UNIVERSITY OF ALBERTA

MAP KINASE SIGNALLING IN CYTOTOXIC T LYMPHOCYTES

BY

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

IN

IMMUNOLOGY

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ABSTRACT

Cytotoxic T lymphocytes (CTL) are cells of the immune system that recognize and destroy infected or altered cells of the body. This cytotoxic response is triggered by stimulation of the T cell receptor (TCR). The biochemical signaling pathways that link the TCR to activation of cytotoxic function are therefore of great interest. One major category of signaling molecules are the mitogen-activated protein kinases (MAP kinases). In the present study, the activation of MAP kinases in cloned CTL was monitored under various conditions. CTL stimulated in suspension exhibited transient activation of MAP kinases ERK1 and ERK2 and no degranulation function while CTL stimulated in an adherent state displayed sustained ERK activity and degranulation function. The biochemical mechanism of ERK activation differed between anchored and suspended cells. In suspended cells, ERK activation was sensitive to inhibitors of conventional protein kinase C (PKC) kinases. In anchored cells, initial ERK activation was sensitive to inhibitors of phospholipase C activity or diacylglycerol function but not to inhibitors of PKC, while sustained ERK activation was sensitive to inhibitors of novel type PKCs. CTL degranulation was blocked by PKC inhibitors.

In other cell types, the integrin receptors also signal through MAP kinases. Surprisingly, ERK signaling was found to be absent following integrin stimulation of CTL. Nevertheless, integrin ligation promotes T cell activation by an as-yet undefined mechanism upstream of ERKs. MAP kinase was involved in changes to cell architecture that occurred in the presence of integrin ligands.

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The novel type PKC isoform, PKC θ plays critical roles in T cell activation and T cells from PKC θ deficient mice are hyporesponsive. In the present study, TCR-mediated PKC θ activity was detected in anchored but not in suspended CTL. PKC θ *in vitro* kinase activity required phosphatidylinositol-3-kinase activity. In T cells that lacked PKC θ, ERK activation in anchored cells utilized conventional PKC activity in place of novel PKC activity, implying that novel PKC θ plays a role in normal TCR-mediated ERK activation.

Collectively, these data demonstrate that MAP kinase and PKC are essential for activation of cytotoxic function in CTL and reveal novel aspects of MAP kinase regulation.

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List of Abbreviations

Ab	Antibody
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
BIM	Bisindolylmaleimide
BIS	N,N'-Methylene-bis-acrylamide
BSA	Bovine Serum Albumin
ConA	Concanavalin A
CPM	Counts per minute
CTL	Cytotoxic T Lymphocyte
DAG	Diacylglycerol
D-PBS	Dulbecco's PBS
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-Signal Regulated Kinase
FAK	Focal Adhesion Kinase
FCS	Fetal calf serum
FN	Fibronectin
GDP	Guanosine 5'-diphosphate
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine 5'-triphosphate

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HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
Ig	Immunoglobulin
IMM	Immobilized
IP	Immunoprecipitate
IP3	Inositol 1,4,5 Tris-Phosphate
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker for activation of T cells
LFA	Leukocyte Function-Associated Antigen
mAb	Monoclonal Antibody
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
MBP	Myelin Basic Protein
MEK	Mitogen-activated, ERK-activating kinase
MHC	Major Histocompatibility Complex
MLB	Magnesium-containing Lysis Buffer
OD	Optical Density
PBL	Peripheral Blood Leukocyte
PBS	Phosphate Buffered Saline
PDK-1	Phosphatidylinositol 3'-phosphate Dependent Kinase 1
PI	Phosphatidylinositol
PIP2	Phosphatidylinositol-4,5-bisphosphate
PI3K	Phosphatidylinositol 3'-hydroxyl kinase
PKC	Protein Kinase C

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PLA2	Phospholipase A2
PLC	Phospholipase C
PLL	Poly-L-Lysine
PMA	Phorbol-12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
Pyk2	Proline rich tyrosine kinase 2
RasGRP	Ras guanylnucleotide-releasing protein
RPMI	Roswell Park Memorial Institute (culture media)
RSB	Reducing sample buffer
SH2	Src homology 2
SH3	Src homology 3
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
SMAC	supramolecular activation cluster
cSMAC	Central SMAC
pSMAC	Peripheral SMAC
TCR	T cell receptor
TPA	Tetradecanoylphorbol 13-acetate
VN	Vitronectin
WB	Western Blot
XL	Cross-linked

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CHAPTER I Introduction

The Adaptive Immune System

The immune system is a system of cells and organs whose primary function is to recognize and eliminate infectious organisms, parasites and other foreign materials that enter the body. The adaptive immune system exhibits specificity and memory in that the system adapts to produce a stronger response against subsequent infections by the same pathogen. The principle cells of the adaptive immune system are the T and B lymphocytes. B lymphocytes produce antibody which can neutralize extracellular pathogens whereas T lymphocytes detect and eliminate cells that harbour intracellular pathogens.

A molecule that triggers an immune reaction is termed an antigen. B cells recognize extracellular antigens directly via membrane immunoglobulin (mIg). In contrast, the T cell receptor (TCR) recognizes intracellular antigens that have been exported to the cell surface by major histocompatibility complex (MHC) proteins. TCR and mIg are clonotypic receptors, meaning that the gene and protein sequence, and therefore the antigen specificity, of these receptors varies between different lymphocyte clones. Each lymphocyte recognizes only one antigen (or a small group of highly similar antigens) but the entire lymphocyte population can collectively recognize millions of different antigens. Clonal expansion of those lymphocytes that recognize encountered pathogens and the elimination of clones that do not, gives the immune system its adaptive properties.

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A. Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes (CTL) are a subset of T lymphocytes that can destroy infected or abnormal cells. CTL circulate through the body surveying the antigens presented by MHC molecules on each cell that they encounter. If a CTL encounters antigen that matches the specificity of its TCR, it will destroy the cell that bears that antigen. Several lines of evidence indicate that CTL are important for combating virus infections *in vivo* (Zinkernagel and Doherty, 1979). CTL numbers and antigen-specific cytotoxic activity increase following virus infection (Marker and Volkert, 1973; Butz and Bevan, 1998; Murali-Krishna et al., 1998). Transfer of CTL from virus-immunized mice to unexposed recipient mice results in lower virus titre and increased survival upon subsequent viral challenge, while destruction of CTL by antibody-mediated cell lysis increases the severity of viral infection (Blanden, 1970; Blanden, 1971; Yap and Ada, 1978b; Yap and Ada, 1978a; Yap et al., 1978). Genetic defects that impair cytotoxic activity lead to chronic infections in both mice and humans (Huizing et al., 2001). CTL also have the potential to recognize tumor cells (Rosenberg, 1997). CTL with anti-tumor activity can be found in melanomas and other tumors removed from patients (Rosenberg et al., 1988). Isolation and ex-vivo expansion of these anti-tumour CTL followed by retransplant back into cancer patients has produced positive clinical results in some cases (Rosenberg et al., 1988; Barth et al., 1990). In all cases, CTL killing depends on detection of specific antigen. The ability of the T cell population to recognize only foreign antigens and not destroy healthy tissue is a result of the manner in which T cells develop.

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T Cell Development

T lymphocytes develop from non-specialized precursor cells produced by hematopoetic stem cells in the bone marrow. Those precursors that are destined to become T cells migrate to the thymus where they become thymocytes. Immature thymocytes initially have no functional TCR gene. In each thymocyte, a single functional TCR gene is assembled by selective recombination from a large pool of alternative TCR gene segments encoded in the germ line. Some segments are always present in the final TCR gene and encode the structural portions of the TCR, whereas the ligand interaction domains differ between individual clones. Therefore, all TCRs have grossly similar properties but differ in their fine specificity. All functional TCRs interact with MHC molecules. MHC class I molecules bind small polypeptides derived from intracellular protein synthesis and display them on the cell surface. Class I MHC is present on most types of cells. MHC class II molecules bind peptides that are derived from phagocytosed material, and are only present on certain cells of the immune system. The TCR interacts with both the MHC surface and the bound peptide. A given TCR will recognize only one type of MHC and only when it is complexed with a particular peptide, or close variants of that peptide. In the mature T cell, recognition of foreign peptide on host MHC will trigger the T cell mediated immune response.

After assembly of the TCR gene, the next stage of thymocyte maturation involves the elimination of any thymocytes whose TCR recognizes MHC-peptide complexes that are present in normal healthy tissue. MHC class I and II are expressed in the thymus and can trigger signaling through the thymocyte TCR.

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Thymocytes that can not recognize MHC and receive no TCR stimulation fail to generate pro-survival signals and do not survive. Thymocytes that efficiently recognize MHC bearing peptides representing normal physiology receive a strong TCR-mediated signal which triggers apoptotic death of the thymocyte. Therefore it is believed that only thymocytes that exhibit moderate interaction with MHC in the thymus receive the appropriate balance between survival and death signals to allow for survival and maturation.

At this stage, thymocytes also differentiate into either $CD4^+$ or $CD8^+$ cells. The ligands for CD4 and CD8 are MHC class II and MHC class I respectively. These receptors bind MHC at sites distinct from the point of TCR interaction and function to stabilize the TCR-MHC interaction. Thus CD4 and CD8 are termed T cell co-receptors. Thymocytes initially express both CD4 and CD8, then lose expression of one co-receptor to become only $CD4^+$ or only $CD8^+$. For a T cell to be functional, the expression of either CD4 or CD8 must correctly match the class of MHC recognized by that T cell's TCR. It is not clear whether this is accomplished by stochastic or instructive means. In a stochastic mechanism, the choice of co-receptor that is retained is determined randomly, then non-functional thymocytes are eliminated. In an instructive mechanism, the MHC class specificity of the TCR somehow instructs the cell to retain one co-receptor or another. One proposal is that Src family kinases associate more strongly with CD4 than with CD8, so that simultaneous engagement of TCR and CD4 by MHC class II in the thymus, triggers a higher level of kinase signaling in the thymocyte that promotes retention of CD4 expression. After these events have occurred, $CD8^+$ cells normally differentiate into cytotoxic T lymphocytes

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(CTL), whereas $CD4^+$ cells normally differentiate into helper T lymphocytes (T_H cells). These cells exit the thymus and are termed immature or naïve T cells.

T Cell Function

Because of the selection process that occurred in the thymus, the immature T cell population consists mainly of cells that can recognize MHC in combination with peptides not encountered in healthy cells. Such peptides would be generated when cells are infected by foreign agents such as viruses or intracellular bacteria. Unlike thymocytes, immature T cells do not apoptose when they encounter a strong TCR stimulus. When an immature T cell encounters an MHC-peptide combination that matches the binding capabilities of its TCR, and appropriate inflammatory co-signals are provided, the T cell proliferates. The resulting T cell progeny all bear TCR that is identical to that of the parent cell. If these mature T cells encounter "target" cells bearing appropriate MHC and peptide, they will again be stimulated through the TCR and will carry out effector functions.

In the case of cytotoxic T lymphocytes (CTL), effector function is killing of the target cell by delivery of a "lethal hit". The principle mechanism for delivery of the lethal hit is directional secretion of cytotoxic materials from internal granular stores (Atkinson and Bleackley, 1995; Barry and Bleackley, 2002). The degranulated material possesses serine protease and cell permeabilizing activities. Cell permeabilization is thought to be mediated by perforin. Membrane permeabilization alone does not necessarily cause target cell death but perforin also appears to directly or indirectly facilitate entry of other granule components into the target cell cytoplasm. Serine protease activity is due to a family of enzymes termed the

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granzymes. The best characterized of these is Granzyme B. Granzyme B triggers apoptosis by proteolytically cleaving and activating pro-caspases within the target cell. Granzymes may also exert pro-apoptotic effects on target cell mitochondria. It is presently thought that granzymes are taken up by receptor-mediated endocytosis and that perforin subsequently facilitates release of granzymes from the endocytic compartment (Barry and Bleackley, 2002). Target cell apoptosis can also be induced through non-degranulation mechanisms such as engagement of Fas receptor on target cells by Fas-ligand on activated CTL (Atkinson and Bleackley, 1995). Inappropriate CTL cytotoxicity directed against healthy tissue is implicated in serious diseases such as diabetes and multiple sclerosis (Santamaria, 2001). As such, the regulation of T cell function is a topic of significant interest.

B. Signaling Through The TCR

Structure of the T Cell Receptor

Recognition of target cells by T lymphocytes is mediated by the T cell receptor (TCR). The TCR exists in a complex of multiple transmembrane proteins termed the TCR-CD3 complex (Qian and Weiss, 1997). The TCR itself is a disulfide-linked heterodimer composed of an α and β chain. This structure mediates MHC and peptide recognition. The associated CD3 complex is composed of multiple dimeric polypeptides of the form CD3γ-CD3ε and CD3δ-CD3ε and is further associated with a disulphide linked ζ-ζ homodimer. The precise stoichiometry of dimers within the complex remains controversial. The TCR α and β chains contain very small intracellular domains suggesting that the TCR is linked to intracellular signaling via the longer intracellular domains of the CD3 and ζ proteins. None of the

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TCR-CD3 chains possesses any detectable enzymatic activity. Ligation of TCR by isolated soluble peptide-MHC molecules does not appear to trigger any T cell response whereas trimeric or tetrameric assemblies of purified peptide-MHC initiate detectable T cell signaling (Boniface et al., 1998). This suggests that TCR clustering is important for initiation of signaling.

TCR-Mediated Signaling

TCR-mediated signaling is required at each stage of T cell development and function. Thymocyte development or deletion, T cell proliferation and maturation, and mature T cell effector function are all triggered by TCR stimulation. Many signaling events that follow TCR engagement have been identified and described in detail. However, the present model of TCR-mediated signaling should be viewed with caution. Many signaling studies have employed immortalized T cell lines or non-lymphocytes transfected with genes of interest. The map of T cell signaling built from these studies may differ from the situation *in vivo*. In particular, current models represent an average view of TCR signaling assembled from experiments on different T cell subsets at various stages of differentiation.

Figure I-1 depicts some of the known intracellular signaling consequences of TCR/CD3 stimulation (Weiss and Littman, 1994; Lin and Weiss, 2001). The earliest detectable signaling event after TCR ligation is tyrosine phosphorylation of various proteins. The Src family protein tyrosine kinase Lck phosphorylates tyrosines in the cytoplasmic tails of the CD3 and ζ molecules at sites termed immune receptor tyrosine based activation motifs (ITAMs) (Weiss and Littman, 1994; Qian and Weiss, 1997). Events at the ζ chain have been best characterized. The Syk family kinase

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ZAP-70 binds to phosphorylated ζ chain and becomes activated by Lck or Fyn-mediated phosphorylation. Activated ZAP-70 phosphorylates the adaptor protein LAT (linker for activation of T cells) at multiple sites (Zhang et al., 1998). Multiple proteins interact with phosphorylated LAT including SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) which recruits additional proteins forming a large signaling complex. One member of this complex Vav, acts as a guanosine nucleotide exchange factor for the Rac and Cdc42 family proteins which in turn regulate cytoskeletal architecture. The LAT/Slp-76 complex recruits both phospholipase C (PLC) and Itk leading to Itk-mediated phosphorylation and activation of PLC. PLC metabolizes the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to produce inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). PIP2 is also a substrate for phosphatidylinositol 3'-hydroxyl kinase (PI3K). PI3K becomes activated following TCR stimulation and phosphorylates inositol lipids at the 3' position. PI3K products can influence the localization ~~or activity~~ of proteins that contain pleckstrin homology (PH) domains such as Vav, and can induce activation of some kinases such as Akt. Thus both PI3K and PLC metabolize PIP2 to generate second messengers that promote T cell activation. Production of IP3 by PLC induces release of calcium from intracellular stores. One major consequence of elevated intracellular calcium is activation of the phosphatase calcineurin. Calcineurin-mediated dephosphorylation of NF-AT leads to nuclear translocation of NF-AT where it acts as a transcription factor for IL-2 and other genes that are important for T cell proliferation. The immunosuppressive drugs cyclosporin A and FK506 mediate their immunosuppressive effects by inhibiting calcineurin function.

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The other major signaling pathway downstream of PLC involves the second messenger DAG, which interacts with enzymes containing C1 domains such as protein kinase C.

Protein Kinase C

Protein Kinase C (PKC) enzymes are a family of serine-threonine kinases involved in multiple aspects of T cell biology including proliferation, cytokine production and TCR downregulation (Szamel and Resch, 1995; Keenan et al., 1997). PKC isozymes are categorized as classical (cPKC α, β, γ), novel (nPKC δ, ε, η, θ) or atypical (Nishizuka, 1995). PKCs contain 4 conserved motifs, termed C1 through C4. The C1 domain binds DAG, resulting in increased PKC activity. The C2 domain is only found in cPKCs and binds phospholipids in a calcium-dependent fashion. cPKC isoforms require both calcium and DAG for membrane localization and kinase activity. nPKCs do not require calcium for their activity. The C3 domain contains the ATP-binding and kinase activity sites of the enzyme. The C4 domain appears to mediate substrate binding. The N-terminal portion of the C1 domain contains a pseudosubstrate motif that occupies the C4 substrate binding site when the kinase is inactive. In addition to DAG, PKC activity requires phosphatidylserine, is promoted by phosphatidylethanolamine, and is inhibited by phosphatidylcholine, suggesting that lipid partitioning may be important to PKC regulation (Nishizuka, 1984).

Both nPKCs and cPKCs can be activated experimentally by the addition of non-hydrolyzable DAG analogues such as PMA (also called TPA). PMA has been much studied owing to its ability to act as a tumor promoter. PMA treatment can exert multiple effects upon cells including activation of the intracellular signaling

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proteins Ras and ERK (Downward et al., 1990; Rayter et al., 1992). The effects of PMA may be Ras-dependent (Rayter et al., 1992) or independent (Burgering et al., 1993) depending on the cell type.

