinase assay buffer (25 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT). Kinase reactions were initiated by adding 30 µL kinase assay buffer containing 2 µg GST-c-Jun (1-79), 20 µM ATP and 10 µCi ^{32}P-γ-ATP. After 15 min at 30°C, reactions were terminated by adding 12 µL 5X SDS-PAGE sample buffer. Samples were separated on 12% SDS-PAGE gels and transferred to nitrocellulose. The Ponceau S-stained GST-c-Jun (1-79) band was excised and ^{32}P incorporation determined by liquid scintillation counting.

In vitro kinase assays on JNK that was precipitated with immobilized GST-c-Jun were performed as described by Coso et al. (187) with slight modifications. Following stimulation, 10^7 cells were solubilized in 350 µL buffer D for 30 min in the cold with rocking. The cell lysates were mixed with 10 µL of GST-c-Jun (1-169) bound to glutathione-Sepharose beads (provided by Dr. S. Pelech, University of British Columbia) for 3 h at 4°C. The beads were washed three times with PBS, 1% NP-40, 2 mM Na_3VO_4, once with 0.1 M Tris-HCl, pH 7.5/0.5 M LiCl, and once with kinase assay buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na_3VO_4). Reactions were initiated by adding 30 µL of kinase assay buffer containing 20 µM ATP and 10 µCi ^{32}P-γ-ATP. After 20 min at 30°C, reactions were terminated by washing the beads with 1 mL of cold 20 mM HEPES, pH 8, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 0.05% Triton X-100. Proteins were eluted from the beads with 40 µL SDS-PAGE sample buffer, separated on 11% SDS-PAGE gels, and transferred to nitrocellulose. The Ponceau S-stained GST-c-Jun (1-169) bands were excised and ^{32}P incorporation determined by liquid scintillation counting.

2.14.3 JNK in-gel kinase assay

Cell lysates (1.5 mg protein in buffer C) were pre-cleared with protein A-Sepharose for 1 h, then immunoprecipitated with 1 μg anti-JNK1 Ab as described above. Immunoprecipitated proteins were separated on a 0.75 mm-thick 10% SDS-PAGE gel in which 1 mg of GST-c-Jun (1-79) was co-polymerized. The gel was then subjected to a denaturation/renaturation procedure (188). The gel was washed in 50 mM Tris-HCl, pH 8, 20% isopropanol (2 x 30 min, 20°C) and then in 50 mM Tris-HCl, pH 8, 5 mM 2-ME (2 x 30 min, 20°C). Proteins were denatured by washing the gel in 50 mM Tris-HCl, pH 8, 5 mM 2-ME/6 M guanidine HCl (1 h, 20°C) and renatured by washing the gel in 50 mM Tris-HCl, pH 8, 5 mM 2-ME, 0.05% Tween 20 (2 x 30 min, 1 x 14 h, 2 x 30 min, all at 4°C). The gel was then washed for 30 min in kinase assay buffer (40 mM sodium HEPES, pH 7.4, 2 mM DTT, 15 mM $MgCl_2$, 1 mM $MnCl_2$, 0.3 mM Na_3VO_4, 0.1 mM EGTA) at room temperature. Kinase reactions were performed by incubating the gel with 16 mL of kinase assay buffer containing 25 μM ATP and 120 μCi ^{32}P-γ-ATP for 1 h at room temperature. Free ^{32}P-γ-ATP was removed by washing the gel with 5% trichloroacetic acid, 1% Na_2PO_7 (12 x 15 min, 20°C). The gel was then dried and exposed to film.

2.14.4 p38 in vitro kinase assay

Cell lysates (500 μg protein in buffer E) were precleared for 1 h at 4°C with 10 μL protein A-Sepharose, then mixed with 5 μg anti-CSBP2 Ab for 2 to 14 h at 4°C. Immune complexes were collected on 10 μL protein A-Sepharose for 1 h and the beads were then washed twice with buffer E and once with kinase assay buffer (25 mM sodium HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4). Reactions were initiated by adding 30 μL kinase assay buffer containing 1 μg GST-ATF2, 50 μM ATP and 10 μCi ^{32}P-γ-ATP. After 30 min at 30°C, reactions were terminated by adding 12 μL 5X SDS-PAGE sample buffer. Proteins

were resolved on 12% SDS-PAGE gels and visualized by Coomassie blue staining. After drying the gels, ^{32}P incorporation into GST-ATF2 was quantitated using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

2.14.5 MAPKAP kinase-2 in vitro kinase assay

Cell lysates (500 μg protein in buffer E) were precleared for 1 h at 4°C with 10 μL protein G-Sepharose, then mixed with 2 μg anti-MAPKAP kinase-2 Ab for 90 min at 4°C. Immune complexes were collected on 10 μL protein G-Sepharose for 1 h and washed as described for the p38 *in vitro* kinase assay. Reactions were initiated by adding 30 μL of kinase assay buffer (25 mM sodium HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4) containing 1 μg Hsp25, 50 μM ATP and 10 μCi ^{32}P-γ-ATP. After 30 min at 30°C, reactions were terminated by adding 12 μL 5X SDS-PAGE sample buffer. Proteins were resolved on 12% SDS-PAGE gels and ^{32}P incorporation into Hsp25 was quantitated using a phosphorimager.

2.15 IκBα phosphorylation and degradation

Cell lysates (40 μg of protein) in buffer A were separated on 12% low-bis acrylamide (12% acrylamide, 0.1% bis-acrylamide, final concentrations) SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked overnight with 5% (w/v) nonfat dry milk, washed with TBST and then incubated overnight with anti-phospho-IκBα Ab (1:1000). After washing the filters for 10 min with TBST, the anti-phospho-IκBα Ab was detected by incubating the filters with protein A-HRP (1:5000) for 1 h. The membranes were washed extensively with TBST and immunoreactive bands were visualized by ECL detection. To reprobe the blots, bound Abs were eluted by incubating the blots for 15 min with TBS, pH 2. The membranes

were blocked as described above and then incubated with the IκBα Ab (1:1000) for 3 h. Immunoreactive bands were visualized as described for the phospho-IκBα Ab.

2.16 Proliferation assays

WEHI-231 cells (1 X 10^4 cells/well) were cultured in 96 well plates in a final volume of 200 μL complete RPMI-1640 medium containing various stimuli. Three to six replicate cultures were set up for each experimental point. After 40 h at 37°C, 1 μCi of [^{3}H]thymidine (Amersham) was added to each well. The cells were harvested 4 h later using a Multiple Automated Sample Harvester (Cambridge, Technologies, Cambridge, MA). The incorporation of [^{3}H]thymidine into DNA was determined by liquid scintillation counting.

CHAPTER 3

Differential Activation of the ERK, JNK, and p38 Mitogen-Activated Protein Kinases by CD40 and the B Cell Antigen Receptor (BCR)

3.1 Introduction

Two key receptors that regulate B cell development and activation are the B cell Ag receptor (BCR) and CD40. The BCR and pre-B cell receptor regulate multiple steps in B cell differentiation. First, the survival and further differentiation of pre-B cells requires signals from the pre-B cell receptor (189,190). Second, Ag binding by the BCR on naive B cells can result in either activation, anergy, or apoptosis depending on the nature of the Ag and whether or not the B cell receives a T cell-derived co-stimulatory signal (1). Finally, signals from the BCR are required for the survival of germinal center B cells that have undergone somatic hypermutation (57).

CD40 also delivers key input at various stages in B cell activation and differentiation. The CD40 ligand (CD40L) is expressed on activated T cells (47) and can deliver the co-stimulatory signal that prevents BCR-induced apoptosis or anergy (55). CD40 signaling synergizes with the BCR to promote the proliferation and differentiation of activated B cells, the survival of germinal center B cells, and Ig secretion (12,29,50,57,191-193). CD40 engagement also promotes Ig class switching (191,192,194).

Of particular interest is how CD40 engagement determines whether BCR signaling leads to activation, anergy, or apoptosis. One model is that BCR signaling causes abortive activation in that it induces responses that promote both activation and apoptosis. In this model, CD40 signaling overcomes the death signal and synergizes with BCR signaling to promote B cell activation and proliferation. Thus, the

interactions between CD40- and BCR-induced signaling pathways are likely to be complex. As co-stimulatory receptors, BCR signaling and CD40 signaling may converge at a single critical signaling component that requires two inputs for activation. Alternatively, BCR- and CD40-stimulated signaling pathways may regulate different sets of transcription factors, both of which are required for cell activation. Inhibition of BCR-induced apoptosis by CD40 may involve down-regulation of BCR-induced signaling events and/or transcriptional activation of genes that prevent the apoptosis program from being carried out.

The WEHI-231 B lymphoma cell line provides a model system for investigating how BCR signaling and CD40 signaling are integrated. Cross-linking the BCR on these cells results in growth arrest in the G_1 phase of the cell cycle followed by apoptosis (63-65). The BCR-induced growth arrest and apoptosis can be prevented by anti-CD40 Abs, transfected fibroblasts expressing CD40L, or a soluble form of CD40L (sCD40L) (28,66,67,178,195). The mechanism by which CD40 protects WEHI-231 cells from BCR-induced apoptosis was not known at the onset of this thesis. There was some suggestion that CD40 may protect WEHI-231 cells from BCR-induced apoptosis by inducing the expression of *bcl-x*$_L$ (67,195,196). Constitutive expression of this *bcl-2* family member blocks BCR-induced apoptosis in WEHI-231 cells (195-197). However, the mechanism by which CD40 signaling induces *bcl-x*$_L$ expression was not known. Moreover, it was not known whether CD40 also inhibits BCR-induced signaling events that promote apoptosis.

In this report, I investigated whether BCR and CD40 signaling are integrated by mitogen-activated protein (MAP) kinases. The MAP kinases are serine/threonine protein kinases that are activated by many receptors and have been implicated in both mitogenic and apoptotic responses (103-106). There are three known MAP kinase families, the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 kinases (Fig. 1.3). The MAP kinases are activated by

dual-specificity kinases called MAP kinase kinases (MKKs) which phosphorylate both the threonine residue and the tyrosine residue in a threonine-X-tyrosine activation motif (109,110). The ERKs, JNKs, and p38 kinases are regulated by different MKKs (109,110), allowing for their independent regulation. Although MAP kinases have numerous substrates, their effects on growth regulation are likely due to their ability to phosphorylate and activate nuclear transcription factors (110,112). The ERK, JNK, and p38 kinases phosphorylate different sets of transcription factors and individual members of each family may also differ in their substrate specificities. Thus, activating different MAP kinases would allow the BCR and CD40 to have distinct effects on B cell activation and differentiation. Moreover, the MAP kinases may provide a mechanism for integrating BCR and CD40 signaling, either by dual regulation of a single MAP kinase family member or by the combinatorial effect of different MAP kinases being activated by the two receptors.

The first MAP kinases to be identified were the ERKs, of which p44 ERK1 and p42 ERK2 are the best characterized. Tyrosine kinase-linked receptors activate ERK1 and ERK2 via the Ras pathway (109,198). Activated Ras promotes the activation of the Raf kinase (199,200). Raf in turn activates MEK1 and MEK2 (201), the MKKs that phosphorylate and activate the ERKs (198). Substrates of the ERKs include the Ets domain-containing transcription factors Elk-1 (116) and Sap1 (117) (Fig. 1.3).

JNK and p38 are related MAP kinases that are activated by environmental stresses such as ultraviolet light (105,119,202) and hyperosmotic shock (203). These kinases are also activated by inflammatory cytokines such as TNFα and IL-1 (105,140,204,205), as well as by LPS (105,206). Although these stimuli activate both JNK and p38, these kinases are regulated by different MKKs, and p38 can be turned on independently of JNK (137,207). For example, p38, but not JNK, is activated in human endothelial cells that have been treated with vascular endothelial growth factor

(137). Unlike the ERKs, JNK and p38 are poorly activated by tyrosine kinase-linked receptors (119,206) (Fig. 1.3).

Ten JNK protein kinases, (also known as stress-activated protein kinases or SAPKs) have been identified. As described in the Introduction (Chapter 1) these kinases are derived by alternative splicing of the transcripts encoded by three different genes (108). The JNK1 (124) and JNK2 (208) members of the JNK family have been studied in detail. JNK2 can bind to c-Jun and phosphorylate it on serine-63 and serine-73 (208). Phosphorylation at these sites greatly increases the transcriptional activity of c-Jun (209). JNK1 can also bind c-Jun and phosphorylate these sites, but its ability to bind c-Jun is 10 to 25 times lower than that of JNK2 (204,208). Thus, *in vivo* JNK1 may primarily phosphorylate other substrates. The JNKs also bind to and activate the ATF2 transcription factor (210). Although not known at the time of this study, components of the kinase cascade that lead to activation of JNK have recently been identified and include MKK4 (also termed SEK1 or JNKK) and MKK7 (131,139) (Fig. 1.3). In addition, the small GTP-binding proteins Rac, Rho and Cdc42 appear to be upstream elements of the JNK signaling pathway that lead from receptors to the activation of MKKs (Fig. 1.3) (121-123).

Four members of the mammalian p38 family have been identified (p38α, p38β, p38δ and p38γ) (125-128). Each of these p38 kinases is encoded by a separate gene. Lee et al. (125) cloned cDNAs encoding two alternatively spliced forms of the p38α gene which they termed CSBP1 and CSBP2, both of which are related to the HOG1 kinase from *Saccharomyces cerevisiae*. The mammalian p38 kinases phosphorylate ATF2 at the same activation sites as JNK, but they do not phosphorylate c-Jun (105). In addition to ATF2, the p38 kinases phosphorylate and activate the transcription factors CHOP (138) and MEF2C (211). Another distinction between the p38 kinases and the JNKs is that the p38 kinases can phosphorylate and activate MAPKAP kinase-2 (140,141), MAPKAP kinase-3 (212) and the p38-regulated/activated protein kinase

(PRAK) (324). These serine/ threonine kinases phosphorylate a heat shock protein called Hsp25 in murine cells and Hsp27 in human cells (140,141). Hsp25/Hsp27 appears to play a role in regulating actin filament dynamics since overexpression of Hsp27 in mouse cells stabilizes microfilament organizations (213). In addition to Hsp25/Hsp27, MAPKAP kinase-2 also phosphorylates and activates the transcription factor CREB (214).

In this report, I have determined which MAP kinases are activated by the BCR and CD40. In addition, I have investigated whether any of the MAP kinases are subject to dual regulation by both the BCR and CD40.

3.2 CD40 does not activate ERKs nor does it influence BCR-induced ERK activation

CD40 may prevent BCR-induced apoptosis in WEHI-231 cells by inhibiting or altering some BCR-induced signaling event. Since the BCR activates ERK2 in WEHI-231 cells (107), I asked whether CD40 inhibited, potentiated, or prolonged BCR-induced ERK2 activation.

I first compared the ability of the BCR and CD40 to activate ERK2. Anti-IgM Abs were used to stimulate BCR signaling while the 1C10 anti-CD40 mAb was used to initiate CD40 signaling. The 1C10 mAb can prevent BCR-induced apoptosis in WEHI-231 cells (178) to the same extent as sCD40L (data not shown). After stimulating the cells for 4 min with these Abs, ERK2 was immunoprecipitated and its activity measured *in vitro* using MBP as a substrate. BCR cross-linking caused a dose-dependent increase in ERK2 activity with maximal (15- to 20-fold) activation at 20 μg/mL (Fig. 3.1*A, B*). In contrast, ligation of CD40 caused very little activation of ERK2 either at 4 min (Fig. 3.1*A, B*) or at later times (Fig. 3.1*C*). The relative ERK2 activity in 1C10-stimulated cells compared to unstimulated cells (= 1.0) was 1.36 $\pm$ 0.30 at 4 min, 1.75

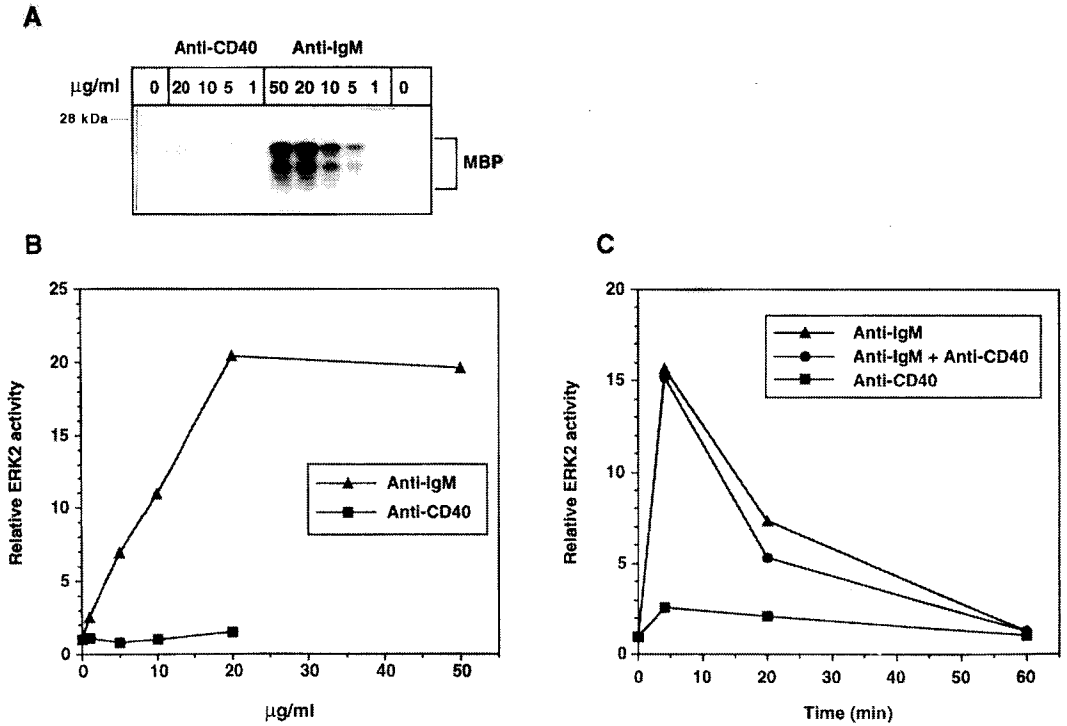


Figure 3.1: ERK2 is activated by anti-IgM but not by anti-CD40.

A and B, WEHI-231 cells were stimulated with the indicated concentrations of anti-CD40 or anti-IgM Abs for 4 min. Cell lysates were immunoprecipitated with anti-ERK2 Abs and *in vitro* kinase assays were performed using MBP as a substrate. After electrophoresis, the labeled MBP bands were visualized by autoradiography (*A*). Ponceau S-stained MBP bands were excised and counted. Relative ERK2 activity (cpm from stimulated sample/cpm from unstimulated samples) is shown in *B*. The ERK2 activity for the unstimulated samples in this experiment was 3591 ± 456 cpm (average ± range, n=2). *C,* WEHI-231 cells were stimulated with 10 µg/ml anti-CD40, 10 µg/ml anti-IgM, or both Abs for 4, 20, or 60 min. *In vitro* kinase assays for ERK2 activity were performed as in *A*. ERK2 activity in unstimulated samples was 5102 ± 450 cpm, n=2. One of three similar independent experiments is shown.

± 0.15 at 20 min, and 1.06 ± 0.02 at 60 min (mean ± SEM, n=3). Thus, ERK2 was activated to a significant extent by the BCR but not by CD40.

To determine if CD40 ligation either inhibited or prolonged BCR-induced ERK2 activation, WEHI-231 cells were treated simultaneously with anti-CD40 and anti-IgM Abs for various times. To maximize the possibility of seeing CD40 effects on anti-IgM-induced ERK2 activation, I used a dose of anti-IgM (10 μg/mL) that caused half-maximal ERK2 activation. I found that CD40 ligation did not significantly affect anti-Ig-induced ERK2 activation (Fig. 3.1*C*) at 4 min, 20 min, or 60 min. The ratio of ERK2 activity in cells treated with both anti-IgM and anti-CD40 to the ERK2 activity in cells treated with anti-IgM alone was 1.05 ± 0.04 at 4 min, 0.93 ± 0.13 at 20 min, and 1.06 ± 0.13 at 60 min (mean ± SEM, n=3). Thus, BCR-induced ERK2 activation was not affected by CD40 engagement.

Our lab has shown previously that BCR cross-linking causes very little activation of ERK1 (107). Therefore, it was possible that CD40 activated ERK1 or that it synergized with the BCR to activate ERK1. To test these models, I immunoprecipitated ERK1 from lysates of anti-IgM- and anti-CD40-stimulated cells and performed *in vitro* kinase assays. Control experiments showed that the anti-ERK1 Ab I used precipitated ERK1 but not ERK2 (data not shown). I found that incubating WEHI-231 cells with anti-IgM Abs for 4 min caused a modest (2- to 3-fold) increase in ERK1 activity (Fig. 3.2). Ligation of CD40 did not activate ERK1 at either 4 min or 20 min and did not potentiate or inhibit BCR-induced ERK1 activation (Fig. 3.2). Thus, ERK1 and ERK2 are not involved in CD40 signaling in WEHI-231 cells and do not represent a site at which CD40 modulates BCR signaling.

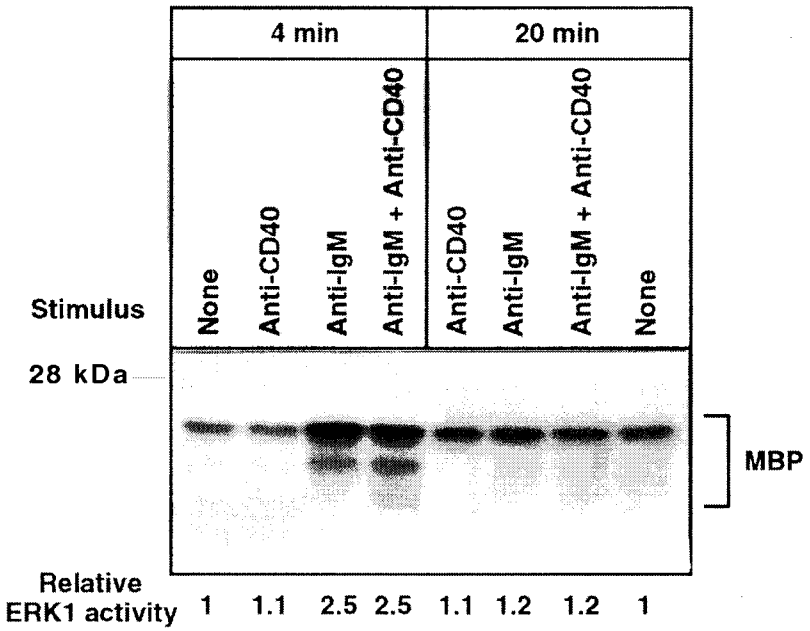


Figure 3.2: ERK1 is activated by anti-IgM but not by anti-CD40.

WEHI-231 cells were stimulated with 10 μg/ml anti-CD40, 10 μg/ml anti-IgM, or both Abs for 4 min or 20 min. Cell lysates were immunoprecipitated with anti-ERK1 Abs and *in vitro* kinase assays were performed using MBP as a substrate. The relative ERK1 activity (cpm from stimulated sample/cpm from unstimulated samples) for each sample is indicated. The ERK1 activity for the unstimulated samples in this experiment was 15,744 ± 311 cpm, n=2.

3.3 CD40 activates JNK

CD40 is structurally homologous to the TNFα and nerve growth factor (NGF) receptors (12). Since TNFα and NGF both activate JNK (119,204,215), I investigated whether CD40 also activates JNK. WEHI-231 cells were stimulated with either anti-CD40 or anti-IgM Abs and then JNK was immunoprecipitated from cell lysates. The anti-JNK Ab used was raised against JNK1, but also recognizes JNK2 to some extent. The activity of the immunoprecipitated JNK was measured in an *in vitro* kinase assay using a GST fusion protein containing the N-terminal 79 amino acids of c-Jun as substrate. JNK phosphorylates c-Jun on serine-63 and serine-73 (208).

Both the 1C10 anti-CD40 mAb and the sCD40L caused a substantial increase in JNK activity in WEHI-231 cells (Fig. 3.3). Maximal stimulation of JNK activity was observed at 15 min (see below, Fig. 3.6*A*). At this time point, 5 μg/mL 1C10 reproducibly caused a 50- to 70-fold increase in JNK activity. The sCD40L also activated JNK in a dose-dependent manner. A 1:4 dilution of tissue culture supernatant containing the sCD40L caused a 25- to 30-fold increase in JNK activity (Fig. 3.3*B*). The ability to stimulate JNK activity with both an anti-CD40 mAb and the sCD40L argues strongly that CD40 activates JNK.

BCR engagement also increased JNK activity, but much less than CD40 stimulation did. Maximal BCR-induced JNK activation (4- to 8-fold) was achieved by stimulating WEHI-231 cells with 20 to 50 μg/mL anti-IgM for 15 to 30 min (Fig. 3.3*A*). The maximal anti-IgM-induced JNK activation was always less than 15% of that caused by the 1C10 anti-CD40 mAb in the same experiment. The BCR-induced JNK activation observed in these *in vitro* kinase assays was not due to precipitation of small amounts of ERK2. The anti-JNK Ab used did not precipitate detectable amounts of ERK2, as judged by immunoblotting (data not shown). Moreover, GST-c-Jun (1-79) does not contain the site at which ERK2 phosphorylates c-Jun (216) and control experiments showed that ERK2 immunoprecipitated from anti-IgM-stimulated cells did

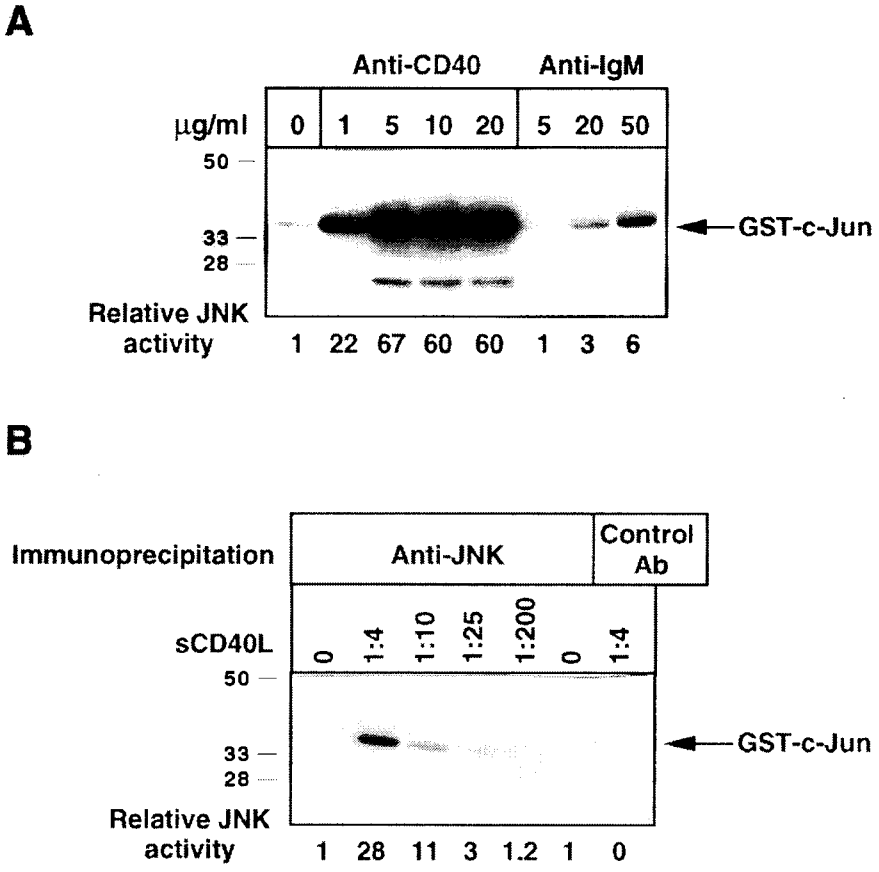


Figure 3.3: Anti-CD40 mAb and sCD40L activate JNK.

A, WEHI-231 cells were stimulated for 15 min with the indicated concentrations of anti-CD40 or anti-IgM Abs. Cell lysates were immunoprecipitated with rabbit anti-JNK1 Abs and then *in vitro* kinase assays were performed using GST-c-Jun (1-79) as a substrate. The relative JNK activity (cpm from stimulated sample/cpm from unstimulated samples) for each sample is indicated. The JNK activity for the unstimulated samples in this experiment was 604 $\pm$ 54 cpm (average $\pm$ range, n=2; note that only one unstimulated sample is shown). A control rabbit Ab did not precipitate any JNK activity (data not shown). *B*, WEHI-231 cells were stimulated for 15 min with dilutions of sCD40L-containing tissue culture supernatant. Cell lysates were immunoprecipitated with rabbit anti-JNK1 and then *in vitro* kinase assays were performed as in *A*. Relative JNK activity for each sample is indicated. The JNK activity for the unstimulated samples in this experiment was 9713 $\pm$ 345 cpm (average $\pm$ range, n=2). Molecular weight standards in kDa are indicated on the left.

not phosphorylate GST-c-Jun (1-79) (data not shown). Thus, the BCR can cause modest activation of JNK.

3.4 CD40 activates both p46 and p54 isoforms of JNK

At the time of this study it was known that p46 and p54 isoforms of both JNK1 and JNK2 existed, however, it was not known whether the p46 and p54 isoforms have different substrate specificities and, therefore, different functions (113,204,208). I used an in-gel kinase assay to determine whether CD40 activates both p46 and p54 isoforms of JNK. GST-c-Jun (1-79) was co-polymerized with the acrylamide in a gel on which anti-JNK1 immunoprecipitates were separated. After renaturing the proteins in the gel, the gel was incubated with ^{32}P-ATP. The renatured enzymes could then phosphorylate the immobilized GST-c-Jun in their immediate vicinity, revealing their molecular masses.

CD40 stimulation of WEHI-231 cells caused the appearance of two bands of 46 kDa and 54 kDa in this in-gel kinase assay (Fig. 3.4). These molecular masses are consistent with those of p46 JNK and p54 JNK. CD40 caused very strong activation of p46 JNK. The p54 JNK isoform was also activated by CD40. Activation of both JNK isoforms was evident after 5 min, was maximal at 15 min, and then declined between 15 and 30 min, consistent with the time course of JNK activation seen in standard *in vitro* kinase assays (see Fig. 3.6*A*). Longer autoradiographic exposures revealed that anti-IgM treatment of WEHI-231 cells weakly activated both p46 JNK and p54 JNK (data not shown).

The in-gel kinase assay indicated that p46 JNK is the major CD40-activated JNK isoform. However, I cannot rule out the possibility that the precipitating Ab reacts more strongly with p46 JNK than with p54 JNK. Since the immunoprecipitating Ab used was raised against JNK1, presumably the p46 and p54 bands correspond to isoforms of JNK1. However, this Ab weakly cross-reacts with JNK2 and, given

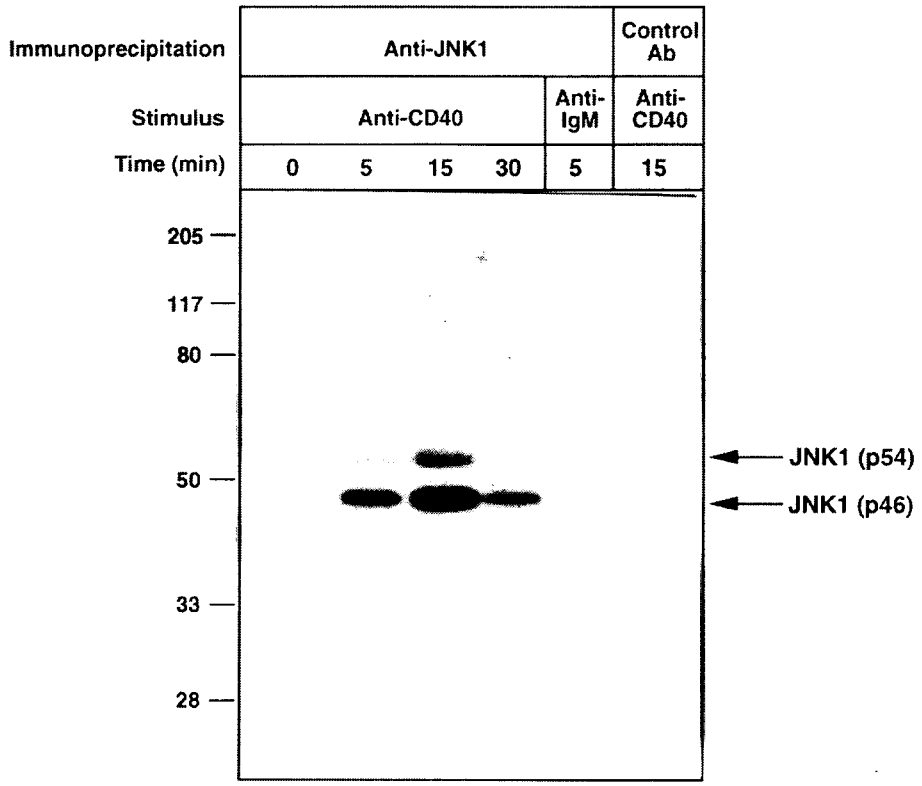


Figure 3.4: Anti-CD40 activates two isoforms of JNK1.

WEHI-231 cells were stimulated with 10 μg/ml anti-CD40 or with 20 μg/ml anti-IgM Abs for the indicated times. Cell lysates were immunoprecipitated with rabbit anti-JNK1 Abs or with a control rabbit Ab (anti-Crk II). Immunoprecipitates were separated on a 10% SDS-PAGE gel which was co-polymerized with GST-c-Jun (1-79) and an in-gel kinase assay was performed as described in the Materials and Methods. The location of immunoprecipitated kinases capable of phosphorylating immobilized GST-c-Jun (1-79) was visualized by autoradiography. Molecular mass standards in kDa are indicated to the left.

sequence similarities between JNK1 and a more recent addition to the JNK family, JNK3, this Ab may also cross-react with the p46 and p54 isoforms of JNK3.

3.5 CD40 activates JNK2

Since JNK1 and JNK2 appear to have different substrate specificities and therefore perhaps different functions (204,208), I asked whether CD40 activates both JNK1 and JNK2. To address this question, I used a GST-c-Jun (1-169) fusion protein bound to glutathione-Sepharose beads to precipitate primarily JNK2. JNK2 binds to c-Jun with 10- to 25-fold greater affinity than JNK1 (204,208). *In vitro* kinase assays were then performed in which the immobilized GST-c-Jun (1-169) also acted as the substrate. In this assay, CD40 ligation caused a 4- to 7-fold increase in the activity of kinases that bind to GST-c-Jun (1-169) (Fig. 3.5). This is in contrast to the 50- to 70-fold increases in JNK activity observed in assays in which the anti-JNK1 Ab was used. The simplest interpretation of these data are that the anti-JNK1 Ab preferentially precipitated JNK1, the GST-c-Jun (1-169) preferentially precipitated JNK2, and CD40 activated JNK1 to a greater extent than JNK2. The BCR also activated JNK1 to a greater extent than JNK2. While anti-IgM caused a 4- to 8-fold increase in the activity of JNK isoforms that bound to the anti-JNK1 Ab, it caused very little (1.5- to 2-fold) activation of JNK isoforms that bound to the GST-c-Jun (1-169) fusion protein. Thus, if the assays used to measure JNK1 and JNK2 activities are comparable, both CD40 and the BCR appear to activate JNK1 to a greater degree than JNK2.

3.6 Anti-IgM potentiates CD40-induced JNK activation

In T cells, significant activation of JNK requires that both the TCR and CD28 be engaged (175). Since CD40 is a co-stimulatory receptor for B cells, analogous to CD28 in T cells, I asked whether BCR signaling could potentiate or prolong CD40-induced JNK activation. To facilitate this analysis, I first determined the kinetics of

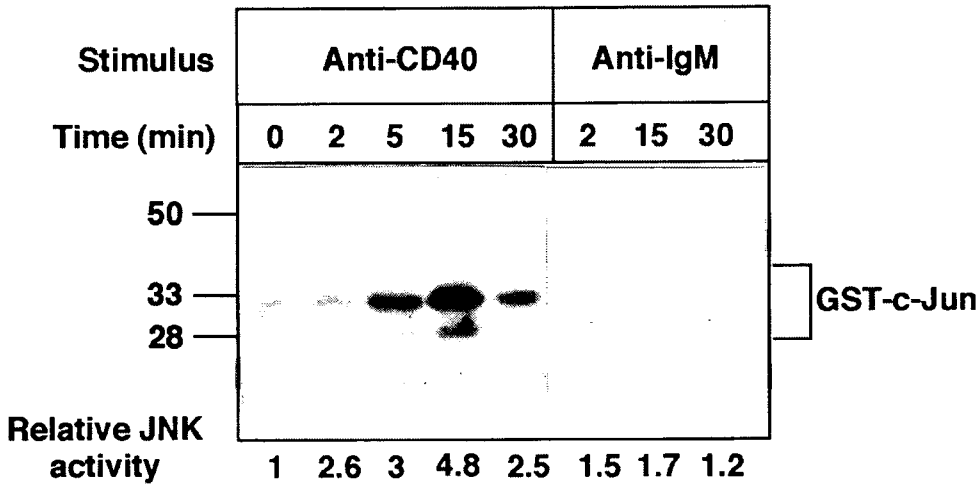


Figure 3.5: Anti-CD40 activates Jun kinases that bind to GST-c-Jun (1-169).

WEHI-231 cells were stimulated for the indicated times with 10 μg/ml anti-CD40 or with 20 μg/ml anti-IgM Abs. Cell lysates were incubated with a GST-c-Jun (1-169) fusion protein immobilized on glutathione-Sepharose beads. *In vitro* kinase assays were then performed in which the GST-c-Jun (1-169) also acted as the substrate. The relative JNK activity for each sample is indicated. Molecular mass standards in kDa are indicated to the left.

CD40-induced JNK activation (Fig. 3.6*A*). JNK activity increased 10- to 25-fold within 5 min of adding 5 μg/mL anti-CD40 to the cells, reached peak levels (50- to 70-fold activation) at 15 min, and declined sharply by 30-60 min. I then analyzed JNK activation when CD40 and the BCR were engaged simultaneously. The combined effects of anti-CD40 and anti-IgM were somewhat more than additive. The JNK activity in cells treated with both anti-IgM and anti-CD40 for 15 min was 40-60% greater than the arithmetic sum of the JNK activity in cells treated with anti-IgM alone and with anti-CD40 alone. Thus, BCR engagement in some way potentiates CD40-induced JNK activation. While anti-IgM increased the magnitude of CD40-induced JNK activation, it did not prolong the activation of JNK by CD40.

In Fig. 3.6*A*, the cells were stimulated with a concentration of the 1C10 anti-CD40 mAb that caused maximal JNK activation. It was possible that potentiation of CD40-induced JNK activation by anti-IgM would be greater at sub-optimal concentrations of 1C10. In Fig. 3.6*B*, WEHI-231 cells were stimulated for 15 min with varying concentrations of 1C10 in the presence or absence of 20 μg/mL anti-IgM. The effect of anti-IgM on CD40-induced JNK activation was somewhat more than additive (40-80% greater than the arithmetic sum of the effects caused by anti-CD40 alone and anti-IgM alone) at all concentrations of anti-CD40 tested, but was slightly more pronounced at suboptimal concentrations (e.g. 1 μg/mL).

3.7 CD40 activates p38

The p38 MAP kinase family appears to have a distinct function in the cell, as its substrate specificity differs from members of the ERK and JNK MAP kinase families. Therefore, I asked whether CD40 activates p38. Since tyrosine phosphorylation is required for activation of all MAP kinases (110), I immunoprecipitated p38 with a rabbit Ab that was raised against the CSBP2 isoform of p38α and then performed anti-phosphotyrosine blots. I found that CD40 ligation stimulated p38 tyrosine

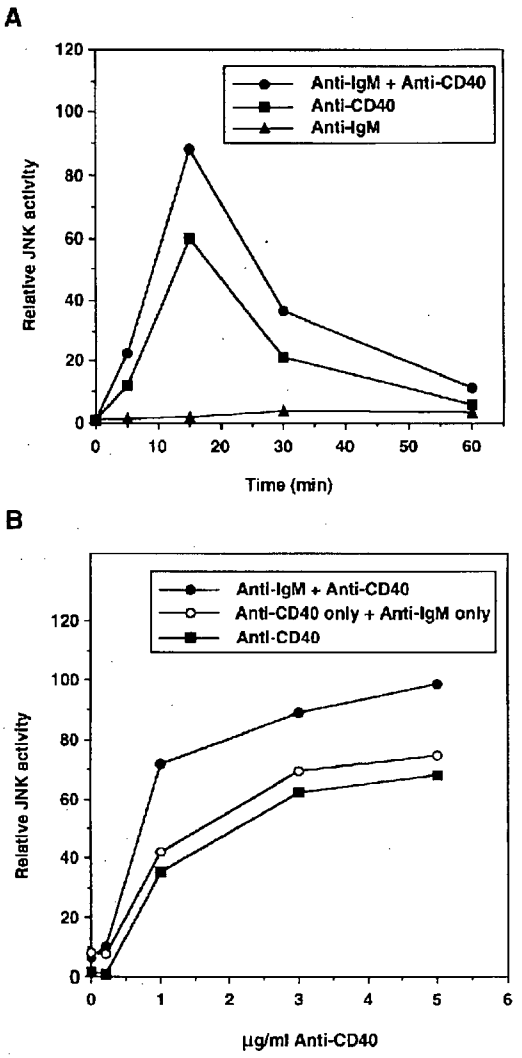


Figure 3.6: Anti-IgM potentiates CD40-stimulated JNK activation.

A, WEHI-231 cells were stimulated with 5 µg/ml anti-CD40, 20 µg/ml anti-IgM, or both Abs for 5, 15, 30 or 60 min. Cell lysates were immunoprecipitated with rabbit anti-JNK1 and *in vitro* kinase assays were performed as in Fig. 3.3. Relative JNK activity for each sample is indicated. The JNK activity for the unstimulated samples in this experiment was 903 ± 186 cpm (average ± range, n=2). *B,* WEHI-231 cells were stimulated for 15 min with the indicated concentrations of the 1C10 anti-CD40 mAb in the presence or absence of 20 µg/ml anti-IgM. Cell lysates were immunoprecipitated with rabbit anti-JNK1 and *in vitro* kinase assays were performed. Relative JNK activity for each sample is indicated. The JNK activity for the unstimulated samples in this experiment was 13,899 ± 974 cpm (average ± range, n=2). The open circles represent the sum of the increases in JNK activity caused by anti-CD40 alone and by anti-IgM alone.

phosphorylation with maximal phosphorylation at 5 to 15 min (Fig. 3.7). BCR ligation also stimulated p38 tyrosine phosphorylation, but not as much as CD40 engagement did (Fig. 3.7).

To directly test whether CD40 and the BCR activated p38, *in vitro* kinase assays were performed on anti-p38 immunoprecipitates using a GST fusion protein containing the N-terminal 96 amino acids of ATF2 (GST-ATF2) as a substrate. GST-ATF2 contains the two residues (threonine-69 and threonine-71) phosphorylated by p38 (105). CD40 cross-linking caused a dose-dependent increase in p38 activity with maximal (4- to 8-fold) activation induced by 1-5 μg/mL anti-CD40 (Fig. 3.8*A*, *D*). p38 activity increased 2-fold within 2 min of adding 5 μg/mL anti-CD40 to the cells, was maximal at 15 min, and then declined (Fig. 3.8*C*). BCR ligation caused a modest (2- to 3-fold) activation of p38 which was maximal when WEHI-231 cells were stimulated with 20 μg/mL anti-IgM for 15 min (Fig. 3.8 *B,C*). A higher concentration of anti-IgM (50 μg/mL) did not cause significantly greater activation of p38. The maximum anti-IgM-induced p38 activation was always less than 25% of that induced by anti-CD40 in the same experiment. Thus, p38 was activated by CD40, and to a lesser extent by the BCR.

To determine if CD40 and the BCR caused synergistic activation of p38, WEHI-231 cells were stimulated with varying concentrations of the 1C10 anti-CD40 mAb in the presence or absence of 20 μg/mL anti-IgM. I found that the simultaneous engagement of CD40 and the BCR had a roughly additive effect at 15 min (Fig. 3.8*D*), when p38 activation was maximal, and at 30 min when the response was declining (data not shown). Thus, BCR signaling did not inhibit, potentiate or prolong CD40-induced p38 activation.

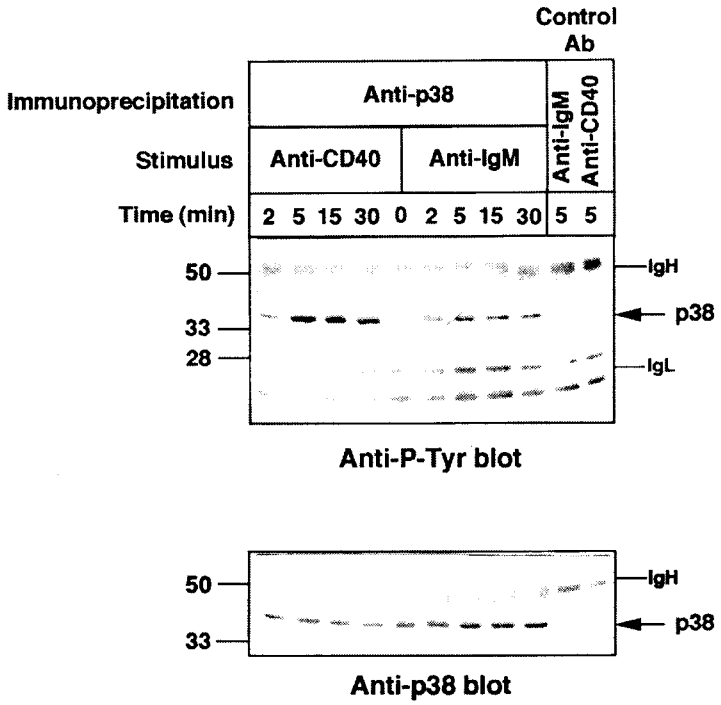


Figure 3.7: Anti-CD40 mAb induces tyrosine phosphorylation of p38.

WEHI-231 cells were stimulated with with 10 μg/ml anti-CD40 or 20 μg/ml anti-IgM for the indicated times. Cell lysates were immunoprecipitated with anti-p38 Abs or with a control rabbit Ab (anti-Crk II). Immunoprecipitates were separated on a 11% SDS-PAGE gel and then transferred to nitrocellulose. Anti-phosphotyrosine blots *(top panel)* were performed to detect tyrosine phosphorylated p38 as described in the Materials and Methods. The blots were reprobed with anti-p38 mAb *(lower panel)* to ensure that similar amounts of p38 were present in each lane. Molecular mass standards in kDa are indicated to the left of each panel. IgH and IgL to the right of each panel refer to the heavy and light chains, respectively, of the immunoprecipitating Ab. This is a representative experiment of three similar independent experiments.

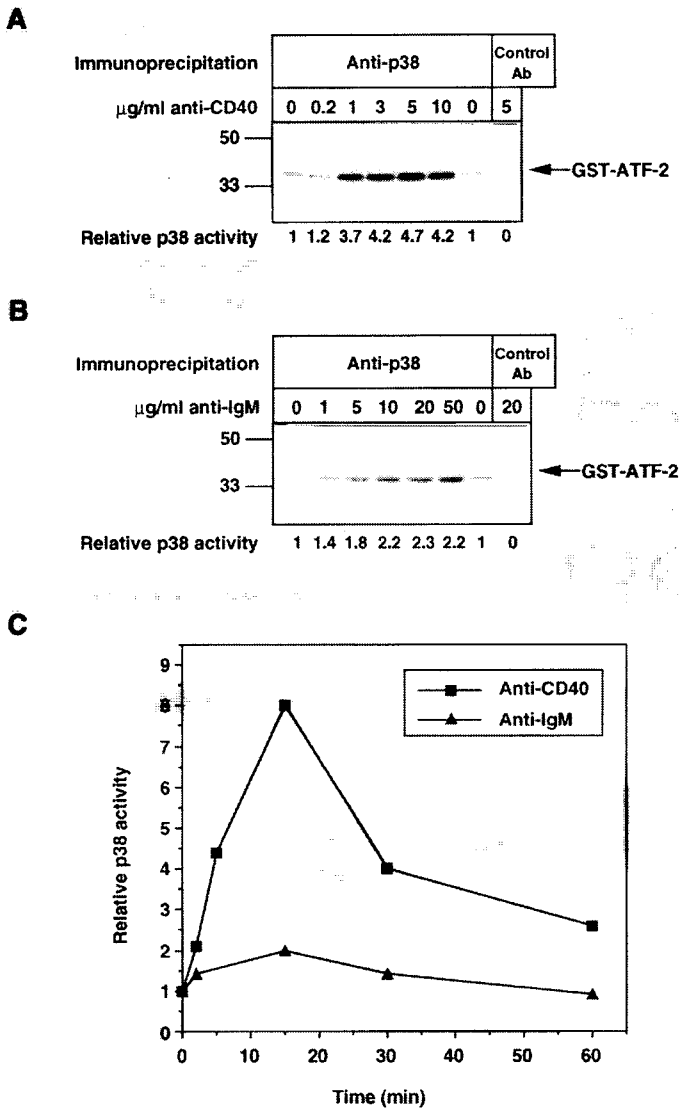


Figure 3.8 A-C: Activation of p38 by anti-CD40 and anti-IgM.

A and B, WEHI-231 cells were stimulated for 15 min with the indicated concentrations of anti-CD40 (*A*) or anti-IgM (*B*) Abs. Cell lysates were immunoprecipitated with anti-p38 Abs or with a control rabbit Ab (anti-Crk II) and then *in vitro* kinase assays were performed using GST-ATF-2 as a substrate. Phosphorylation of GST-ATF-2 was quantitated with a phosphorimager. The relative p38 activity for each sample is indicated. Note that *A* and *B* represent independent experiments. Molecular mass standards in kDa are indicated to the left of each panel. *C*, WEHI-231 cells were stimulated with 10 μg/ml anti-CD40 or 20 μg/ml anti-IgM for the indicated times. *In vitro* kinase assays were performed on anti-p38 immunoprecipitates as in *A*.

D

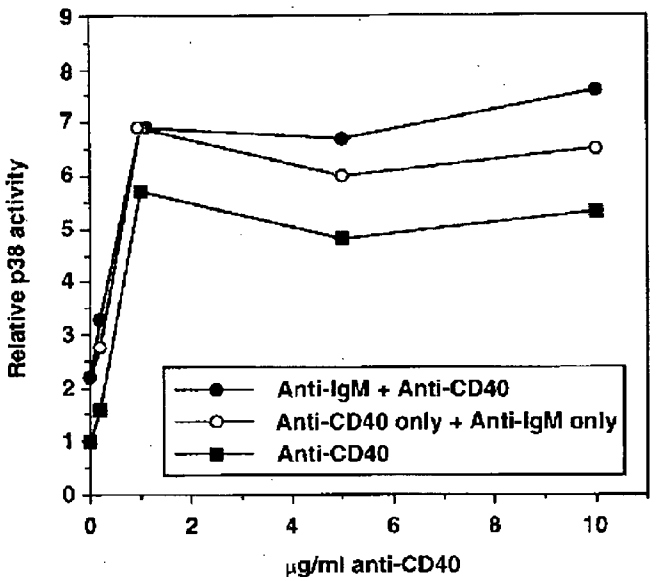


Figure 3.8 D: Activation of p38 by anti-CD40 and anti-IgM.

D, WEHI-231 cells were stimulated for 15 min with the indicated concentrations of the 1C10 anti-CD40 mAb in the presence or absence of 20 µg/ml anti-IgM. Anti-p38 *in vitro* kinase assays were performed as in *A*. The open circles represent the sum of the increases in p38 activity caused by anti-CD40 alone and by anti-IgM alone.

3.8 CD40 activates MAPKAP kinase-2

To extend my findings on the p38 pathway, I asked whether CD40 also activated MAPKAP kinase-2, a downstream target of p38. MAPKAP kinase-2 was immunoprecipitated from cell lysates and in *vitro* kinase assays were performed using recombinant Hsp25 as a substrate. I found that CD40 caused a 10- to 15-fold increase in MAPKAP kinase-2 activity, with maximal activation at 15 min (Fig. 3.9). Thus, the time course of CD40-induced MAPKAP kinase-2 activation was similar to that for activation of p38 by CD40. In contrast to CD40, 20 μg/mL anti-IgM caused only a 2- to 3-fold increase in MAPKAP kinase-2 activity, consistent with the weak activation of p38 by the BCR. While 50 μg/mL anti-IgM caused a slightly larger increase in MAPKAP kinase 2-activity in some experiments (e.g. Fig. 3.9*A*), this increase was always less than 20% of that caused by CD40 in the same experiment. Finally, the effect of simultaneously engaging CD40 and the BCR on MAPKAP kinase-2 activity was only slightly more than additive (Fig. 3.9*A*). This is consistent with my observation that CD40 and the BCR did not cause synergistic activation of p38 (Fig. 3.8*D*).

3.9 CD40-induced activation of MAPKAP kinase-2 is dependent on p38

p38 activity has been shown to be required for the activation of MAPKAP kinase-2 in response to IL-1, cellular stresses and bacterial endotoxin (140). To determine whether CD40-induced activation of MAPKAP kinase-2 is entirely dependent on p38 MAP kinase activity, I determined whether a specific inhibitor of p38 MAP kinase, SB 203580 (140), blocked the ability of CD40 to activate MAPKAP kinase-2 in WEHI-231 cells. I found that pretreatment of WEHI-231 cells with SB 203580 completely blocked the ability of CD40 to activate MAPKAP kinase-2 (Fig. 3.10 A) but had no effect on the ability of CD40 to activate JNK (Fig. 3.10 B). These results demonstrate that CD40-induced activation of MAPKAP kinase-2 is entirely dependent on p38 MAP kinase activity.

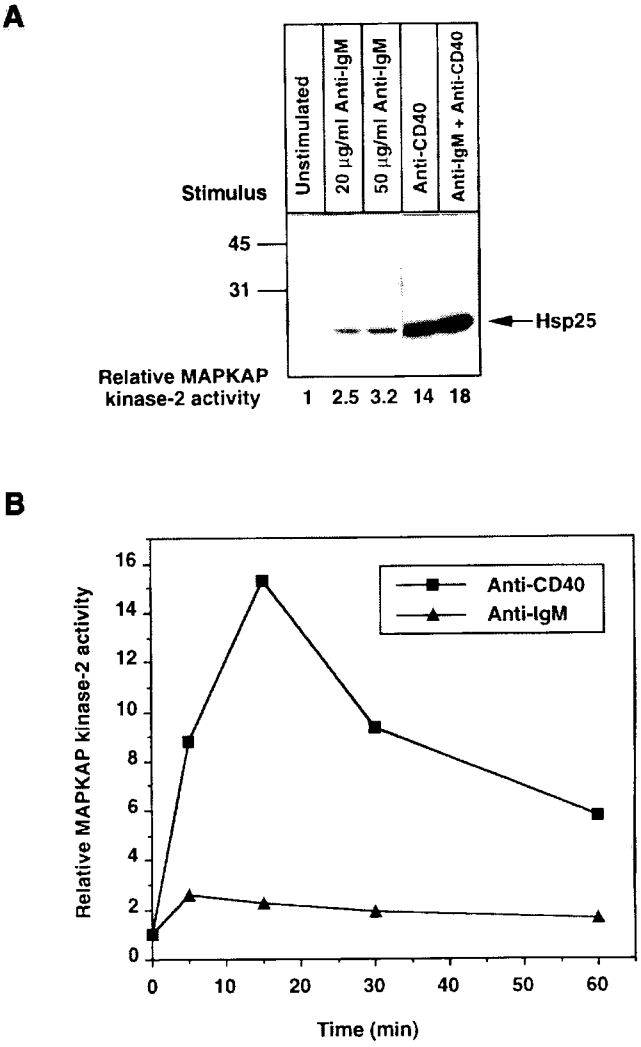


Figure 3.9: CD40 activates MAPKAP kinase-2.

A, WEHI-231 cells were stimulated for 15 min with 20 or 50 μg/ml anti-IgM, 5 μg/ml of the 1C10 anti-CD40 mAb, or the combination of 20 μg/ml anti-IgM and 5 μg/ml 1C10. Cell lysates were immunoprecipitated with the anti-MAPKAP kinase-2 Ab and *in vitro* kinase assays were performed using Hsp25 as a substrate. Phosphorylation of Hsp25 was quantitated with a phosphorimager. The relative MAPKAP kinase-2 activity for each sample is indicated. Molecular mass standards in kDa are indicated to the left. *B*, WEHI-231 cells were stimulated for the indicated times with 20 μg/ml anti-IgM or 5 μg/ml of the 1C10 anti-CD40 mAb. Anti-MAPKAP kinase-2 *in vitro* kinase assays were performed as in *A*. One of three similar independent experiments is shown.

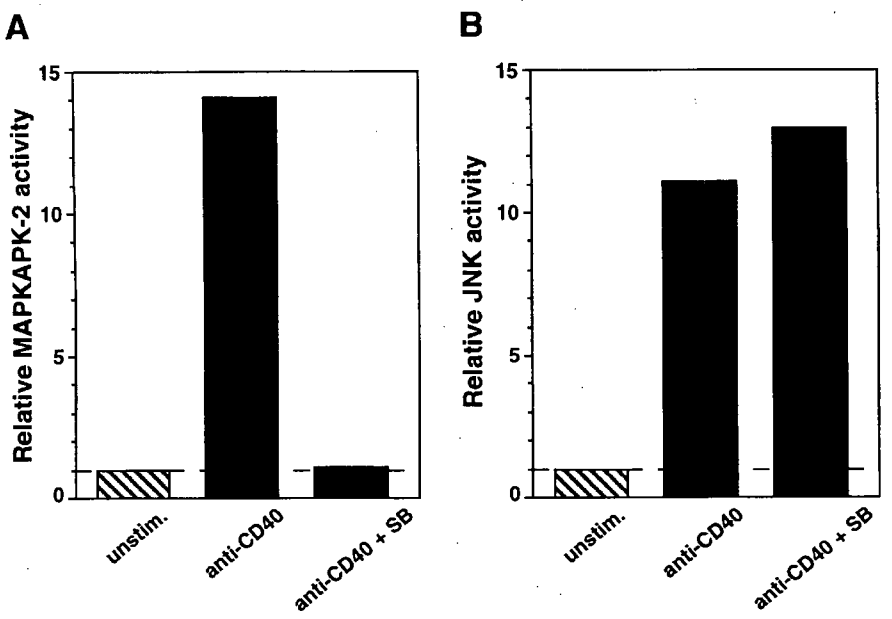


Fig. 3.10: CD40-induced activation of MAPKAP kinase-2 is dependent on p38.

WEHI-231 cells were pretreated with 50 μM SB 203580 (p38 inhibitor) for 30 min and then stimulated with 5 μg/mL anti-CD40 Ab for 15 min. *A*, Cell lysates were immunoprecipitated with anti-MAPKAP kinase-2 Ab, and *in vitro* kinase assays were performed using Hsp25 as a substrate. Phosphorylation of Hsp25 was quantitated with a PhosphorImager. The relative MAPKAP kinase-2 activity for each sample is indicated. *B*, Cell lysates were immunoprecipitated with rabbit anti-JNK1, and *in vitro* kinase assays were performed as described in Figure 3.3. The relative JNK activity for each sample is indicated.

3.10 Discussion

3.10.1 MAP kinase activation by the BCR and CD40

I have investigated the regulation of MAP kinases by the BCR and CD40 in WEHI-231 cells. I found that the BCR strongly activated ERK2 and weakly stimulated ERK1, JNK, and p38. In contrast, CD40 did not activate ERK1 or ERK2 but caused a very large increase in JNK activity as well as an increase in p38 activity. By activating different MAP kinases, the BCR and CD40 can direct the phosphorylation of different substrates and regulate the activity of different transcription factors.

Although ERK1 and ERK2 are both expressed at high levels in WEHI-231 cells, only ERK2 was strongly activated by the BCR. The basis for this selective activation is not known. The MKK that phosphorylates and activates the ERKs is regulated by the Raf kinase (Fig. 3.11). Activation of Raf by the BCR is mediated by activation of both Ras and PKC (107,217-219).

In contrast to the ERKs, I found that CD40 strongly activated JNK and p38 as well as MAPKAP kinase-2, a downstream target of p38 (140,141) that may regulate actin filament dynamics (220). During the progress of this study, Sakata et al. (221) and Berberich et al. (222) reported that CD40 activates JNK. However, this was the first report that CD40 activates p38 and MAPKAP kinase-2. My data suggest that CD40 activates JNK1 very strongly and JNK2 to a lesser degree. JNK2 binds to c-Jun with 10 to 25 times higher affinity than JNK1 (204,208) and may be the major c-Jun kinase *in vivo*. Consistent with this idea, overexpressing JNK2, but not JNK1, increases transcription of a c-Jun-dependent reporter gene in HeLa cells (208). JNK1 may be more similar in function to p38 since a *hog1* deletion in yeast can be complemented by JNK1 but not JNK2 (204).