Ras and MAP Kinases

Mitogen-activated protein kinases (MAP kinases) are a family of serine-threonine kinases involved in numerous eukaryotic signal transduction processes including those controlling cell proliferation, cell differentiation, yeast pheromone responses, and cytokine biosynthesis (Cobb, 1999). MAP kinases function as the distal element of three-step kinase cascades (Figure I-2). The best studied mammalian MAP kinases are ERK1 and ERK2. ERKs are activated by dual phosphorylation on tyrosine and threonine residues (Thr202 and Tyr204 in human ERK2) (Canagarajah et al., 1997) by the tyrosine/threonine kinases MEK1 and MEK2. The MEK enzymes are themselves activated by phosphorylation by the serine/threonine kinase Raf (Cobb and Goldsmith, 1995; Robinson and Cobb, 1997; English et al., 1999). Raf activity is thought to be regulated principally by Ras-mediated recruitment of Raf to the cell membrane, although the mechanism of Raf activation at the membrane is ill-defined (Morrison and Cutler, 1997). Ras molecules act as biochemical switches. They can exist in an "inactive" state bound to a molecule of guanosine 5'-diphosphate (GDP), or an "active" conformation when bound to guanosine 5'-triphosphate (GTP). The "active" conformation binds to effector molecules such as Raf. The ratio of GTP-bound Ras to GDP-bound Ras is modulated by GTPase-activating proteins (GAPs) and guanylnucleotide exchange factors (GEFs) (Boguski and McCormick, 1993). GAPs decrease Ras effector

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function by enhancing the intrinsic GTPase activity of Ras, while GEFs promote Ras effector function by accelerating the release of GDP from Ras, allowing GTP to bind.

The best characterized Ras-MAPK activation pathway is that stimulated through the receptor protein tyrosine kinase (RPTK) family of growth factor receptors (Figure I-2); reviewed in: (McCormick, 1993). When ligated, these receptors become autophosphorylated, allowing for the binding of the adaptor molecule Grb2 (Lowenstein et al., 1992). This interaction is mediated by the SH2 (Src homology 2) domain of Grb2 which recognizes motifs containing phosphorylated tyrosine. In some cases recruitment of Grb2 to phosphorylated tyrosine occurs indirectly through the adaptor protein Shc, which recognizes phosphotyrosine motifs and also binds Grb2. Grb2 additionally contains two SH3 (Src homology 3) domains which bind to proline-rich motifs. Through the SH3 domains, Grb2 associates with a ubiquitous RasGEF called Sos, and the recruitment of Grb2-Sos complexes to the membrane activates membrane-associated Ras (Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993).

Mechanism of TCR-mediated MAP kinase activation

The TCR was the first receptor shown to regulate Ras under physiologically relevant conditions (Downward et al., 1990). However, the mechanism of TCR-mediated Ras and MAPK activation has been a subject of controversy with several different models successively dominating the field over the past 12 years (Figure I-3).

It was originally shown that both TCR stimulation and addition of PMA induce accumulation of GTP-bound Ras in T cells (Downward et al., 1990). Since protein kinase C (PKC) was the major known receptor for phorbol esters, it was

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thought that TCR stimulation must be linked to the Ras-MAP kinase pathway by PKC (Downward et al., 1990). Since the rate of ^{32}P-GTP to GDP conversion on purified recombinant Ras appeared to be slower in the presence of lysate from PMA-treated lymphoblasts, it was thought that PKC activated Ras by inhibiting RasGAPs (Downward et al., 1990; Downward et al., 1992).

The PKC model of Ras-MAPK activation was soon challenged by studies which found no Ras inhibition when PKC activity was inhibited by a PKC pseudosubstrate peptide, indicating that PKC-independent pathways for TCR-mediated Ras activation also exist (Downward et al., 1992; Izquierdo et al., 1992). With the discovery that Grb2-Sos complexes linked growth factor receptor tyrosine phosphorylation to activation of Ras (Figure I-2) (Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993), it was thought that an analogous process would occur in T cells following phosphorylation of the TCR ζ chain. Several studies have reported an increase in Grb2-Sos association and/or increased Sos activity upon TCR ligation (Nel et al., 1995; Ravichandran et al., 1995; Li et al., 1996). One early study reported direct association between Shc-Grb2 complexes and TCR ζ chain following anti-TCR and anti-CD4 co-stimulation of By155.16 T cell hybridomas (Ravichandran et al., 1993). Another study detected Fyn-Shc and Shc-Grb2 complexes in anti-CD3 stimulated HBC T cell hybridomas (Li et al., 1996). However, studies in human PBL showed that Shc can not compete effectively with ZAP-70 for binding to dually-phosphorylated ζ chain (Osman et al., 1995) and Grb2-Shc association is not detected in all T cell lines (Buday et al., 1994; Nel et al., 1995). Although detection of Grb2 in

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association with TCR is not consistently observed (Koretzky, 1997), several studies detected complexes of Grb2 and a 36 kDa phosphoprotein (Buday et al., 1994; Sieh et al., 1994; Nel et al., 1995) that was later identified as LAT (linker for the activation of T cells) (Zhang et al., 1998). LAT is a transmembrane protein containing Grb2-binding sites that become phosphorylated in response to TCR stimulation and could therefore recruit Grb2-Sos complexes to the membrane (Cantrell, 1998; Zhang et al., 1998). These observations were assumed to explain MAPK activation in T cells. However, later studies would show that the Grb2 binding sites could be mutated without loss of TCR-mediated ERK activation (Zhang et al., 2000). LAT binds additional proteins including PLC-γ and SLP-76 which contributes to PLC-γ activation (see Figure I-1). Interestingly, loss of PLC recruitment or of SLP-76 function eliminates TCR-mediated MAP kinase activation (Yablonski et al., 1998; Zhang et al., 2000). One consequence of PLC activity is production of diacylglycerol (DAG) and activation of PKC (Nishizuka, 1995) suggesting that the older PKC-mediated model of Ras activation might yet have merit.

Recently, a non-PKC receptor for diacylglycerol and phorbol ester was characterized that possesses intrinsic RasGEF activity. Ras guanylnucleotide releasing protein (RasGRP), also known as CalDag-GEFII, is a novel RasGEF expressed in neuronal and lymphoid cells (Ebinu et al., 1998; Kawasaki et al., 1998; Tognon et al., 1998). RasGRP contains a C1 DAG-binding domain and can mediate Ras activation in response to PMA (Ebinu et al., 1998; Tognon et al., 1998). Clearly, earlier studies using PMA as a PKC activator must be re-evaluated in light of these findings. In Jurkat T cells, TCR stimulation induces RasGRP association with the

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membrane and RasGRP overexpression enhances Ras-ERK activation in response to TCR stimulation (Ebinu et al., 2000).

The precise mechanism or mechanisms of TCR-mediated MAP kinase activation in T lymphocytes remains controversial. The relative contributions of PKC, Grb2-Sos and RasGRP to TCR-mediated ERK activation is unknown. The mechanisms of TCR-mediated signaling may differ at each stage of the T cell life cycle and between T cell subsets. The nature and relative contributions of PKC and non-PKC mechanisms of TCR-mediated ERK activation in mature effector CTL is the subject of CHAPTER V and CHAPTER VI

C. Integrin Signaling

Integrins

Integrins are a large family of receptors with no intrinsic kinase activity that function in cell-to-cell and cell-to-matrix adhesion (Springer, 1990; Hynes, 1992). Integrins are heterodimeric transmembrane proteins composed of α and β chains, and are often classified by the form of the β chain, although both chains contribute to ligand specificity. T cells express β1, β2 and β3 -containing integrins. The β2 integrin LFA-1 (leukocyte function-associated antigen 1) binds to ICAM-1 (intracellular adhesion molecule1) and is a major mediator of T cell to target cell adhesion (Springer, 1990). β1 and β3 integrins mediate adhesion to extracellular matrix proteins such as fibronectin and vitronectin (Chan et al., 1991; Moulder et al., 1991; Ma et al., 1997). Contact with extracellular matrix proteins significantly diminishes the degree of TCR stimulation required to achieve T cell activation (Matsuyama et al., 1989; O'Rourke and Mescher, 1992; Ostergaard and Ma, 1995;

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Ybarrondo et al., 1997). Integrins are also important in non-lymphocytes. Healthy mammalian cells generally do not proliferate in response to mitogenic signals in the absence of integrin-mediated adhesion (Folkman and Moscona, 1978; Miranti and Brugge, 2002). As such, identifying the intracellular signals produced by integrins is a topic of great interest.

The best characterized integrin signaling pathway involves the non-receptor tyrosine kinase FAK (focal adhesion kinase) and Grb2-Sos mediated Ras-ERK activation (see Figure I-4) (reviewed in: Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999; Miranti and Brugge, 2002). In this pathway, β1 family integrins can interact with FAK via the cytoplasmic tail of the β1 chain. Upon integrin ligation and clustering FAK autophosphorylation occurs which allows recruitment of Src family kinases and further phosphorylation of FAK (Hanks et al., 1992; Schlaepfer et al., 1994). Tyrosine phosphorylation of FAK at amino acid position 925 provides a binding site for Grb2 which is proposed to cause Ras activation in a manner similar to that described for receptor tyrosine kinases (Schlaepfer et al., 1994). Indeed, exposure of fibroblasts to extracellular matrix results in transient activation of MAP kinases (Chen et al., 1994; Schlaepfer et al., 1994; Morino et al., 1995). This raises the possibility that integrin-mediated MAPK activation might contribute to the phenomenon of integrin enhancement of T cell activation.

Interestingly, TCR ligation elicits phosphorylation of both FAK and a closely related kinase, Pyk2 (Maguire et al., 1995; Berg and Ostergaard, 1997). One study using Jurkat T leukemia cells showed an association between phosphorylated Pyk2 and Grb2, and proposed this as a mechanism of TCR-mediated MAP kinase

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activation (Ganju et al., 1997) Figure I-3. These results further suggest that Grb2 recruitment to phosphorylated FAK and Pyk2 might represent a common pathway shared by both integrins and TCR and thus explain the enhancement of T cell stimulation seen in the presence of integrin ligands. Whether or not these pathways play a role in CTL is the topic of CHAPTER IV .

D. Study Objectives

The MAP kinase signaling pathway is important for controlling proliferation and differentiation in a variety of cell types and represents a potential target for the therapeutic disruption of harmful cell proliferation as in cancer, autoimmunity, or transplant rejection. Therefore it is desirable to learn as much as possible about the regulation of this pathway. The goal of the present study is to further our understanding of the mechanism of MAP kinase activation in the context of CTL effector function. The following questions were specifically addressed:

1) What is the relationship between cell anchorage, MAP kinase, and CTL effector function?
2) What is the relationship between integrin ligation and MAP kinase activity in CTL?
3) What intracellular signaling mechanisms are important for TCR-mediated MAP kinase regulation in CTL? Specifically, what role does PKC play in MAPK kinase activation? If PKC is relevant, what isoforms of PKC are involved?

Addressing these questions will aid our understanding of a cell type that plays an important role both in disease states and in the maintenance of normal health.

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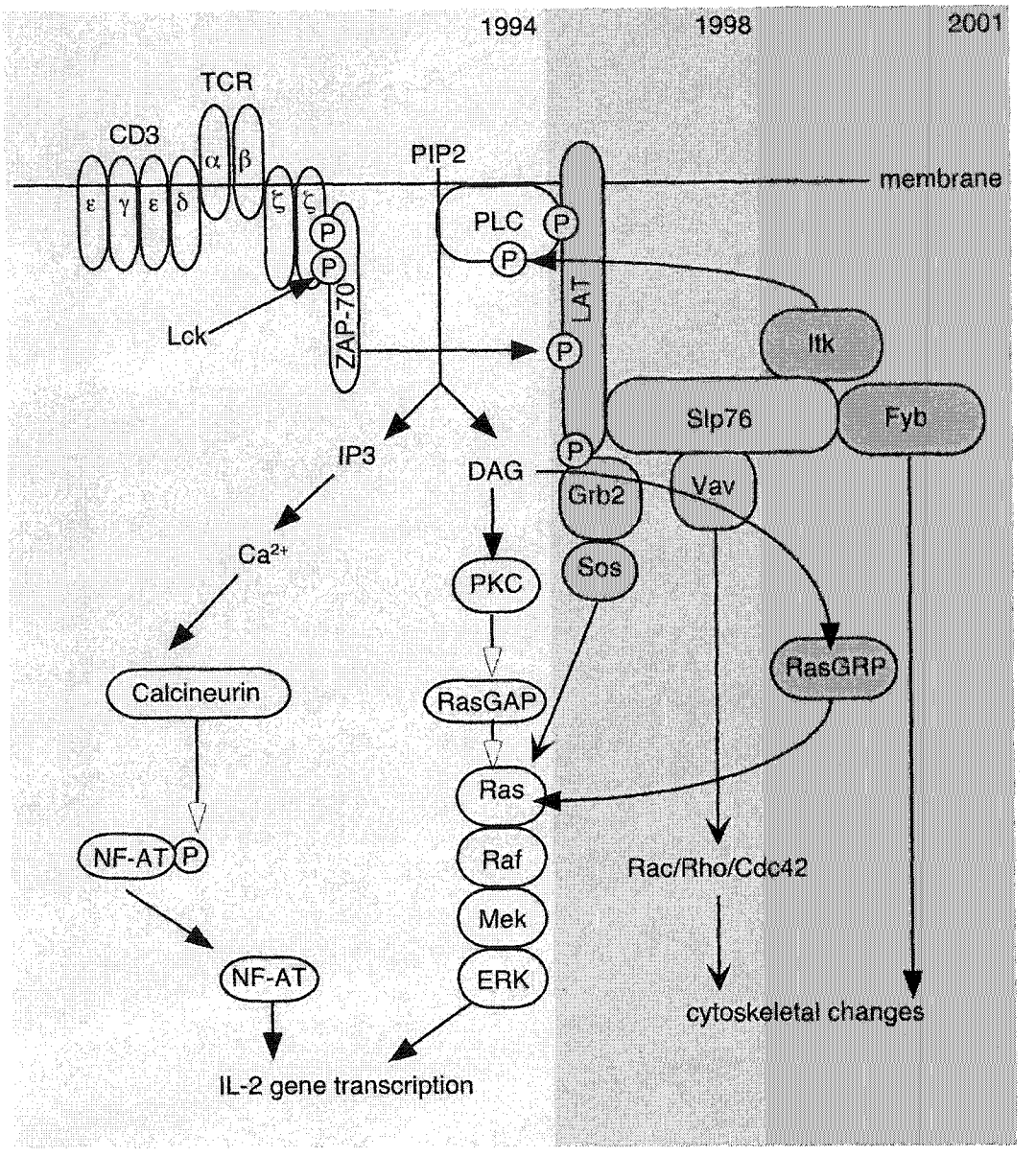

Figure I-1 Major TCR signaling pathways.
To highlight how the understanding of TCR-mediated signaling has changed, this figure summarizes information from reviews published in 1994 (Weiss and Littman, 1994), 1998 (Cantrell, 1998) and 2001 (Lin and Weiss, 2001) in successive columns. Circled "P" indicates phosphorylation. Open arrowheads indicate inhibitory effects. Note that Ras is membrane-associated but is depicted away from the membrane for clarity. Slp76 binds LAT via an adaptor protein intermediate such as Gads or Grb2 (not shown). Activation of Ras via PKC-mediated inhibition of RasGAP is controversial. Although LAT binds Grb2 there is no direct evidence that Grb2-Sos functions to activate Ras in T lymphocytes.

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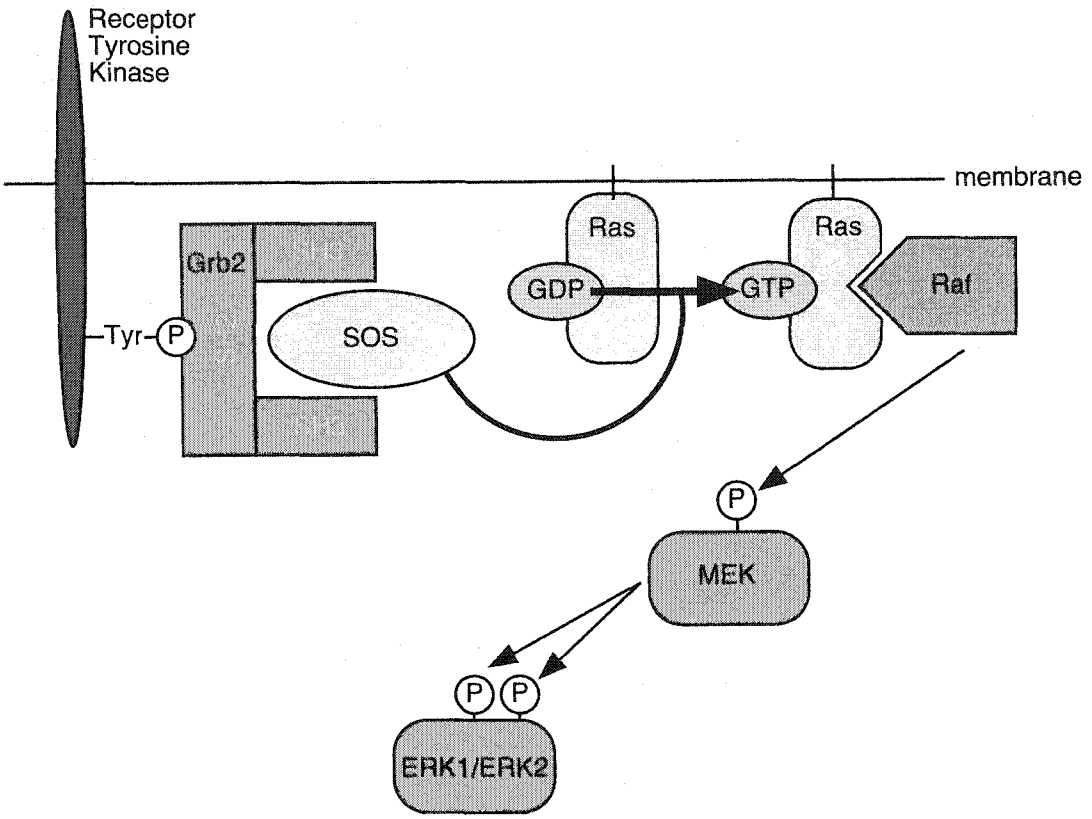

Figure I-2 Canonical growth factor – Ras – MAP kinase pathway. Phosphorylation of a receptor on tyrosine provides a binding site for proteins containing SH2 domains, such as Grb2. Grb2 is complexed with the RasGEF Sos which promotes exchange of Ras-bound GDP for GTP. Ras-GTP interacts with Ras effectors such as Raf, leading to induction of Raf kinase activity. Raf phosphorylates MAP kinase kinases such as MEK, which in turn become active and phosphorylate MAP kinases such as ERK1 and ERK2.