The mammalian p38 kinases include p38α (CSBP1 and CSBP2 isoforms) (125), p38β (126), p38δ (127) and p38γ (128). The p38 kinases differ in their substrate specificities and thus likely have different functions. All four p38 homologues are

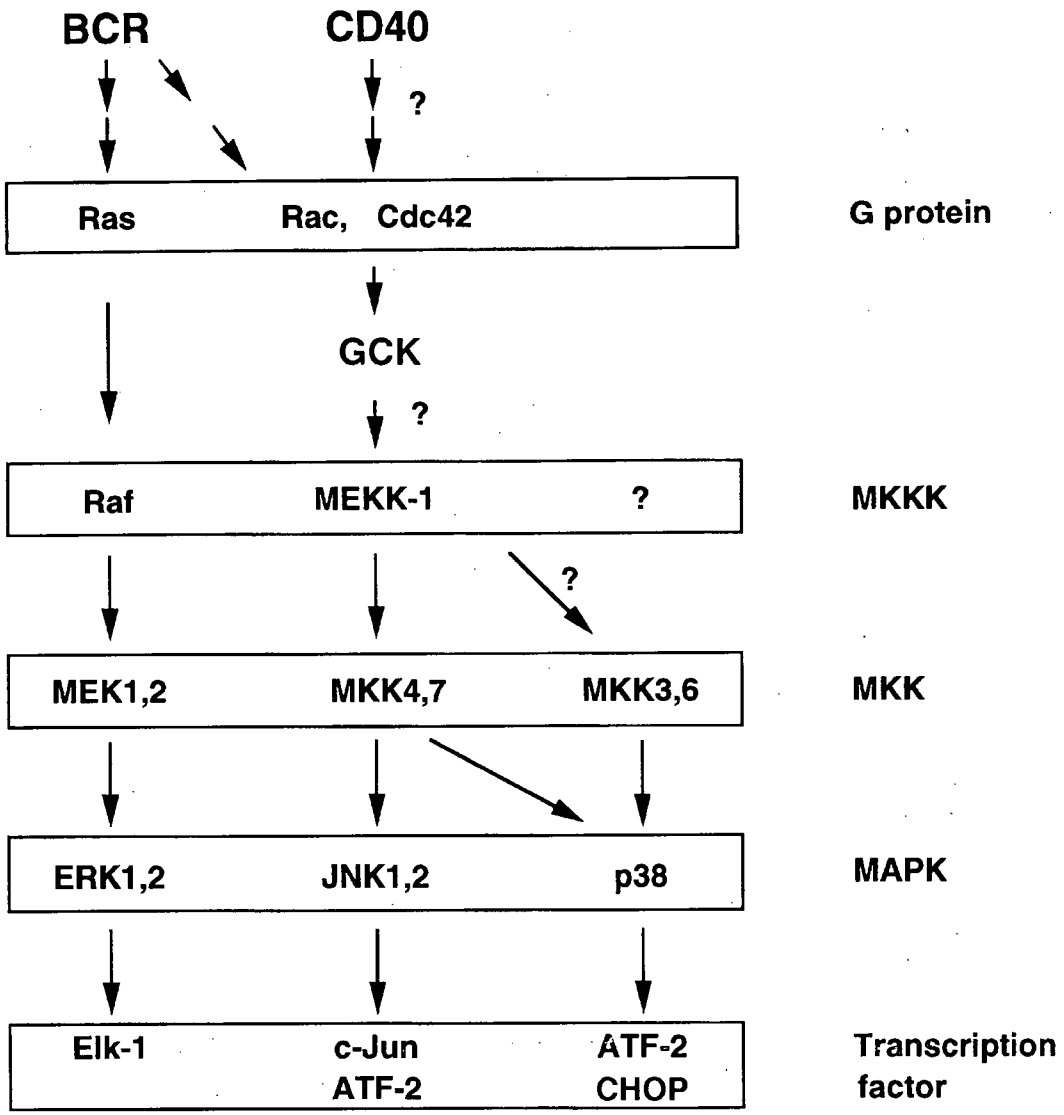


Figure 3.11: Proposed scheme for the regulation of MAP kinases by the BCR and CD40. See the discussion for details.

capable of phosphorylating ATF2, however, only p38α and p38β induce significant phosphorylation of MAPKAP kinase-2 and MAPKAP kinase-3 (223). In addition, only p38α and p38β are sensitive to the p38 inhibitor, SB 203580 (223,224). Although the anti-p38 Ab used in this study was raised against CSBP2, I cannot rule out the possibility that this polyclonal Ab cross-reacts with the other p38 kinases. Thus, I cannot be sure which p38 kinase was responsible for phosphorylating ATF2, and whether it was p38α or p38β that activated MAPKAP kinase-2. Homologue-specific Abs are needed to determine which p38 kinases are activated by CD40.

I found that BCR ligation in WEHI-231 cells caused a 4- to 8-fold increase in JNK activity and a small increase in p38 activity. This is the first report of BCR-induced activation of JNK. In contrast to my results in WEHI-231 cells, Sakata et al. (221) reported that BCR cross-linking does not activate JNK in the Ramos human B cell line or in human tonsillar B cells. This could reflect differences in the activation state or differentiation stage of the cells. Alternatively, it may reflect the type of JNK *in vitro* kinase assay used. Sakata et al. (221) immunoprecipitated c-Jun kinases with GST-c-Jun which preferentially binds JNK2. Using this assay, I also found that the BCR caused very little activation of JNK2. However, when I used the anti-JNK1 Ab, I was able to detect significant JNK1 activation by the BCR. Thus, the BCR may be able to increase the transcriptional activity of c-Jun and ATF-2 to a small extent. However, the much higher levels of JNK and p38 activation caused by CD40 may be required to induce significant changes in the activity of these transcription factors. The ability of the BCR and CD40 to phosphorylate and thus activate c-Jun and ATF2 could be tested by immunoblotting with phospho-JNK and phospho-ATF2-specific Abs that have recently become commercially available.

In contrast to my findings in WEHI-231 cells, Li et al. (225) and Purkerson et al. (226) reported that CD40 ligation results in activation of ERK as well as JNK in resting murine splenic B cells. This discrepancy on the ability of CD40 to activate ERK could

reflect differences in the activation state or differentiation stage of the cells. Both Ras-dependent and PKC-dependent pathways contribute to the activation of ERK by the BCR (1). The mechanism by which CD40 activates ERK in splenic B cells remains to be determined but is likely mediated by Ras and not PKC. This hypothesis is based on the finding that Ras is activated in response to CD40 engagement on Daudi B cells (227). Furthermore, PKC inhibitors have no effect on CD40-mediated activation of ERK in resting murine splenic B cells (225).

3.10.2 Signaling pathways that link the BCR and CD40 to JNK and p38

The signaling pathways that link CD40 and the BCR to activation of JNK and p38 are only partly understood (Fig. 3.11). The dual specificity kinase MKK4 phosphorylates and activates JNK, and to a lesser extent, p38 (207,228,229). In contrast, MKK7 selectively activates JNK (230), whereas MKK3 and MKK6 selectively activate p38 (137,207). While this indicates that JNK and p38 can be regulated independently, most stimuli that activate p38 also activate JNK. The MKKs are phosphorylated and activated by serine/threonine kinases called MEKKs. Monomeric G proteins are upstream of MEKKs (231) and appear to be responsible for the ability of the BCR to activate JNK. A recent study demonstrated that a dominant negative form of Rac1 (Rac1N17) markedly inhibits both JNK and p38 activation after BCR-crosslinking on DT40 chicken cells, whereas expression of RasN17 had no effect (219). These results indicate that, at least in DT40 cells, Rac1 but not Ras is required for activation of JNK and p38 by the BCR. The finding that BCR-induced activation of JNK and p38 is also abolished in phospholipase C-γ2 (PLC-γ2)-deficient DT40 cells indicates that in addition to Rac1, a PLC-γ2 signal appears to be required for activation of JNK and p38 by the BCR (219).

It remains to be determined whether in WEHI-231 cells CD40 stimulates MEKK-1 or activates JNK via a different MEKK isoform such as MEKK2 or MEKK3 (Fig. 3.11).

In either case, a monomeric G protein is likely to link CD40 to the MEKKs. Stimulation of Daudi B cells with anti-CD40 Ab induces activation of both Ras and Rac1 (227). Furthermore, the TNFα receptor, a member of the same superfamily as CD40, appears to be linked to JNK by the Rac and Cdc42 G proteins. Dominant negative versions of Rac and Cdc42 block JNK activation by TNFα (121,122,232). Future work could investigate whether dominant negative versions of Rac block CD40-stimulated JNK activation.

Rac and Cdc42 do not activate MEKK directly, but via serine/threonine kinases called p21-PAKs which bind to activated Rac or Cdc42 (122,134). Pombo et al. (233) have identified two p21-activated kinases (PAKs) that are highly expressed in B cells. Interestingly, one of them, GCK, mediates TNFR1-induced activation of JNK but not p38 (233,234). It remains to be determined whether CD40 activates JNK via GCK and p38 via another PAK.

3.10.3 Dual regulation of the MAP kinases by the BCR and CD40

In addition to determining which MAP kinases were activated by the BCR and CD40, I investigated whether any of these enzymes were regulated by both receptors. Dual regulation of a single MAP kinase by two receptors is seen in T cells where the TCR and CD28 each cause very little JNK activation by themselves but together cause a very large increase in JNK activity (175). While BCR signaling potentiated CD40-induced JNK activation in WEHI-231 cells, this may not be biologically significant since CD40 causes a very large increase in JNK activity by itself. Future work could test whether JNK activation in resting B cells from mouse spleen shows greater synergy between CD40 and the BCR. It is possible that activation of JNK by CD40 requires that cells be primed in some way and that this priming signal is always present in a proliferating cell line like WEHI-231. Consistent with this notion, Berberich et al. (222) found that CD40 ligation failed to stimulate JNK activity in tonsillar B cells from some

individuals unless the cells were first treated with phorbol esters, IL-4, or anti-IgM Abs. In contrast to JNK, the effect of simultaneously engaging the BCR and CD40 in WEHI-231 cells was simply additive for p38 activation. Moreover, CD40 ligation had no effect on BCR-induced ERK2 activation. Thus, it is likely that signals from the BCR and CD40 are integrated at the level of transcription factors as opposed to dual regulation of a single MAP kinase.

Activating different MAP kinases allows the BCR and CD40 to control the activity of different transcription factors and thereby exert different effects on B cell activation and differentiation. ERK2 activation by the BCR should result in phosphorylation of Elk-1 (112) (Fig. 3.10). Phosphorylation of Elk-1 that is complexed with serum response factor stimulates transcription of genes such as c-*fos* whose promoter contains a serum response element (SRE) (235,236). BCR engagement has been shown to induce c-*fos* transcription in WEHI-231 cells and in murine splenic B cells (237) (Fig. 3.12). CD40, which does not activate ERKs, does not upregulate c-*fos* expression or promote transcription of SRE-dependent reporter genes (222). Instead, activation of JNK and p38 by CD40 should lead to phosphorylation and activation of c-Jun and ATF2 (112,238) (Fig. 3.12). c-Jun/ATF2 heterodimers bind to the c-*jun* promoter. Phosphorylation of both members of this complex stimulates c-*jun* transcription (112). The activation of different MAP kinases by the BCR and CD40 not only allows these two receptors to regulate different transcription factors but also provides a mechanism by which the combination of BCR and CD40 signaling can have unique synergistic effects by promoting the formation of active AP-1 complexes (Fig. 3.12). CD40-induced c-Jun synthesis, coupled with BCR-induced c-Fos production, would result in increased formation of c-Fos/c-Jun AP-1 complexes which strongly activate the transcription of a number of genes (239). Moreover, CD40-mediated phosphorylation of c-Jun would further increase AP-1 activity (240).

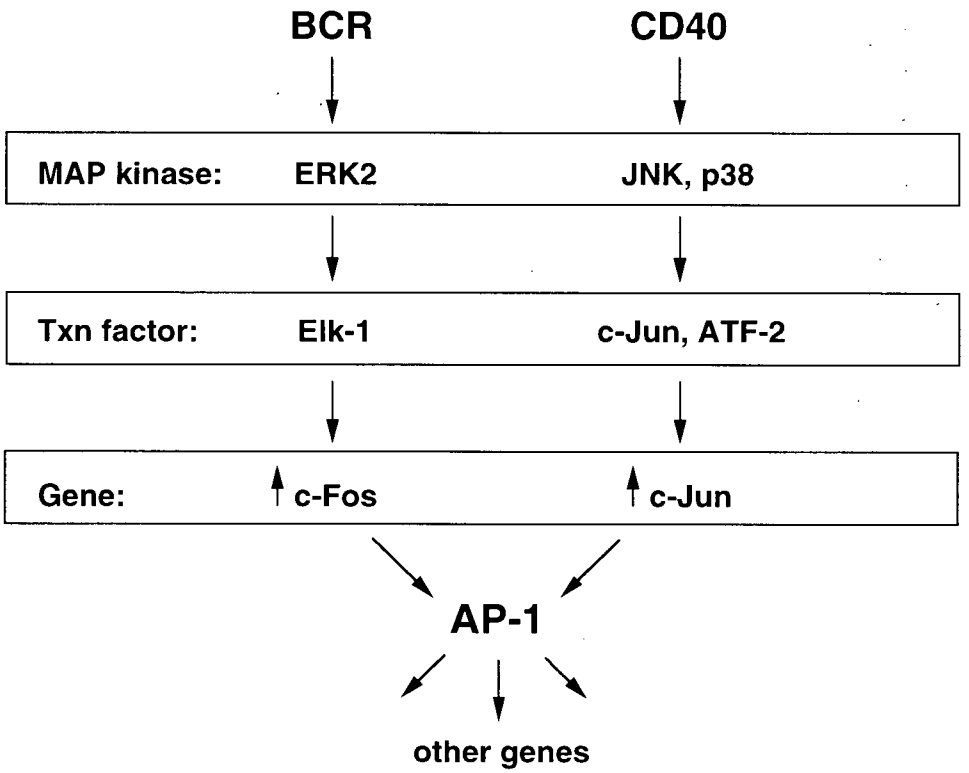


Fig. 3.12 MAP kinases can integrate BCR and CD40 signaling.

3.10.4 Possible roles of the MAP kinases in BCR-induced apoptosis and CD40-mediated survival

In WEHI-231 cells, BCR signaling causes programmed cell death which can be overcome by CD40 ligation. Thus, activation of ERK2 correlates with apoptosis whereas strong activation of all three MAP kinases correlates with survival and continued proliferation. While there is no direct evidence that MAP kinases regulate apoptosis in WEHI-231 cells, it should be possible to test this hypothesis by manipulating the upstream activators of the MAP kinases and selectively activating or inhibiting either ERK, JNK, or p38. It is possible that other signaling events are responsible either wholly or in part for the regulation of apoptosis in WEHI-231 cells.

While ERK activation correlates with apoptosis and JNK/p38 activation correlates with survival in WEHI-231 cells, the reverse is true in PC-12 cells. Apoptosis of PC-12 cells caused by NGF withdrawal correlates with activation of JNK and p38 (106). Moreover, activating JNK or p38 by overexpressing upstream activators of these kinases causes apoptosis. In contrast, apoptosis due to NGF withdrawal can be prevented by expressing a constitutively-active form of the MKK that activates ERKs. Thus, in PC-12 cells, ERK activation promotes survival whereas JNK/p38 activation causes apoptosis. This supports the idea that MAP kinases regulate both cell proliferation and apoptosis, but suggests that the relative roles of ERK, JNK, and p38 differ depending on the cell type.

In summary, I have shown that CD40 strongly activates JNK and p38 whereas the BCR activates primarily ERK2. By phosphorylating different substrates and regulating different transcription factors, ERK, JNK, and p38 may account for the distinct effects of BCR and CD40 signaling on B cells and may also provide a means by which the two receptors synergistically regulate B cell activation and differentiation. However, the role of MAP kinases in regulating BCR-induced apoptosis and CD40-mediated protection from BCR-induced apoptosis remains to be determined.

CHAPTER 4

An 11 Amino Acid Sequence in the Cytoplasmic Domain of CD40 is Sufficient for Activation of JNK and MAPKAP kinase-2, Phosphorylation of IκBα, and Protection of WEHI-231 Cells from BCR-Induced Growth Arrest

4.1 Introduction

CD40 is a receptor on B cells that regulates proliferation, survival, Ig class switching, and memory cell formation (92). The ligand for CD40 (CD40L) is expressed on activated $CD4^+$ T cells (48). The essential role of the CD40/CD40L interaction in the development of humoral immunity is exemplified by X-linked hyper-IgM syndrome. B lymphocytes from patients with this immunodeficiency disease fail to undergo Ig class switching and do not form germinal centers (241,242). Similarly, mice lacking CD40 or CD40L are unable to generate secondary humoral immune responses to T cell-dependent Ags (243,244).

Engagement of CD40 by CD40L or anti-CD40 Abs activates multiple signaling pathways including the kinase cascade that activates NF-κB, the JAK3/STAT3 pathway, the phosphatidylinositol 3-kinase pathway, and the kinase cascades that lead to activation of the ERK, JNK, and p38 mitogen-activated protein (MAP) kinases (95,96,221,222,245,246). The roles of individual signaling pathways in mediating the effects of CD40 on B cells for the most part remain to be elucidated.

MAP kinases are key signaling intermediates that have been implicated in both mitogenic and apoptotic responses to receptor signaling (208). Upon activation, MAP kinases translocate to the nucleus where they phosphorylate and activate transcription factors. The three families of MAP kinases, the ERK, JNK, and p38 MAP kinases each phosphorylate and activate a different set of transcription factors. The ERKs

phosphorylate ETS domain-containing transcription factors such as Elk-1; JNK phosphorylates c-Jun and ATF2; and p38 MAP kinase phosphorylates ATF2, MEF2C, and CHOP (138,208,210,211). p38 MAP kinase also phosphorylates and activates MAPKAP kinase-2 (140,141), a serine/threonine kinase whose targets include the Hsp25 heat shock protein and the CREB transcription factor (214).

I have previously shown that CD40 activates the JNK and p38 MAP kinases as well as MAPKAP kinase-2 in WEHI-231 B lymphoma cells (245). The mechanism by which CD40 activates these kinases is not completely understood. JNK and p38 are activated by dual specificity kinases called MAP kinase kinases (MKKs), which phosphorylate both the threonine and tyrosine residue in a conserved threonine-X-tyrosine activation motif (109). The MKKs that phosphorylate JNK and p38 are activated by upstream kinases which are regulated by the Rac and Cdc42 GTPases (122,232). Several MKKs can phosphorylate both JNK and p38 and many stimuli activate both of these MAP kinases (105), indicating that activation of JNK and p38 reflects the bifurcation of a single pathway. However, some MKKs preferentially activate only JNK (247,248) or only p38 (137) and certain stimuli can activate p38 without the concomitant activation of JNK (129,130). This raises the possibility that CD40 could use distinct pathways to activate JNK and p38.

CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily and has no intrinsic enzymatic activity. This suggests that CD40 interacts with adapter proteins that couple it to signaling pathways. Indeed, four members of the TNFR associated factor (TRAF) family of adapter proteins, TRAF2, TRAF3, TRAF5 and TRAF6, can bind to the cytoplasmic domain of CD40 (157-160). When overexpressed in fibroblasts, TRAF2, TRAF5 and TRAF6 can activate both JNK and NF-κB (157,159,160,162,249-251). The ability of these TRAF proteins to activate the p38/MAPKAP kinase-2 pathway has not been examined. In addition to the TRAF proteins, two other proteins that associate with CD40 may also link CD40 to signaling

pathways. A novel 23-kDa transmembrane protein associates with the extracellular domain of CD40 (252) while the JAK3 tyrosine kinase has been reported to bind to the cytoplasmic domain of human CD40 (95).

To determine which of these CD40-associated proteins might mediate activation of JNK and p38, as well as activation of NF-κB and pro-survival pathways in B cells, my approach was to map the regions of the CD40 cytoplasmic domain that are responsible for activating these signaling pathways. The cytoplasmic domain of murine CD40 contains 74 amino acids while that of human CD40 contains 62 amino acids. Amino acids 31-62 (numbering from the inside of the plasma membrane) of the murine and human CD40 cytoplasmic tails are completely identical (167,253). *In vitro* studies have shown that amino acids 35-51 of the CD40 cytoplasmic domain contain sequences required for binding TRAF2 and TRAF3 (165,166,254). TRAF5 appears to bind to the same site (159). In contrast, TRAF6, which can also activate NF-κB, JNK and perhaps ERK, binds to residues 15-23 of the human CD40 cytoplasmic domain (160,163,254) which is homologous to amino acids 19-28 of the murine CD40 cytoplasmic domain. Like TRAF6, JAK3 has been reported to bind to the membrane proximal region of the human CD40 cytoplasmic domain. While the JAK3 binding site has been mapped to amino acids 5-14 of the human CD40 cytoplasmic domain (95), it has not been shown that JAK3 binds to murine CD40.

I have used a gain-of-function approach to identify the minimal regions of the CD40 cytoplasmic domain that can activate the JNK, p38, NF-κB, and pro-survival pathways in B cells. I expressed in WEHI-231 cells chimeric receptors consisting of the extracellular and transmembrane domains of CD8 fused to progressively smaller portions of the murine CD40 cytoplasmic domain. I found that an 11 amino acid linear sequence corresponding to amino acids 35-45 of the murine CD40 cytoplasmic tail was sufficient for maximal activation of the JNK and the p38/MAPKAP kinase-2 pathways. Amino acids 35-45 of the CD40 cytoplasmic tail were also sufficient for

activation of NF-κB and for protection of WEHI-231 cells from anti-IgM-induced growth arrest. These results suggest that amino acids 35-45 of the CD40 cytoplasmic domain constitute a minimal TRAF2/3/5-binding motif and are consistent with the idea that TRAF2, TRAF3, or TRAF5 couple CD40 to the JNK and p38 MAP kinase pathways, to NF-κB activation, and to pro-survival pathways.

4.2 Construction of CD8α/CD40 chimeric receptors

A plasmid containing cDNA encoding human CD8α in which a *Bgl II* site had been inserted after the fourth codon of the cytoplasmic domain (180) was a gift from Dr. A. Weiss (Univ. of California, San Francisco). The CD8 cDNA was excised from this vector and subcloned into the pLXSN retroviral expression vector (181) as described in the Materials and Methods (Chapter 2). cDNAs encoding the full length cytoplasmic domain of murine CD40 (amino acids 1-74), a region corresponding to amino acids 26-63, and a region corresponding to amino acids 26-53 were produced by RT-PCR using WEHI-231 B cell mRNA as a template. The primers used added a *Bgl II* site at the 5' end of the amplified cDNAs and a stop codon followed by a *Bgl II* site at the 3' end (Table 4.1). The smaller CD40 segments were created by annealing together synthetic oligonucleotides that contained the relevant CD40 sequences as well as a *Bgl II* site at the 5' end and a stop codon followed by a *Bgl II* site at the 3' end (Table 4.2). The CD40 cDNA fragments were digested with *Bgl II* and ligated into pLXSN-CD8α at the *Bgl II* site (Fig. 2.2). Plasmids were digested with *Bgl II* to screen for the presence of inserts corresponding to the CD40 cytoplasmic tail. The sequence of each CD8α/CD40 chimeric cDNA was confirmed by DNA sequencing, as described in the Materials and Methods (Chapter 2), using a primer corresponding to codons 177-183 of the CD8 sense strand.

Table 4.1: Primers used for PCR amplification of portions of the CD40 cytoplasmic domain.

The *Bgl II* sites used for cloning the PCR product into pLXSN-CD8α are underlined. The stop codons are italicized.

[a]The resulting PCR product encoded amino acids 1-74 of the CD40 cytoplasmic domain (i.e. the complete CD40 cytoplasmic domain) as well as the endogenous stop codon from the CD40 mRNA and 54 bases of 3' untranslated sequence. Thus, the antisense primer did not contain an additional stop codon in this case.

CD40 cytoplasmic domain codons amplified	*Oligo type*	*Oligonucleotide sequence (5' to 3')*
1 - 74 [a]	sense	CGG GAA GAT CTA AAA AGG TGG TCA AGA AAC CAA AGG ATA ATG AAA TGT TA
1 - 74 [a]	antisense	CTT GGC AGA TCT CAA ACT TCA AAA GGT CAG
26-63	sense	CCC CAG AGA TCT GAA GAT TAT CCC GGT CAT
26-63	antisense	TAT GAG ATC TGT *CTA* CTG CCG CTC CTG CAC TAG
26 - 53	sense	CCC CAG AGA TCT GAA GAT TAT CCC GGT CAT
26 - 53	antisense	CTG AGA TCT GAC T*CT* ATT TAC CAT CCT CCT GTG T

Table 4.2: Oligonucleotides used to generate murine CD40 cytoplasmic domain fragments.

The corresponding sense and antisense oligonucleotides were annealed, cleaved with *Bgl II* and cloned into pLXSN-CD8α that had been digested with *Bgl II*. The *Bgl II* sites are underlined and the stop codons are italicized. The base changes used to create the 35-53 T40A mutation are in bold.

CD40 tail amino acids	*Oligo type*	*Oligonucleotide sequence (5' to 3')*
1 - 25	sense	CGG GAA GAT CTA AAA AGG TGG TCA AGA AAC CAA AGG ATA ATG AAA TGT TAC CCC CTG CGG CTC GAC GGC AAG ACC CCC AGG AGA TG *T GAA* GAT CTT CCG G
1 - 25	antisense	CCG GAA GAT CT *T CAC* ATC TCC TGG GGG TCT TGC CGT CGA GCC GCA GGG GGT AAC ATT TCA TTA TCC TTT GGT TTC TTG ACC ACC TTT TT A GAT CTT CCC G
15 - 30	sense	CGG GAA GAT CTC CTG CGG CTC GAC GGC AAG ATC CCC AGG AGA TGG AAG ATT ATC CCG GT *T GAA* GAT CTT CCG G
15 - 30	antisense	CCG GAA GAT CT *T CAA* CCG GGA TAA TCT TCC ATC TCC TGG GGA TCT TGC CGT CGA GCC GCA GG A GAT CTT CCC G
35 - 53	sense	CGG GAA GAT CTG CTC CAG TGC AGG AGA CAC TGC ACG GGT GTC AGC CTG TCA CAC AGG AGG ATG GTA AA *T GAA* GAT CTT CCG G
35 - 53	antisense	CCG GAA GAT CT *T CAT* TTA CCA TCC TCC TGT GTG ACA GGC TGA CAC CCG TGC AGT GTC TCC TGC ACT GGA GC A GAT CTT CCC G
35 - 53 T40A	sense	CGG GAA GAT CTG CTC CAG TGC AGG AG **G** CAC TGC ACG GGT GTC AGC CTG TCA CAC AGG AGG ATG GTA AA *T GAA* GAT CTT CCG G
35 - 53 T40A	antisense	CCG GAA GAT CT *T CAT* TTA CCA TCC TCC TGT GTG ACA GGC TGA CAC CCG TGC AGT G **C**C TCC TGC ACT GGA GC A GAT CTT CCC G
26 - 44	sense	CGG GAA GAT CTG AAG ATT ATC CCG GTC ATA ACA CCG CTG CTC CAG TGC AGG AGA CAC TGC ACG GGT GT *T GAA* GAT CTT CCG G
26 - 44	antisense	CCG GAA GAT CT *T CAA* CAC CCG TGC AGT GTC TCC TGC ACT GGA GCA GCG GTG TTA TGA CCG GGA TAA TCT TC A GAT CTT CCC G
35 - 45	sense	CGG GAA GAT CTG CTC CAG TGC AGG AGA CAC TGC ACG GGT GTC AG *T GAA* GAT CTT CCG G
35 - 45	antisense	CCG GAA GAT CT *T CAC* TGA CAC CCG TGC AGT GTC TCC TGC ACT GGA GC A GAT CTT CCC G
45 - 63	sense	CGG GAA GAT CTC AGC CTG TCA CAC AGG AGG ATG GTA AAG AGA GTC GCA TCT CAG TGC AGG AGC GGC AG *T GAA* GAT CTT CCG G
45 - 63	antisense	CCG GAA GAT CT *T CAC* TGC CGC TCC TGC ACT GAG ATG CGA CTC TCT TTA CCA TCC TCC TGT GTG ACA GGC TG A GAT CTT CCC G
43 - 53	sense	CGG GAA GAT CTG GGT GTC AGC CTG TCA CAC AGG AGG ATG GTA AA *T GAA* GATCTT CCG G
43 - 53	antisense	CCG GAA GAT CT *T CAT* TTA CCA TCC TCC TGT GTG ACA GGC TGA CAC CC A GAT CTT CCC G

4.3 Expression of chimeric CD8α/CD40 receptors in WEHI-231 cells

To identify signaling motifs in the CD40 cytoplasmic domain, I constructed a set of chimeric CD8α/CD40 receptors in which segments of the murine CD40 cytoplasmic domain were fused onto the C-terminus of a truncated human CD8α protein consisting of the extracellular domain, transmembrane region, and first four cytoplasmic amino acids of CD8α (Fig. 4.1). Chimeric receptors were constructed containing the full length CD40 cytoplasmic domain (amino acids 1-74 of the murine CD40, counting from the inside face of the plasma membrane), the membrane-proximal portion of the CD40 cytoplasmic domain (amino acids 1-25), or amino acids 26-63 which is the "homology box" region that is nearly identical in the murine and human CD40 proteins. The homology box region of the CD40 cytoplasmic domain was also subdivided into smaller regions in chimeric receptors that contained amino acids 26-53, 35-53, 26-44, 35-45, 45-63, or 43-53 of the CD40 cytoplasmic domain. Finally, I constructed a chimeric receptor containing residues 35-53 of the CD40 tail in which the threonine residue at position 40 was changed to an alanine. I was interested in determining whether this threonine was required for CD40-induced survival as well as activation of JNK, MAPKAP kinase-2 and NF-κB, since changing this threonine residue to an alanine in human CD40 abrogates several responses to CD40 engagement including upregulation of CD23, B7.1 and Fas (168,255-257). These ten chimeric receptors were stably expressed in WEHI-231 cells, as was the truncated CD8α protein (CD8/). For each receptor expressed, clones were screened for CD8α expression and clones with similar levels of expression were selected for further study (Fig. 4.2).

4.4 Mapping the portion of the CD40 cytoplasmic domain required for activating JNK and MAPKAP kinase-2

I have previously shown that in WEHI-231 cells CD40 strongly activates the JNK and p38 MAP kinases, as well as MAPKAP kinase-2, a downstream target of p38

Figure 4.1: Schematic representation of the CD8α/CD40 chimeric receptors.

The CD8/ protein contains the extracellular and transmembrane domains of human CD8α as well as the first four amino acids of the CD8 cytoplasmic domain. For the chimeric receptors, various portions of the murine CD40 cytoplasmic domain were fused to the C-terminus of CD8/. The amino acid sequence of the CD40 cytoplasmic domain is shown and the residues are numbered starting at the inner face of the membrane. The numbers in brackets indicate which portions of the CD40 cytoplasmic domain have been fused to CD8α for each chimeric receptor. In the CD8/(35-53 T40A) chimeric receptor, the threonine residue at position 40 was changed to an alanine.

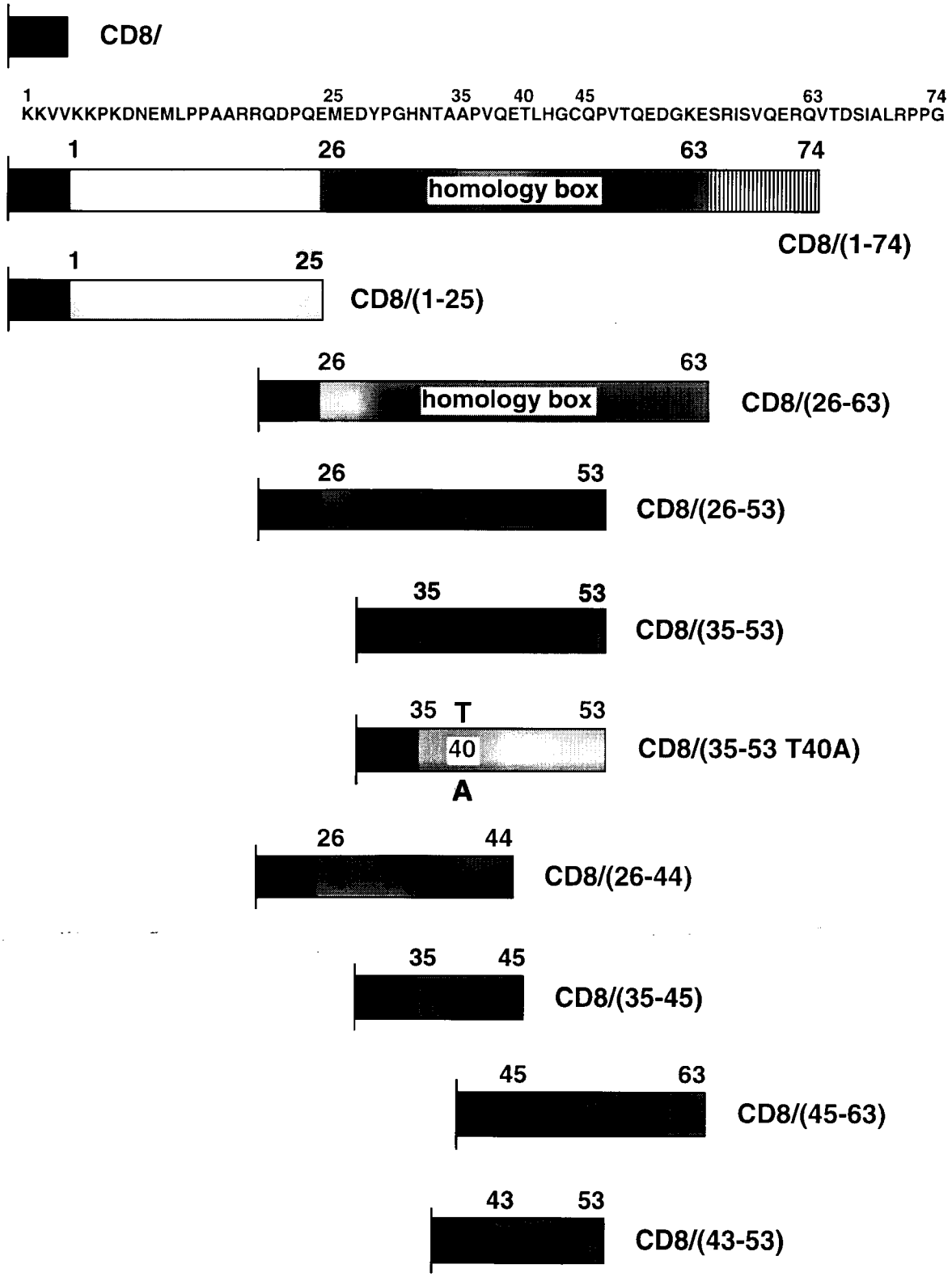
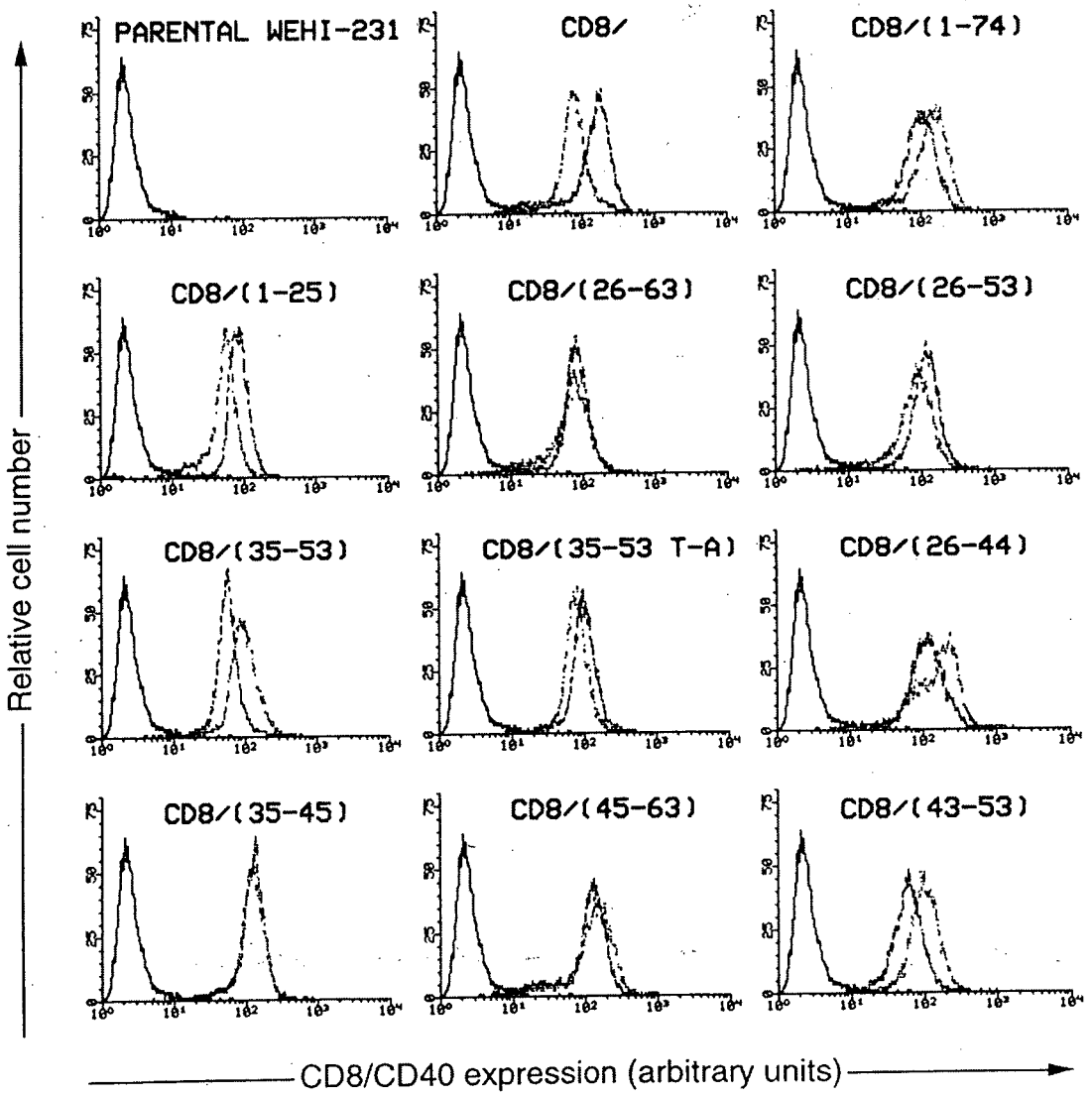

CD8/
1 25 35 40 45 63 74
KKVVKKPKDNEMLPPAARRQDPQEMEDYPGHNTAAPVQETLHGCQPVTQEDGKESRISVQERQVTDSIALRPPG
1 26 63 74
homology box
CD8/(1-74)
1 25
CD8/(1-25)
26 63
homology box
CD8/(26-63)
26 53
CD8/(26-53)
35 53
CD8/(35-53)
35 T 53
40
A
CD8/(35-53 T40A)
26 44
CD8/(26-44)
35 45
CD8/(35-45)
45 63
CD8/(45-63)
43 53
CD8/(43-53)

Figure 4.2: Expression of CD8α/CD40 chimeric receptor proteins in WEHI-231 cells.

Untransfected parental WEHI-231 cells (solid lines) and two stable clones expressing each CD8/CD40 chimeric receptor (dotted lines) were stained with the anti-human CD8α-specific OKT8 mAb followed by anti-mouse IgG-FITC. The numbers in brackets denote which amino acids of the CD40 cytoplasmic domain were present in each chimeric receptor. Note that the CD8/(35-53 T40A) chimeric receptor is referred to as CD8/(35-53 T-A) in this figure.


PARENTAL WEHI-231
CD8/
CD8/(1-74)
CD8/(1-25)
CD8/(26-63)
CD8/(26-53)
CD8/(35-53)
CD8/(35-53 T-A)
CD8/(26-44)
CD8/(35-45)
CD8/(45-63)
CD8/(43-53)
Relative cell number
CD8/CD40 expression (arbitrary units)

(Chapter 3 and (245)). In order to identify proteins that may link CD40 to activation of these kinase signaling pathways, I used the chimeric CD8α/CD40 receptors to map the portion of the CD40 cytoplasmic domain responsible for activating the JNK and p38/MAPKAP kinase-2 pathways. I chose to use MAPKAP kinase-2 activation as an indirect measure of p38 activation since MAPKAP kinase-2 is usually activated to a greater extent than p38 (245). This gave me a larger range with which to quantitate the relative abilities of different regions of CD40 to activate the p38/MAPKAP kinase-2 pathway. In WEHI-231 cells, CD40-stimulated activation of MAPKAP kinase-2 is entirely dependent on p38 activity. Fig. 3.10 (Chapter 3) shows that a specific inhibitor of p38, SB 203580 (140), completely blocked the ability of CD40 to activate MAPKAP kinase-2 while having no effect on activation of JNK by CD40.

I first determined whether a chimeric receptor containing the entire cytoplasmic domain of CD40 (amino acids 1-74) could activate JNK and MAPKAP kinase-2 when expressed in WEHI-231 B lymphoma cells. I found that clustering this CD8/(1-74) chimeric receptor with a biotinylated anti-CD8 mAb (51.1-biotin) and avidin routinely caused a 15-fold increase in JNK activity (Fig. 4.3) and a 10-fold increase in MAPKAP kinase-2 activity (Fig. 4.4). This is similar to the magnitude of JNK activation and MAPKAP kinase-2 activation by treating parental WEHI-231 cells with the 1C10 anti-CD40 mAb or with soluble CD40 ligand ((245) and data not shown). Thus, interactions mediated by the CD40 cytoplasmic domain are sufficient to fully activate JNK and MAPKAP kinase-2 in the absence of any interactions mediated by the extracellular or transmembrane domains of CD40.

My next goal was to map the portion of the CD40 cytoplasmic domain responsible for activating JNK and MAPKAP kinase-2. Amino acids 26-63 of the cytoplasmic domains of murine and human CD40 are 92% identical whereas the membrane-proximal region of CD40 diverges considerably between mouse and human (167,253). This indicated that the homology box region (amino acids 26-63)

Figure 4.3: Amino acids 35-45 of the CD40 cytoplasmic domain constitute a signaling motif that is sufficient for the activation of JNK.

WEHI-231 clones expressing the indicated CD8α/CD40 chimeric receptors were stimulated with 10 μg/mL biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin for 15 min. Cell lysates were immunoprecipitated with the anti-JNK1 Ab and *in vitro* kinase assays were performed using GST-c-Jun(1-79) as a substrate. The JNK activity induced by engaging CD8/(1-74), the chimeric receptor containing the full length CD40 cytoplasmic domain, was assigned a value of 100%. The dashed line indicates the JNK activity from CD8/(1-74)-expressing cells which were not stimulated. The average value for this basal JNK activity was 7% of the JNK activity induced by engaging the CD8/(1-74) chimeric receptor, i.e. engaging CD8/(1-74) caused a 15-fold increase in JNK activity. The basal levels of JNK activity in clones expressing other chimeric receptors were very similar. Activation of JNK caused by engaging the other chimeric receptors is expressed as a percent of the JNK activation caused by engaging CD8/(1-74). The data represent the mean and standard deviation from a total of three or more independent experiments using two different clones expressing that particular chimeric receptor. Avidin alone did not stimulate JNK activity (data not shown).

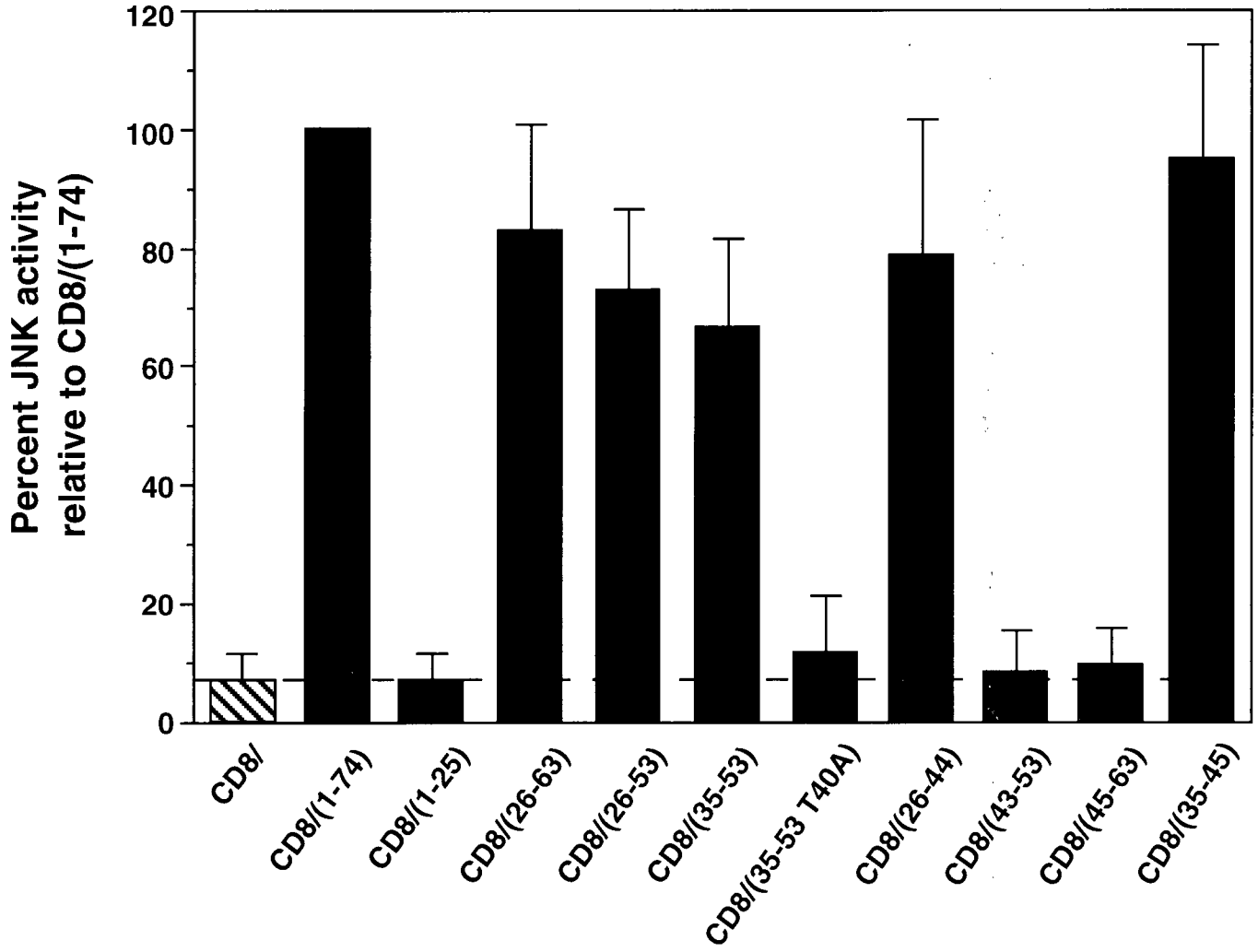
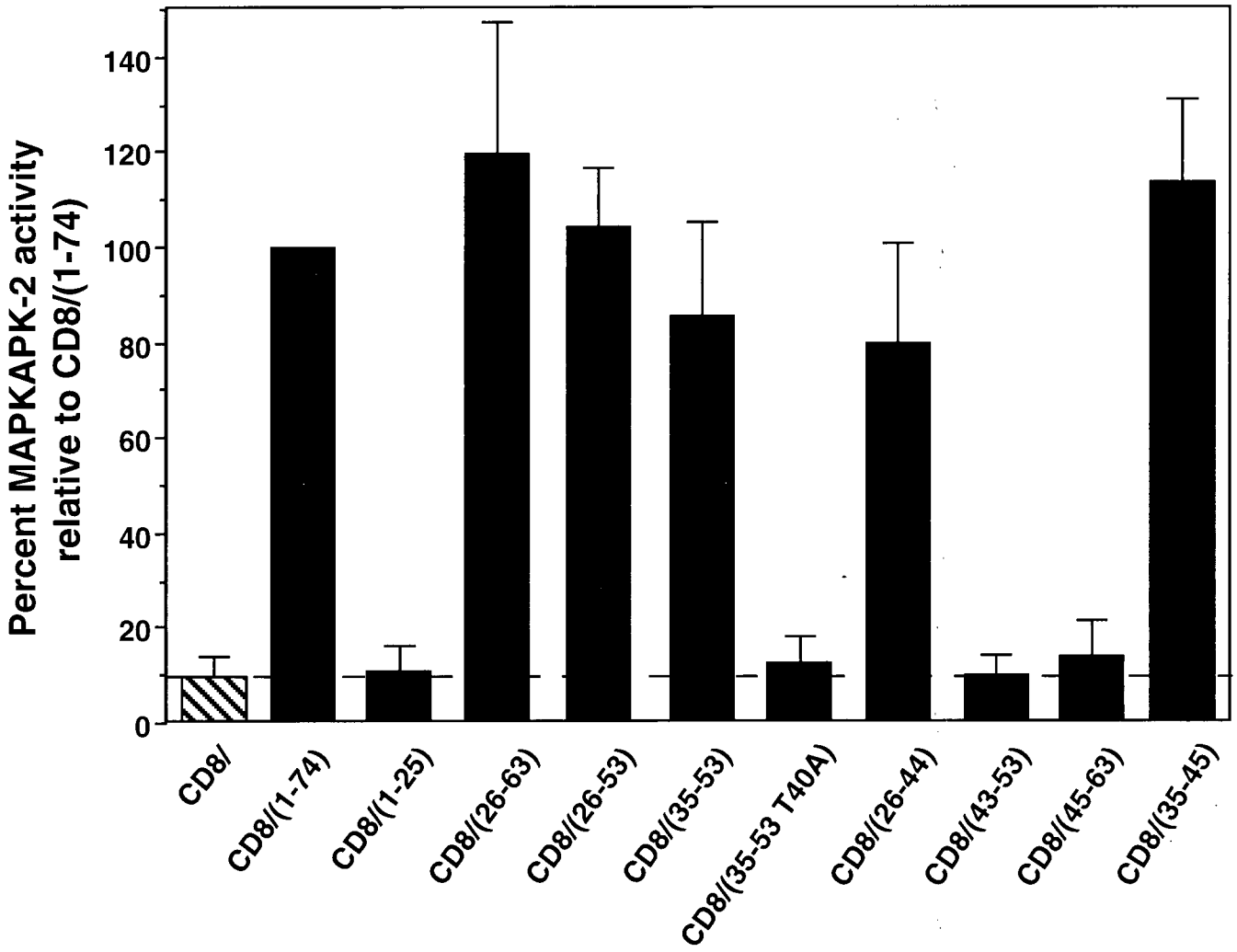

Percent JNK activity
relative to CD8/(1-74)
120
100
80
60
40
20
0
CD8/
CD8/(1-74)
CD8/(1-25)
CD8/(26-63)
CD8/(26-53)
CD8/(35-53)
CD8/(35-53 T40A)
CD8/(26-44)
CD8/(43-53)
CD8/(45-63)
CD8/(35-45)

Figure 4.4: Amino acids 35-45 of the CD40 cytoplasmic domain constitute a signaling motif that is sufficient for the activation of MAPKAP kinase-2.

WEHI-231 clones expressing the indicated CD8α/CD40 chimeric receptors were stimulated with 10 μg/mL biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin for 15 min. Cell lysates were immunoprecipitated with the anti-MAPKAP kinase-2 Ab and *in vitro* kinase assays were performed using Hsp25 as a substrate. The MAPKAP kinase-2 activity induced by engaging CD8/(1-74), the chimeric receptor containing the full length CD40 cytoplasmic domain, was assigned a value of 100%. The dashed line indicates the MAPKAP kinase-2 activity from cells expressing CD8/(1-74) which were not stimulated. The average value for this basal MAPKAP kinase-2 activity was 9.6% of the activity induced by engaging the CD8/(1-74) chimeric receptor, i.e. engaging CD8/(1-74) caused about a 10-fold increase in MAPKAP kinase-2 activity. The basal levels of MAPKAP kinase-2 activity in clones expressing other chimeric receptors were very similar. Activation of MAPKAP kinase-2 caused by engaging the other chimeric receptors is expressed as a percent of the MAPKAP kinase-2 activation caused by engaging CD8/(1-74). The data represent the mean and standard deviation from a total of three or more independent experiments using two different clones expressing that particular chimeric receptor. Avidin alone did not stimulate MAPKAP kinase-2 activity (data not shown).


Percent MAPKAPK-2 activity relative to CD8/(1-74)
0
20
40
60
80
100
120
140
CD8/
CD8/(1-74)
CD8/(1-25)
CD8/(26-63)
CD8/(26-53)
CD8/(35-53)
CD8/(35-53 T40A)
CD8/(26-44)
CD8/(43-53)
CD8/(45-63)
CD8/(35-45)

might contain the important CD40 signaling motifs. Consistent with this idea, a chimeric receptor containing the membrane-proximal 25 residues of the CD40 cytoplasmic domain (CD8/(1-25)) did not activate JNK (Fig. 4.3) or MAPKAP kinase-2 (Fig. 4.4). In contrast, CD8/(26-63), a chimeric receptor containing the homology box region activated both JNK (Fig. 4.3) and MAPKAP kinase-2 (Fig. 4.4) to a similar extent as the chimeric receptor containing the full length CD40 cytoplasmic tail. These results show that residues 64-74 of the murine CD40 tail are not required for activation of the JNK and p38 pathways. Human CD40 terminates after residue 62, consistent with the idea that this C-terminal extension in murine CD40 is unlikely to carry out important signaling functions. When expressed in murine cells, human CD40 can mediate the same responses as the endogenous murine CD40 (28,255,256,258).

To further delineate the region of CD40 responsible for activation of JNK and MAPKAP kinase-2, I constructed chimeric receptors containing progressively smaller portions of this CD40 homology box. I first tested whether the last 10 residues of the homology box were required for activating these kinases. I found that the CD8/(26-53) chimeric receptor activated JNK and MAPKAP kinase-2 to similar extents as CD8/(26-63) (Figs. 4.3, 4.4). Thus, the last 10 residues of the homology box (residues 54-63) are not needed for activating the JNK and p38 MAP kinase signaling pathways.

My results thus far had indicated that the JNK and p38 activation motifs were contained within residues 26-53 of the CD40 cytoplasmic domain. *In vitro* studies with fusion proteins have shown that TRAF2 can bind to peptides corresponding to amino acids 35-51 of murine CD40 (165,166). TRAF3 and TRAF5 appears to associate with an identical or overlapping region of CD40 (159,254). When overexpressed in fibroblasts, TRAF2 and TRAF5 can activate JNK while TRAF3 overexpression does not (162). Although the ability of TRAF proteins to activate the p38/MAPKAP kinase-2 pathway has not been examined, expressing a dominant negative form of TRAF3 in the RAMOS human B cell line has been shown to completely block CD40-induced

activation of p38 but only partially block activation of JNK (164). Thus, TRAF2 or TRAF5 may couple CD40 to JNK while TRAF3 appears to preferentially couple CD40 to p38. To determine if the proposed TRAF2/3/5 binding region of CD40 corresponds to the region capable of activating JNK and MAPKAP kinase-2, I made a chimeric receptor containing residues 35-53 of the CD40 cytoplasmic domain. I found that CD8/(35-53) strongly activated JNK (Fig. 4.3) and MAPKAP kinase-2 (Fig. 4.4), consistent with the idea that TRAF2, TRAF3, or TRAF5 might couple CD40 to activation of JNK and p38 in B cells.

To further refine the CD40 signaling motif required for activation of JNK and MAPKAP kinase-2, I first used chimeric receptors containing either the N- or C-terminal portions of the region spanning amino acids 35-53 of the CD40 cytoplasmic domain. I found that CD8/(26-44), a chimeric receptor containing the N-terminal half of this region could activate JNK and MAPKAP kinase-2 (Figs. 4.3, 4.4) while two chimeric receptors containing the C-terminal half of this region, CD8/(45-63) and CD8/(43-53), were incapable of activating JNK and MAPKAP kinase-2 (Figs. 4.3, 4.4). Since only CD8/(26-44) and CD8/(35-53) out of this set of chimeric receptors could activate JNK and MAPKAP kinase-2, it indicated that residues other than 35-44 in the CD40 cytoplasmic domain are dispensable for CD40-induced activation of the JNK and p38/MAPKAP kinase-2 pathways. To determine if this region was sufficient for activation of these kinases, I constructed a chimeric receptor containing only residues 35-45 of the CD40 cytoplasmic domain. CD8/(35-45) was able to activate these kinases to the same extent as the chimeric receptor containing the full length CD40 tail (Fig. 4.3, 4.4). Thus, residues 35-45 of murine CD40 constitute a JNK/p38 MAP kinase activation motif and may be the minimal TRAF2/3/5 binding site.

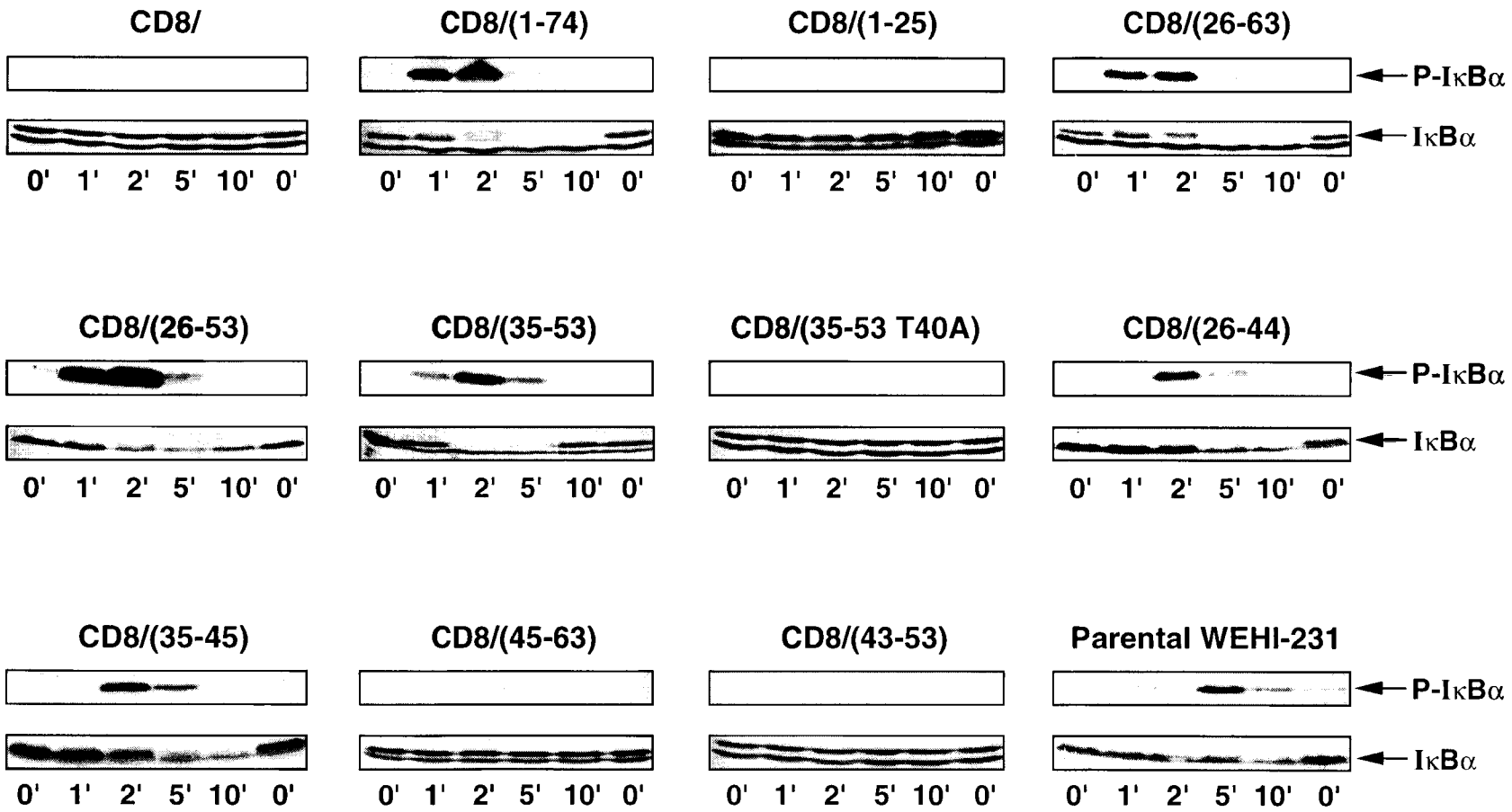
4.5 Residues 35-45 of the murine CD40 cytoplasmic domain mediate activation of the NF-κB pathway and protection from anti-IgM-induced growth arrest

CD40 engagement activates the NF-κB transcription factor (96). NF-κB is retained in the cytosol in an inactive state, bound to the inhibitory IκB proteins (150). NF-κB activation occurs via phosphorylation of IκBα at serines 32 and 36 (259). This targets IκBα for degradation and allows NF-κB to translocate to the nucleus (150,259,260). When overexpressed in fibroblasts, TRAF2, TRAF5, and TRAF6 can all activate NF-κB (157,159,160,162,261). However, it is not clear whether all of these TRAF proteins can link CD40 to NF-κB activation in B cells. TRAF2 and TRAF5 bind to amino acids 35-51 of CD40 (159,166) while TRAF6 binds to the membrane proximal region of CD40 (160). To determine which regions of CD40 activate NF-κB in B cells, I tested the ability of my chimeric receptors to induce phosphorylation and degradation of IκBα.

Cross-linking the CD8/(1-74) chimeric receptor caused a time-dependent increase in IκBα phosphorylation which was readily detectable after 1 min and maximal by 2 min after receptor engagement (Fig. 4.5). Consistent with the phosphorylation kinetics, IκBα degradation was apparent within 2 min of CD8/(1-74) engagement and complete within 5 min. Similar results were observed when the 1C10 anti-CD40 mAb was used to engage the endogenous CD40 in parental WEHI-231 cells although the kinetics of IκBα phosphorylation and degradation were slightly slower (Fig. 4.5). In contrast, ligation of CD8/(1-25) did not cause IκBα phosphorylation or degradation after 1 to 10 min (Fig. 4.5) or at 40 min (data not shown). Thus, my data indicates that the membrane-proximal region of the CD40 cytoplasmic tail is incapable by itself of causing significant activation of NF-κB in WEHI -231 cells. The CD8/(26-63), CD8/(26-53), CD8/(35-53) and CD8/(26-44) chimeric receptors all induced marked phosphorylation of IκBα within 1 to 2 min of engagement

Figure 4.5: Residues 35-45 of the murine CD40 cytoplasmic domain are sufficient to induce IκBα phosphorylation and degradation.