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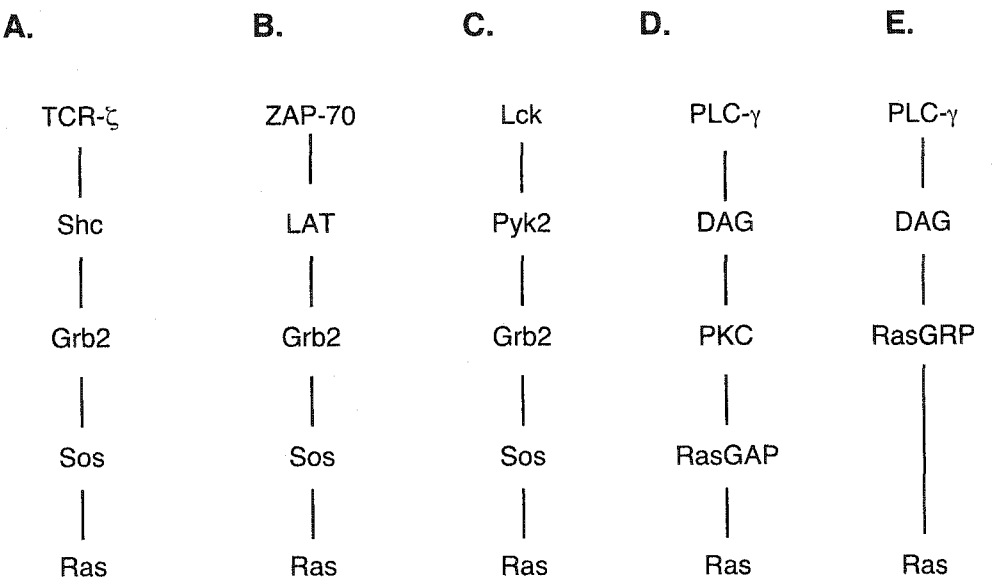

Figure I-3 Potential mechanisms of TCR-mediated Ras activation. TCR stimulation triggers tyrosine phosphorylation of TCR-ζ, activation of Lck and ZAP-70 kinases, and activation of PLC-γ. Which pathway leads to activation of Ras, and consequently of ERK, is controversial. Models A-C propose Ras activation pathways that involve protein-protein interactions and the RasGEF Sos. Models D and E propose pathways that utilize the second messenger DAG to either inhibit GAPs (D) or activate GEFS (E).

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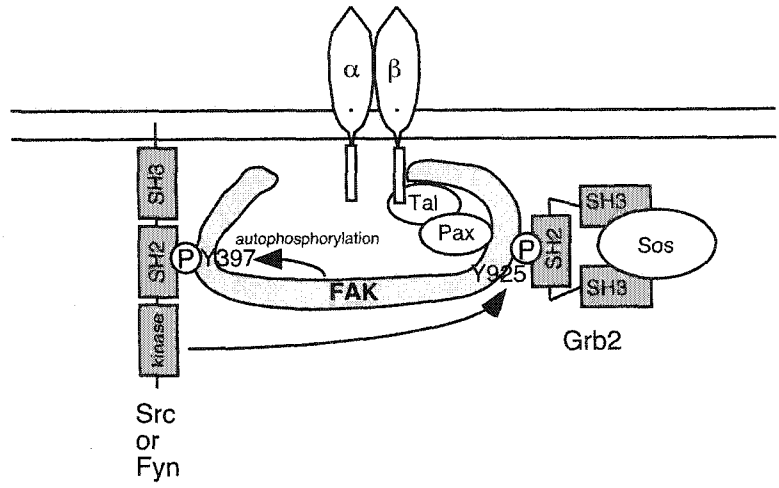

Figure I-4 Integrin signaling through FAK and Grb2.
Integrin clustering results in autophosphorylation of FAK at Tyr397. This residue can bind the SH2 domain of Src or Fyn kinases which phosphorylate FAK at position Tyr925. In fibroblasts, phosphorylated Tyr925 interacts with Grb2, which activates Ras via the exchange factor Sos. Abbreviations: Pax; paxillin; Tal, talin; Y, tyrosine. Diagram adapted from (Giancotti and Ruoslahti, 1999).

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CHAPTER II Materials and Methods

Cell lines

The murine $CD8^+$ anti-H-$2K^b$ allo-specific CTL clone AB.1 was originally derived by limiting dilution from a BALB/c anti-C57BL/6 mixed lymphocyte culture (Kane et al., 1989). Murine CTL clone 11 was originally derived from peritoneal exudates cells of (B10.BRxB10.D2)F_1 mice (Kane et al., 1989). CTL clones were maintained in RPMI with 8%FCS, supplemented with L-glutamine, penicillin, streptomycin, sodium pyruvate, non-essential amino acids, and 0.05 mM 2-mercaptoethanol. Clone growth was maintained by weekly stimulation with allogeneic irradiated splenocytes from C57BL/6 (H-2^b) mice (Jackson Laboratory, Bar Harbour, ME) and IL-2, provided 4 to 7 days prior to experiment use.

Generation of ConA Blasts

Spleens were removed from C57BL/6 mice that had been humanely sacrificed. Spleens were homogenized and treated with ammonium chloride to lyse red blood cells. Splenocytes were stimulated to proliferate by the addition of 2 μg/ml concanavalin A in RPMI with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol. Cells were allowed to proliferate for 2 days before use.

Preparation of Splenic T Cells

Spleens were removed from C57BL/6 mice (Jackson Laboratory, Bar Harbour, ME) or PKC $\theta^{-/-}$ mice (a kind gift of Dr. D. Littman, Skirball Institute, N.Y.) that had been humanely sacrificed. Spleens were homogenized and treated with ammonium chloride to lyse red blood cells. The remaining splenocytes were washed

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and resuspended in PBS with 2% defined calf serum. Non-T cells were removed by passing the cell suspensions through mouse T cell columns (CedarLane Laboratories, Honby, Ontario) according to the instructions supplied by the column manufacturer.

Antibodies

The hybridoma producing 145-2C11 (anti-CD3ε) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The hybridoma producing PY-72 (anti-phosphotyrosine) was obtained from Dr. B. Sefton (The Salk Institute, La Jolla, CA). Anti-Pyk2 antisera was generated by Dr. Nancy Berg (Berg and Ostergaard, 1997). m199 (anti-RasGRP) mAb was kindly provided by Dr. J. Stone. Anti-PKC θ antiserum was kindly provided by Dr. C. Arendt (The Skirball Institute, NY). Anti-MAP kinase (ERK1+ERK2) mAb was purchased from Zymed (San Francisco, CA). Anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibodies 9101, 9105 and 9106 were purchased from New England Biolabs (Beverly, MA). Antibodies H-7 (anti-PKC α), C-16 (anti-PKC β1), C-17 (anti-PKC δ), E-5 (anti-PKC ε) and C-18 (anti-PKC θ) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An additional anti-PKC ε antibody was purchased from BD Biosciences Canada (Mississauga, ON). RAS10 (anti-Ras) antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-CD29 (anti-β1) and anti-CD61 (anti-β3) mAb were purchased from Pharmingen (San Diego, CA). Anti-Grb2 mAb was purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-hamster Ig, goat anti-hamster Ig, and anti-mouse Ig^{HRP} Abs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

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Protein reagents

Human cellular fibronectin was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Ras Binding Domain fusion protein bound to glutathione agarose was obtained from Upstate Biotechnology (Lake Placid, NY). Myristoylated pseudosubstrate peptide inhibitors of PKC (Ward and O'Brian, 1993) were purchased from Calbiochem (San Diego, CA).

Chemical reagents

PMA was purchased from Sigma Chemical Company (Mississauga, Ontario). Wortmannin, Ly-294002, U73343, U73122 and the PKC inhibitors bisindolylmaleimide I, calphostin C, Gö-6983, Gö-6976 and Ro-31-8220 were purchased from Calbiochem (San Diego, CA). The MEK (MAP kinase kinase) inhibitor PD98059 was purchased from Calbiochem (San Diego, CA).

Antibody immobilization

Ninety-six well flat bottom Falcon 3912 microtitre plates (Becton Dickinson, Oxnard, CA) were coated with 40 µl of 10 µg/ml 145-2C11 in PBS overnight at 4°C. Wells were washed twice with PBS, then blocked with 2% BSA in PBS for 30-60 minutes at 37°C, then washed twice with PBS before use.

Antibody+ Fibronectin immobilization

Various concentrations of 145-2C11 alone or in combination with 15-20 µg/ml of fibronectin, were diluted in PBS and incubated overnight at 4°C in ninety-six-well flat bottom Nunc Maxisorp immuno-plates. Prior to use, wells were washed twice with PBS, blocked with 2% BSA/PBS for 30-60 minutes at 37°C, then washed twice with PBS.

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Cell Stimulation

CTL clones were harvested and washed in Dulbecco's PBS (D-PBS) (Life Technologies, Rockville, MD). Cells were stimulated in one of three ways. For acute PMA stimulation, 100 ng/ml PMA was added and cells were harvested after incubation at 37°C for 10 minutes or the time indicated in each figure. For immobilized anti-CD3 stimulation, cells were added to microtitre wells in which 145-2C11 had been bound as described above under *Antibody Immobilization,* and incubated at 37°C for the times indicated in each figure. For soluble, cross-linked stimulation, cells were incubated with 10 μg/ml 145-2C11 for 15 minutes on ice, then washed and resuspended in D-PBS (with drug or carrier control if required), then 5 μg/ml rabbit anti-hamster antibody was added to cross-link the 145-2C11. Cells were aliquoted into BSA-blocked microtitre plate wells and incubated at 37°C for the times indicated in each figure. In each case, $1x10^5$ cells in a volume of 40 μl were used for each sample.

Western Blot

After stimulation, cells were lysed at the indicated times by the addition of 2X Laemmli reducing sample buffer and boiled for 3 minutes. 80 μl samples representing equal cell numbers were separated on the basis of protein weight by standard SDS-PAGE methodology. Samples were transferred to Immobilon-P (Millipore Corporation, Bedford, MA) in a wet transfer tank using a current of 150 mA for 4 hours. Immunoblotting was performed using the appropriate primary antibody and anti-mouse^{HRP} or protein-A^{HRP} and visualized by Enhanced Chemiluminescence (NEN Life Science Products, Boston, MA).

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PKC downregulation by chronic PMA exposure

PMA was added to cell cultures at 10 ng/ml, 50 ng/ml or 100 ng/ml, 18 hours before cells were used, to downregulate PKC expression.

Enzyme Inhibitors

Cells in D-PBS were preincubated with the appropriate inhibitor or carrier control (DMSO or ethanol), at the indicated concentration(s), for 20-30 minutes at 37°C except for calphostin C which was activated by exposure to light for 30 minutes, then incubated with cells for 5-10 minutes. Experiments using rottlerin were performed in the presence of 1% serum to maintain cell viability. Cell viability was assessed at the end of the assay by trypan blue exclusion. None of the drugs used had a significant impact on cell viability over the duration of the assay.

MAP kinase (ERK1 and ERK2) analysis

Cells were lysed at the indicated times after stimulation by the addition of 2X Laemmli reducing sample buffer and boiled for 3 minutes. Whole cell lysates were subjected to SDS-PAGE using a 15% low-Bis (175:1 acrylamide:Bis) gel. The activated forms of MAP kinases ERK1 (p44) and ERK2 (p42) experience reduced mobility under these conditions. Proteins were transferred to Immobilon P (Millipore Corporation, Bedford, MA). Immunoblotting was performed using anti-MAP kinase (ERK1+ERK2) mAb (Zymed, San Francisco, CA) and anti-mouse-Ig^{HRP} and visualized by Enhanced Chemiluminescence (NEN Life Science Products, Boston, MA). In some cases the amount of active and inactive MAP kinase was quantitated by scanning optical densitometry using an Agfa Duoscan T1200 scanner and the public domain NIH Image software program (developed at the U.S. National

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Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The percentage of active ERK was calculated from O.D. measurements using the formula (active ERK)/(active ERK+inactive ERK)x100%.

Immunoprecipitation from fibronectin-stimulated cells

Cells were stimulated as described above except that fibronectin and/or 145-2C11 mAb were immobilized on Falcon 1147 non-tissue culture treated 24 well plates. $1.5x10^6$ cells were stimulated for each condition and lysed by the addition of an equal volume of 2X lysis buffer (2% NP-40, 20mM Tris, 150mM NaCl). Postnuclear lysates were incubated with anti-Pyk2 antisera for 15 minutes on ice, then for 1-2 hours with protein A Sepharose (30 µl of a 50% slurry) at 4°C to capture immune complexes. Precipitated material was washed 4 times in 0.5% NP-40 lysis buffer, resuspended in Laemmli reducing sample buffer, and boiled for 3 minutes. Samples were analyzed by Western blotting as described above.

Adhesion Assay

Adhesion was measured under static conditions by a standard assay (Mobley and Shimizu, 1999). Briefly, cells were labeled with ^{51}Cr, then treated with PD98059 or DMSO carrier control for 30 minutes at 37°C. 100 µl cell aliquots were added to wells of a ninety-six well Maxisorp™ plate that had been coated with 145-2C11 and/or fibronectin as described above under *Protein Immobilization*. Plates were incubated for one hour at 37°C, then washed with cold media to remove unbound cells. After removal of all media, 125 µl of Supermix™ (Wallac, Finland) was added and ^{51}Cr counts measured on a Trilux 1450 Microβ liquid scintillation counter.

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Results were determined in triplicate and the average and standard deviation are shown.

Ras Assay

Cells were resuspended at a density of 2.5 x 10^7 cells/ml in D-PBS and treated with 2 μg/ml of BIM or DMSO (carrier control) for 30 minutes prior to cell stimulation. For each sample 10^7 cells in D-PBS were stimulated in 60x15 mm non-tissue-treated polystyrene dishes (Fisher, Nepean) that had been coated with 10 μg/ml 145-2C11 and blocked with 2% BSA in PBS, or in dishes coated with 2% BSA only. Dishes were incubated at 37°C for the indicated times, then the cells were lysed in 1 ml of Magnesium-containing Lysis Buffer (MLB: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 2 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF) (Taylor and Shalloway, 1996). Nuclei were removed by brief centrifugation. Aliquots of post-nuclear lysates were saved for assessment of total Ras expression and ERK1/2 activity and the remainder of the lysate was used for Raf-1 affinity precipitation. 15 μl of Raf-1 Ras Binding Domain fusion protein bound to glutathione agarose, obtained from Upstate Biotechnology (Lake Placid, NY), was added to each sample. This construct binds to the biologically active GTP-bound form of Ras. Samples were incubated with rotation at 4°C for 30 minutes, then the beads were washed 3 times with MLB and resuspended in Laemmli sample buffer. The entire sample was electrophoresed on a 12% SDS-PAGE gel, transferred to Immobilon-P and immunoblotted with RAS10 antibody (Upstate Biotechnology, Lake Placid, NY) as described in the Raf-1-RBD-agarose product bulletin.

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Degranulation Assay

Cytotoxic degranulation was measured using a published method (Pasternack and Eisen, 1985; Kane et al., 1989). 1.5×10^5 cells in 150 µl of RPMI 1640 with 2% newborn calf serum were added to each well of a 96 well microtitre plate that had been prepared as described above in *Antibody Immobilization*. After 4-5 hours of incubation at 37°C, 25 µl of supernatant was mixed with 150 µl of substrate (4.8 mg Nα-benzylocarbonil-L-lysine thiobenzyl ester (BLT) + 4.8 mg 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) in 50 ml PBS). CTL degranulation releases esterases. In this assay, benzylocarbonil-L-lysine thiobenzyl ester esterase activity produces a coloured product that can be assayed by measuring optical absorbance at 405 nm. Results shown are the average and standard deviation of samples prepared in triplicate or quadruplicate.

Cell Fractionation

$2\text{-}5\text{x}10^7$ cells were resuspended in ice cold hypotonic lysis buffer (10 mM Tris pH 7.4, 2 mM EDTA). Cells were frozen in liquid N_2 and rapidly thawed. After two freeze-thaw cycles, cells were resuspended using a dounce. Sucrose was added to a final concentration of 0.25 M and tubes were centrifuged at 4600 x g for 10 minutes. The supernatant was saved and the pellet resuspended in hypotonic buffer with 0.25 M sucrose with 20 strokes of the dounce homogenizer. After a second centrifugation, the supernatants were pooled and centrifuged at 19,200 x g for one hour. The resulting supernatant was taken as the cytosolic fraction and the resulting pellet was resuspended in 1% NP-40 in PBS and taken as the membrane fraction. To confirm

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effective fractionation, samples were probed by Western blotting for CD45 and paxillin as markers of membrane and cytosol respectively.

Total PKC Activity Assay

Cells that had been treated for 18 hours with PMA or with ethanol carrier control were resuspended at 10^7 cells/ml and lysed in extraction buffer containing 25 mM Tris, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 mM PMSF. Post-nuclear lysates were assayed for PKC activity using the SignaTECT™ PKC assay system from Promega Corporation (Madison, WI) as described in the product bulletin. This system uses the biotinylated peptide Neurogranin as a substrate. The ratio of ^{32}P CPM incorporation into the peptide using extracts assayed in the presence of phosphatidylserine and diacylglycerol, to the incorporation using extract assayed in the absence of activators, was determined. Three samples from each cell lysate were analyzed and the standard deviation between samples is shown.

PKC θ Immunoprecipitation

1-2x10^7 cells were collected and stimulated by either TPA, immobilized anti-CD3 mAb, or soluble cross-linked anti-CD3 mAb as described above under *Cell Stimulation*. Non-adherent cells were collected and resuspended in 100 µl of lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4) while the remaining plate-bound cells were harvested by the addition of 900 µl of lysis buffer to the plates. These fractions were pooled and nuclei removed by centrifugation at 16,000 x g for 5-10 minutes. 40 µl of postnuclear supernatant was reserved. 5 µl of anti-PKC θ antiserum was added to the remaining

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material. Samples were incubated on ice for 15-45 minutes before addition of protein A-sepharose and incubation on a tube rotator for 1-2 hours at 4°C. Samples were washed 3 times in lysis buffer, then protein was released from the beads by the addition of 70 µl of reducing sample buffer and boiling.

PKC θ in vitro Kinase Activity Assay

PKC θ immunoprecipitations were washed twice with lysis buffer, then once with kinase buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.1 mM EGTA), and resuspended in 20 µl of reaction mix (100 µM ATP, 10 µCi γ-^{32}P-ATP, 2µg MBP in kinase buffer). Each reaction was incubated for 20 minutes at 30°C, then stopped by the addition of 20 µl of 2X reducing sample buffer. Samples were boiled for 5 minutes, then loaded on a standard 15% SDS-PAGE gel. Proteins were transferred to Immobilon P (Millipore Corporation, Bedford, MA) and ^{32}P labeled protein was detected by autoradiography.

Reproducibility of Results

Representative data is shown. In all cases a minimum of three experiments were performed giving similar results.

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CHAPTER III MAPK Activity in CTL: Stimulation of Anchored *vs.* Suspended Cells

A. Introduction

The primary function of cytotoxic T lymphocytes is to eliminate infected or altered cells that the CTL encounters. One major mechanism of cytotoxicity is the release of cytotoxic granules. Degranulation can be elicited by plating CTL onto plastic-immobilized anti-CD3 or anti-TCR antibodies. In striking contrast, no CTL degranulation is triggered by ligation of TCR/CD3 in suspension cultures of CTL (Berg et al., 1998). However CTL in suspension culture can be triggered to degranulate by treatment with PMA and ionomycin (Berg et al., 1998). PMA treatment mimics the effects of diacylglycerol (DAG) while ionomycin increases intracellular calcium. Thus, PMA and ionomycin treatments mimic the effects of PLC-γ activation while bypassing earlier signaling events (see Figure I-1). This suggested that CTL stimulated in suspension fail to degranulate due to a blockage in signaling rather than a blockage of the degranulation machinery. This phenomenon is reminiscent of the anchorage-dependent signaling that has been described for growth factor receptor signaling. In fibroblasts and other cell types, serum growth factor mediated ERK activation is dependent upon a state of cell anchorage (reviewed in: Howe et al., 2002). This suggested that ERK activation might also be differentially regulated in anchored *vs.* suspended CTL, and that this may impact CTL function. To address the role of MAP kinases in CTL function, ERK1 and ERK2 MAP kinase activation was examined in anchored and suspended CTL.

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B. Results

MAPK activation is required for CTL degranulation

Although a role for MAP kinase in the transactivation of IL-2 gene transcription is well established (Rayter et al., 1992; Weiss and Littman, 1994), the role of MAP kinase in cytotoxic T cell functions has not been studied. To examine whether MAP kinase is required for cytotoxic T cell effector function, TCR-triggered degranulation was measured from CTL clones that had been pretreated with the MEK inhibitor PD98059 (Figure III-1). Degranulation was inhibited with increasing concentrations of PD98059. CTL-mediated killing does not require new protein synthesis (Ostergaard and Clark, 1989). Therefore, the role of MEK/ERK in CTL-mediated killing is distinct from its established role in activating gene transcription.

Immobilized vs. suspension stimulation

In fibroblasts and other cell types, ERK activation is dependent on a state of cell anchorage (Howe et al., 2002). Interestingly, CTL stimulated by plastic-immobilized anti-CD3 or anti-TCR mAb release cytotoxic granules whereas CTL stimulated by anti-CD3 crosslinking in suspension do not (Berg et al., 1998).

To examine how MAP kinases responded to different forms of TCR/CD3 stimulation, CTL were stimulated by plate-bound "immobilized" 145-2C11 (anti-CD3) mAb, a situation abbreviated hereafter as "IMM", or were stimulated in suspension with soluble 145-2C11 and anti-hamster-immunoglobulin Ab as a crosslinking agent - this method is abbreviated hereafter as "XL". These methods are described in detail in chapter II. XL stimulation resulted in transient activation of the ERK1 and ERK2 MAP kinases. Activation was detectable by 2-5 minutes and

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returned to basal levels after 15-20 minutes (Figure III-2). In contrast, IMM stimulation resulted in sustained activation of ERK1 and ERK2. Erk activation was detectable approximately 20 minutes after addition of cells to antibody-coated plastic and was sustained for at least 45 minutes (Figure III-2 and additional data not shown).

In cultured fibroblasts, Erk activation in response to serum growth factors is strong in cells grown on an extracellular matrix protein substrate, but weak or absent in suspended cells (Cybulsky and McTavish, 1997; Renshaw et al., 1997; Howe et al., 2002). To determine whether CTL exhibited similar anchorage-dependent signalling, I examined whether the transient MAPK activation elicited by XL treatment could be made to resemble the extended pattern of IMM-stimulated MAPK activation by providing adhesion separately from the TCR/CD3 signal.

Anchorage to extracellular matrix is mediated by the integrin family of cell surface receptors (Hynes, 1992). CTL express a β3 integrin fibronectin receptor (Berg and Ostergaard, 1995; Ostergaard and Ma, 1995). To examine whether β3 integrin-mediated adhesion would extend MAP kinase activation by non-immobilized anti-CD3 mAb, cells were stimulated by XL treatment and immediately placed in fibronectin-coated wells. This treatment is known to trigger fibronectin-mediated adhesion (O'Rourke and Mescher, 1992) and did so in our cells (Figure III-3) but failed to extend the duration of MAPK signaling (Figure III-4).

Lymphocytes express additional adhesive and costimulatory molecules. LFA-1 interacting with target cell ICAM-1 provides a major adhesive interaction between T cells and target cells (Springer, 1990), but contributes little to signaling in CTL (Berg and Ostergaard, 1995). CD28 is a T cell co-receptor that promotes T cell

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activation when it encounters B7 molecules expressed on specialized antigen-presenting cells (Linsley and Ledbetter, 1993). CD28 appears to signal by triggering tyrosine phosphorylation events and recruiting various SH2 domain-containing signaling proteins (Linsley and Ledbetter, 1993; Rudd, 1996). I examined whether the ligation of either of these known T cell co-receptors would facilitate degranulation when applied in conjunction with TCR/CD3 crosslinking. CTL clones were stimulated by XL treatment in wells that had been coated with anti-CD28 or anti-LFA-1 mAb. The XL method had to be modified because the anti-hamster-Ig antibody used to crosslink 145-2C11 also recognized the anti-CD28 antibody. This would result in indirect immobilization of the 145-2C11 via an anti-hamster-Ig antibody "bridge" between the free 145-2C11 and plate-bound anti-CD28 mAb. To avoid this situation, biotinylated 145-2C11 was prepared and crosslinked using streptavidin. Biotinylated 145-2C11 retained its ability to recognize CD3 as it was able to trigger degranulation when used in plate-bound form. Degranulation in response to soluble, crosslinking treatment was not increased in the presence of immobilized anti-CD28 or anti-LFA-1 (Figure III-5).

C. Discussion

CTL stimulated by the XL method do not degranulate (Berg et al., 1998) and one requirement for degranulation is MAPK activation (Figure III-1). MAPK activation was of significantly shorter duration under XL conditions as compared to degranulation-inducing IMM stimulation (Figure III-2). Immobilized *vs.* suspension anti-CD3 treatment also impacts the duration of PKC activity (Manger et al., 1987). Experimental interruption of signaling by addition of Src family kinase inhibitor PP1

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or cytochalasins at various times after TCR stimulation showed that sustained signaling is a requirement for CTL activation (Shen & Ostergaard, unpublished observations). These results suggest that sustained activation of ERK1/2 is one of the determinants of the outcome of TCR ligation. The duration of MAP kinase signaling has also proven to be significant to biological outcome in a neuronal cell model (Marshall, 1995).

Combining XL stimulation with plating onto various adhesive or co-stimulatory substrates did not trigger full activation. This may indicate that XL stimulation does not generate sufficient signaling upstream of the point at which adhesive signals and TCR signals are integrated. In NIH 3T3 cells, expression of activated FAK is sufficient to permit growth factor mediated activation of ERK in suspended cells, suggesting that FAK mediates the permissive "adhesion signal" (Renshaw et al., 1999). Both IMM and XL stimulation trigger phosphorylation of FAK in CTL (Berg and Ostergaard, 1997) although the kinase activity of FAK under each condition was not examined. Other studies suggest that Raf activation (Lin et al., 1997b) or MEK activation (Renshaw et al., 1997) is the anchorage-dependent step in Ras-MAPK signaling. Therefore, anchorage signals may not be able to assist the TCR-mediated signals if XL stimulation does not induce a strong enough signal at or above the level of Ras.

An alternate view is that antigenic (TCR/CD3) and anchorage signals are intertwined rather than separate. In the case of T cells, activation in response to an APC or target cell normally involves the reorientation of cell surface receptors and intracellular structures towards the target cell (Kupfer et al., 1986; Kupfer et al.,

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1987). Therefore the inability of soluble antibody to trigger CTL activation may reflect the lack of polarization or directionality inherent in this mode of stimulation. Thus cell polarization may not only be a result of T cell activation but part of the activation mechanism itself, as suggested by studies of the "immunological synapse". The immunological synapse is a specific arrangement of signaling and adhesive molecules that forms at the tight interface between CD4+ T cells and antigen presenting cells (APC) (Monks et al., 1998; Grakoui et al., 1999). Signaling molecules including TCR, Lck and PKC θ are found in the center of the synapse in a structure termed the cSMAC (central supramolecular activation cluster), whereas adhesive and structural components such as LFA-1 and talin are concentrated in a ring around the signaling molecules termed the pSMAC (peripheral SMAC). Some have suggested that the purpose of this structure is to concentrate signaling proteins and generate a high "effective molarity" of second messengers (Penninger and Crabtree, 1999). In the context of IMM *vs.* XL, generation of small amounts of second messenger throughout the cell by XL stimulation may be less effective than generating the same total amount of second messenger within a single region of the cell by IMM stimulation. The importance of the SMAC may not be limited to increasing signal strength. A recent study demonstrated that in CTL-target cell conjugates, the immunological synapse consists of a ring of adhesion molecules formed at the CTL-target cell interface which encircles two distinct domains, one concentrated in signaling molecules and another rich in CTL granules (Stinchcombe et al., 2001). This structure formed at the point where CTL centrioles were reoriented

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towards the target cell, suggesting that polarization, signaling, and degranulation are tightly coordinated.

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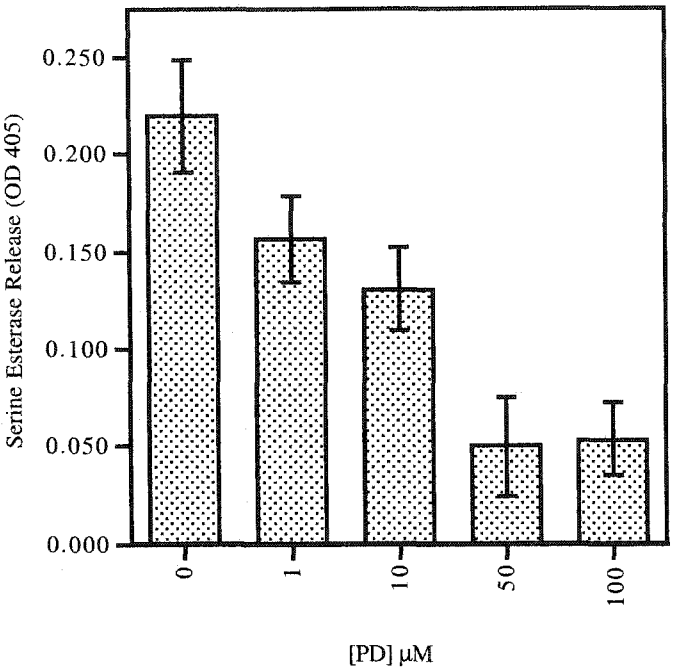

Figure III-1 Effect of PD98059 on CTL degranulation.
CTL clones were stimulated by immobilized 145-2C11 anti-CD3 mAb in the presence of the indicated concentrations of PD98059 [PD] and degranulation was measured by detection of serine esterase activity in the supernatant.

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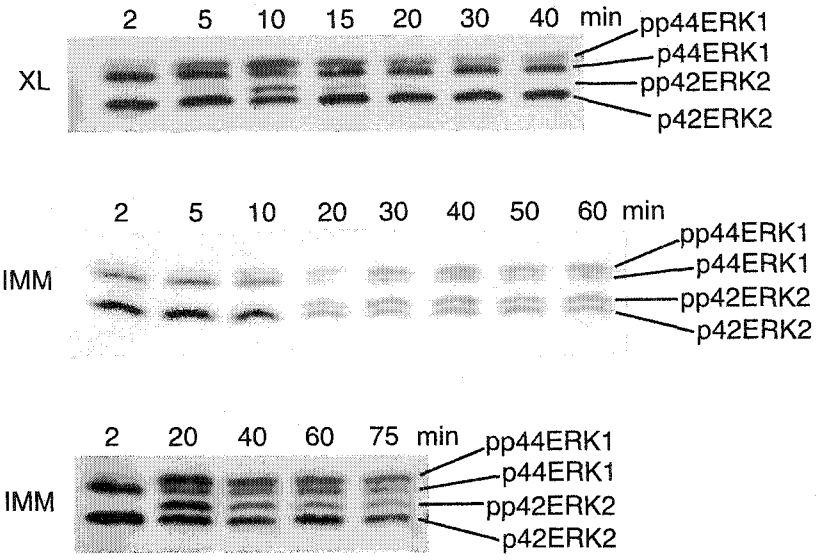

Figure III-2 Immobilized *vs.* soluble, crosslinked anti-CD3 stimulation triggers sustained *vs.* transient MAP kinase activation.
Clone AB.1 CTL were stimulated by soluble, crosslinked (XL) 145-2C11 (anti-CD3) mAb or by plastic-immobilized (IMM) mAb. Samples were analyzed by a gel mobility shift assay. The positions of inactive and phosphorylated activated p44ERK1 and p42ERK2 MAP kinases are indicated.

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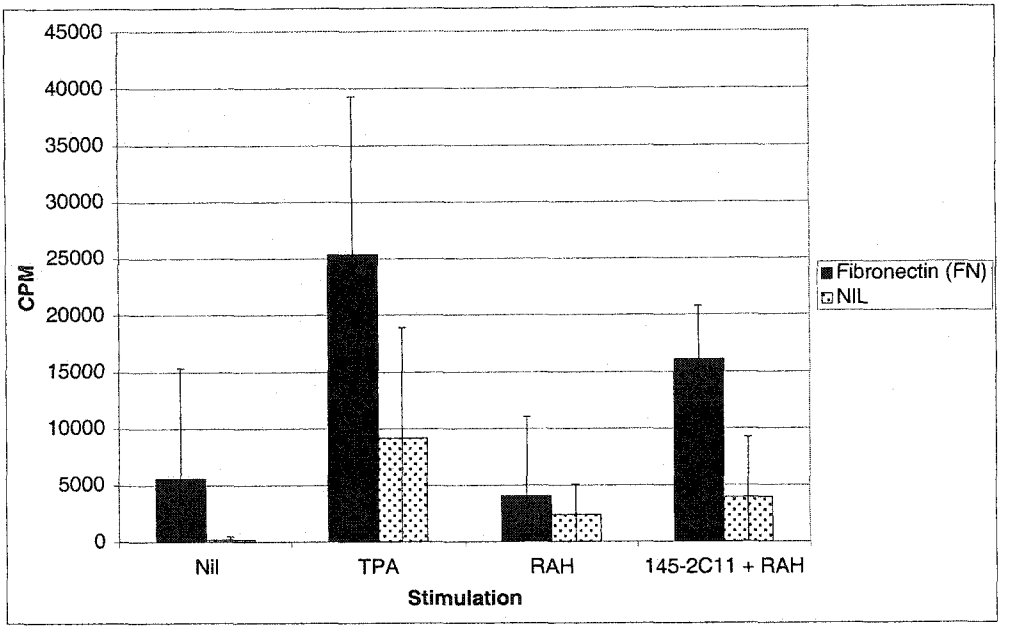

Figure III-3 CD3 crosslinking triggers adhesion to fibronectin. ^{51}Cr-labeled CTL were stimulated by 145-2C11 (anti-CD3) mAb crosslinked by rabbit anti-hamster Ig (RAH) or by RAH alone as a control, then plated on fibronectin and BSA, or BSA alone. Wells were washed and the number of cells remaining were measured by the amount of ^{51}Cr radioactivity remaining. CTL did not adhere significantly to fibronectin until stimulated by CD3 crosslinking or TPA (PMA) treatment. Error bars represent standard deviation between four replicate samples within a single experiment.