WEHI-231 cell clones expressing the indicated CD8α/CD40 chimeric receptors were stimulated with 10 μg/mL biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin for 1 to 10 min. The parental WEHI-231 cells (lower right panel) were stimulated with 10 μg/mL of the 1C10 anti-CD40 mAb. In the *upper panel* of each pair, cell lysates were analyzed by immunoblotting with an Ab specific for the phosphorylated form of IκBα (P-IκBα). The filters were then stripped and reprobed with an anti-IκBα Ab *(lower panels)* to assess IκBα degradation. Two different WEHI-231 clones expressing each chimeric receptor were analyzed in at least three independent experiments. Representative results are shown.


CD8/
CD8/(1-74)
CD8/(1-25)
CD8/(26-63)
P-IκBα
IκBα
0' 1' 2' 5' 10' 0'
CD8/(26-53)
CD8/(35-53)
CD8/(35-53 T40A)
CD8/(26-44)
CD8/(35-45)
CD8/(45-63)
CD8/(43-53)
Parental WEHI-231

and this was followed by degradation of IκBα (Fig. 4.5), indicating that the minimal NF-κB activation motif was contained within residues 35-44 of the CD40 cytoplasmic domain. Consistent with this idea, the CD8/(35-45) chimeric receptor was capable of inducing IκBα phosphorylation and degradation. Since residues 35-45 of the CD40 cytoplasmic domain participate in the binding of TRAF2 and TRAF5 (254), my data indicate that TRAF2 and/or TRAF5 mediate CD40 activation of NF-κB in WEHI-231 cells.

While amino acids 35-45 of the CD40 cytoplasmic domain were sufficient to induce IκBα phosphorylation and degradation, additional sequences appear to be required for maximal activation of NF-κB by CD40. Fig. 4.5 shows that the IκBα phosphorylation caused by CD8/(1-74), the chimeric receptor containing the full length CD40 cytoplasmic domain, was significantly stronger than that caused by the CD8/(35-45) chimeric receptor. CD8/(1-74)-induced IκBα phosphorylation was also more rapid than that caused by CD8/(35-45). CD8/(1-74) caused near maximal phosphorylation of IκBα after 1 min while CD8/(35-45) did not induce significant IκBα phosphorylation until 2 min. Comparing the magnitude and kinetics of IκBα phosphorylation induced by the other chimeric receptors allowed me to determine which CD40 sequences were required for maximal IκBα phosphorylation. I found that the CD8/(26-53) chimeric receptor could induce IκBα phosphorylation to the same extent and with the same rapid kinetics as CD8(1-74). Thus, residues 26-34 and/or 46-53 of the CD40 cytoplasmic domain contribute to the ability of CD40 to induce IκBα phosphorylation and degradation. Both of these flanking sequences may be required for maximal IκBα phosphorylation and degradation since CD8/(26-53) induced significantly stronger and more rapid IκBα phosphorylation than either CD8/(26-44) or CD8/(35-53), both of which induced the slower and less robust response characteristic of CD8/(35-45). Moreover the CD8/(45-63) and CD8/(43-53) chimeric receptors were unable to activate NF-κB, suggesting that if the C-terminal flanking regions contribute to NF-κB

activation, they do so by cooperating with residues 35-45 as opposed to independently binding activators of NF-κB.

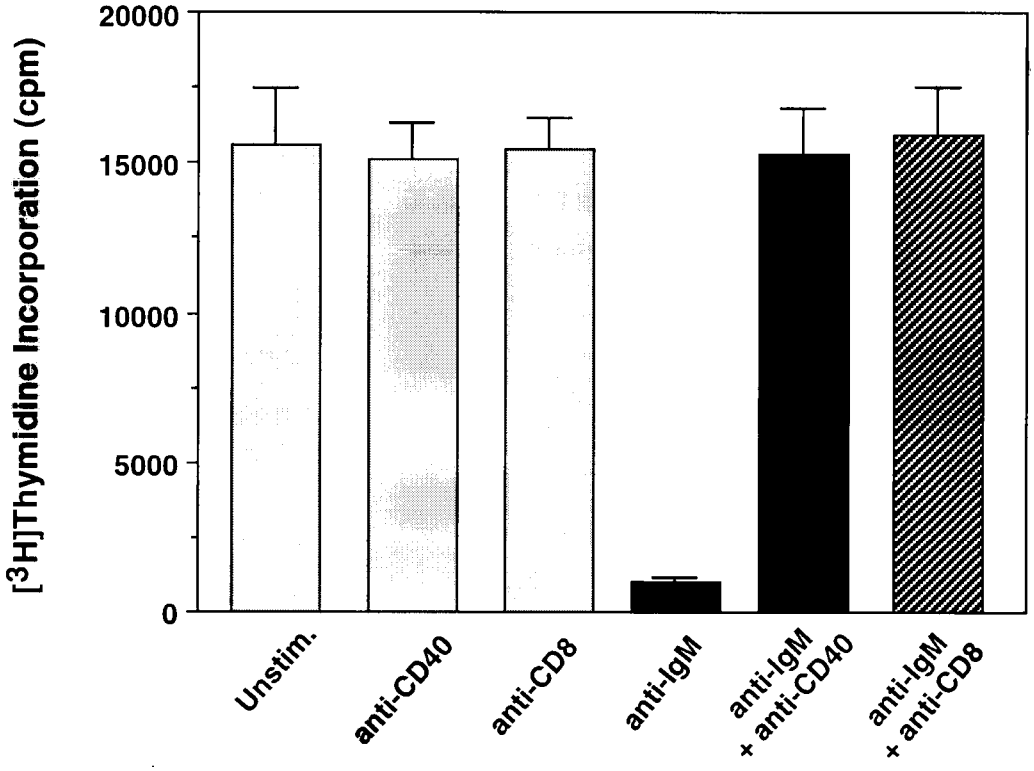
Recent work by Sonenshein and colleagues has shown that activation of NF-κB is essential for CD40 to prevent BCR-induced growth arrest and apoptosis in WEHI-231 cells (146). I found that engaging the CD8/(35-45) chimeric receptor with anti-CD8 Abs could completely protect WEHI-231 cells from anti-IgM-induced growth arrest (Fig. 4.6). This indicates that the CD8/(35-45) chimeric receptor can activate NF-κB to a biologically significant extent even though it does not induce IκBα phosphorylation to the same extent as the chimeric receptor containing the full length CD40 cytoplasmic domain.

4.6 Threonine-40 is essential for CD40 signaling

The threonine residue at position 40 of the human CD40 cytoplasmic region has previously been shown to be important for CD40 signaling (168,255-257). I asked whether changing this residue in murine CD40 would affect its ability to signal. I found that this threonine to alanine mutation completely abolished the ability of the murine CD40 cytoplasmic domain to activate JNK and MAPKAP kinase-2. The CD8(35-53) chimeric receptor was fully active whereas the CD8/(35-53 T40A) chimeric receptor in which residue 40 was changed to an alanine did not activate JNK (Fig. 4.3) or MAPKAP kinase-2 (Fig. 4.4). The CD8/(35-53 T40A) chimeric receptor also did not induce IκBα phosphorylation or degradation (Fig. 4.5). Thus, threonine-40 is essential for murine CD40 to activate the JNK, p38/MAPKAP kinase-2 and NF-κB signaling pathways. Presumably this residue interacts with proteins that link CD40 to these signaling pathways. This threonine residue has recently been shown to be important for CD40 to bind TRAF2, TRAF3, and TRAF5 (158,159,254), again consistent with the idea that these TRAF proteins link CD40 to activation of JNK, p38, and NF-κB.

Figure 4.6: Residues 35-45 of the CD40 cytoplasmic tail are sufficient to protect WEHI 231 cells from anti-IgM-induced growth arrest.

A WEHI-231 clone expressing the CD8/(35-45) chimeric receptor were cultured with or without 3 μg/mL anti-IgM Ab in the presence of medium alone, 10 μg/mL of the 1C10 anti-CD40 mAb, or 10 μg/mL each of biotinylated 51.1 (anti-CD8 mAb) and avidin. [^{3}H]thymidine was added after 40 h and 4 h later the incorporation of [^{3}H]thymidine into DNA was determined by liquid scintillation counting. All determinations were carried out in triplicate and the mean and standard deviation for each data point are shown. This is one of three similar experiments performed with two different clones expressing CD8/(35-45).


[3H]Thymidine Incorporation (cpm)
20000
15000
10000
5000
0
Unstim.
anti-CD40
anti-CD8
anti-IgM
anti-IgM + anti-CD40
anti-IgM + anti-CD8

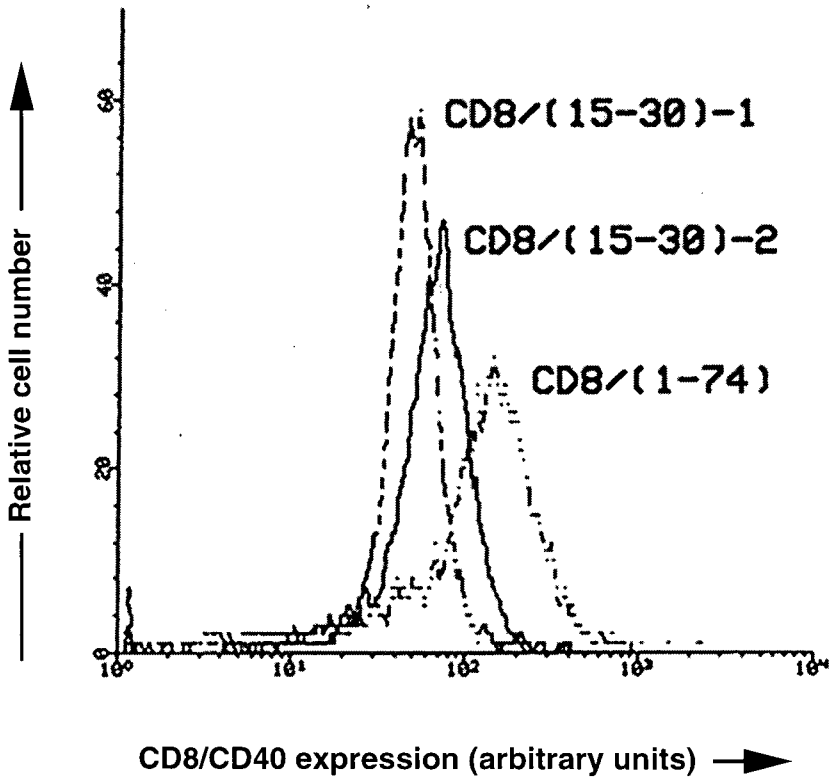
4.7 The isolated TRAF6 binding site of CD40 is not sufficient for signaling in WEHI-231 cells

My results show that amino acids 35-45 of the CD40 cytoplasmic domain contain a signaling motif that can mediate the activation of JNK and MAPKAP kinase-2 and induce the phosphorylation and degradation of IκBα. In contrast, chimeric receptors containing other regions of CD40 were unable to induce these signaling events. Most notably, both the CD8/(1-25) and CD8/(45-63) chimeric receptors were incapable of activating JNK and MAPKAP kinase-2 or inducing the phosphorylation and degradation of IκBα (Figs. 4.3-4.5). The inability of the CD8/(1-25) chimeric receptor to signal was surprising since it appeared to contain the minimal binding site for TRAF6 and TRAF6 has been shown to activate JNK and NF-κB when overexpressed in fibroblasts (160). However, more detailed mapping studies by Pullen et al. (254) have recently shown that the optimal TRAF6 binding site corresponds to amino acids 19-28 of the murine CD40 cytoplasmic domain. Since my CD8/(1-25) chimeric was missing key residues of the TRAF6 binding site, I constructed a new chimeric receptor that contained amino acids 15-30 of the murine CD40 cytoplasmic domain and expressed this chimeric receptor in WEHI-231 cells.

FACS analysis showed that the cell surface expression of the CD8/(15-30) chimeric receptor was lower than that of the other CD8 chimeric receptors I had expressed. Twelve WEHI-231 clones expressing the CD8/(15-30) chimeric receptor were analyzed by staining with anti-CD8 Abs. For the two clones expressing the highest levels of the CD8/(15-30) chimeric receptor, the mean fluorescence intensity of anti-CD8 staining was 38% and 52%, respectively, of that for a CD8/(1-74)-expressing WEHI-231 clone that I had used in my previous experiments (Fig. 4.7). However, WEHI-231 clones expressing similar levels of the CD8/(35-53) chimeric receptor (i.e. 40-50% that of the CD8/(1-74) clone) showed strong activation of JNK, MAPKAP kinase-2, and NF-κB in response to anti-CD8 Abs (data not shown). Thus, the level of

Figure 4.7: Expression of the CD8/(15-30) chimeric receptor in WEHI-231 cells.

Two stable WEHI-231 clones expressing the CD8/(15-30) chimeric receptor as well as a CD8/(1-74)-expressing clone used in previous figures were stained with the human CD8α-specific OKT8 mAb followed by anti-mouse IgG-FITC. Of the 12 CD8/(15-30)-expressing clones analyzed, the two shown in this figure had the highest expression. The level of CD8/(15-30) expression on the surface of these two clones (mean fluorescence intensity) was 38% (clone 1) and 52% (clone 2) of the surface expression of CD8(1-74) on the WEHI-231 CD8/(1-74) clone shown in this figure.


CD8/(15-30)-1
CD8/(15-30)-2
CD8/(1-74)
Relative cell number
CD8/CD40 expression (arbitrary units)

cell surface expression of the CD8/(15-30) chimeric receptor should not be a limiting factor in its ability to initiate signals.

The CD8/(15-30) chimeric receptor contains the TRAF6 binding site but not the TRAF2/3/5 binding site. In Fig. 4.8 I analyzed the ability of this receptor to activate JNK and MAPKAP kinase-2 and to induce the phosphorylation and degradation of IκBα. Although overexpression of TRAF6 has been reported to activate JNK and NF-κB in fibroblasts (160,162), I found that the CD8/(15-30) chimeric receptor caused little or no activation of JNK or MAPKAP kinase-2 in WEHI-231 cells (Fig. 4.8A) and did not induce IκBα phosphorylation or degradation (Fig. 4.8B). Although I cannot rule out the possibility that the cytoplasmic domain of the CD8/(15-30) chimeric receptor was improperly folded and unable to interact with TRAF6, the simplest interpretation of this data is that the TRAF6 binding motif of murine CD40 is not sufficient by itself to mediate these responses, at least in WEHI-231 cells.

4.8 Discussion

4.8.1 Identification of a major signaling motif in the cytoplasmic domain of CD40

In this study, I have used chimeric receptors containing different portions of the CD40 cytoplasmic domain to identify a region in the CD40 cytoplasmic domain that is responsible for (i) activating JNK, (ii) activating the p38/MAPKAP kinase-2 pathway, and (iii) and inducing the phosphorylation and degradation of IκBα. I found that amino acids 35-45 of the CD40 cytoplasmic tail constitute a signaling motif that is sufficient for activation of these signaling pathways in B cells. Other regions of the CD40 cytoplasmic domain were unable to mediate activation of JNK, MAPKAP kinase-2, or NF-κB. I also showed that the same 11 amino acid region of the CD40 cytoplasmic domain is sufficient to fully protect WEHI-231 cells from anti-IgM-induced growth arrest. My findings demonstrate that oligomerization of CD40 is sufficient to initiate these

Figure 4.8A: The CD8/(15-30) chimeric receptor causes little or no activation of JNK or MAPKAP kinase-2.

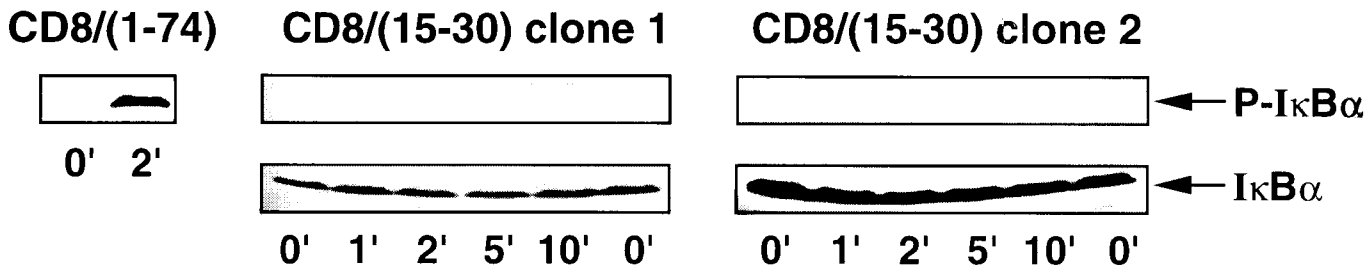
Two different WEHI-231 clones expressing CD8/(15-30) as well as WEHI-231 clones expressing either the truncated CD8α (CD8/) or the CD8/(1-74) chimeric receptor containing the full length CD40 cytoplasmic domain were stimulated with 10 μg/mL biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin for 15 min. Cell lysates were immunoprecipitated with the anti-JNK Ab *(upper panel)* or the anti-MAPKAP kinase-2 Ab *(lower panel)* and *in vitro* kinase assays were performed using GST-c-Jun as a substrate for JNK and Hsp25 as a substrate for MAPKAP kinase-2. The JNK or MAPKAP kinase-2 activity induced by engaging CD8/(1-74) was assigned a value of 100%. The data represent the mean and standard deviation from three independent experiments.

Percent JNK activity relative to CD8/(1-74)
120
100
80
60
40
20
0
CD8/(1-74)
CD8/
CD8/(15-30) clone 1
CD8/(15-30) clone 2

Percent MAPKAPK-2 activity relative to CD8/(1-74)
120
100
80
60
40
20
0
CD8/(1-74)
CD8/
CD8/(15-30) clone 1
CD8/(15-30) clone 2

Figure 4.8B: The CD8/(15-30) chimeric receptor does not induce activation of NF-κB.

The two WEHI-231 clones expressing CD8/(15-30) were stimulated with 10 μg/mL biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin for 1 to 10 min. In the *upper panel* of each pair, cell lysates were analyzed by immunoblotting with an Ab specific for the phosphorylated form of IκBα (P-IκBα). The filters were then stripped and reprobed with an anti-IκBα Ab *(lower panels)* to assess IκBα degradation. Lysates of CD8/(1-74)-expressing cells were used as a positive control for IκBα phosphorylation.


CD8/(1-74)
CD8/(15-30) clone 1
CD8/(15-30) clone 2
P-IκBα
IκBα
0' 2'
0' 1' 2' 5' 10' 0'
0' 1' 2' 5' 10' 0'

CD40 responses and that interactions mediated by the extracellular or transmembrane domains of CD40 are not required for these events.

This is the first report directly identifying the region of CD40 that activates the JNK and p38/MAPKAP kinase-2 pathways. I have also shown that this same region, amino acids 35-45 of the CD40 cytoplasmic domain, is sufficient for CD40 to induce the phosphorylation and degradation of IκBα. Studies by other groups have shown that amino acids 36-52 of the CD40 cytoplasmic domain are sufficient to activate NF-κB in 293 cells (165) and that amino acids 32-41 are necessary for CD40 to activate NF-κB in B cells (150). Taken together, these results indicate that amino acids 36-41 of CD40 (PVQETL) are critical for CD40 to activate NF-κB. This is consistent with recent findings that a PVQET motif is essential for the CD40-related Epstein-Barr virus LMP1 protein to activate NF-κB (262).

4.8.2 Threonine-40 is essential for CD40 signaling

The threonine residue at position 40 of the CD40 cytoplasmic domain appears to be particularly important for CD40 signaling. I found that changing this threonine residue to an alanine abolished the ability of the CD8/(35-53) chimeric receptor to activate NF-κB, JNK and MAPKAP kinase-2. Although further mutational analysis is required to determine whether the PVQET motif is essential for CD40 to activate JNK and MAPKAP kinase-2, the threonine residue is essential for most of the responses initiated by CD40 engagement. Changing this residue to an alanine abrogates the ability of CD40 to activate NF-κB (257), and to induce homotypic aggregation, Ab secretion, and upregulation of B7.1, Fas, and CD23 (255,256). Thus, either the PVQET motif or another overlapping signaling motif containing threonine-40 is responsible for the majority of CD40-induced signaling events including, as I have shown, activation of the JNK and the p38/MAPKAP kinase-2 pathways. An important question that remains to be determined is whether threonine-40 is phosphorylated and

whether phosphorylation of this residue affects the ability of CD40 to bind to adapter proteins and to signal.

4.8.3 TRAF proteins may bind to residues 35-45 of the CD40 cytoplasmic tail and mediate CD40 signaling

My results indicate that the activation of JNK, p38, and NF-κB by CD40, as well as protection of WEHI-231 cells from anti-IgM-induced growth arrest, is mediated by proteins that bind to residues 35-45 of the CD40 cytoplasmic domain. TRAF2, TRAF3, TRAF5 and TRAF6 can bind directly to the cytoplasmic region of CD40 via their highly-related C-terminal TRAF domains (157-160). *In vitro* binding assays have shown that TRAF2 and TRAF3 can bind to fusion proteins or peptides corresponding to amino acids 36-52 of murine CD40 (165,166,254) while TRAF5 binds to an identical or overlapping region of CD40 (159). The CD40 signaling motif I have identified, residues 35-45 of the CD40 cytoplasmic domain, may therefore contain the essential elements for binding TRAF2, TRAF3, and TRAF5. My findings are consistent with the idea that TRAF2, TRAF3 or TRAF5 mediate the ability of CD40 to activate NF-κB, JNK, and the p38/MAPKAP kinase-2 pathway as well as the ability of CD40 to protect WEHI-231 cells from anti-IgM-induced growth arrest. Several lines of evidence support this conclusion. First, overexpression of TRAF2 or TRAF5 in fibroblasts results in activation of both NF-κB and JNK (157,159,162,249-251,261). Second, expressing a truncated (i.e. dominant-negative) form of TRAF2 in B cells blocks the ability of CD40 to activate JNK (263), implicating the portion of CD40 that binds TRAF2, TRAF3, and TRAF5 in this response. Similarly, it has recently been shown that expressing a dominant-negative form of TRAF3 in B cells blocks activation of p38 by CD40 (164). Finally, changing the threonine residue in the PVQET motif of human CD40 to an alanine not only prevents CD40 signaling but also abolishes the ability of CD40 to bind TRAF2, TRAF3 and TRAF5 (158,159,165). Although I cannot rule out the involvement of other

proteins that bind to residues 35-45 of the CD40 cytoplasmic domain, these data all support the idea that residues 35-45 of CD40 constitute a minimal TRAF-binding motif and that TRAF2, TRAF3 or TRAF5 couple CD40 to activation of JNK, p38 and NF-κB.

4.8.4 Signaling components that connect the TRAF proteins to NF-κB, JNK and p38

TRAF2 and TRAF5 can activate both NF-κB and JNK when overexpressed in fibroblasts (159,162,261), whereas a dominant negative TRAF3 fully blocks activation of p38 and partially blocks activation of JNK in CD40-stimulated human B cells (164). These studies suggest that TRAF2, TRAF3 and/or TRAF5 mediate activation of NF-κB, JNK and p38. My finding that the TRAF2/TRAF3/TRAF5 binding site of CD40 is sufficient for activation of NF-κB, JNK and p38 is consistent with these studies. TRAF2 binds NIK (162,264), a kinase that binds to and presumably activates the IκB kinase (IKK) complex (154) which is responsible for phosphorylating IκBα. The link between TRAF proteins and JNK/p38 activation is not as well understood but involves a family of kinases (MEKK1-4, MLK, and GCK) that phosphorylate and activate the MKKs which activate JNK and p38. It is not known how TRAF proteins are coupled to the MKK kinases but they are thought to be activated by upstream kinases called PAKs which in turn are regulated by GTPases such as Rac and Cdc42 (see Fig. 3.11) (121,122,232).

4.8.5 The TRAF6 binding site of CD40 is not sufficient for CD40 signaling in WEHI-231 cells

Unlike the other TRAFs, TRAF6 binds to a membrane-proximal region of CD40, residues 14-23 of human CD40 or residues 19-28 of murine CD40 (160,254). Although TRAF6 can activate NF-κB and JNK when overexpressed in fibroblasts (160,162), I found that the CD8/(15-30) chimeric receptor, which contains the TRAF6 binding site, was unable to activate NF-κB, JNK, or MAPKAP kinase-2 when

expressed in WEHI-231 cells (Fig. 4.8). This suggests that TRAF6 does not make a major contribution to these responses, at least in WEHI-231 cells. Although WEHI-231 cells express TRAF6 mRNA (160), they may not express sufficient amounts of the TRAF6 protein to allow this region of CD40 to activate these signaling pathways. Alternatively, the CD8/(15-30) chimeric receptor may not be able to interact efficiently with TRAF6 either because it is misfolded or because additional sequences are required. While I cannot rule out that the cytoplasmic domain of CD8/(15-30) is misfolded, Pullen et al. showed that a peptide containing only residues 18-28 of CD40 can bind TRAF6 with high affinity *in vitro* (254). Thus if folded properly, the CD8/(15-30) chimeric receptor should be able to bind TRAF6. Further work is required to determine the relative contribution of TRAF6 to the ability of CD40 to activate NF-κB, JNK, and p38 in B cells.

4.8.6 Residues 35-45 of the CD40 tail mediate protection of WEHI-231 cells from anti-IgM-induced growth arrest

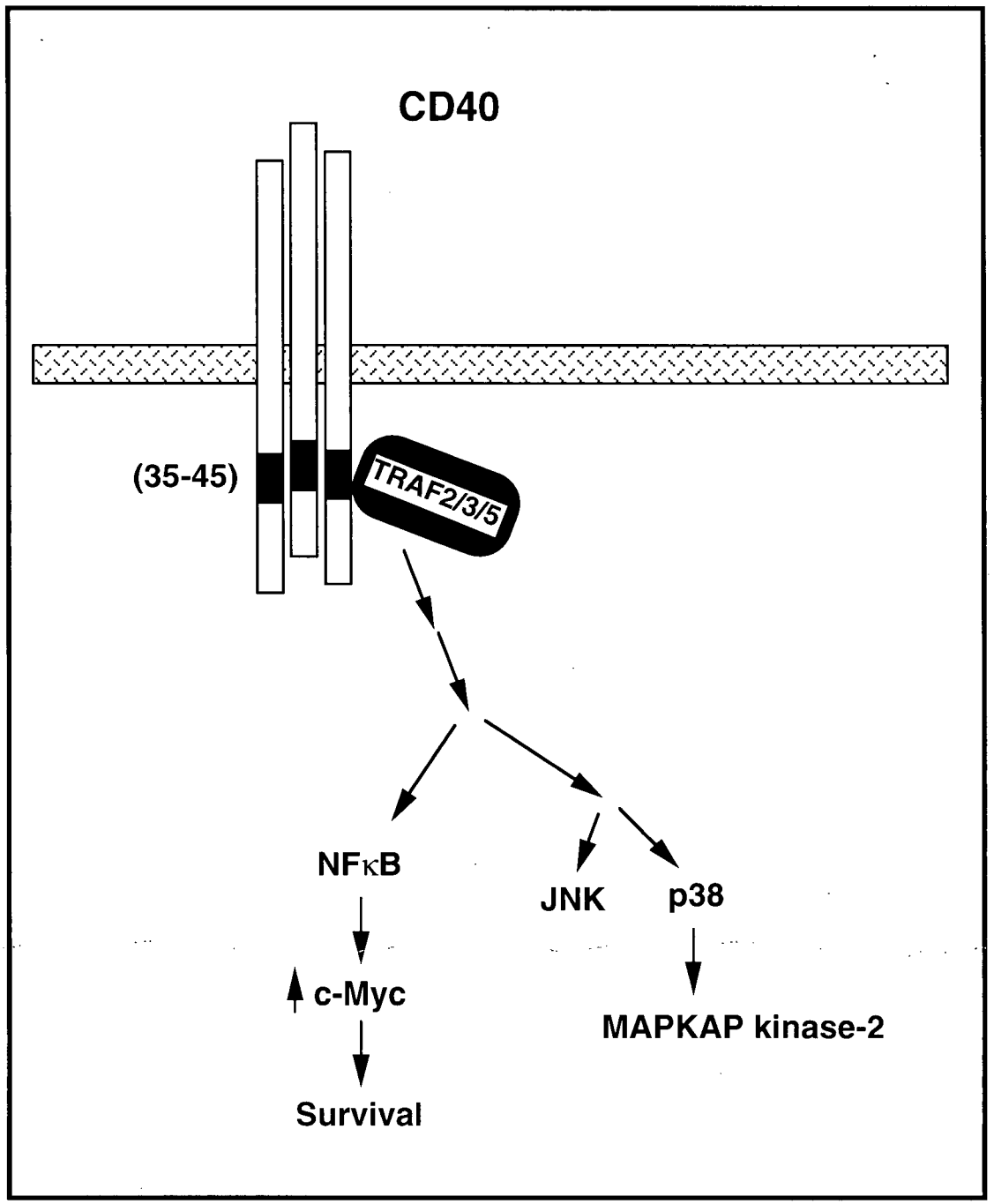
In addition to activating JNK, p38 and NF-κB, we found that signaling mediated by amino acids 35-45 of the CD40 cytoplasmic domain was sufficient to completely protect WEHI-231 cells from anti-IgM-induced growth arrest. The finding that the same region of CD40 initiates both NF-κB activation and an anti-apoptotic signal is consistent with work showing that CD40 prevents apoptosis in WEHI-231 cells via an NF-κB-dependent pathway that prevents catastrophic decreases in the level of c-Myc (146). Moreover, our finding that the TRAF2/TRAF3/TRAF5 binding site of CD40 is sufficient to protect B cells from apoptosis is consistent with the role of TRAF2 in activating NF-κB. Reports showing that thymocytes from TRAF2-deficient mice are hypersensitive to pro-apoptotic stimuli (265) further support the role of TRAF2 in coupling receptors to survival pathways.

4.8.7 Summary

In summary, I have used a gain-of-function approach to define a major signaling motif in the cytoplasmic domain of CD40 that is sufficient for activation of JNK and MAPKAP kinase-2, phosphorylation and degradation of IκBα, and for protection of WEHI-231 cells from anti-IgM-induced growth arrest. This is the first report in which the CD40 JNK activation motif and p38 activation motif have been mapped. I found that all of these functions mapped to the same region of CD40 that bind TRAF2, TRAF3, and TRAF5. My results are consistent with a model in which TRAF2, TRAF3 and/or TRAF5 link CD40 to two distinct signaling pathways; a series of kinases that activate NF-κB by phosphorylating its inhibitor, IκB, and a kinase cascade that activates the JNK and p38/MAPKAP kinase-2 pathways (Fig. 4.9). While other studies using loss-of-function approaches such as expression of truncated CD40 proteins or CD40 with point mutations have shown that amino acids 35-45 of the CD40 cytoplasmic domain are important for some CD40 functions, they could not rule out that other regions of CD40 were also required. My results show that amino acids 35-45 of the CD40 cytoplasmic domain are both necessary and sufficient for these responses whereas the membrane proximal region of CD40 which binds TRAF6 is incapable of initiating these responses. Moreover, any interactions mediated by the extracellular or transmembrane domains of CD40 are not necessary for activation of JNK, MAPKAP kinase-2 or NF-κB-mediated protection of WEHI-231 cells from anti-IgM-induced growth arrest.

Figure 4.9: Model for CD40-induced activation of JNK, p38/MAPKAP kinase-2 and NF-κB, as well as protection of WEHI-231 cells from BCR-induced growth arrest.