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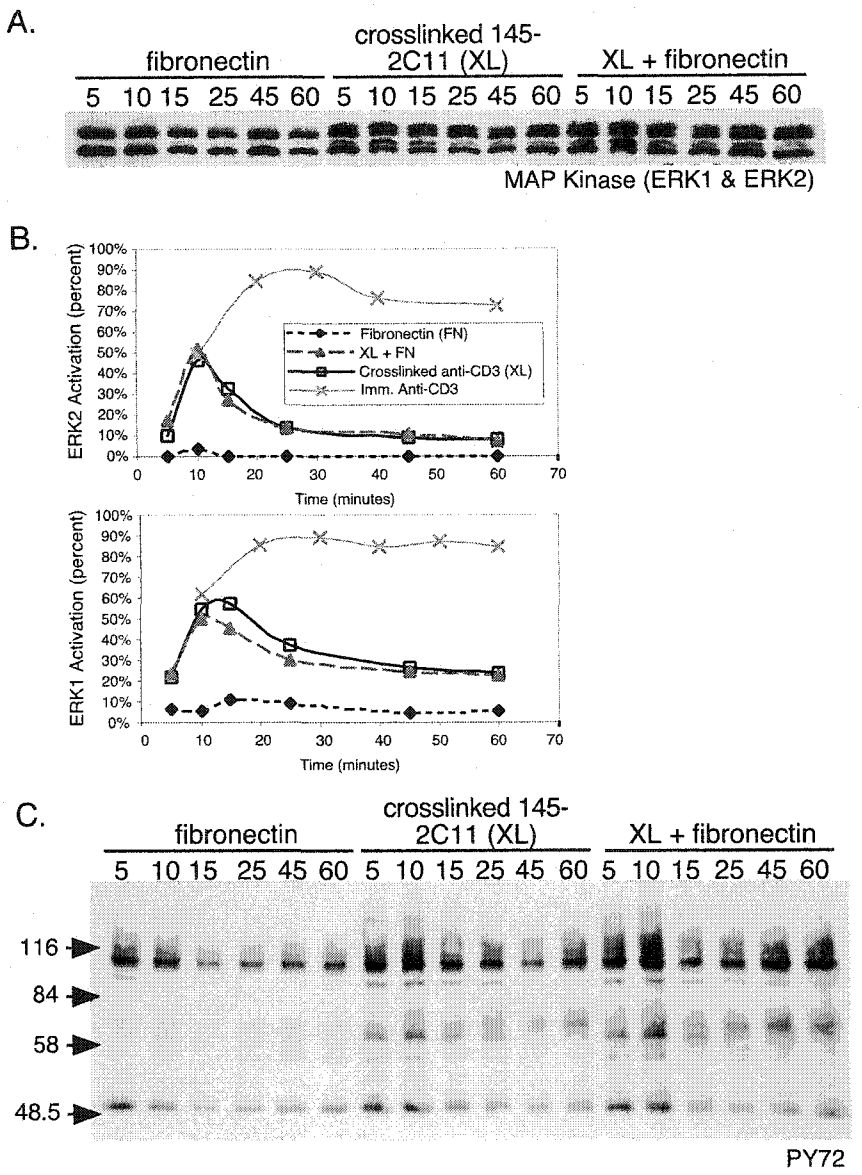

Figure III-4 Fibronectin does not enhance MAPK activation downstream of CD3 crosslinking by soluble anti-CD3 mAb.
Clone AB.1 cells were stimulated by 145-2C11 mAb mediated CD3 crosslinking or left unstimulated, then plated onto fibronectin. (A), MAPK activation was assayed at the indicated times (minutes) by gel mobility shift assay. ERK activation by fibronectin alone was no greater than by BSA alone (see Figure IV-1) (B), Bands shown in *A* were quantitated by scanning optical densitometry using NIH Image software. The percentage of active ERK was calculated from O.D. measurements by the formula (active ERK)/(active ERK+inactive ERK)x100%. Results from *A* were compared against a sample profile of MAPK activation triggered by immobilized (Imm.) anti-CD3. (C), Cell lysates from the same pool of cells were analyzed by SDS-PAGE on a 7.5% gel and immunoblotted with PY72 anti-phosphotyrosine antibody.

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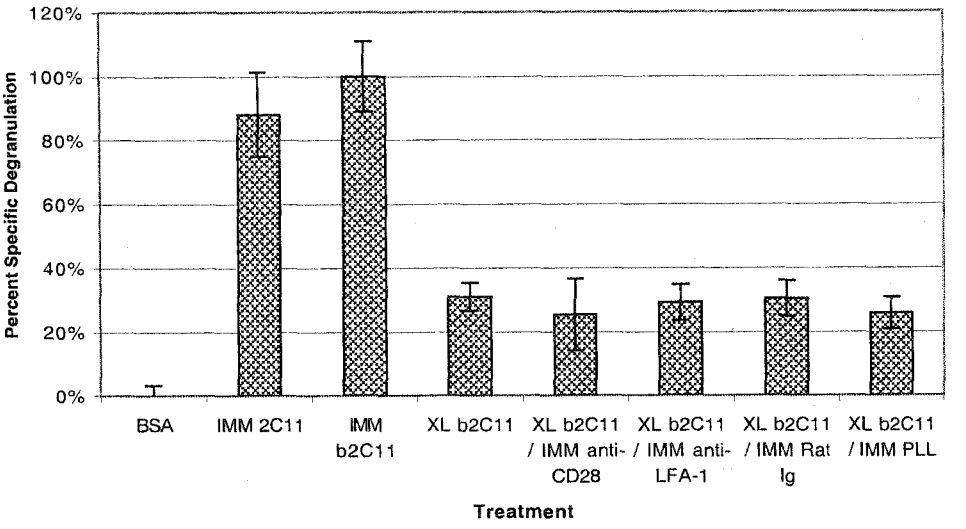

Figure III-5 Neither LFA-1 ligation nor CD28 ligation enhances degranulation when combined with soluble anti-CD3 mAb.
Clone AB.1 cells were stimulated by immobilized (IMM) or soluble crosslinked (XL) 145-2C11 (2C11) or biotinylated 145-2C11 (b2C11) in the presence of the indicated immobilized (IMM) antibodies. Cytotoxic granule release was measured as described in materials and methods and was expressed as percent specific degranulation ((sample release –spontaneous release)/(maximum release – spontaneous release)x100%) where the BSA condition was considered to be spontaneous release and immobilized b2C11 was considered to be maximal degranulation. Poly-L-lysine (PLL) was included as a receptor-independent adhesion-promoting substrate.

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CHAPTER IV Regulation of MAP Kinase by β1 and β3 Integrins

A. Introduction

Integrins are a family of proteins that mediate vital lymphocyte functions including adhesion, motility, and extravasation (Springer, 1990; Hynes, 1992). Integrins are heterodimeric transmembrane proteins composed of an α and β chain. Multiple integrins are expressed by T lymphocytes. β1 integrins are expressed in murine $CD4^+$ (Chan et al., 1991) and $CD8^+$ (Ma et al., 1997) T cell clones and human $CD4^+$ T cells (Shimizu et al., 1990). β1 integrins mediate adhesion to fibronectin in $CD4^+$ (Chan et al., 1991) but not in $CD8^+$ cells (Ma et al., 1997; Ybarrondo et al., 1997). β3 integrins are expressed in murine CTL clones (Ma et al., 1997) and ex-vivo T cells (Moulder et al., 1991) and mediate binding to both fibronectin and vitronectin.

Integrins can generate downstream signals (outside-in signaling) but can also respond to cytosolic signals by altering their avidity for ligand (inside-out signaling) (Springer, 1990; Hynes, 1992). Integrin ligation can enhance or modulate the effects of other receptors, including growth factor receptors (Renshaw et al., 1997) and the TCR (Matsuyama et al., 1989; O'Rourke and Mescher, 1992; Ostergaard and Ma, 1995; Ybarrondo et al., 1997). The nature of integrin-mediated signals is not well characterized. In fibroblasts and lymphocytes, contact with integrin ligands results in tyrosine phosphorylation of the non-receptor protein tyrosine kinase FAK (Hanks et al., 1992; Kornberg et al., 1992; Maguire et al., 1995; Ma et al., 1997). In fibroblasts this leads to activation of MAP kinases (Chen et al., 1994; Schlaepfer et al., 1994; Miyamoto et al., 1995; Morino et al., 1995). FAK can interact with the cytoplasmic

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tail of the β1 integrin subunit and becomes tyrosine phosphorylated upon integrin clustering (Figure I-4). This creates binding sites for the adapter protein Grb2 (Schlaepfer et al., 1994). Grb2 can recruit the Ras guanylnucleotide exchange factor Sos, which promotes release of GDP from Ras in exchange for GTP (see Figure I-2). The GTP-bound form of Ras recruits the kinase Raf to the membrane proximal region where Raf is activated. Activated Raf phosphorylates the MAP kinase kinase MEK, which in turn phosphorylates and activates the MAP kinases ERK1 and ERK2. However, expression of a dominant negative form of FAK does not block β1 integrin-mediated activation of MAP kinase indicating that other pathways to MAP kinase activation exist in fibroblasts (Lin et al., 1997a). The GTPase Rho may be involved in integrin-mediated Ras activation in certain cell types (Renshaw et al., 1996). Fibronectin adhesion in CTL is mediated by β3 rather than β1 integrins and it is not known whether β3 integrins also signal through ERKs.

The avidity of integrins for ligand can be regulated independently of changes in expression level (Springer, 1990). In T lymphocytes, TCR stimulation triggers binding of the β2 integrin LFA-1 to its ligand ICAM-1 (Springer, 1990) and of the β1 integrins (in $CD4^+$ T cells) and β3 integrins (in $CD8^+$ T cells) to fibronectin (Shimizu et al., 1990; Chan et al., 1991; O'Rourke and Mescher, 1992; Ma et al., 1997). This process is referred to as integrin activation. The TCR-to-integrin signals responsible for integrin activation are not well understood, although PI3K and Itk have been shown to play a role in the activation of β1 integrins (Woods et al., 2001) and cells from mice lacking Slap-130/Fyb are defective in TCR-triggered binding to ICAM-1 or fibronectin (Griffiths et al., 2001; Peterson et al., 2001). In addition, there is

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evidence that Ras and MAP kinases play a role in the activation of the β2 integrin LFA-1. Transfection of a thymocyte cell line with a dominant negative form of p21*ras* was seen to inhibit TCR-triggered adhesion of LFA-1 to ICAM-1 while transfection with constitutively active *ras* enhanced adhesion (O'Rourke et al., 1998). Whether β1 and β3 integrin function were also affected was not reported. Therefore, MAP kinases have been implicated in both "inside-out" and "outside-in" integrin signaling.

Integrin ligands such as fibronectin can synergize with sub-optimal TCR stimuli to trigger activation of $CD4^+$ T cells (Matsuyama et al., 1989) and CTL (Ostergaard and Ma, 1995; Ybarrondo et al., 1997). However, the exact mechanism by which fibronectin binding promotes CTL activation is unknown. It was previously shown that exposure of CTL clones to fibronectin, vitronectin, or anti-β1 or anti-β3 antibodies induces phosphorylation of the non-receptor protein tyrosine kinases FAK and PYK2 (Ma et al., 1997). In other cell types, phosphorylation of FAK or PYK2 appears to lead to MAP kinase activation via recruitment of Grb2 (see Figure I-4) (Schlaepfer et al., 1994; Lev et al., 1995; Dikic et al., 1996; Ganju et al., 1997). Since MAP kinase activation is required for CTL degranulation (Figure III-1) (Berg et al., 1998), I hypothesized that integrin stimulation promotes T cell activation by activating MAP kinases.

B. Results

Integrin ligation does not stimulate detectable MAP kinase activation in CTL

Plating fibroblasts onto extracellular matrix (ECM) induces MAP kinase (ERK1 and ERK2) activation via integrin-mediated signaling (Chen et al., 1994;

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Miyamoto et al., 1995; Morino et al., 1995). Surprisingly, plating CTL onto fibronectin did not result in increased activation of MAP kinases ERK1 or ERK2 (Figure IV-1). ERK activation was equivalent to plating on an irrelevant protein (BSA) (Figure IV-1), and no increase in ERK activation could not be detected over a range of fibronectin concentrations (data not shown). Plating onto fibronectin induced phosphorylation of Pyk2 (Figure IV-3) indicating that the fibronectin was biologically active under these conditions.

Neither β1 nor β3 integrins induce MAP kinase activation in CTL

$CD8^+$ T cells adhere to fibronectin via β3 integrins (Moulder et al., 1991; Ma et al., 1997). Tight adhesion is not constitutive but is induced by TCR stimulation. Therefore, the inability of fibronectin alone to induce MAP kinase activation (Figure IV-1) could reflect failure of integrins to efficiently interact with this ligand. To address this, CTL were stimulated by anti-β3 mAb to ensure efficient integrin ligation. Integrin ligation by mAb did not induce ERK1/2 activation (Figure IV-2) even though this treatment induces FAK and Pyk2 phosphorylation (Ma et al., 1997). CTL also express β1 integrins, which have been shown to stimulate MAP kinase activation in non-lymphocytes (Chen et al., 1994; Schlaepfer et al., 1994; Miyamoto et al., 1995; Morino et al., 1995). Surprisingly, anti-β1 mAb also did not activate ERK1 or ERK2 MAP kinase (Figure IV-2). Therefore, ligation of β1 or β3 integrins by mAb or natural ligand is not sufficient to activate the ERK1/2 MAP kinases in CTL.

Pyk2 does not associate with Grb2 in CTL clones

β3 ligation by fibronectin or mAb induces tyrosine phosphorylation of Pyk2 in

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CTL (Ma et al., 1997). Pyk2 phosphorylation has been linked to MAPK activation by a mechanism of Grb2 recruitment to Pyk2 in PC12 neuronal (Lev et al., 1995; Dikic et al., 1996) and Jurkat T leukemia cells (Ganju et al., 1997). Since β1 and β3 integrin ligation failed to activate MAP kinase in T cell clones (Figure IV-1& Figure IV-2), I examined whether or not Grb2 interacted with Pyk2 in these cells. Pyk2 was immunoprecipitated from AB.1 cells that had been plated on immobilized fibronectin alone or in combination with a substimulatory concentration of anti-CD3 mAb, and probed with anti-phosphotyrosine and anti-Grb2 mAbs (Figure IV-3A). Tyrosine phosphorylation of Pyk2 was readily detected in cells plated on fibronectin (Figure IV-3A, upper panel). However, no Grb2 was co-precipitated with Pyk2 (Figure IV-3A, middle panel), even though Grb2 was readily detectable in pre-IP lysates (Figure IV-3A, lower panel).

Co-immunoprecipitation of Pyk2 and Grb2 after CD3 crosslinking has been reported in Jurkat T leukemia cells (Ganju et al., 1997). AB.1 T cells were stimulated on plates coated with 10 μg/ml 145-2C11 anti-CD3 mAb and Pyk2 was immunoprecipitated (Figure IV-3B). As expected, Pyk2 was strongly tyrosine phosphorylated under these conditions (Figure IV-3B), (Berg and Ostergaard, 1997). Grb2 was present in cell lysate but was not detectable in Pyk2 immunoprecipitates at any of the time points examined (Figure IV-3B, lower panel).

Integrin ligands synergize with low levels of TCR stimulation to activate MAP kinase

As previously reported (Matsuyama et al., 1989; Ostergaard and Ma, 1995; Ybarrondo et al., 1997), co-immobilization of integrin and TCR/CD3 ligands greatly

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reduces the amount of TCR/CD3 ligand required for T cell activation (Figure IV-4A). This further confirmed that fibronectin was biologically functional in our experimental system. For murine clone AB.1 CTL, an anti-CD3 mAb concentration as low as 0.1 μg/ml was sufficient, in the presence of fibronectin, to induce degranulation. Significantly, a similar pattern was observed for MAP kinase activation. Although neither fibronectin alone nor 0.1 μg/ml 145-2C11 alone was sufficient to activate ERK1 or ERK2, the combination of substimulatory 145-2C11 and fibronectin induced significant ERK activation (Figure IV-4B). Identical results were obtained using vitronectin (data not shown). This phenomenon was not unique to CTL clones. When T cell blasts were prepared from C57BL/6 mouse spleen cells by ConA treatment, these T cells also failed to activate ERK1/ERK2 in response to plating on fibronectin (Figure IV-5). Neither low concentration anti-CD3 mAb alone nor fibronectin alone triggered ERK activation whereas co-immobilization of the two stimuli induced robust ERK1 and ERK2 activation (Figure IV-5).

Fibronectin-mediated cell spreading is PD98059-sensitive, but triggered adhesion is not

Rather than acting downstream of integrins, there is also evidence that MAP kinase plays a role in inducing the high-avidity state of β2 integrins in response to mitogenic stimuli (O'Rourke et al., 1998). To determine whether MAP kinase is important for the affinity activation of the β3 integrins that mediate CTL adhesion to fibronectin, adhesion assays were performed in the presence or absence of the MEK inhibitor PD98059. As previously reported (O'Rourke and Mescher, 1992), CTL do not strongly adhere to fibronectin unless a TCR/CD3 stimulus is also present (Figure

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III-3 and Figure IV-6). Triggered adhesion was only slightly diminished by PD98059, implying that activation of MAP kinase is not absolutely required for triggered adhesion to fibronectin (Figure IV-6).

Consistent with the adhesion measurements, cell spreading was observed in the presence of high concentrations of immobilized anti-CD3 mAb (Figure IV-7F) or of substimulatory anti-CD3 co-immobilized with fibronectin (Figure IV-7B), but not in the presence of substimulatory anti-CD3 (Figure IV-7A) or fibronectin (Figure IV-7E) alone. CTL on fibronectin alone exhibited an extended morphology typical of migrating cells. Cell spreading on high concentrations of immobilized anti-CD3 mAb was not strongly affected by treatment with MEK inhibitor PD98059 (Figure IV-7G-H). Surprisingly, triggered cell spreading on fibronectin in the presence of substimulatory amounts of anti-CD3 mAb was strongly inhibited by PD98059 (Figure IV-7C-D) even though static adhesion was only slightly affected (Figure IV-6). The same results were obtained when CTL clones were stimulated in suspension by soluble cross-linked anti-CD3 prior to plating on fibronectin (Figure IV-8). These data suggest that MAP kinase activation is not required for the activation of tight binding to fibronectin, but does play a role in fibronectin-mediated changes in cell morphology.

C. Discussion

Integrin ligands synergize with TCR stimulation through an unknown mechanism to promote CTL degranulation (Ostergaard and Ma, 1995; Ybarrondo et al., 1997). In fibroblasts, β1 integrins can signal through the Ras-MAP kinase pathway downstream of FAK phosphorylation (Schlaepfer et al., 1994). MAP kinase

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activation is a requirement for CTL degranulation (Berg et al., 1998), and it was previously shown that integrin stimulation of CTL induces phosphorylation of not only FAK but also the related kinase PYK2 (Ma et al., 1997). However, the present study shows that integrin ligation alone is not sufficient to induce detectable activation of the MAP kinases ERK1 or ERK2 in murine CTL clones.

Integrin affinity for ligand is not constitutively high but is regulated (Springer, 1990; Hynes, 1992). Therefore, integrin-mediated MAP kinase signaling might not occur until integrin avidity was first activated by signals from the TCR. Two observations argue against this explanation. Firstly, direct integrin ligation by antibodies did not result in MAP kinase signals (Figure IV-2). Secondly, the presence of fibronectin, or integrin ligation by mAbs, resulted in tyrosine phosphorylation of FAK and Pyk2 indicating that integrins were functionally engaged (Figure IV-3 and Figure III-4) (Ostergaard and Ma, 1995; Ma et al., 1997).

In Jurkat T leukemia and PC12 neuronal cells, tyrosine phosphorylation of Pyk2 results in recruitment of Grb2 and consequent activation of MAP kinases downstream of Ras (Lev et al., 1995; Dikic et al., 1996; Ganju et al., 1997). The present study shows that in cloned CTL, β1 and β3 integrins trigger Pyk2 tyrosine phosphorylation but are uncoupled from MAP kinase activation, likely at the point of Grb2 recruitment. Furthermore, I found no evidence of Pyk2-Grb2 association after TCR stimulation, an association which had been proposed to be a mechanism of TCR-mediated ERK activation based on a study of Jurkat T leukemia cells (Ganju et al., 1997). In CTL, Pyk2 might preferentially bind partners other than Grb2. This may be related to the expression of an alternatively spliced form of Pyk2 in most

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hematopoietic cells, which lacks a 42-amino acid portion of the C-terminal domain, whereas Jurkat T cells and non-hematopoetic cells express full-length Pyk2 (Dikic and Schlessinger, 1998; Li et al., 1998; Xiong et al., 1998).

The Ras-MAP kinase pathway has been reported to be involved in triggering avidity of the β2 integrin LFA-1 for its ligand ICAM-1 (O'Rourke et al., 1998). To determine whether this pathway is important for β3-mediated triggered binding to fibronectin, adhesion assays were performed in the presence or absence of the MEK inhibitor PD98059. This inhibitor only slightly inhibited triggered binding to co-immobilized fibronectin and anti-CD3 mAb (Figure IV-6). This slight decrease might be attributed to reduced cell spreading in the presence of PD98059 (Figure IV-7). Interestingly, a combination of both PD98059 and the PI3K inhibitor wortmannin is required to completely inhibit the triggered binding of LFA-1 to ICAM-1 in mature $CD4^+$ T cells (O'Rourke et al., 1998). One effect of PI3K products is activation of Vav, and thymocytes from $Vav^{-/-}$ mice exhibit decreased adhesion to extracellular matrix (Krawczyk et al., 2002).

Although PD98059 did not prevent triggered adhesion under static conditions (Figure IV-6), it inhibited triggered cell spreading on fibronectin (Figure IV-7). A difference in ERK involvement between spreading and adhesion has also been reported for REF52 cells adhering to fibronectin in the presence of serum (Fincham et al., 2000) where MEK inhibition prevented cell spreading while not altering net adhesion. Similarly, PMA-mediated downregulation of PKC in fibroblasts inhibited cell spreading but not adhesion (Miranti et al., 1999). In that system Raf, MEK, and ERK activity were decreased but FAK phosphorylation was unaltered, suggesting that

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FAK can also be uncoupled from MAPK in non-T cells under some circumstances. Interestingly, ERKs have been observed to colocalize with integrins at points of ECM contact in fibroblasts (Miyamoto et al., 1995; Fincham et al., 2000). Together, these data support a role for MAP kinases not in the regulation of integrin affinity but in regulating the location of points of cell-to-ECM contact.

MAP kinase activity does not appear to be necessary for the cell spreading which occurs on high concentrations of anti-CD3 mAb alone (Figure IV-7F-H), a result also reported for Jurkat T cells (Bunnell et al., 2001). In the case of strong TCR stimulus, the exchange factor Vav is thought to link TCR stimulation to the Rho family kinases Rac, CDC42 and RhoA which in turn mediate cytoskeletal changes (Penninger and Crabtree, 1999; Borroto et al., 2000). Lack of ERK involvement in spreading on high concentration TCR/CD3 ligand is consistent with this model.

Co-immobilization of anti-CD3 mAb with fibronectin or anti-integrin antibodies facilitated CTL activation by amounts of anti-CD3 mAb that would otherwise be sub-stimulatory. At least three possible mechanisms may account for the synergy between fibronectin and TCR/CD3 ligation: increased TCR/CD3 occupancy, integrin-mediated signals that co-operate with CD3 signaling, or non-integrin signals that result from cell anchorage that co-operate with CD3 signaling. Increased TCR/CD3 occupancy probably makes a minor contribution. The presence of fibronectin facilitated degranulation at ten-fold lower concentrations of anti-CD3 (Figure IV-4), whereas fibronectin-mediated cell spreading only increases the surface area contacted by about 2.25-fold (Figure IV-7). However, fibronectin-mediated adhesion probably also increases the duration of contact with CD3 ligand, which may

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be significant. The presence of fibronectin can enhance degranulation from CTL stimulated by anti-CD3 coated beads even though the integrin and CD3 ligands are on different surfaces in this situation (Ostergaard and Ma, 1995). This observation is most consistent with co-operation via signaling rather than co-operation via increased TCR/CD3 occupancy. CD3 and integrin ligation both induce phosphorylation of FAK and Pyk2 (Maguire et al., 1995; Berg and Ostergaard, 1997; Ma et al., 1997), suggesting that synergism can occur at the level of these kinases, although the role of FAK and Pyk2 phosphorylation in T cells remains unclear. Lastly, it is possible that CD3 signaling is enhanced as an indirect consequence of integrin-mediated cell anchorage. In several systems, it has been demonstrated that cell shape rather than integrin ligation *per se* is the requisite factor for responsiveness to other stimuli (Folkman and Moscona, 1978; Miranti and Brugge, 2002).

In summary, although stimulation of β1 and β3 integrins leads to phosphorylation of FAK and Pyk2, this does not result in MAP kinase activation in CTL as it does in other types of cells. MAP kinase activation was not essential for increasing β3-mediated adhesion to fibronectin but may be important for cell spreading on a fibronectin substrate. Therefore, in addition to facilitating adhesion to target cells, integrin ligation promotes CTL activation; it allows cell spreading and increased target contact, and directly or indirectly initiates intracellular signals that co-operate synergistically with TCR/CD3 mediated signals upstream of MAP kinase.

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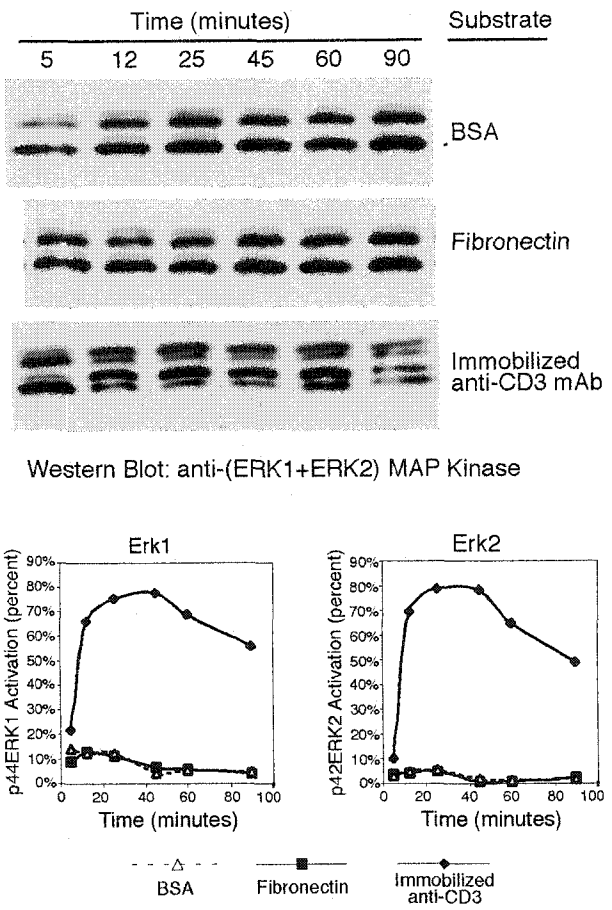

Figure IV-1 Fibronectin contact does not mediate MAPK activation in CTL. Clone AB.1 CTLs were plated on BSA, fibronectin or 10 μg/ml immobilized anti-CD3 mAb. MAPK activation was determined by the gel mobility shift method. Results were quantitated by scanning optical densitometry using NIH Image software and plotted as percent of ERK1/ERK2 activated out of total ERK1/ERK2 recovered.

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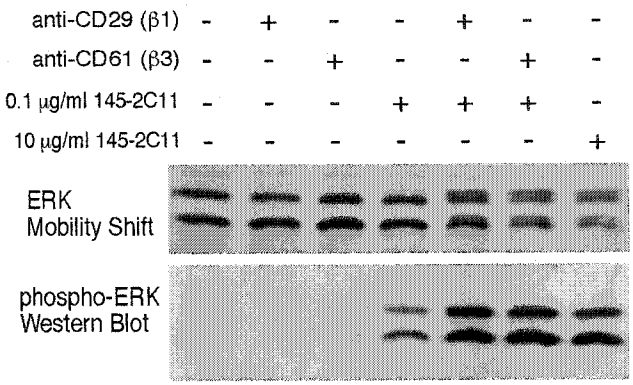

Figure IV-2 Ligation of β1 or β3 integrin does not activate MAP kinases. Clone AB.1 CTL were plated on the indicated substrates alone or in combination and MAPK activation was measured by both gel mobility shift assay (top panel) and by Western blotting for Thr202/Tyr204 phosphorylated ERK1 and ERK2 (bottom panel).

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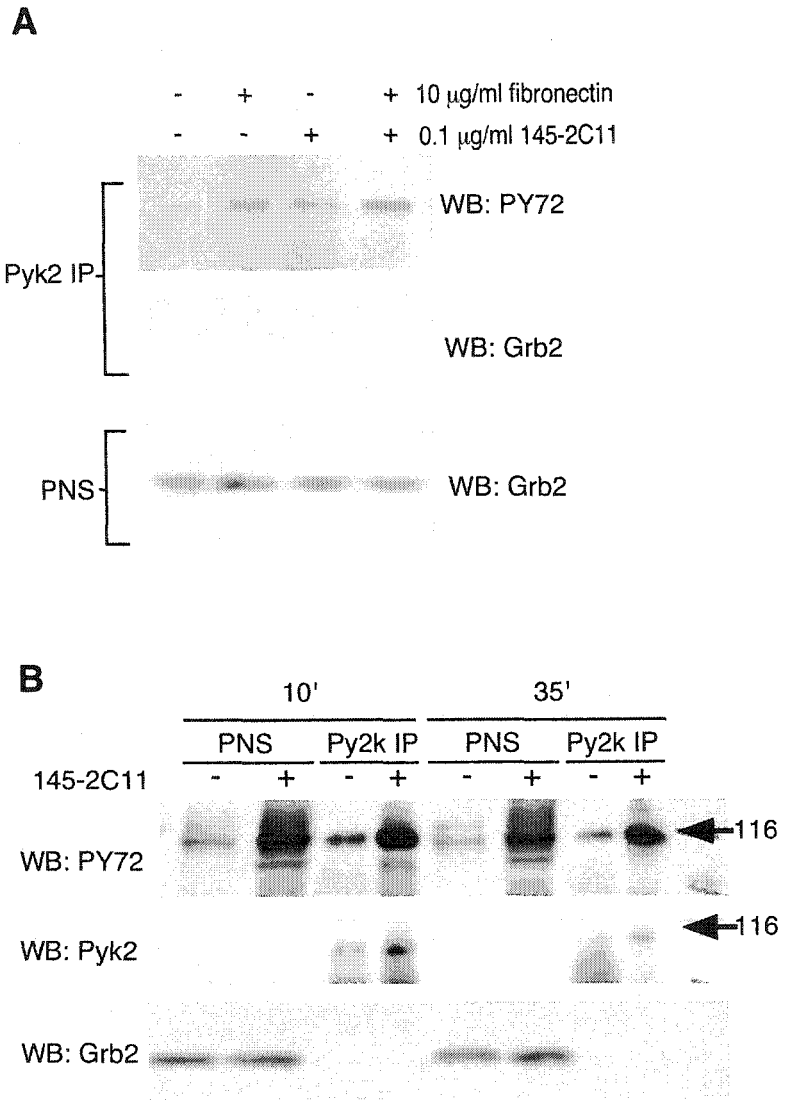

Figure IV-3 Fibronectin contact or CD3 ligation causes Pyk2 phosphorylation but without co-precipitation of Grb2.
(A) Clone 11 CTL were stimulated by 145-2C11 and fibronectin, either alone or in combination. Pyk2 was immunoprecipitated (IP) and probed by Western blotting (WB) using PY72 mAb (anti-phosphotyrosine) and anti-Grb2 mAb. Post-nuclear supernatant (PNS) was also probed to confirm efficient detection of Grb2. (B) CTL were stimulated by immobilized 145-2C11 (+) or left unstimulated (-). Post-nuclear supernatants (PNS) and Pyk2 immunoprecipitates (IP) were prepared and probed by Western blot (WB) using PY72 (anti-phosphotyrosine), anti-Pyk2 and anti-Grb2 mAbs.

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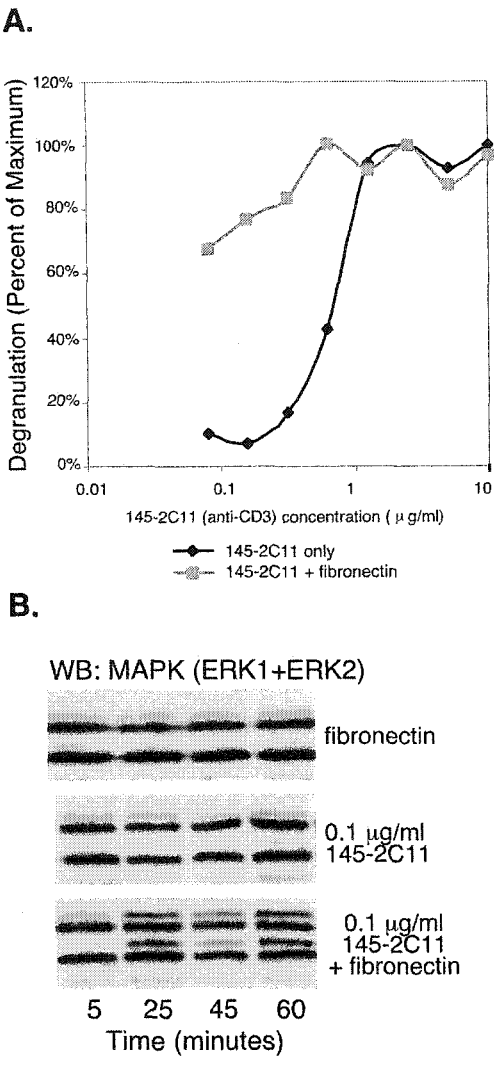

Figure IV-4 Fibronectin facilitates MAPK activation and CTL degranulation in co-operation with immobilized anti-CD3 mAb.
(A), Plates were prepared with the indicated concentrations of 145-2C11 (anti-CD3) mAb in the presence or absence of fibronectin and clone AB.1 degranulation was measured. (B), Clone AB.1 cells were added to fibronectin-coated microtitre wells in the absence or presence of a substimulatory concentration (0.1μg/ml) of 145-2C11. ERK1 and ERK2 MAP kinases were detected from whole cell lysates by Western blotting under conditions in which active MAP kinase can be distinguished by its reduced mobility.

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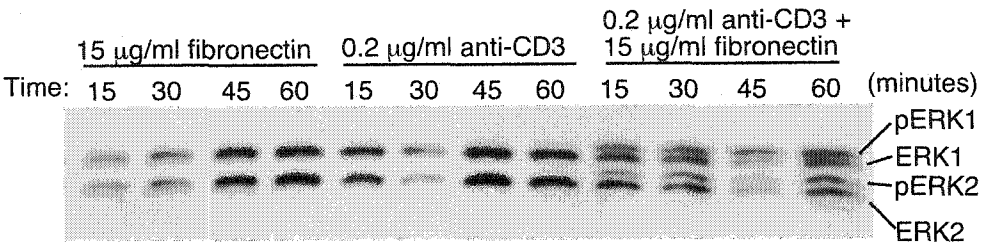

Figure IV-5 Fibronectin and CD3 stimulation synergize to trigger MAPK activation in T cell blasts.
T cell blasts were prepared from C57BL/6 mouse splenocytes by stimulation with concanavalin A. Cells were plated on the indicated concentrations of fibronectin or 145-2C11 anti-CD3 mAb, alone or in combination. ERK1 and ERK2 activation were measured by gel mobility shift assay.