Upon crosslinking of CD40 by its trimeric ligand, the TRAF2, TRAF3 and/or TRAF5 adapter proteins are recruited to the receptor complex. Residues 35-45 of the CD40 cytoplasmic tail are sufficient to mediate this recruitment. TRAF2, TRAF3 and/or TRAF5 link CD40 to two distinct signaling pathways; a series of kinases that activate NF-κB by phosphorylating its inhibitor, IκB, and a kinase cascade that activates the JNK and p38/MAPKAP kinase-2 pathways.


CD40
(35-45)
TRAF2/3/5
NFκB
JNK
p38
c-Myc
MAPKAP kinase-2
Survival

CHAPTER 5

Signaling by Another TRAF-Associated Receptor (ATAR) in B Cells

5.1 Introduction

Members of the tumor necrosis factor receptor (TNFR) superfamily play important roles in regulating lymphocyte development and function (20,155). For example, CD40 regulates Ig class switching, cytokine production and survival in B cells (26), TNFR2 stimulates the proliferation of thymocytes (266), and Fas triggers apoptosis of activated lymphocytes (60,267). TNFR superfamily members are characterized by an extracellular domain containing multiple repeats of a cysteine-rich motif (20). However, the cytoplasmic regions of these receptors share limited sequence homology with the exception of the "death domain" found in TNFR1, Fas and DR3 (22,268-270).

TNFR superfamily members associate with two main classes of adapter proteins that couple these receptors to signaling pathways. The "death domain"-containing adapter proteins TRADD, RIP and FADD associate with the death domain of TNFR1 and Fas. FADD, in turn, recruits Caspase-8 (Flice), an ICE-like cysteine protease. The recruitment of Caspase-8 initiates a protease cascade that results in apoptosis (271-273). The second class of adapter proteins, the TNFR-associated factors (TRAFs), bind directly to TNFR2, CD40, CD30 and the lymphotoxin-β receptor (156,274-276). The cytoplasmic domains of these TRAF-associated receptors share a few conserved amino acids that mediate TRAF binding. The TRAF proteins connect TNFR superfamily members to activation of the NF-κB transcription factor and to JNK (159,160,162,261), a kinase that contributes to activation of the AP-1 transcription factor (112,239). Both NF-κB and AP-1 induce the expression of many lymphocyte

activation-associated genes including ones encoding cytokines, cytokine receptors and cell adhesion molecules (150,155).

Recently, another TRAF-associated receptor (ATAR) was identified as a novel member of the TNFR superfamily (171). Human ATAR is over 99% homologous to the Herpes virus entry mediator (HVEM) that is expressed on human B and T cells (172,173) and is believed to be the same protein. ATAR/HVEM has recently been shown to bind the Herpes simplex virus (HSV) gD protein and mediate entry of (HSV) type-1 into human T and B cells (172,173). Other ligands for ATAR/HVEM include LIGHT, a new member of the TNF superfamily that is expressed on the surface of activated T cells, as well as lymphotoxin-α (LTα), a cytokine secreted by activated T cells.

Since ATAR binds ligands made by activated T cells, ATAR may play a role in T cell-dependent activation of B cells, similar to CD40. In this thesis we tested the hypothesis that signaling by ATAR induces many of the same responses in B cells as signaling by CD40, a TNFR superfamily member that is a key regulator of B cell activation and function. A study in which ATAR and the TRAF proteins were coexpressed in 293 fibroblasts demonstrated that, like CD40, ATAR initiates signaling events by binding TRAF2 and TRAF5 (171). Moreover, ATAR also activates NF-κB, a target of CD40 signaling, in transiently transfected 293 cells (171). However, it is not known whether ATAR can activate these signaling pathways under physiological conditions or in B cells. It also remained to be tested whether ATAR mediates activation of the JNK and p38 MAP kinases in B cells or protects B cells from antigen receptor-induced apoptosis as does CD40.

We have used the approach of making chimeric receptors to induce ATAR signaling since antibodies to ATAR are unavailable. Furthermore, the use of natural ligands to study ATAR signaling may be difficult given the promiscuity of members of the TNF superfamily. For example, LTα binds TNFR1 (277,278), TNFR2 (279) and

HVEM (173), while LIGHT binds HVEM and the LTβ receptor (173). These complex receptor-binding patterns exhibited by TNF superfamily members make it difficult to use natural ligands to study the signaling events that result from engaging a single TNFR superfamily member. The use of chimeric receptors to study ATAR signaling avoids such difficulties.

This work was performed in collaboration with Kirsten Mattison, an undergraduate student who worked in our laboratory under my direction. We chose to work with the murine form of ATAR since we have a successful, retroviral-mediated transfection system for expressing proteins in murine B cell lines. The murine and human forms of ATAR share 51% and 25% identity within their extracellular and intracellular domains, respectively (171). Chimeric receptors consisting of the extracellular and transmembrane domains of human CD8α fused to all or part of the murine ATAR cytoplasmic domain were expressed in WEHI-231 B lymphoma cells. Two CD8/ATAR chimeric receptors were generated. The first contained the entire 46 amino acid residue ATAR cytoplasmic tail. A second chimeric receptor containing only the C-terminal 20 amino acids of ATAR was generated to determine which ATAR-induced signaling events were mediated by the TRAF2 and TRAF5 adapter proteins. This C-terminal portion of ATAR contains the TRAF2 and TRAF5 interaction domain of ATAR (171).

Anti-CD8 mAbs were used to selectively induce signaling by the CD8/ATAR chimeric receptors. We found that the cytoplasmic tail of ATAR could activate NF-κB, JNK and p38, as well as prevent BCR-induced apoptosis in WEHI-231 cells. In addition, we found that the C-terminal portion of the ATAR tail was sufficient for inducing these signaling events in B cells. These results suggest that TRAF2 and/or TRAF5 may link ATAR to NFκB activation, to MAP kinase activation, and to protection of B cells from BCR-induced apoptosis. Thus, ATAR is capable of signaling in B cells

and, at least for the signaling events tested, ATAR appears to signal in a similar manner to CD40.

5.2 Construction of CD8/ATAR chimeric receptors

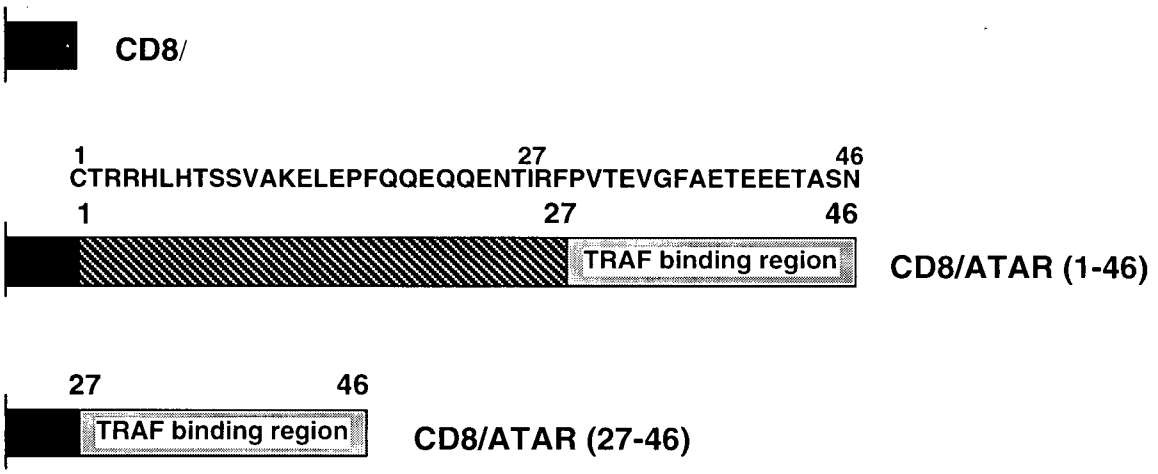
To evaluate ATAR signaling in B cells, two chimeric CD8α/ATAR receptors consisting of the extracellular and transmembrane domains of CD8α fused to fragments of the murine ATAR cytoplasmic domain were constructed. The ATAR fragments included the full length ATAR cytoplasmic domain (amino acids 1-46; numbering from the start of the ATAR cytoplasmic domain) and the C-terminal 20 amino acids of the ATAR cytoplasmic domain (amino acids 27-46) (Fig. 5.1). We were interested in determining whether this C-terminal portion of ATAR, which contains the TRAF2/TRAF5 binding site (171), was sufficient for ATAR signaling. The sequences of the complementary oligonucleotides used to construct the ATAR tail segments are listed in Table 5.1. Four oligonucleotides were used to construct the full length ATAR cytoplasmic tail. These four oligonucleotides were annealed together to create two overlapping sets of complementary oligonucleotides. These overlapping fragments were then ligated to each other in equimolar ratios to construct the full length ATAR cytoplasmic tail. Once formed, the double-stranded ATAR cDNA fragments were cut with *Bgl II* and ligated into the *Bgl II* site of CD8 in the pLXSN/CD8 retroviral expression vector (see Chapter 2 for details). Plasmids were cut with *Bgl II* to screen for the presence of inserts corresponding to the ATAR cytoplasmic domain. The sequence of each CD8/ATAR chimeric cDNA was confirmed by DNA sequencing as described in Chapter 2.

5.3 Expression of CD8/ATAR chimeric receptors in WEHI-231 B cells

After infecting WEHI-231 murine B cells with retroviral particles carrying pLXSN-CD8/ATAR (1-46) or pLXSN-CD8/ATAR (27-46), G418-resistant clones were screened

Figure 5.1: Schematic representation of the CD8/ATAR chimeric receptors.

The CD8/ protein contains the extracellular and transmembrane domains of human CD8α as well as the first four amino acids of the CD8α cytoplasmic domain. The solid black box represents the cytoplasmic domain of the truncated CD8α. For the chimeric receptors, either the entire 46 amino acid murine ATAR cytoplasmic domain or the C-terminal 20 amino acids of the ATAR cytoplasmic domain were fused to the C-terminus of CD8/. The amino acid sequence of the ATAR cytoplasmic domain is shown and the residues are numbered starting at the inner face of the plasma membrane. The numbers in brackets indicate which portions of the ATAR cytoplasmic domain have been fused to CD8α for each chimeric receptor.


CD8/
1
27
46
CTRRHLHTSSVAKELEPFQQEQQENTIRFPVTEVGFAETEEETASN
1
27
46
TRAF binding region
CD8/ATAR (1-46)
27
46
TRAF binding region
CD8/ATAR (27-46)

ATAR tail amino acids	*Oligo type*	*Oligonucleotide sequence (5' to 3')*
1 - 29 (for 1-46 construct)	sense	CGG GAA GAT CTT GTA CAC GAA GAC ACC TGC ATA CAT CAA GTG TGG CTA AGG AGC TGG AGC CCT TCC AGC AGG AGC AGC AGG AGA ACA CAA TCC GAT TC
30-46 (for 1-46 construct)	sense	CCT GTG ACA GAG GTG GGT TTC GCT GAG ACA GAG GAG GAG ACA GCT TCC AAC TAG *TGA* GGA TCC AGA TCT TTC GG
1 - 23 (for 1-46 construct)	antisense	CTG CTG CTC CTG CTG GAA GGG CTC CAG CTC CTT AGC CAC ACT TGA TGT ATG CAG GTG TCT TCG TGT ACA AGA TCT TCC CG
24-46 (for 1-46 construct)	antisense	CCG GAA GAT CTG GAT CC*T CAC TA*G TTG GAA GCT GTC TCC TCC TCT GTC TCA GCG AAA CCC ACC TCT GTC ACA GGG AAT CGG ATT GTG TTC TC
27-46	sense	CGG GAA GAT CTA TCC GAT TCC CTG TGA CAG AGG TGG GTT TCG CTG AGA CAG AGG AGG AGA CAG CTT CCA ACT AG *T GA*G GAT CCA GAT CTT CCG G
27-46	antisense	CCG GAA GAT CTG GAT CC*T CA*C TAG TTG GAA GCT GTC TCC TCC TCT GTC TCA GCG AAA CCC ACC TCT GTC ACA GGG AAT CGG AT A GAT CTT CCC G

Table 5.1: Oligonucleotides used to generate murine ATAR cytoplasmic tail fragments. *Bgl II* sites underlined, stop codons italicized. (See text for details)

for CD8/ATAR expression by flow cytometry. WEHI-231 clones expressing the parental CD8α molecule which contains only the extracellular and transmembrane domains of CD8α (CD8/), were also generated. For each receptor expressed; clones with similar levels of expression were chosen for further study (Fig. 5.2).

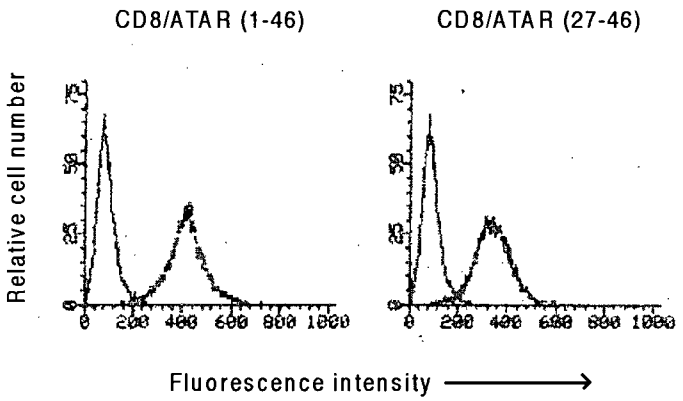
5.4 ATAR can activate JNK and p38 in B cells

MAP kinases are serine/threonine protein kinases which appear to be involved in both mitogenic and apoptotic responses to receptor signaling (106,131). These kinases are activated by dual phosphorylation of threonine and tyrosine residues in a threonine-X-tyrosine activation motif (109,110). Three families of MAP kinases have been identified and include the c-Jun amino terminal kinases (JNKs), the p38 kinases and the extracellular signal-regulated kinases (ERKs). When activated, these kinases translocate to the nucleus where they phosphorylate and activate different sets of transcription factors (110,112). I have previously shown that CD40 strongly activates the JNK and p38 MAP kinases in WEHI-231 cells (245). In addition, HVEM/ATAR has been shown to activate JNK when transiently transfected into 293 cells (280). We asked whether, similar to CD40, the cytoplasmic domain of ATAR could activate the JNK and p38 MAP kinases in WEHI-231 B cells.

We first determined whether the chimera containing the full length ATAR tail, CD8/ATAR (1-46), was capable of activating JNK and p38 in WEHI-231 cells. Biotinylated anti-CD8 mAb plus avidin was used to stimulate signaling by the CD8/ATAR chimeric receptors. After stimulating the cells for various times, cell lysates were immunoblotted with antibodies specific for either phospho-JNK or phospho-p38. These antibodies detect the dually phosphorylated threonine-X-tyrosine activation motif in JNK or p38, respectively. The appearance of these dually phosphorylated forms of JNK and p38 indicates that these kinases have been activated since phosphorylation is essential for MAP kinase activity (109,110). We found that

Figure 5.2: Expression of CD8/ATAR chimeric receptors in WEHI-231 cells.

Untransfected parental WEHI-231 cells (peak on the left hand side of each histogram) as well as a stable clone expressing either the CD8/ATAR (1-46) or CD8/ATAR (27-46) chimeric receptor were stained with the anti-human CD8 mAb, OKT8, followed by anti-mouse IgG-FITC. The numbers in brackets denote which amino acids of the ATAR cytoplasmic domain were present in each chimeric receptor.


CD8/ATAR (1-46)
CD8/ATAR (27-46)
Relative cell number
0
25
50
75
0
200
400
600
800
1000
Fluorescence intensity

clustering the full length ATAR cytoplasmic tail stimulated phosphorylation of both the p46 and p54 isoforms of JNK (Fig. 5.3), as well as p38 (Fig. 5.4). Maximal phosphorylation of the JNK and p38 MAP kinases occurred within 5 to 10 min of receptor engagement (Fig. 5.3 and 5.4). These results suggest that transcription factors targeted by JNK and/or p38, such as c-Jun (112), AP-1 (112), ATF2 (210), CHOP (138), and CREB (214) may be activated by ATAR and mediate functional responses in B cells.

CD8/ATAR (27-46) also induced phosphorylation of p38 (Fig. 5.4), although with slower kinetics than CD8/ATAR (1-46). Thus, the TRAF binding site of ATAR is sufficient to mediate ATAR-induced activation of p38. This finding suggests that TRAF proteins mediate ATAR-induced activation of p38. However, we cannot rule out the possibility that another, unidentified molecule associates with this region of ATAR and mediates activation of p38. Our finding that it takes several minutes longer for CD8/ATAR (27-46) to induce phosphorylation of p38 compared to CD8/ATAR (1-46) suggests that the adapter molecule(s) that mediate activation of p38 may bind better to CD8/ATAR (1-46) compared to CD8/ATAR (27-46). It remains to be determined whether the C-terminal 20 amino acids of ATAR are sufficient for mediating activation of JNK.

5.5 The cytoplasmic domain of ATAR can mediate activation of NF-κB in B cells

When overexpressed in fibroblasts, ATAR activates the transcription factor NF-κB (171). This activation appears to be mediated by the TRAF proteins, since a mutated ATAR that cannot bind TRAF2 or TRAF5 fails to activate NF-κB (171). However, it is not known whether ATAR can activate NF-κB under physiological conditions or in B cells. To address these questions, we tested the ability of our CD8/ATAR chimeric receptors to induce phosphorylation and subsequent degradation

Figure 5.3: The cytoplasmic domain of ATAR mediates phosphorylation of the p46 and p54 isoforms of JNK1.

A WEHI-231 cell clone expressing the CD8/ATAR (1-46) chimeric receptor was stimulated with 10 μg/mL biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin for 15 min. Cell lysates were analyzed for JNK activation by immunoblotting with an Ab specific for the phosphorylated form of JNK1 (phospho-JNK). The 46 kDa and 54 kDa forms of JNK1 are indicated by the arrows. Molecular weight standards are indicated on the right.

CD8/ATAR (1-46)

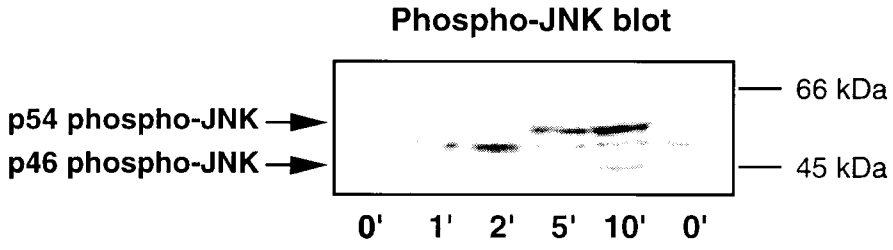
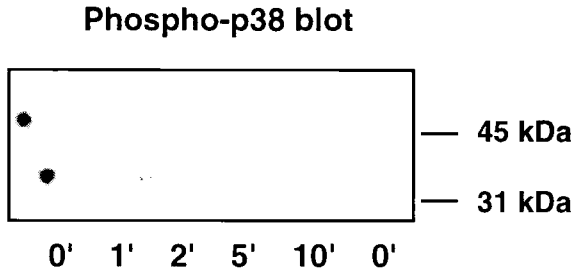

Phospho-JNK blot
p54 phospho-JNK
p46 phospho-JNK
66 kDa
45 kDa
0'
1'
2'
5'
10'
0'

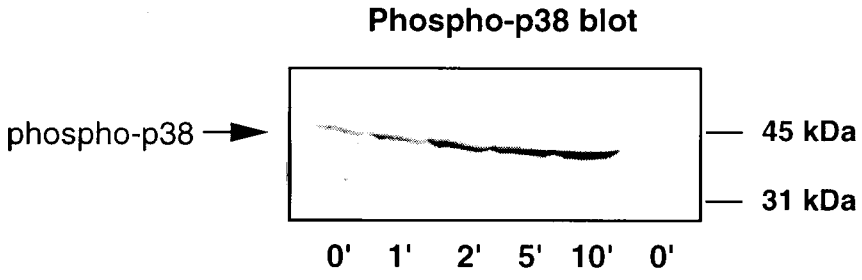
Figure 5.4 The cytoplasmic domain of ATAR mediates phosphorylation of p38.

WEHI-231 clones expressing CD8/, CD8/ATAR (1-46) or CD8/ATAR (27-46) were stimulated with 10 μg/mL biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin for 15 min. Cell lysates were analyzed by immunoblotting with an Ab specific for the phosphorylated form of p38 (phospho-p38).

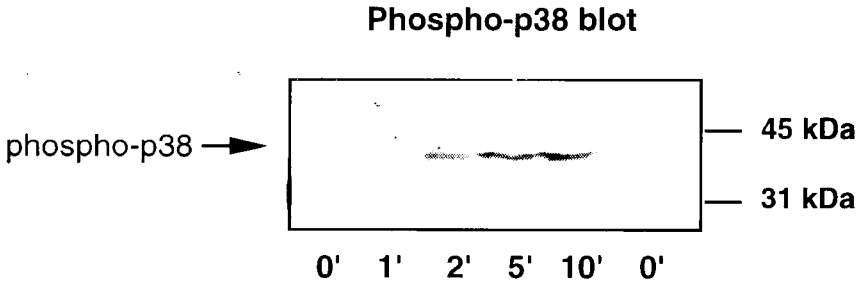
CD8/



CD8/ATAR (1-46)



CD8/ATAR (27-46)



of IκBα, an inhibitor of NF-κB. In its normal, unphosphorylated state, IκBα inhibits NF-κB activity by sequestering NF-κB in the cytoplasm (150). Phosphorylation of IκBα targets the inhibitor for degradation, thus freeing NF-κB to translocate to the nucleus (150,259). Biotinylated anti-CD8 mAb plus avidin was used to stimulate ATAR signaling. After stimulating the cells for various times, cell lysates were immunoblotted with antibodies specific for either phospho-IκBα or IκBα. As expected, CD8/ failed to induce IκBα phosphorylation or degradation (Fig. 5.5). However, cross-linking the chimera containing the full length ATAR cytoplasmic tail, CD8/ATAR (1-46), induced marked phosphorylation of IκBα within 2 min of receptor engagement (Fig. 5.5). Degradation of phosphorylated IκBα was evident 5 min after CD8/ATAR (1-46) engagement (Fig. 5.5). This indicates that NF-κB was free to translocate to the nucleus and activate transcription (152). Our results demonstrate that the cytoplasmic tail of ATAR mediates activation of NF-κB under physiological conditions and that this activation can occur in B cells.

CD8/ATAR (27-46) also mediated phosphorylation and degradation of IκBα (Fig. 5.5). However, CD8/ATAR (27-46)-induced IκBα phosphorylation and degradation occurred more slowly and was less marked than that induced by CD8/ATAR (1-46) (Fig. 5.5). These results demonstrate that the last 20 amino acids of ATAR, which contain the TRAF interaction domain, are sufficient to mediate activation of NF-κB. However, the N-terminal portion of the ATAR cytoplasmic tail appears to enhance the binding of adapter proteins to the last 20 amino acids of ATAR. Our results are consistent with the idea that the TRAF2 and/or TRAF5 adapter proteins couple ATAR to activation of NF-κB (171).

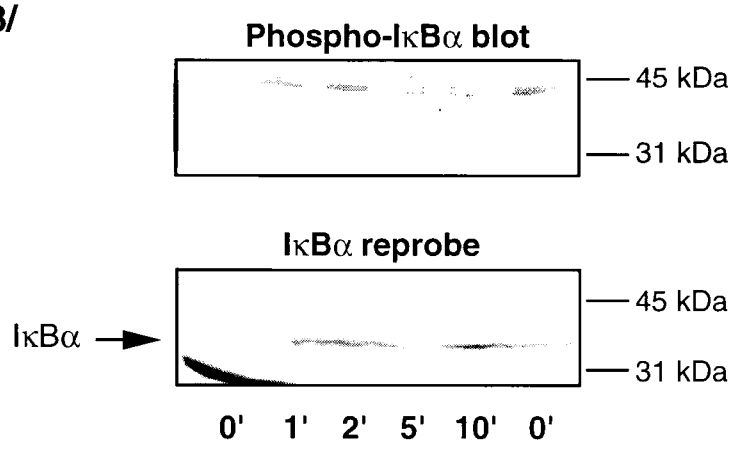
5.6 ATAR can prevent BCR-induced growth arrest in WEHI-231 cells

Antigen binding to the BCR on naive B cells can result in activation, anergy or apoptosis depending on whether the cell receives a second, costimulatory signal

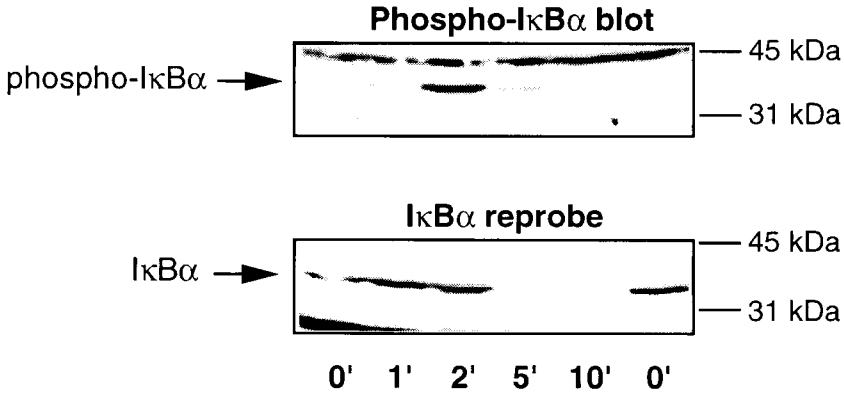
Figure 5.5: The cytoplasmic domain of ATAR mediates phosphorylation and degradation of IκBα.

CD8/ATAR chimeric receptor-expressing WEHI-231 clones were stimulated with 10 μg/mL biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin for the indicated times. In the *upper panels* for each chimera, cell lysates were analyzed by immunoblotting with an Ab specific for the phosphorylated form of IκBα (phospho-IκBα). The membranes were then stripped and reprobed with an anti-IκBα Ab *(lower panels)* to detect degradation of IκBα.

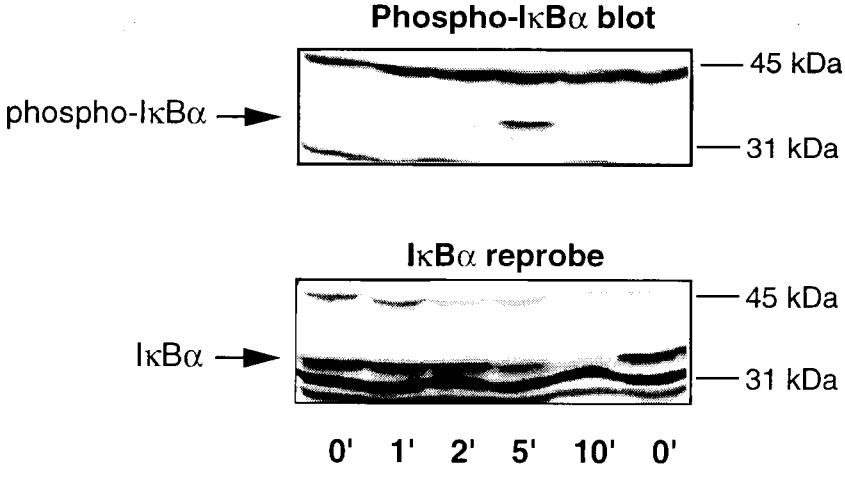
CD8/



CD8/ATAR (1-46)



CD8/ATAR (27-46)



through CD40 (1). Engagement of the BCR on WEHI-231 B cells induces growth arrest followed by apoptosis (63-65). However, if these cells receive a second signal through CD40, the BCR-induced growth arrest/apoptosis is abrogated (28,66,67,195). CD40 protects B cells from BCR-induced apoptosis through NF-κB dependent transcription of the *c-myc* gene (146,147). Since we have shown that ATAR is capable of activating NF-κB in WEHI-231 cells, we asked whether ATAR can substitute for CD40 and protect these cells from anti-IgM-induced growth arrest. To test this hypothesis, WEHI-231 clones expressing CD8/ATAR chimeric receptors were cultured with various stimuli, and 48 hours later the ability to incorporate [^{3}H]-thymidine was evaluated (Fig. 5.6). We found that signaling through CD8/ATAR (1-46) abrogated anti-IgM-induced growth arrest to a similar extent as signaling through endogenous CD40 (Fig. 5.6). Similar results were found with CD8/ATAR (27-46) (Fig. 5.6). As expected, CD8/ failed to confer protection from anti-IgM treatment (Fig. 5.6). These results demonstrate that ATAR could substitute for CD40 in providing a costimulatory signal to B cells.

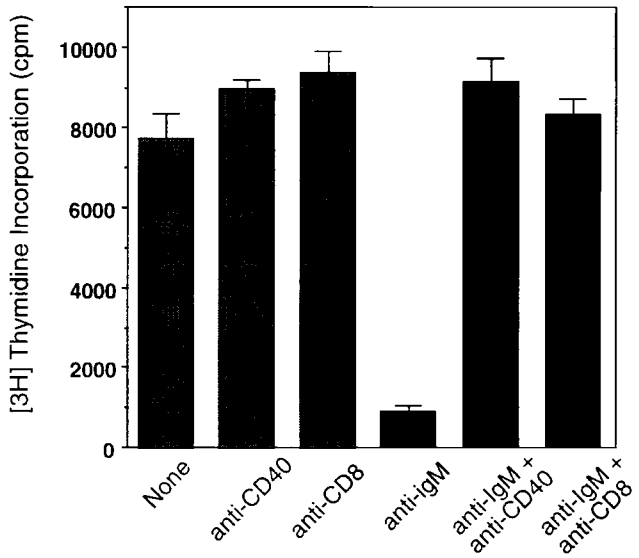
5.7 Discussion

We constructed CD8/ATAR chimeric receptors and expressed these receptors in WEHI-231 cells in order to study ATAR signaling. We found that the ATAR cytoplasmic domain activated the JNK and p38 MAP kinases, and induced phosphorylation and degradation of IκBα. We also showed that signaling mediated by the ATAR cytoplasmic domain could protect WEHI-231 cells from anti-IgM-induced growth arrest to a similar extent as CD40 (Fig. 5.6). Finally, the C-terminal 20 amino acids of ATAR, which contain the TRAF2/TRAF5 binding site (171), was sufficient for activation of p38, phosphorylation and degradation of IκBα, and protection of WEHI-231 cells from anti-IgM-induced growth arrest. Our results demonstrate that clustering of the ATAR cytoplasmic tail initiates these ATAR signaling events, that the

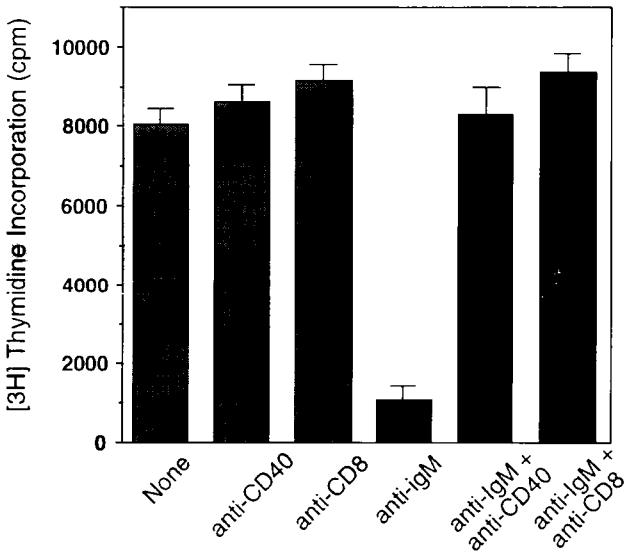
Figure 5.6: The cytoplasmic domain of ATAR mediates protection of WEHI-231 cells from anti-IgM-induced growth arrest.