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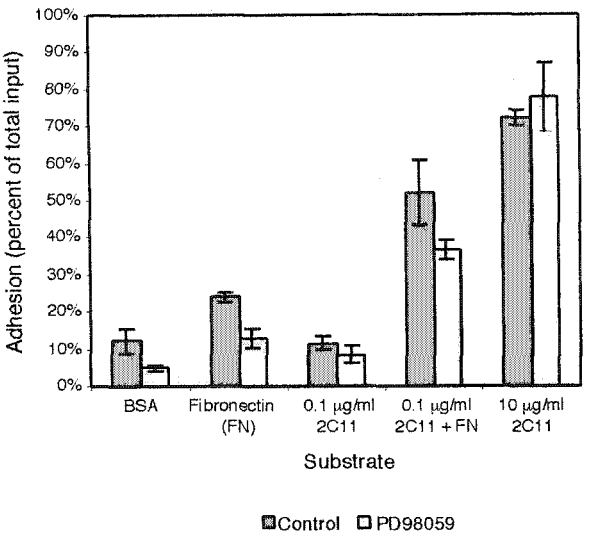

Figure IV-6 MAP kinase activation is not essential for triggered adhesion to fibronectin.
Wells of a Nunc Maxisorp™ plate were coated with 20 µg/ml of fibronectin or the indicated concentrations of 145-2C11 alone or in combination. Adhesion of AB.1 T cells was measured in the presence or absence of 5 µg/ml (18 µM) of the kinase inhibitor PD98059 which prevents MEK activation. Error bars represent standard deviation between three replicate samples within a single experiment.

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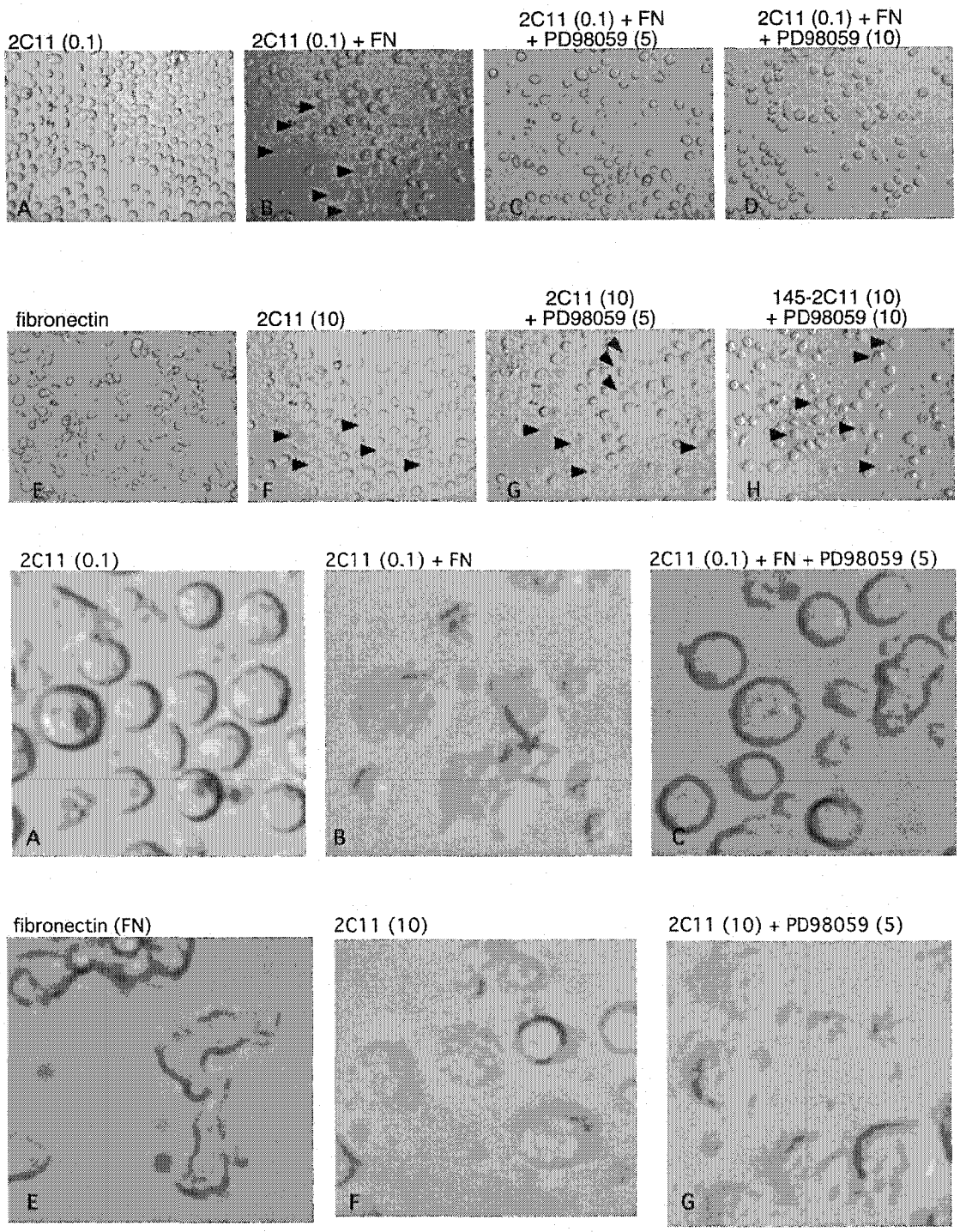

Figure IV-7 PD98059 inhibits fibronectin-mediated cell spreading but not anti-CD3 mAb mediated cell spreading.

Clone AB.1 T cells were plated in microtitre wells coated with 0.1 μg/ml 145-2C11 (A-D), 20 μg/ml fibronectin (B-E), or 10 μg/ml 145-2C11 (F-H). Cells were treated with DMSO carrier control (A,B,E,F) or with 5 μg/ml (18 μM) PD98059 (C and G) or 10 μg/ml (37 μM) PD98059 (D and H). Cells were observed after 1 hour of incubation. Spread cells exhibited a "fried egg" appearance with the large nucleus surrounded by a radially extended cytoplasm. Examples of cells exhibiting a highly spread and flattened morphology are indicated by filled arrowheads.

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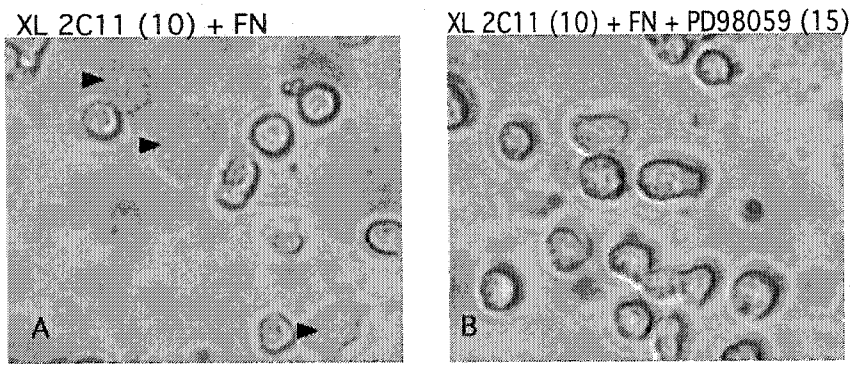

Figure IV-8 CD3 crosslinking triggers cell spreading on fibronectin that is inhibited by PD98059.
Clone AB.1 cells were stimulated by anti-CD3 cross-linking prior to plating on fibronectin. Cells were pre-treated with DMSO alone (A) or 15 μg/ml (56 μM) PD98059 in DMSO (B). Highly spread cells are indicated by filled arrowheads.

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CHAPTER V PKC-Dependendent and Independent Activation of MAP Kinases in Cytotoxic T Lymphocytes

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A. Introduction

In T lymphocytes, ligation of the T cell receptor (TCR) results in activation of Ras, Raf and ERK (Su and Karin, 1996). This pathway is required for T cell function, including IL-2 production (Rayter et al., 1992; Su and Karin, 1996) and CTL degranulation (Berg et al., 1998). The mechanism linking TCR stimulation to activation of the Ras-Raf-MEK-ERK pathway in T cells is unclear although several mechanisms have been proposed.

Both TCR stimulation and addition of PMA induce accumulation of GTP-bound Ras in T cells (Downward et al., 1990). Since both treatments activate protein kinase C (PKC), it was thought that TCR stimulation might be linked to the Ras-MAP kinase pathway by PKC (Downward et al., 1990). The rate of ^{32}P-GTP incorporation was identical in PMA-treated and untreated permeabilized lymphocytes, whereas the rate of ^{32}P-GTP to GDP conversion appeared to be slower in the presence of lysate from PMA-treated lymphoblasts. This suggested that PKC activated Ras by inhibiting RasGAPs rather than by activating RasGEFs (Downward et al., 1990;

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Downward et al., 1992). However, studies in which PKC was inhibited by a pseudosubstrate peptide indicated that PKC-independent pathways for TCR-mediated Ras activation also exist (Downward et al., 1992; Izquierdo et al., 1992).

Other studies indicated that PKC can act downstream of Ras at the level of Raf. TCR-mediated activation of Raf-1 has been reported to be PKC-dependent (Siegel et al., 1990), and at least one isoform of PKC is capable of activating Raf-1 by direct phosphorylation (Kolch et al., 1993), although mutation of the PKC phosphorylation site does not block activation of Raf by PMA (Whitehurst et al., 1995).

A second model for activation of the Ras-MAP kinase pathway in T cells is that, analogous to the mechanism of Ras activation by growth factor receptor protein tyrosine kinases, Ras activation occurs when the Ras-GEF Sos is recruited to the cell membrane by protein-protein interactions (Koretzky, 1997). In T cells, this is thought to be mediated by the adapter protein LAT (linker for the activation of T cells). LAT is a transmembrane protein containing multiple Grb2-binding sites that become phosphorylated in response to TCR stimulation and could therefore recruit Grb2-Sos complexes to the membrane (Cantrell, 1998; Zhang et al., 1998). However, it is not known whether Sos is the physiologically relevant RasGEF in T cells (Koretzky, 1997).

Ras guanylnucleotide releasing protein (RasGRP), also known as CalDag-GEFII, is a novel RasGEF expressed in neuronal cells and lymphoid cells (Ebinu et al., 1998; Kawasaki et al., 1998; Tognon et al., 1998). RasGRP contains a C1 DAG binding domain and can mediate Ras activation in response to PMA (Ebinu et al.,

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1998; Tognon et al., 1998). In Jurkat T cells, TCR stimulation induces RasGRP association with the membrane and RasGRP overexpression enhances Ras-ERK activation in response to TCR stimulation (Ebinu et al., 2000). Furthermore, RasGRP has been shown to be essential for T cell development (Dower et al., 2000). Therefore, DAG production downstream of TCR stimulation likely mediates effects through RasGRP as well as through PKC. Additional mechanisms of MAP kinase regulation in T cells have been suggested, including direct recruitment of Shc-Grb2-Sos complexes to phosphorylated CD3ζ (Koretzky, 1997; Chau and Madrenas, 1999), interactions between Lck and MAP kinases (August and Dupont, 1996), modulation by nitric oxide (Lander et al., 1996), and regulation of phosphatases (Blanco-Aparicio et al., 1999).

In the present study, we evaluate the role of PKC in the regulation of the MAP kinases ERK1 and ERK2 in antigen and IL-2 dependent cytotoxic T lymphocyte (CTL) clones. We demonstrate that dependence on PKC for ERK1/2 activation varies with stimulation conditions and varies over the time course of stimulation. Our results indicate that there are PKC-independent but diacylglycerol-dependent mechanisms, in addition to a PKC-dependent pathway, regulating MAP kinases in T cells.

B. Results

Effect of bisindolylmaleimide on MAP kinase activation

T cell receptor (TCR) stimulation results in the activation of multiple signaling proteins including phospholipase-C-γ1 (PLC-γ1) (Qian and Weiss, 1997). PLC-γ1 converts phosphatidylinositol 4,5 bisphosphate (PIP2) into inositol 1,4,5-

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trisphosphate (IP3) and diacylglycerol (DAG). IP3 activates calcium-dependent pathways while DAG activates PKC. PKC activation has been reported to promote MAP kinase activation in a number of cell types including T cells. As expected, treatment of CTL clone AB.1 with the DAG analogue PMA resulted in a rapid increase in the phosphorylation of ERK1 and ERK2 and this response was completely inhibited by the PKC inhibitor bisindolylmaleimide (BIM) (Figure V-1A).

To examine whether ERK activation in response to TCR/CD3 stimulation was also PKC-dependent, we stimulated CTL clones in suspension with anti-CD3 mAb and cross-linking antibody, or in anti-CD3 coated assay plates and in the presence or absence of BIM. CTL clones stimulated by soluble, cross-linked anti-CD3 mAb exhibited a transient activation of ERK1 and ERK2 that was maximal at 5 minutes and was inhibited by BIM, indicating that ERK activation under these conditions is PKC-dependent (Figure V-1).

In dramatic contrast to the results obtained with crosslinked anti-CD3 antibody, ERK phosphorylation resulting from immobilized anti-CD3 stimulation comprised an initial phase from 15 to 25 minutes after plating that was resistant to BIM, followed by a phase that was BIM-sensitive (Figure V-2, B-D). The drug was effective in these cells as BIM treatment inhibited the degranulation response in a dose-dependent manner (Figure V-3). The delay in inhibition of ERK activation was not due to slow uptake of BIM since this drug completely inhibited the much higher level of ERK phosphorylation induced by PMA after only 10 minutes (Figure V-2A). The decline in ERK activation was not due to cell death since BIM treated cells exhibited no significant decrease in viability as measured by trypan blue exclusion

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after 120 minutes of exposure, and remained viable when replated in growth media and cultured overnight. Similar results were observed using CTL clone 11 and with Con A blasts generated from C57BL/6 spleen cells (Figure V-4).

Effect of bisindolylmaleimide on Ras activation

To determine if BIM exerted its effect upstream or downstream of Ras, we determined the degree of Ras activation after stimulation with immobilized anti-CD3 in the presence or absence of BIM. As shown in Figure V-5, Ras activation, as measured by the ability to bind a Raf-1 fusion protein, is observed in the presence of BIM, particularly at the 15 minute time point. By 30 minutes there is a significant decrease in the level of Ras activity in the 145-2C11-stimulated cells in the presence of BIM compared to control cells. The BIM was effective in this experiment as PMA stimulation of Erk in the same pool of cells used for the Ras assay was inhibited by BIM (Figure V-5B). These results suggest that there is BIM-sensitive and BIM-resistant activation of Ras that mirrors the results observed in Figure V-2 with ERK1/2 activation.

PLC activity and DAG production are required for ERK activation

BIM inhibits PKC activity by interfering with its ATP-binding domain. We next examined the effect of the inhibitor calphostin C, which interacts with the C1 DAG binding domain found in DAG-responsive PKC isoforms and in RasGRP (Lorenzo et al., 2000). Phosphorylation of ERK1 and ERK2 in response to immobilized anti-CD3 stimulation was drastically reduced in the presence of calphostin C (Figure V-6). In striking contrast with the effects of bisindolylmaleimide, inhibition was evident at all time points. This result suggests

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that all phases of ERK activation following immobilized anti-CD3 stimulation require one or more DAG binding proteins even though the early phase is bisindolylmaleimide-resistant.

To confirm that the inhibition of ERK phosphorylation observed with calphostin C was due to a requirement for DAG, we utilized the PLC-γ1 inhibitor U73122. U73122 but not its inert analogue U73343 completely inhibited ERK1 and ERK2 phosphorylation at all time points examined (Figure V-7). Taken together, the effects of these inhibitors suggested the existence of an activity upstream of MAP kinases ERK1 and ERK2 that was PLC-γ1 dependent and DAG-dependent, but independent of BIM-inhibitable PKC activity.

PMA-mediated downregulation of PKC

As an alternate method to examine the role of PKC in ERK activation, PKC expression was downregulated by overnight exposure of cells to PMA. This treatment is known to downregulate the DAG-responsive PKC isoforms. Consistent with the effects of BIM (Figure V-1A), the ERK phosphorylation normally induced by acute PMA treatment did not occur when cells were pretreated overnight with 100 ng/ml of PMA (Figure V-8A). Also consistent with the BIM inhibition data, early ERK activation in response to immobilized anti-CD3 stimulation was not significantly inhibited by this treatment (Figure V-8). When PMA-pretreated cells were stimulated with immobilized anti-CD3 mAb, degranulation was partially inhibited at 10 and 50 ng/ml and substantially inhibited at 100 ng/ml of PMA in the overnight treatment (Figure V-8B). Antibodies to CD45, which do not trigger degranulation were used as a control to show that the overnight PMA treatment did

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not cause the cells to non-specifically degranulate. The incomplete inhibition in cells treated with the higher concentrations of PMA is likely due to the re-expression of PKC during the 4-5 hours of incubation required for the degranulation assay. These results also confirm that PKC activity is required for optimal degranulation as was observed with the PKC inhibitor studies (Figure V-3).

To confirm that overnight treatment of PMA did downregulate PKC activity, the activity in total extracts from control and PMA treated cells was measured. The ratio of activity measured in the extracts assayed in the presence of PKC activators to the activity measured in the absence of activators was determined. A ratio of one would be predicted in cells that had no DAG-stimulated PKC activity. The results in Figure V-9A demonstrate that total PKC activity was diminished in the presence of PMA pretreatment, particularly at the 50 and 100 ng/ml concentrations. To further confirm that DAG-responsive PKC isozymes were downregulated after overnight PMA exhaustion, lysates from the PMA pretreated and control cells were probed with antibodies to various PKC isozymes. As shown in Figure V-9, PKC α, PKC βI, PKC δ, PKC ε and PKC θ were all significantly downregulated in response to PMA. We could not detect PKC βII or PKC γ in these cells (data not shown). Very little of each isozyme remained in the cells after overnight treatment with 50 ng/ml or 100 ng/ml PMA. Taken together with the lack of PMA-stimulated ERK1/2 activity, the decreased degranulation and the decreased enzymatic activity, our results strongly suggest that overnight PMA treatment substantially depletes the cells of DAG-responsive PKC isozymes. In spite of the significant decrease in PKC activity, there is still substantial anti-CD3-stimulated ERK1/2 activation (Figure V-8).