WEHI-231 cell clones expressing the CD8/ATAR (1-46) chimeric receptor or the CD8/ATAR (27-46) chimeric receptor were cultured in medium containing 3 μg/mL anti-IgM Ab, 10 μg/mL of biotinylated 51.1 (anti-CD8 mAb) and 10 μg/mL avidin, 3 μg/mL anti-IgM and 10 μg/mL each of 51.1-biotin and avidin, or 5 μg/mL 1C10 (anti-CD40 mAb) for 40 h. The incorporation of [^{3}H] thymidine into DNA was determined by liquid scintillation counting. All determinations were carried out in triplicate. Error bars represent the mean $\pm$ the standard error of replicate samples.

CD8/ATAR (1-46)



CD8/ATAR (27-46)



extracellular and transmembrane regions of ATAR are not required for these events, and that ATAR can induce these events in B lymphocytes.

Our finding that ATAR is capable of activating the MAP kinase JNK is consistent with a report that transient transfection of HVEM/ATAR into 293 cells induces activation of JNK (280). Similar to CD40 (245), ATAR induces activation of both the p46 and p54 isoforms of JNK1. This is the first report that ATAR/HVEM activates p38, the other MAP kinase activated by CD40 in WEHI-231 cells (245). Since JNK and p38 activate a variety of transcription factors, including c-Jun, ATF2, CHOP and CREB (112,138,210,214), activation of JNK and p38 should enable ATAR/HVEM to regulate the expression of a number of genes. We predict that transcription of some of these genes may contribute to B cell activation. Although highly expressed in WEHI-231 cells, ERK1 and ERK2, members of the third MAP kinase family, are not activated by CD40 in these cells. Future work could test whether ATAR induces activation of ERK.

ATAR is likely linked to activation of JNK and p38 through its association with the TRAF2 and TRAF5 adapter proteins (171). This notion is supported by the finding that TRAF2 and TRAF5 can activate JNK when overexpressed in fibroblasts (159,162). Furthermore, we found that the C-terminal 20 amino acids of ATAR, which contain the TRAF2/TRAF5 interaction domain, are sufficient to mediate activation of p38. However, we cannot rule out the possibility that other, unidentified, molecules bind to this C-terminal region of ATAR and are responsible for mediating p38 activation. It remains to be tested whether the C-terminal 20 amino acids of ATAR are sufficient for ATAR-induced activation of JNK. Our finding that the region of ATAR that binds the TRAF proteins is sufficient to mediate ATAR-induced activation of p38 is not surprising given that the TRAF proteins appear to mediate activation of JNK and p38 by several other TNFR superfamily members, including CD40 (164). TRAF proteins are thought to be coupled to JNK and p38, through monomeric GTPases (121,122,232) which in turn activate a group of serine/threonine kinases known as p21-associated kinases

(PAKs) (122,134). The PAKs appear to be upstream of another group of kinases (ASK-1 and MEKK1-4) that activate the MAP kinase kinases (MKKs) which in turn activate JNK and p38 (131,281). Future work could test whether our CD8/ATAR chimeric receptors induce activation of Ask-1, MEKKs and MKKs in WEHI-231 cells.

In addition to activating JNK and p38, we found that ATAR induces phosphorylation and degradation of IκBα. Moreover, the C-terminal 20 amino acids of ATAR which contain the TRAF2/TRAF5 binding site were sufficient to mediate these events. These findings are consistent with a report that overexpression of ATAR in fibroblasts activates NF-κB (171) and that coexpression of ATAR with TRAF5 results in synergistic activation of NF-κB (171). The TRAF proteins appear to be linked to IκBα by binding NIK (162,264), a kinase that associates with and is thought to activate the IκB kinase (IKK) complex (154). IKK, in turn, phosphorylates IκBα, thereby targeting IκBα for ubiquitin-mediated degradation by proteasomes (154). Whether TRAF2 and/or TRAF5 links ATAR to IκBα inactivation by this pathway remains to be determined.

The finding that ATAR induces activation of NFκB and mediates protection of WEHI-231 cells from BCR-induced growth arrest is consistent with reports that NFκB induces transcription of a gene, or a group of genes, that prevent cells from undergoing apoptosis in response to a variety of signals (282). Indeed, CD40 protects WEHi-231 cells from BCR-induced apoptosis via NF-κB-dependent induction of *c-myc* (146). It will be interesting to determine whether ATAR rescues WEHI-231 cells from BCR-induced apoptosis by a similar mechanism as CD40, or whether it activates an entirely different survival pathway.

Although CD8/ATAR (27-46) mediated phosphorylation of p38 and IκBα, as well as degradation of IκBα, these events were induced with slightly slower kinetics compared to CD8/ATAR (1-46). Since these two chimeric receptors were expressed at similar levels (Fig. 5.2), the C-terminal 20 amino acid portion of the ATAR tail appears

to signal less efficiently than the full length ATAR tail. It is possible that the N-terminal portion of the ATAR tail stabilizes the ATAR/TRAF interaction. Alternatively, maximal activation of these signaling pathways may depend on concomitant binding of TRAF to the C-terminal portion of the ATAR tail and binding of another, unknown molecule to the N-terminal portion. A third possibility to explain the slower signaling kinetics of CD8/ATAR (27-46) compared to CD8/ATAR (1-46), is that in this truncated receptor the TRAF binding site is too close to the plasma membrane to allow efficient TRAF binding.

At least for the signaling events tested, ATAR appears to signal in B cells in much the same way as CD40 does. Both of these receptors associate with TRAF2 and TRAF5, activate JNK and p38, induce phosphorylation and degradation of IκBα, and protect WEHI-231 cells from anti-IgM-induced growth arrest. Our results suggest that ATAR could regulate B cell activation and survival in a similar manner as CD40. ATAR may provide an alternative way for B cells to obtain the second signal that promotes B cell activation as opposed to anergy or apoptosis. Future work will assess whether ATAR and CD40 are redundant in function or whether they have unique functions. Differences in signaling functions may exist, given that ATAR, unlike CD40, does not appear to bind the TRAF3 adapter protein (171). TRAF3 appears to play a role in CD40-induced activation of p38 (164). However, we have shown that ATAR can activate p38 in the apparent absence of TRAF3 binding. This indicates that other TRAF proteins may substitute for TRAF3 signaling function(s).

The results presented in this study improve our understanding of ATAR signaling. Furthermore, the use of CD8/ATAR chimeric receptors provides a valuable tool for mapping the amino acid residues that mediate different ATAR signaling events. However, to determine whether ATAR actually plays a role in B cell activation and function, we need to confirm that endogenous ATAR can induce the same signaling events as our CD8/ATAR chimeras and that these events are not an artifact of chimera overexpression. Antibodies to ATAR are needed to evaluate signaling by endogenous

ATAR. Antibodies to ATAR can be produced by immunizing rats with a peptide, corresponding to a portion of the extracellular domain of murine ATAR, linked to the carrier, KLH. In addition to being used to study ATAR signaling, these antibodies could be used to analyze the pattern of ATAR expression on B cells at different stages of development.

The role of HSV infection of B and T lymphocytes in the etiology of Herpes virus disease remains to be determined. HSV-1 and HSV-2 infect a variety of cell types in culture. In the natural host, infection is characterized by lesions in the epidermis of mucosal surfaces. The virus then spreads to the peripheral nervous system where it establishes latent infections in neurons (172,173). Binding of HSV-1 or HSV-2 to cells is mediated primarily by interaction of virion glycoprotein C (gC) with heparan sulfate on cell surface proteoglycans. Subsequent fusion between the viral envelope and the cell membrane requires the glycoproteins gB, gD, gH and gL (reviewed in (283)). By binding to gD, HVEM/ATAR (recently designated as HveA (284)) is the principal receptor for entry of HSV into B and T lymphocytes but not into other cell types (172). It remains to be determined whether the gD/ATAR interaction simply represents a mechanism for the entry of HSV into lymphocytes or whether cell-associated gD causes immune modulation of B or T cells. For example by binding ATAR, gD could prevent the binding of ATAR's natural ligands such as LTα and LIGHT and thereby down modulate the immune response. Our finding that ATAR can protect WEHI-231 cells from BCR-induced growth arrest suggests that if gD can induce ATAR signaling this may be a benefit for viral replication. Alternatively, like CD40, ATAR may induce Fas expression and thereby allow infected B cells to be killed by activated T cells.

In conclusion, we have found that ATAR induces activation of the JNK and p38 MAP kinases as well as phosphorylation and degradation of IκBα. ATAR also protects WEHI-231 cells from anti-IgM-induced growth arrest. These events may be mediated by the TRAF2 and/or TRAF5 adapter proteins since the C-terminal portion of the ATAR

cytoplasmic tail which contains the TRAF-interaction domain was found to be sufficient to mediate these responses. These results suggest that ATAR could mimic some of the effects of CD40 on B cells and thereby regulate B cell activation and function. The results presented here provide a framework on which to further explore the role of HVEM/ATAR in B cell development and activation.

CHAPTER 6

Mitogen-Activated Protein Kinase Activation by CD40 and LPS in Murine Dendritic Cells

6.1 Introduction

Dendritic cells (DCs) are unique antigen presenting cells (APCs) that are essential for the initiation of T-cell dependent immune responses (reviewed in (3,4)). The special ability of mature DCs to activate naive $CD4^+$ and $CD8^+$ T cells is not completely understood but is probably related to their relatively high expression (compared to other APCs) of major histocompatibility (MHC) molecules, adhesion molecules such as ICAM-1 and costimulatory molecules such as B7.1 and B7.2 once they are activated by inflammatory stimuli (285,286). DCs also produce IL-1β and IL-12 which activate T cells and influence Th1 versus Th2 differentiation (287-289). In addition to activating naive T cells, DCs also have major effects on extrafollicular B cell growth and differentiation (290).

DCs originate from a $CD34^+$ bone marrow precursor that, depending on the cytokine conditions, also gives rise to granulocytes and macrophages (3,80). During their life cycle, DCs undergo phenotypic and functional changes that are reflective of their maturation state. In the tissues, DCs are present in an immature state in which they are very efficient at capturing and processing antigens as well as forming MHC-peptide complexes but are poor activators of T cells (3,4). In response to a variety of inflammatory signals including the Gram negative bacterial cell wall component lipopolysaccharide (LPS), DCs undergo an initial maturation stage. During this stage, DCs become efficient T cell activators by upregulating surface expression of MHC class II antigens and the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) and by secreting cytokines such as IL-1β and TNFα (80,177,291). LPS also induces DCs

to migrate from the tissues to the T cell-rich areas of lymphoid organs (3,177,292). Terminal maturation of DCs is completed upon interaction with T cells and is characterized by loss of phagocytic ability as well as increased expression of costimulatory molecules and production of cytokines (80,177,287,293). This T-cell dependent maturation of DCs is mediated by T cell-derived cytokines such as interferon-γ, and by CD40 (293) which binds CD40L expressed on activated T cells. In addition to maintaining high level expression of MHC class II, B7.1 and B7.2 molecules on DCs (73), CD40 engagement increases the surface expression of CD40L and CD25 on DCs (73). Expression of CD25, the IL-2Rα chain, is characteristic of mature, interdigitating DCs found in secondary lymphoid organs (73). CD40 also induces DCs to secrete TNFα and IL-12 as well as the chemokines IL-8, macrophage inflammatory protein (MIP)-1α and MIP-1β (73). In light of these findings, a two-step model of DC maturation and activation has been proposed. Signals from the environment, such as LPS, initiate the maturation of DCs while signals from T cells, such as CD40L, are required to complete DC maturation and activation (80).

While stimuli such as LPS and CD40 play important roles in regulating DC maturation and activation, the signaling events triggered by these stimuli have not been characterized. A major limitation in analyzing the signaling events that mediate transition from the immature to mature state has been the low numbers of DCs found in normal tissues and the absence of long-term DC lines. Recently, Ricciardi-Castagnoli and colleagues have generated a murine DC line (D1 cells) by using a retroviral vector to transduce an *env-myc* fusion gene into splenic DCs from newborn mice (176). D1 cells can be propagated in a growth factor-dependent immature state by supplementing the growth medium with a conditioned medium (CM) consisting of fibroblast-derived growth factors and GM-CSF (177). In the absence of these growth factors, D1 cells undergo apoptotic cell death (294). However, in addition to inducing DC maturation, LPS can protect D1 cells from growth factor withdrawal-induced

apoptosis (294). Thus, D1 cells provide a system for analyzing the signaling events that mediate DC activation, maturation and survival.

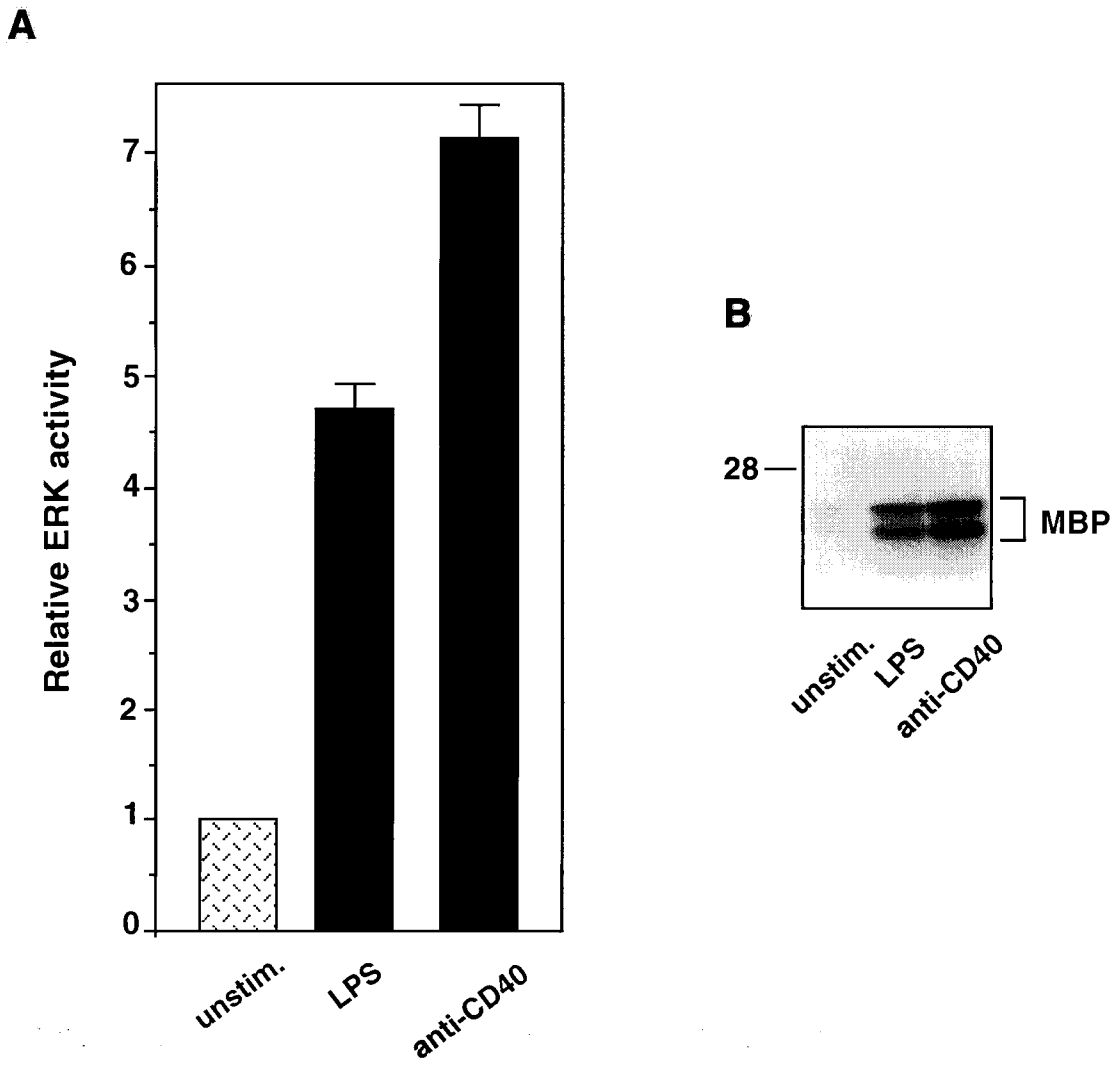
In this study, we have used the D1 DC system to determine whether the mitogen activated protein kinases (MAP kinases) are activated by LPS and CD40 in D1 cells. These serine/threonine kinases are activated by many receptors, as well as by environmental stresses, and have been shown to mediate cell proliferation, differentiation and survival (131,295). Three families of MAP kinases are involved in signal transduction, the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 kinases. The MAP kinases are activated by MAP kinase kinases (MKKs) which phosphorylate threonine and tyrosine residues in the threonine-X-tyrosine activation motif (103,110). Upon activation, the ERK, JNK and p38 translocate to the nucleus where they phosphorylate and activate different sets of transcription factors, and thereby regulate gene expression (103,110). Here, we have determined whether the ERK, JNK and p38 MAP kinase pathways are activated by LPS and CD40 in D1 cells. Our collaborators, M. Rescigno and M. Martino, under the direction of Dr. P. Ricciardi-Castagnoli (University of Milano, Italy) have extended these findings by evaluating the role of ERK in mediating LPS-induced D1 cell maturation and survival.

6.2 LPS activates ERK in D1 DCs

Since LPS has been shown to activate the ERK, JNK, and p38 MAP kinases in murine macrophages (206,296), we investigated whether LPS activates ERK, JNK and MAPKAP kinase-2, a downstream target of p38 (140,141), in the D1 DC line. D1 cells were incubated with LPS and *in vitro* kinase assays were performed to measure ERK, JNK, and MAPKAP kinase-2 activities as described in Materials in Methods (Chapter 2). We found that treating D1 cells with LPS for 15 min resulted in a 5-fold increase in ERK activity (Fig. 6.1). ERK activity began to decline after 30 minutes of LPS treatment

Figure 6.1: LPS and CD40 both activate ERK in D1 DCs

D1 cells were incubated with or without 10 μg/mL LPS or 10 μg/mL anti-CD40 mAb for 15 min. *In vitro* kinase assays were performed on anti-ERK immunoprecipitates using myelin basic protein (MBP) as a substrate. *(A)* ERK activity relative to that in unstimulated D1 cells (which is defined as 1.0). The data represent the average and range of the relative ERK activities from two independent experiments. *(B)* A representative autoradiograph showing the phosphorylation of MBP by immunoprecipitated ERK.


A
Relative ERK activity
0
1
2
3
4
5
6
7
unstim.
LPS
anti-CD40
B
28
MBP
unstim.
LPS
anti-CD40

(data not shown). The Ab used to immunoprecipitate ERK from D1 cell lysates (Ab C-14) was raised against ERK2 but also weakly cross-reacts with ERK1. Thus, it remains to be determined whether the ERK1 or ERK2 isoform is the main transducer of LPS signaling in D1 cells. In contrast to ERK, LPS did not cause significant activation of JNK (Fig. 6.2) and, depending on the experiment, caused either little or no activation of MAPKAP kinase-2 (Fig. 6.3).

6.3 CD40 activates ERK in D1 DCs

I have previously shown that CD40 activates the JNK and p38 MAP kinases, as well as MAPKAP kinase-2, a downstream target of p38 in the WEHI-231 murine immature B cell line (Chapter 3) (245). To determine whether CD40 induces MAP kinase activity in immature DCs, D1 cells were treated with the rat FGK 45.5 anti-murine CD40 mAb for various times. After stimulating the cells with this anti-CD40 mAb, the cells were lysed and ERK, JNK and MAPKAP kinase-2 *in vitro* kinase assays were performed as described in Materials and Methods (Chapter 2). We found that incubating D1 cells with anti-CD40 mAb caused a 4-fold increase in ERK activity at 5 min and a 7-fold increase in ERK activity 15 min (Fig. 6.1). No increase in ERK activity was observed in D1 cells treated for 15 min with an isotype-matched control rat mAb (data not shown). This finding indicates that the increased ERK activity in response to anti-CD40 mAb is a specific effect of CD40 engagement. In contrast to WEHI-231 cells, CD40 triggering did not induce significant activation of JNK (Fig. 6.2) or MAPKAP kinase-2 (Fig. 6.3) in D1 cells. Thus, CD40 appears to strongly activate ERK in D1 DCs but causes very little or no activation of the JNK and p38 MAP kinase pathways. These findings indicate that CD40 activates a different spectrum of MAP kinases in DCs than in WEHI-231 B cells.

Figure 6.2: LPS and CD40 do not significantly activate JNK in D1 DCs

D1 cells were incubated with or without 10 μg/mL LPS or 10 μg/mL anti-CD40 mAb for 15 min. As a positive control for activation of JNK, D1 cells were exposed to high osmolarity conditions by treating them with 0.6 M sorbitol for 15 min. *(A) In vitro* kinase assays were performed on anti-JNK immunoprecipitates using GST-c-Jun (1-96) as a substrate JNK activity relative to that in unstimulated D1 cells (which is defined as 1.0). The data represent the average and range of the relative JNK activities from two independent experiments. *(B)* A representative autoradiograph showing the phosphorylation of GST-c-Jun (1-96) by immunoprecipitated JNK.

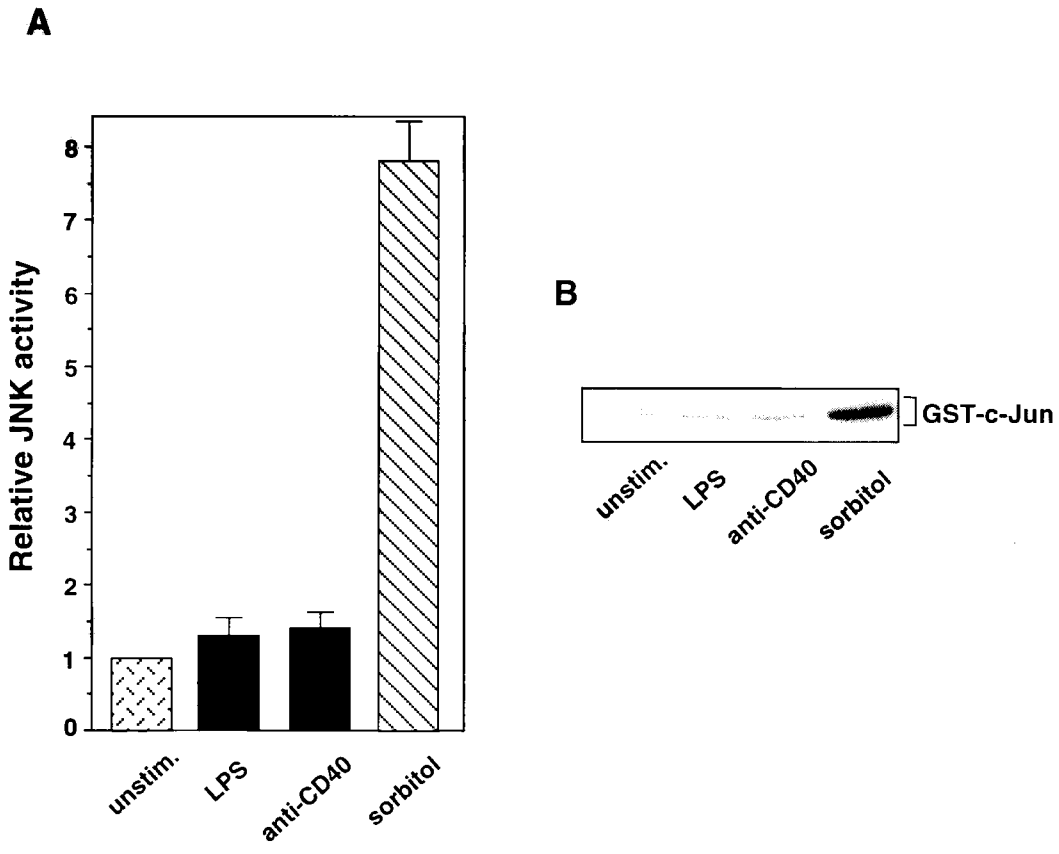
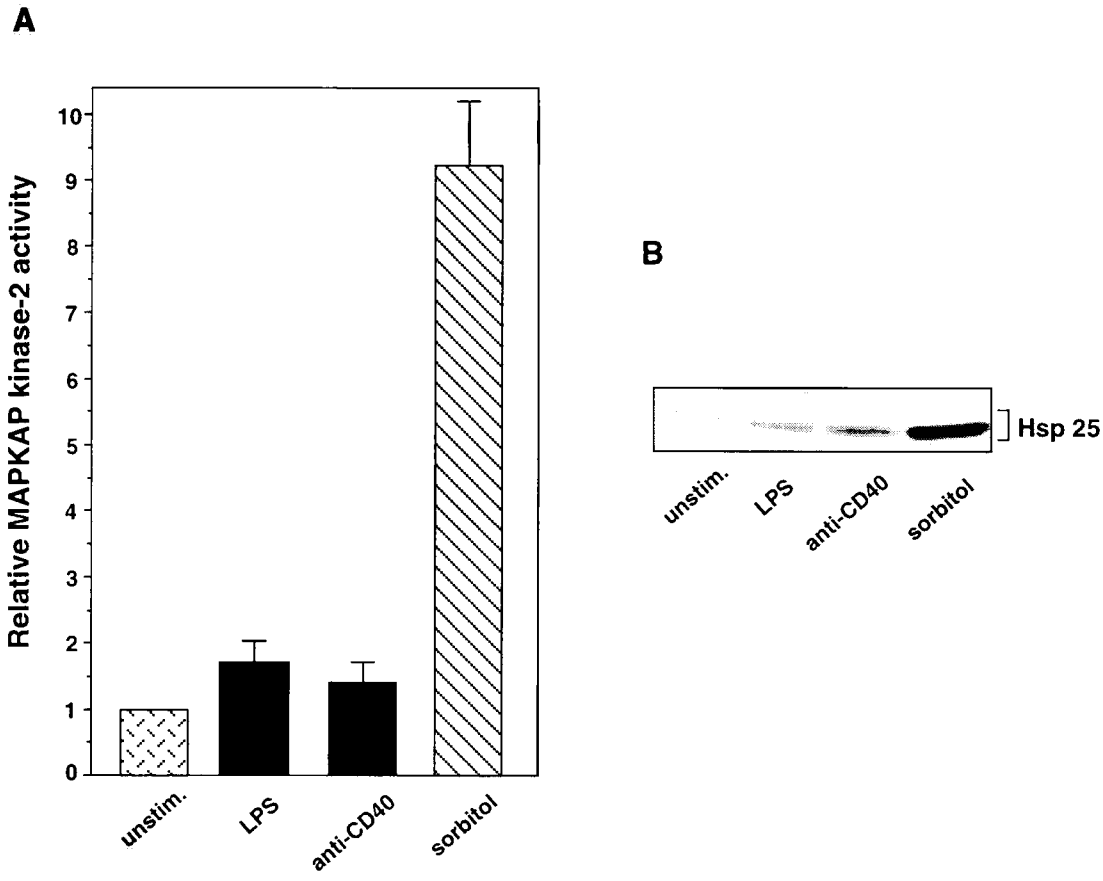

A
Relative JNK activity
0
1
2
3
4
5
6
7
8
unstim.
LPS
anti-CD40
sorbitol
B
GST-c-Jun
unstim.
LPS
anti-CD40
sorbitol

Figure 6.3: LPS and CD40 do not markedly activate MAPKAP kinase-2 in D1 DCs

D1 cells were incubated with or without 10 μg/mL LPS or 10 μg/mL anti-CD40 mAb for 15 min. As a positive control for activation of MAPKAP kinase-2, D1 cells were exposed to high osmolarity conditions by treating them with 0.6 M sorbitol for 15 min. *(A) In vitro* kinase assays were performed on anti-MAPKAP kinase-2 immunoprecipitates using Hsp25 as a substrate MAPKAP kinase-2 activity relative to that in unstimulated D1 cells (which is defined as 1.0). The data represent the average and range of the relative MAPKAP kinase-2 activities from two independent experiments. *(B)* A representative autoradiograph showing the phosphorylation of Hsp25 by immunoprecipitated MAPKAP kinase-2.


A
Relative MAPKAP kinase-2 activity
0
1
2
3
4
5
6
7
8
9
10
unstim.
LPS
anti-CD40
sorbitol
B
Hsp 25
unstim.
LPS
anti-CD40
sorbitol

6.4 The role of ERK in LPS-induced DC maturation and survival

To evaluate the role of ERK in both DC survival and maturation, our collaborators have investigated the effect of a highly selective inhibitor of the ERK pathway on D1 cells. PD98059 (297) is a specific inhibitor of MEK, the kinase that phosphorylates and activates ERK1 and ERK2. DC maturation correlates with the upregulation of a variety of cell surface markers including MHC class II antigens and costimulatory molecules (80). Using FACS analysis, our collaborators found that pre-treating D1 cells with the MEK inhibitor PD98059 had no effect on the ability of LPS to increase the surface expression of MHC class II and the B7.2 costimulatory molecule (294). Thus, ERK activation is not required for DC maturation.

In contrast, it was found that MEK activity was essential for LPS-mediated DC survival following CM (fibroblast-derived conditioned medium) withdrawal. Using annexin V-FITC staining to detect the appearance of the early apoptotic marker phosphatidylserine on the cell surface, our collaborators found that the ability of LPS to prevent apoptosis of D1 cells was markedly reduced when the cells were pretreated with the MEK inhibitor PD98059 (294). While PD98059 blocked the ability of LPS to prevent apoptosis of D1 cells, it did not block the ability of CM to prevent apoptosis. This result shows that the MEK inhibitor is not toxic to D1 cells and that its ability to block LPS-induced survival of D1 cells is a specific effect. Thus, activation of the MEK/ERK pathway is required for LPS, but not CM, to prevent apoptosis of D1 cells due to growth factor withdrawal. Our collaborators also found that PD98059 blocked the ability of LPS to induce TNFα production by D1 cells (294). Since TNFα has been shown to maintain the viability of Langerhans cells in culture (298), TNFα production may also promote DC viability and may be the basis for the ability of LPS to prevent apoptosis in D1 cells. From these results a model can be proposed in which LPS blocks D1 cell death from growth factor withdrawal by inducing TNFα production via an ERK-dependent pathway.

6.5 Discussion

LPS and CD40 both induce maturation of immature DCs (3) while LPS also promotes DC survival in the absence of growth factors (294). However, the signaling events involved in these processes remain largely unknown. In this chapter, we have investigated the regulation of MAP kinases by LPS and CD40 in the D1 immature DC line. We found that LPS treatment or CD40 ligation in D1 cells caused significant increases in ERK activity. This is the first report of LPS or CD40-induced activation of ERK in DCs. In contrast to ERK, JNK or MAPKAP kinase-2, a downstream target of p38 (140,141), were not activated to appreciable extents by LPS or CD40 in D1 cells.

CD40 induces its effects on DCs by binding to the CD40 ligand expressed on activated $CD4^+$ T cells (73,293). However, the mechanism by which DCs respond to LPS is less clear. Previous studies have shown that DCs do not express CD14, a cell surface protein that binds LPS (299) and which mediates LPS-induced activation of monocytes and neutrophils (300,301). Consistent with these reports, our collaborators did not detect any CD14 expression on the surface of D1 cells by FACS analysis (data not shown). However, in addition to being expressed as a glycosyl-phosphatidylinositol-anchored membrane protein (mCD14), CD14 also exists as a soluble protein (sCD14) in plasma that forms complexes with LPS and the serum protein LPS-binding protein (LBP). These trimolecular complexes have been shown to mediate the effects of LPS on CD14-negative cells such as epithelial cells and endothelial cells (302-304). Human peripheral blood DCs, and hence presumably D1 cells, also appear to respond to LPS via a sCD14-dependent pathway (305). The mechanism by which CD14-negative cells recognize these LPS/LBP/sCD14 complexes presumably involves other receptors. Recent work has indicated that Toll-like receptor 2 (TLR2) binds LPS/LBP/mCD14 complexes and mediates LPS-induced cellular signaling (306). However, it remains to be determined whether TLR2 also binds sCD14-containing trimolecular complexes.

The signaling pathways by which CD40 and LPS activate ERK are only partly resolved. The CD40 cytoplasmic tail interacts with several members of the tumor necrosis factor receptor-associated factor (TRAF) family of adapter proteins, including TRAF2 (157), TRAF3 (158), TRAF5 (159) and TRAF6 (160). Studies in 293 cells suggest that CD40 activates ERK by both a Ras-dependent pathway and a Ras-independent pathway in which TRAF6 could be involved (163). CD40 ligation has also been found to induce phosphorylation of the Ras guanine nucleotide exchange factor, Son of sevenless (Sos) in murine splenic B cells (225). However, it remains to be determined whether this phosphorylation activates Sos, or alternatively, inhibits the interaction of Sos with Grb2, an adapter protein that recruits Sos to the plasma membrane. Many different receptors including the BCR, activate Ras which in turn controls a protein kinase cascade that leads to activation of ERK (Fig. 1.3). In this cascade, Ras activates the serine/threonine kinase Raf-1 (200) which in turn phosphorylates and activates MEK (201), the kinase that activates ERK (307). However, studies in splenic B cells have shown that CD40 uses an unidentified protein kinase A-insensitive MEK kinase, rather than Raf-1, to regulate ERK activity (226). Whether CD40 uses a similar pathway to activate ERK in D1 cells remains to be determined. The pathway by which LPS activates ERK is largely unknown. However, in human astrocytes LPS has been shown to strongly activate both Raf-1 and ERK2 (308). This finding suggests that LPS-induced activation of ERK may be mediated by the traditional Ras/Raf-1 pathway.

Our observation that CD40 strongly activates ERK in D1 cells is in contrast to our findings in WEHI-231 murine B cells where CD40 activates JNK and p38 but not the ERKs (245). We have also found that LPS strongly activates JNK in WEHI-231 cells (data not shown) but has little effect on JNK activity in D1 cells. Our findings that LPS does not significantly activate the JNK and p38 pathways in D1 cells also differ from reports in macrophages that LPS induces activation of all three MAP kinase

pathways (206,296). Thus, CD40 and LPS appear to activate different MAP kinases and, therefore, presumably elicit different responses, depending on the cell type and/or maturation stage. The basis for the activation of different MAP kinases in different cell types remains to be determined but may involve differential expression or regulation of signaling molecules that connect CD40 to activation of this kinases.

After we identified which MAP kinases are activated by LPS and CD40 in D1 cells, our collaborators evaluated the role of ERK in both LPS-induced DC maturation and survival by investigating the effect of PD98059, a specific inhibitor of the ERK pathway (297), on D1 cells. Rescigno et al. found that blocking LPS-induced activation of ERK with PD98059 had no apparent effect on DC maturation. However, the ERK pathway was found to be essential for LPS to prevent apoptosis of D1 cells due to growth factor withdrawal (294). The role of ERK in promoting the survival of D1 cells is consistent with the finding that activation of ERK is also essential for preventing apoptosis of PC-12 neuronal cells from growth factor withdrawal (106). The mechanism by which ERK promotes cell survival in the absence of growth factors is unknown but presumably involves activation of transcription factors that mediate induction of anti-apoptotic genes. Upon activation, ERK translocates to the nucleus (113), where it can phosphorylate and activate several different transcription factors. For example, ERK phosphorylates the ternary complex factor, Elk-1/p62^{TCF}, which in turn stimulates transcription of genes such as c-*fos* (235,236,309). ERK also phosphorylates NF-IL6, which induces expression of several genes including IL-6 and IL-8 (310,311). Whether ERK mediates survival from growth factor withdrawal through activation of these transcription factors or others is a question for further investigation.

The role of ERK in CD40 signaling in D1 cells remains to be determined. Similar studies as those using the MEK inhibitor to investigate the role of ERK in LPS-signaling could be done to investigate the role of ERK in CD40-stimulated responses

such as increased surface expression of CD40L and CD25 molecules, and secretion of the cytokines TNFα and IL-12 as well as the chemokines IL-8, MIP-1α and MIP-1β.

CHAPTER 7

Final Discussion/Future Studies

Several questions and suggestions for future work on CD40 have evolved from the findings presented in this thesis as well as from recent findings from other groups. These areas of interest are briefly discussed below.

7.1 How is CD40 signaling initiated?

7.1.1 Aggregation of CD40 proteins is a critical initiating step for CD40 signaling

It is unclear at this stage whether before ligand binding CD40 molecules are present as monomers or as oligomers such as dimers or trimers on the cell surface. Biochemical studies have shown that CD40 from normal B cells or from the Burkitt lymphoma line Raji can be found as disulfide-linked homodimers on the cell surface (312). However, it is possible that the structure of CD40 may differ depending on the cell type.

By analogy with the crystal structure of the human TNFR1/TNFβ complex, where each complex contains three receptors bound to one ligand trimer (21), it is expected that ligand-dependent activation of TNFR superfamily members involves receptor trimerization. A recent study has shown that native sCD40L is a biologically active trimer (19). This indicates that the binding of CD40L trimers to CD40 is sufficient to effectively aggregate CD40 molecules and induce CD40 signaling events (26). Several studies indicate that multimerization of CD40 molecules on the cell surface is an essential step for the initiation of CD40 signaling. For example, monovalent (Fab) anti-CD40 Ab fragments fail to induce proliferation of TPA-treated tonsillar B cells or peripheral blood B cells whereas $F(ab')_2$ fragments can induce B cell proliferation to a

similar degree as intact antibody (169). Although the minimal CD40 stimulatory aggregate is still unknown, anti-CD40 mAbs presumably initiate CD40 signaling by aggregating CD40 molecules together in chains.

The CD8/CD40 chimeric receptors constructed in this thesis should have been present as disulfide-linked homodimers, given that CD8α typically forms homodimers through disulfide bonding in its extracellular domain (313,314). My finding that the CD8/CD40 chimeric receptors were not constitutively active and needed to be cross-linked with anti-CD8 Abs in order to signal suggests that dimerization of CD40 cytoplasmic tails is not sufficient to initiate CD40 signaling. Rather, more extensive aggregation of CD40 molecules appears to be required for significant CD40 signaling. Consistent with this idea, I found that my CD8/CD40 chimeric receptors signaled more strongly when the cells were incubated with biotinylated anti-CD8 mAb and avidin than with biotinylated anti-CD8 mAb only. Since each avidin molecule binds four biotin molecules, the addition of avidin would have caused more extensive aggregation of the CD8/CD40 chimeras.

7.1.2 Assembly and regulation of the CD40 receptor complex

Members of the TRAF family of adapter proteins including TRAF2, TRAF3, TRAF5 and TRAF6 bind to CD40 and mediate CD40 signaling events (26,254). Presently, it is not clear whether these proteins associate constitutively with CD40 or whether they are recruited to the receptor upon stimulation with CD40L. As discussed above, signaling by CD40 appears to require aggregation of CD40 molecules which is presumably induced upon binding of CD40 to its trimeric ligand. The aggregation of CD40 cytoplasmic domains may serve to generate composite sites or to induce conformational changes that initiate CD40 signaling either by activating constitutively-associated TRAF proteins or by recruiting TRAF proteins to the receptor complex.

The model that postulates that CD40 associates constitutively with the TRAF proteins is supported by findings from *in vitro* binding studies. For example, no known modifications are required for the binding of a GST fusion protein containing the murine CD40 cytoplasmic tail to TRAF2 or TRAF3 proteins obtained from lysates of 293 cells engineered to overexpress these TRAF proteins (165). In addition, TRAF2, TRAF3, TRAF5 and TRAF6 bind *in vitro* to peptides corresponding to different regions of the human CD40 cytoplasmic domain (254). Although these *in vitro* binding studies suggest that modifications are not required for association of CD40 with TRAF proteins, it is possible that the CD40 tail regions were so highly overexpressed that they aggregated spontaneously, in the absence of CD40 stimulation. In support of the TRAF recruitment model, binding of sCD40L to CD40 on DND39 human B cells induces the recruitment of both TRAF2 and TRAF3 to CD40, whereas immunoprecipitation of CD40 from unstimulated DND39 cells does not reveal constitutively-associated TRAF2 or TRAF3 (161). Future work should clarify whether CD40-associated adapter proteins such as the TRAFs are constitutively associated with CD40, are recruited to CD40 upon CD40 engagement or whether a combination of these events occurs. This question has been difficult to address because TRAF proteins are expressed at low levels in cells.

7.2 Pathways that link CD40 to JNK, p38 and NF-κB

In this thesis I found that CD40 strongly activates the JNK and p38 MAP kinases as well as the NF-κB pathway in WEHI-231 B cells. As discussed in Chapter 4, members of the TRAF family of adapter proteins associate with the CD40 cytoplasmic domain and are believed to mediate CD40-induced activation of JNK, p38 and NF-κB. TRAF2 binds NIK (162,264), a kinase that binds to and presumably activates the IκB kinase (IKK) complex (154) which is responsible for phosphorylating IκBα (Fig. 7.1). The TRAF proteins are upstream of kinases called MKKs which phosphorylate and

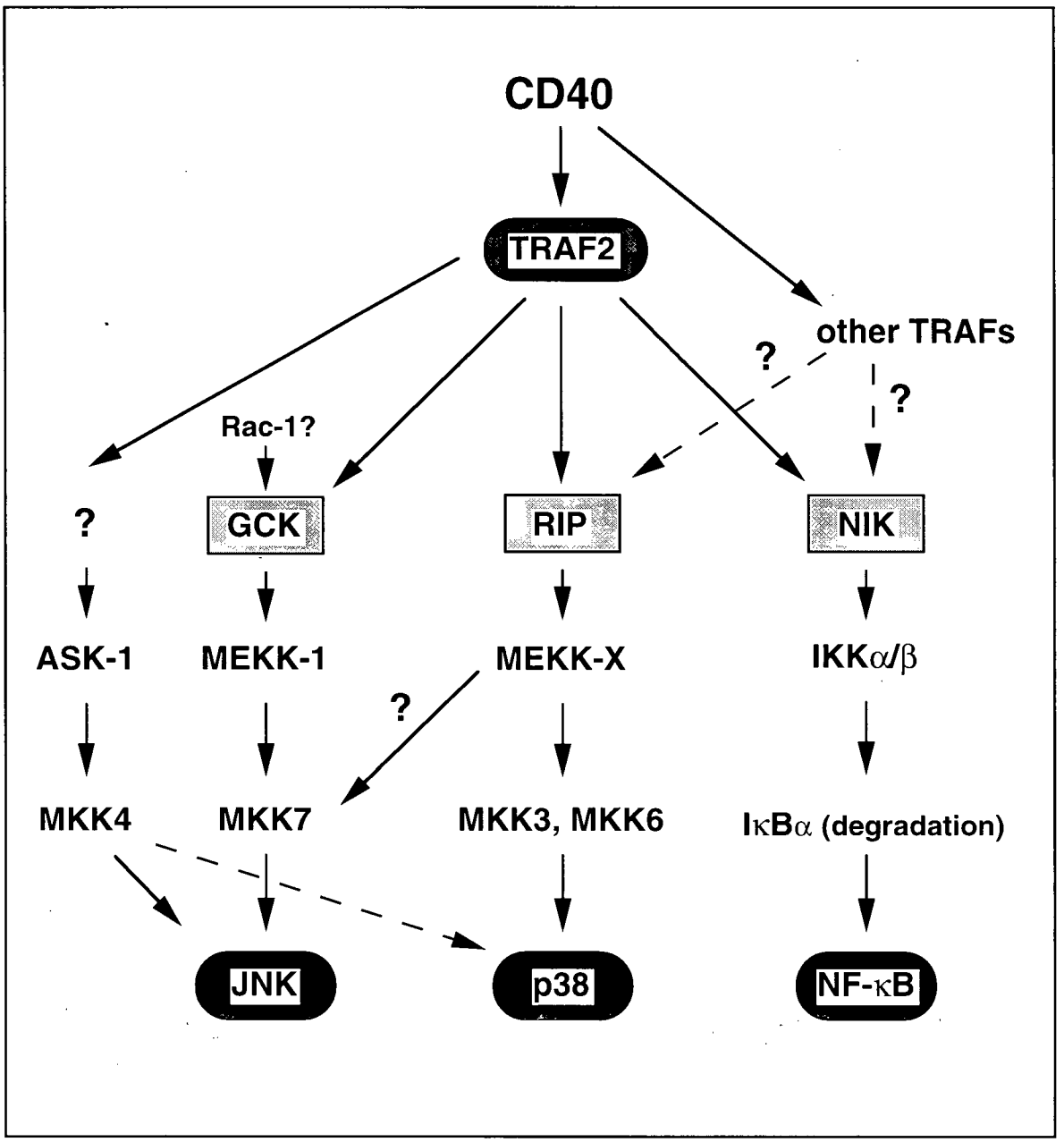


Fig. 7.1: Proposed scheme for CD40-induced activation of JNK, p38 and NF-κB. (based on recent findings with TNFR1).

activate JNK and p38. MKK4 activates both JNK and p38 (108) whereas MKK7 is specific for JNK (247,248), and MKK3 and MKK6 are specific for p38 (131). Until recently the link between TRAF proteins and the MKKs was not well understood.

A recent study showed that in TNFR1 signaling, germinal center kinase (GCK) interacts with TRAF2 and couples TRAF2 to MEKK1, a kinase that is upstream of MKK7 and JNK (Fig. 7.1) (234). In addition to GCK, a second serine/threonine kinase called receptor interacting protein (RIP) was shown to bind TRAF2 and couple TNFR1 to JNK and p38 (234). Interestingly, RIP was found to be required for TRAF2 activation of p38 but not JNK. RIP was shown to be constitutively associated with an MEKK that is upstream of MKK6 and p38 (Fig. 7.1). Thus, TRAF2 mediates activation of JNK and p38 by binding to GCK and RIP. It remains to be determined whether CD40 is linked to JNK and p38 by the same pathways that are used for TNFR1 signaling. Given that TNFR1 and CD40 are both members of the TNFR superfamily, similar signaling mechanisms likely exist. Future work could determine whether dominant negative forms of GCK and RIP affect CD40-induced activation of JNK and p38 in WEHI-231 B cells.

It is not yet known how GCK and RIP are regulated. GCK belongs to the PAK family of serine/threonine kinases. Members of the PAK family are regulated by GTPases such as Rac and Cdc42 (122,232). Dominant negative versions of Rac and Cdc42 block JNK activation by TNFα (122,232), whereas the small GTPase Rho has been implicated in p38 activation (157). Whether GCK and RIP are regulated by small G proteins in B cells and whether this regulation affects the JNK and p38 pathways remains to be determined.

One of the goals of my thesis was to map the regions in the CD40 cytoplasmic domain responsible for activating the JNK and p38 pathways. My finding that the same 11 amino acid sequence in CD40 activates both JNK and p38 is consistent with the idea that the JNK and p38 pathways share the same proximal signaling events.

These new findings that the JNK and p38 pathways diverge at the point of GCK and RIP may explain how these two kinases can be independently regulated even though they share the same proximal signaling events.

It remains to be determined whether additional members of the TRAF family, TRAF3, TRAF5 and TRAF6 which have been implicated in JNK and p38 activation are required for CD40 signaling to JNK and p38 and whether they also associate with GCK and RIP or whether they activate JNK and p38 by other mechanisms. The apoptosis signal-regulating kinase (ASK1) has recently been shown to interact with TRAF2, TRAF5 and TRAF6 and to be activated by overexpression of these TRAF proteins in 293 cells (281). ASK1 is essential for TNF-induced activation of JNK by TRAF2 and constitutes a GCK and MEKK1-independent signaling pathway to JNK activation (281,315). However, the role of ASK-1 in TRAF5 and TRAF6-mediated activation of JNK remains to be determined.

7.3 Mechanisms of CD40-mediated protection from BCR-induced apoptosis

Although genetic and biochemical studies indicate that JNK and p38 regulate cellular proliferation or apoptosis in some situations (106,108), recent studies indicate that JNK and p38 are either not involved in or at least do not play a major role in BCR-induced apoptosis or CD40-mediated survival. For example, Salmon et al. (316) found that inhibiting p38 MAP kinase activity in WEHI-231 cells with the p38 inhibitor SB 203580 had no effect on either BCR-induced apoptosis or anti-CD40-mediated suppression of apoptosis. Rather, the major player in CD40-mediated rescue of WEHI-231 cells from BCR-induced apoptosis appears to be the transcription factor NF-κB (146). CD40 engagement was found to prevent the decrease in the level of nuclear NF-κB complexes that occurs in response to BCR cross-linking. CD40-mediated maintenance of NF-κB activity was, in turn, shown to prevent the drop in c-Myc levels

that follows BCR cross-linking and which is responsible for these cells undergoing apoptosis (146-149). Similar to the finding that NF-κB protects B cells from anti-IgM-induced apoptosis, Karin et al. (317) found that NF-κB protects human breast carcinoma cells from TNFα-induced apoptosis. Furthermore, although TNFα also activates JNK, JNK activation is not involved in TNFα-induced apoptosis of these cells (317).

In addition to activating NF-κB, it appears that CD40-induced cooperation between the Bcl-x_L, cdk4 and cdk6 proteins may play a key role in CD40-mediated protection of WEHI-231 cells from anti-IgM induced apoptosis (258). CD40 signaling was found to prevent the decreases in Bcl-x_L, cdk4 and cdk6 protein levels that occur following BCR cross-linking. However, while constitutive expression of Bcl-x_L blocked anti-IgM induced apoptosis of G1 arrested cells, it failed to prevent arrest of these cells in the G1/S phase (258). One of the CD40 signaling events required for entering S phase could be linked to the maintenance of cdk4 and cdk6 protein levels, which are suppressed by BCR signaling (258). It remains to be determined whether constitutive expression of cdk4 and cdk6 in WEHI-231 cells would be sufficient to block anti-IgM-induced growth arrest and subsequent apoptosis. It also remains to be determined whether Bcl-x_L-mediated protection of WEHI-231 cells is linked to c-Myc expression or whether it represents an NF-κB-independent survival pathway.

7.4 Identification of genes regulated by CD40 signaling

Our lab is now doing subtractive cDNA hybridization studies in WEHI-231 cells to identify genes that are upregulated in response to CD40 engagement. Over 100 cDNA clones representing genes whose expression may be regulated by CD40 signaling have been obtained. Northern blot analysis is currently being performed to determine which of these genes are in fact regulated by CD40. These potential CD40-regulated genes include ones encoding the chemokine receptor CXCR4, the kinase

sgk, and an mRNA splicing factor 9G8. A future goal of the lab will be to determine the role of these gene products in CD40 effects on B cells. Since I have determined that CD40 strongly activates the JNK and p38 MAP kinases in WEHI-231 cells and since MAP kinases provide a direct link to gene regulation through activation of specific transcription factors, an extension of this goal will be to determine the effects of p38-specific and JNK-specific inhibitors on CD40-induced gene expression.

7.5 Role of ERK in BCR-induced apoptosis

A role for ERK in BCR-induced apoptosis has yet to be confirmed. I have shown that ERK2 activation correlates with anti-IgM-induced apoptosis in WEHI-231 cells. Consistent with the idea that ERK2 plays a role in mediating anti-IgM-induced apoptosis of WEHI-231 cells, Lee et al. (318) found that overexpression of mitogen-activated protein kinase phosphatase-1 (MKP-1), a protein that dephosphorylates and subsequently reduces ERK2 activity (319), in WEHI-231 cells abrogated anti-IgM-induced apoptosis. Whether ERK represents another pathway to apoptosis or is involved in mediating the anti-IgM-induced drop in c-Myc levels is not known. It should be possible to directly determine whether ERK activation is involved in BCR-induced apoptosis of WEHI-231 cells by testing the effects of constitutively active and dominant negative forms of upstream activators of ERK on anti-IgM-induced apoptosis. Alternatively, the role of ERK could be tested by determining whether a specific inhibitor of the ERK pathway, PD98059 (BIOMOL, Plymouth Meeting, PA), blocks anti-IgM-induced apoptosis in WEHI-231 cells.

Since the onset of this thesis, knowledge in the apoptosis field has increased dramatically. It is now known that apoptosis induced by several receptors including Fas, TNFR1 and TNFR2 is mediated by the activation of intracellular proteases, of which the caspase family of cysteine proteases is the best characterized (reviewed in (320,321)). A recent study presented by Dr. J. Monroe at the 1998 Midwinter

Conference of Immunologists in Asilomar, CA showed that engagement of the BCR on WEHI-231 cells induces activation of caspase-3. However, it remains to be determined whether caspase-3 activation is involved in anti-IgM-induced apoptosis. Given the kinetics of caspase activation and the drop in c-Myc levels, presumably anti-IgM-induced activation of caspases in WEHI-231 cells is a secondary event to the anti-IgM-induced drop in c-Myc levels.

7.6 Does the BCR or CD40 activate other MAP kinase pathways?

In addition to the ERK, JNK and p38 families, a fourth MAP kinase family, ERK5 (also termed BMK1) has recently been identified (322,323). Like JNK and p38, ERK5 is activated in response to environmental stresses (322,323). ERK5 interacts with MEK5 (323). This interaction suggests that MEK5 may activate ERK5. However, little else is known about the ERK5 signaling pathway. Furthermore, it is not known whether ERK5 phosphorylates and activates transcription factors. Future work could address whether ERK5 is activated by the BCR in B cells or by CD40 in B cells, dendritic cells and macrophages.

7.7 Is threonine-40 in the CD40 tail phosphorylated?

The threonine residue at position 40 in the murine CD40 cytoplasmic region, which corresponds to threonine at position 39 in human CD40, is essential for CD40 signaling. I found that changing threonine-40 to an alanine abolished the ability of the CD8/(35-53) chimeric receptor to activate NF-κB, JNK, and MAPKAP kinase-2. Substituting this threonine residue with an alanine also abrogates the ability of CD40 to induce homotypic aggregation, Ab secretion, and upregulation of B7.1, Fas, and CD23 (255,256). The recent finding that changing this threonine residue to an alanine in human CD40 not only prevents CD40 signaling but also destroys the ability of CD40

to bind TRAF2, TRAF3 and TRAF5 (158,159,165) suggests that the threonine-39 CD40 mutant fails to signal because it is no longer able to bind TRAF proteins.

Two possible reasons could explain the importance of the threonine at position 40 for the ability of CD40 to signal. First, it is possible that the presence of a threonine residue at this position is critical for the proper folding of the CD40 cytoplasmic region. If this region of CD40 is improperly folded then perhaps the TRAF proteins are unable to bind to CD40. A second possibility is that threonine-40 becomes phosphorylated upon CD40 engagement and phosphorylation of this residue affects the ability of CD40 to bind to adapter proteins such as the TRAFs and to signal.

It remains to be determined whether threonine-40 is phosphorylated and whether phosphorylation of this residue affects the ability of CD40 to bind to adapter proteins and to signal. Initial studies indicate that human CD40 from tonsillar B cells and from human CD40-transfected murine M12 B cells is constitutively phosphorylated, however the phosphorylation site(s) is not known (168,170). Although it remains to be determined whether CD40 engagement causes increased phosphorylation of CD40, both IL-6 and PMA have been found to increase the phosphorylation state of human CD40 expressed in the B lymphoblastoid cell line CESS (170). Presumably phosphorylation of CD40 occurs on serine and threonine residues such as threonine-40, as human CD40 has no tyrosines in its cytoplasmic domain.

Five phosphorylatable residues including three threonines and two serines are conserved between murine and human CD40 (167). These five residues are located in the homology box region of the CD40 cytoplasmic domain (Figure 4.1). A study in which the B lymphoma cell line M12 was transfected with mutant human CD40s in which an alanine had been substituted in turn for each of the serine and threonine residues in the CD40 tail found that only threonine-39 in human CD40 was essential for CD40-mediated growth inhibition of these cells (168). Thus, together with my

findings and those of others it appears that, at least for the signaling events tested, only the threonine at position 40 in murine CD40 appears to be essential for CD40 signaling. Although it remains to be tested, I hypothesize that phosphorylation of threonine-40 is not important for CD40 signaling. This prediction is based on *in vitro* binding studies where it was found that peptides corresponding to regions of the CD40 tail could bind to recombinant TRAF proteins in the absence of any apparent covalent modification (254). It should be possible to determine whether threonine-40 is phosphorylated by labeling WEHI-231 cell clones expressing the CD8/(35-45) chimeric receptor with [^{32}P]orthophosphate and then immunoprecipitating the chimera with anti-CD8 Abs. Threonine-40 is the only phosphorylatable residue within this 11 amino acid signaling motif. In addition to determining whether threonine-40 is constitutively phosphorylated, it should be possible to determine whether engagement of CD8/(35-45) with anti-CD8 Abs induces increased phosphorylation of threonine-40.

7.8 Identification of a second signaling motif in the CD40 cytoplasmic domain

My studies show that residues 35-45 of the CD40 cytoplasmic domain contain a major signaling motif that is able to mediate full activation of JNK and p38 MAP kinase as well as substantial activation of NF-κB. Work by Hostager et al. (256), together with recent experiments we have performed, indicate that there is a second signaling motif in the CD40 cytoplasmic domain which partially overlaps the NF-κB/JNK/p38 activation motif contained within residues 35-45 of the CD40 cytoplasmic domain. Hostager et al. showed that residues 41-62 of the CD40 cytoplasmic domain as well as the threonine at position 40 are required for CD40 to induce expression of the cell surface markers CD23, Fas, and B7.1 in the M12.4.1 murine B cell line (256).

Using my chimeric receptors, Yvonne Yang in our lab has shown that the CD8/(1-74) chimeric receptor could induce expression of CD23 in M12.4.1 cells but

that the CD8/(35-45) and CD8/(43-53) chimeric receptors could not (data not shown). This indicates that induction of CD23 expression is mediated by a CD40 signaling motif that is not entirely contained within residues 35-45 of the CD40 cytoplasmic domain. Thus, the CD40 cytoplasmic domain appears to contain two overlapping but distinct signaling motifs. The first motif, which I have defined in this thesis, is contained within residues 35-45 and mediates activation of NF-κB, JNK and p38. The second signaling motif requires the threonine at position 40 as well as other amino acids contained within residues 41-62 and is responsible for upregulation of CD23 and other cell surface markers. The definition of this second CD40 signaling motif, as well as the identification of adapter proteins that bind differentially to the two motifs, are the next steps in elucidating the molecular basis of CD40 signaling.

7.9 Role of HVEM/ATAR in B cell development

As discussed in Chapter 5, we found that the cytoplasmic domain of ATAR activates the JNK, p38 and NF-κB pathways in WEHI-231 cells. ATAR was also shown to protect WEHI-231 cells from anti-IgM-induced growth arrest. Our results indicate that ATAR could mimic some of the effects of CD40 on B cells and thereby regulate B cell activation and function. A future goal of the lab will be to further explore the role of ATAR in B cell development and activation. Given that ATAR specifically mediates entry of HSV-1 and HSV-2 into B and T cells, it will be interesting to determine the role of lymphocyte infection in the etiology of HSV disease.

7.10 CD40 signaling in dendritic cells

As discussed in Chapter 6, LPS and CD40 both regulate key steps in DC maturation and activation, however, little is known about the signaling events triggered by these stimuli. In this thesis, I found that LPS and CD40 both strongly activate ERK2 in D1 DCs but have little effect on JNK and p38. Our collaborators, M. Rescigno and

M. Martino extended these findings by evaluating the role of ERK in mediating LPS-induced D1 DC maturation and survival. They found that blocking ERK activation with PD98059, a specific inhibitor of the kinases that phosphorylate and activate ERK (297), blocked the ability of LPS to protect D1 DCs from growth factor withdrawal-induced apoptosis, but had no effect on LPS-induced DC maturation. Future work could address the role of ERK in mediating CD40 effects on DCs. In particular, similar experiments as were done with LPS could be performed by pretreating D1 DCs with PD98059 and determining whether CD40-mediated events are affected. Such events include CD40-mediated upregulation of the cell surface proteins CD40L and CD25, as well as secretion of the TNFα and IL-12 cytokines and the chemokines IL-8, MIP-1α and MIP-1β.

In addition to evaluating the role of ERK in CD40 signaling, future studies could determine which regions of the CD40 cytoplasmic tail mediate various CD40 signaling events in DCs. These studies could be performed by expressing the CD8/CD40 chimeric receptors that I have constructed in D1 cells using retroviral-mediated gene transfer. Once clones of D1 cells expressing the various chimeras are generated, one could determine which region(s) of the CD40 cytoplasmic domain mediate CD40-induced signaling events in DCs. Identifying the region(s) of the CD40 tail that mediate CD40 signaling events in DCs will provide clues as to which adapter proteins link CD40 to these pathways. It will be interesting to determine whether the same 11 amino acid motif in CD40 that I found to be responsible for mediating CD40 signaling events in WEHI-231 cells also mediates CD40 signaling events in DCs.

7.11 CD40 signaling in macrophages

CD40 has recently been shown to be expressed on monocytes and macrophages following exposure to cytokines such as IFN-γ, IL-3 and GM-CSF (33,72). CD40 engagement induces monocytes to secrete proinflammatory cytokines

such as IL-1, and TNFα (72,73). CD40 engagement also induces macrophages to secrete matrix metalloproteinases (75), enzymes which are believed to cause joint degradation in rheumatoid arthritis. Given the role of CD40 in monocyte/macrophage effector functions and its important clinical ramifications, a future aim of the lab should be to understand the molecular basis of CD40 signaling in macrophages. One approach to improving our understanding of CD40 signaling in macrophages is to express my CD8/CD40 chimeric receptors in macrophage cell lines in order to map the regions of CD40 that mediate CD40 responses in macrophages. Elucidating the molecular mechanism by which CD40 signals in these cells as well as other cell types may permit the development of tools to screen for autoimmune diseases or immunodeficiency diseases or the development of therapeutic reagents to combat such diseases.

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