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The surprising activation of ERK in response to anti-CD3 in these CTL clones treated overnight with PMA suggested that a factor activated by anti-CD3, but not downregulated in response to chronic PMA stimulation, was acting upstream of ERK phosphorylation. The novel RasGEF, RasGRP is expressed in CTL clones (unpublished observations), and is regulated by DAG (Ebinu et al., 1998; Tognon et al., 1998; Lorenzo et al., 2000). We therefore examined the expression of RasGRP in cells that had been chronically treated with PMA. The PKC α blot shown in Figure V-9B was stripped and re-probed with an antibody specific for RasGRP. RasGRP expression was not decreased by PMA pretreatment (Figure V-9C). The anti-RasGRP mAb m199 detects a doublet, the molecular basis for which is presently unknown. These results indicate that unlike PKC, RasGRP is not downregulated in response to chronic PMA treatment. It is interesting that downregulation of PKC resulted in the loss of PMA-triggered ERK activity but not of TCR/CD3-triggered ERK. The reason for this difference is unclear but may suggest that under TCR/CD3 stimulation conditions RasGRP is subject to additional regulation beyond that triggered solely by addition of DAG or PMA.

C. Discussion

Several mechanisms have been proposed for the regulation of MAP kinases in T lymphocytes. Activation of the MAP kinases ERK1 and ERK2 in response to TCR stimulation is generally thought to be mediated either by protein kinase C (PKC) (Downward et al., 1992) or by recruitment of Grb2-Sos complexes to phosphorylated LAT (Cantrell, 1998). In our present study, when T cells were stimulated in suspension by PMA or by cross-linked anti-CD3 mAb, ERK activation was

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completely blocked by the PKC inhibitor bisindolylmaleimide (BIM). In contrast, when cells were stimulated by immobilized anti-CD3 antibody, we identified BIM-sensitive and BIM-resistant phases of Ras and ERK activation.

CTL that are stimulated in suspension do not degranulate whereas CTL degranulation is effectively stimulated by immobilized anti-CD3. These disparate outcomes correlate with the duration of signaling through various pathways including ERKs (Berg et al., 1998). The difference in duration of signaling between the two conditions might reflect quantitative differences in the duration of TCR stimulation, or qualitative differences in the pathways activated. Here we have found that each condition activates different pathways upstream of ERK1 and ERK2 as indicated by a difference in sensitivity to the PKC inhibitor BIM.

The initial increase in ERK activity in response to immobilized anti-CD3 stimulation was relatively insensitive to the presence of BIM whereas the sustained phase was inhibited by the drug. This pattern of regulation for the Raf-MEK-ERK pathway has also been found in other cell types. Only the late phase of integrin-mediated Raf activation is PKC-dependent in fibroblasts, whereas the initial phase is dependent on Raf membrane localization, induced either artificially or by Ras-Raf interaction (Howe and Juliano, 1998). There is evidence that B lymphocytes also have both PKC-dependent and PKC-independent mechanisms for ERK activation (Gold et al., 1992).

Ras activation in T cells has been proposed to be mediated by the recruitment of Grb2-Sos complexes to the cell membrane via LAT (Cantrell, 1998; Zhang et al., 1998). This process is solely dependent on protein-protein interactions involving

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inducible protein tyrosine phosphorylation. However, ERK activation is strongly inhibited by the PLC-γ1 inhibitor U73122 in Jurkat T cells (Ebinu et al., 2000) and CTL clones (Figure V-7). LAT is involved in the recruitment and activation of PLC-γ1 following TCR stimulation (Cantrell, 1998; Yablonski et al., 1998; Zhang et al., 1998). Interestingly, a mutant of LAT that is unable to bind PLC-γ1 but is still capable of binding Grb2 is unable to support MAP kinase activation suggesting that a PLC-γ1-dependent pathway is important for the regulation of MAP kinase activity in Jurkat cells (Zhang et al., 2000). In Slp-76 deficient T cells, which recruit Grb2 to LAT but do not activate PLC-γ1, Ras and ERK2 are not activated (Yablonski et al., 1998). Taken together, these data suggest that the major function of LAT with respect to ERK activation is to activate PLC-γ1, and that PLC-γ1 is essential for TCR-mediated ERK activation, most likely due to a requirement for DAG. Our results do not necessarily rule out a role for Grb2-Sos in TCR-induced signaling but suggest that the bulk of MAP kinase activity is regulated by both DAG-dependent but PKC-independent and PKC-dependent mechanisms.

Our data indicate that although DAG is essential for ERK activation in T cells, the major DAG receptor, PKC, is not required for the initial period of ERK activation as indicated by insensitivity to BIM. These results suggest the existence of a DAG-responsive, but non-PKC, component upstream of ERK activation in T cells. The novel Ras-guanylnucleotide exchange factor, RasGRP contains a DAG binding domain and can mediate Ras and ERK activation in response to PMA (Ebinu et al., 1998; Tognon et al., 1998). We found that long-term PMA exposure did not downregulate RasGRP expression, suggesting that it could be responsible for the

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CD3-stimulated ERK activation observed following chronic PMA treatment. RasGRP can mediate Ras activation in response to TCR cross-linking in Jurkat T cells and RasGRP overexpression enhances TCR-mediated activation of ERK (Ebinu et al., 2000). RasGRP is therefore likely to be one of the mediators of DAG-dependent, PKC-independent activation of ERK in T cells.

In summary, the extended period of ERK activation required for CTL degranulation consists of an early PKC-independent phase and a late PKC-dependent phase. The early phase, while PKC-independent, is PLC-γ1-dependent and DAG-dependent and may involve non-PKC DAG-responsive signaling elements such as RasGRP.

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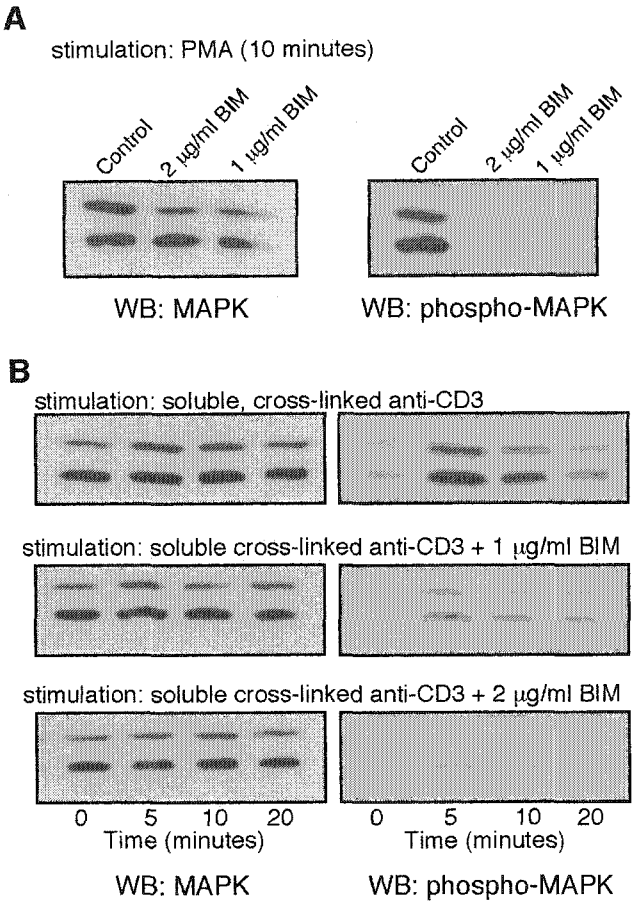

Figure V-1 ERK activation is bisindolylmaleimide-sensitive when CTL are stimulated in suspension.
CTL clone AB.1 cells were stimulated by (*A*) PMA or (*B*) soluble, cross-linked 145-2C11, in the presence of the indicated concentrations of bisindolylmaleimide (BIM) or DMSO carrier control. Activated p44ERK1 and p42ERK2 were detected by Western blotting (WB) with mAb 9105 which is specific for the Thr202/Tyr204 phosphorylated form of these MAP kinases (right panels). Equal total amounts of ERK1 and ERK2 was confirmed by reblotting with anti-MAP kinase (ERK1+ERK2) mAb (left panels). Similar results were obtained with CTL line Clone 11 (not shown).

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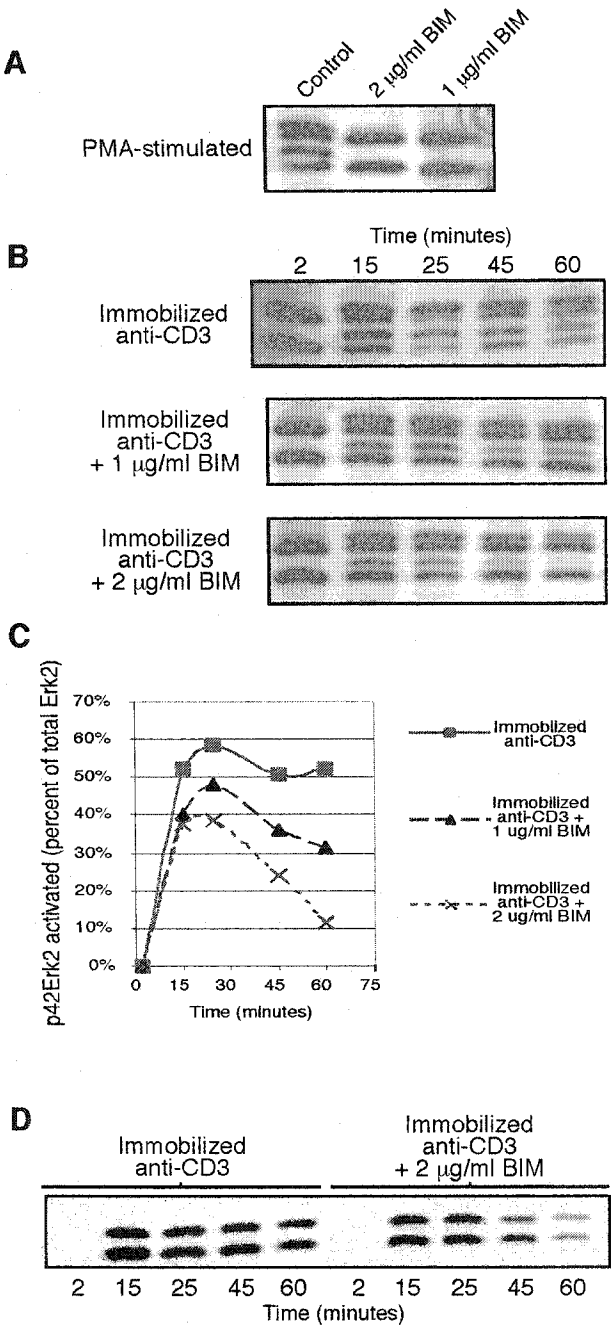

Figure V-2 ERK activation in response to immobilized anti-CD3 stimulation comprises bisindolylmaleimide-resistant and -sensitive phases. *(A),* Effectiveness of bisindolylmaleimide (BIM) treatment was verified by inhibition of ERK activation in response to acute PMA stimulation. Activated ERK1 (pp44) and ERK2 (pp42) were distinguished from their inactive forms by mobility shift assay as described in *Materials and Methods*. *(B),* Clone AB.1 cells were added to wells coated with anti-CD3 mAb 145-2C11 and lysed after the indicated times. Active and inactive ERK1 and ERK2 were visualized as in *A*. *(C),* The ratio of activated to inactive ERK2 was quantitated from *B,* using NIH Image, and plotted as the percent of ERK2 in the active form over time. *(D),* The identification of the mobility-shifted bands shown in *B* as activated ERK1 and ERK2 was confirmed by reprobing the same membrane with phospho-p44/42 MAP kinase (Thr202/Tyr204) specific mAb 9105.

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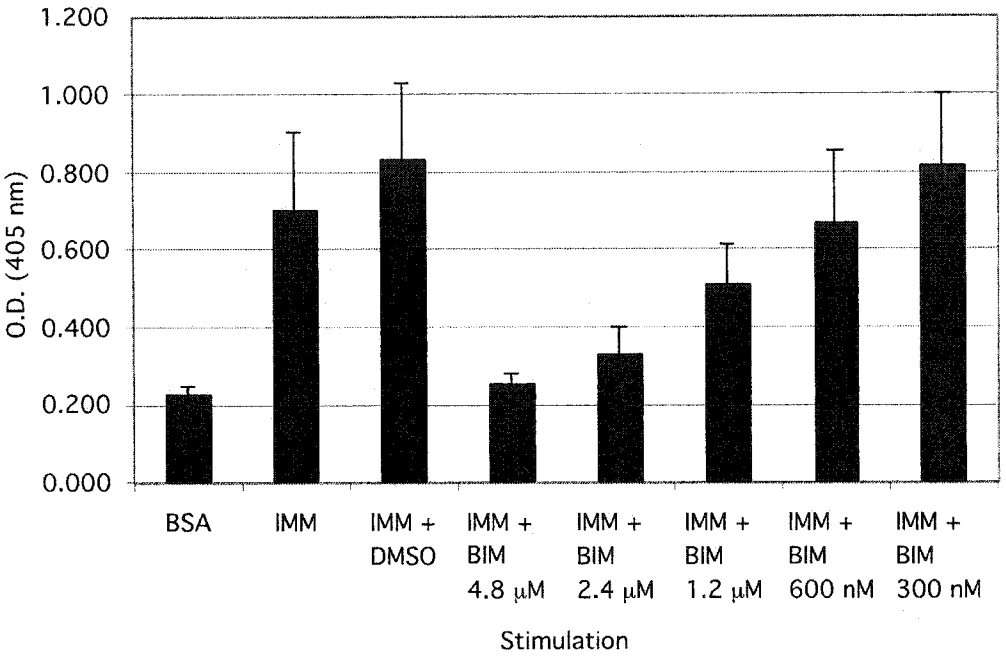

Figure V-3 Bisindolylmaleimide (BIM) inhibits anti-CD3 mediated degranulation.
CTL were pretreated with the indicated concentrations of the PKC inhibitor bisindolylmaleimide, then plated on immobilized 145-2C11 anti-CD3 mAb (IMM). Degranulation was measured by serine esterase release assay.

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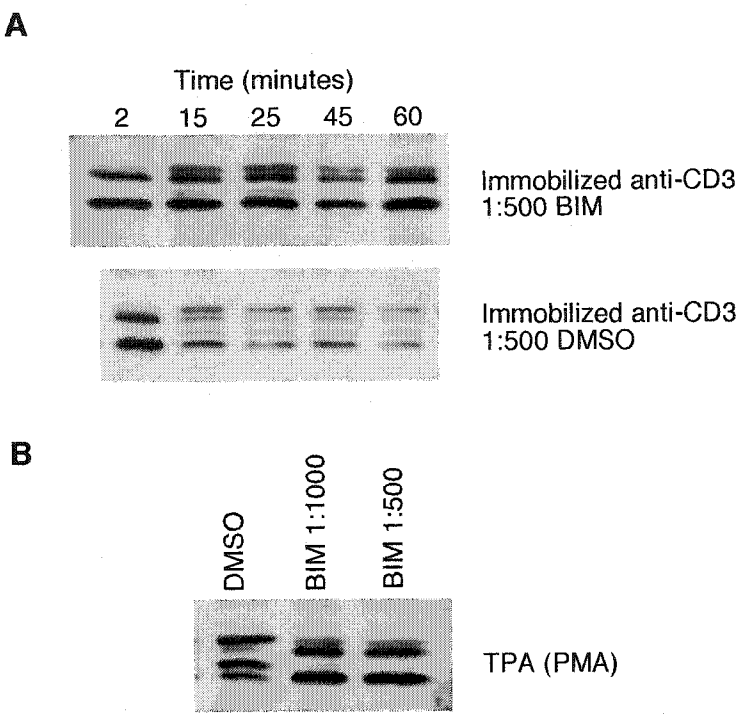

Figure V-4 Bisindolylmaleimide inhibits immobilized anti-CD3 mAb mediated (A) and PMA-mediated (B) MAPK activation in *ex vivo* splenocytes.
ConA activated splenocytes were prepared from C57BL6 mice and stimulated by immobilized 145-2C11 anti-CD3 mAb or PMA in the presence of the indicated concentrations of bisindolylmaleimide (BIM) or DMSO control. MAPK activation was measured by gel mobility shift assay.

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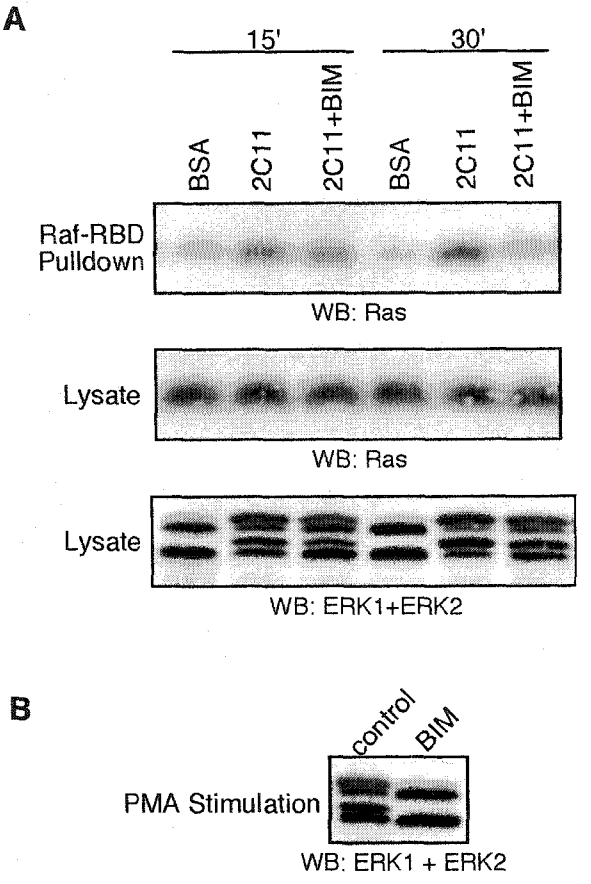

Figure V-5 Ras activation in response to immobilized anti-CD3 is partially inhibited by BIM.
A, AB.1 were incubated for the indicated time on plates immobilized with BSA or 145-2C11. Cells were pretreated for 30 minutes with 2 μg/ml BIM or with DMSO before addition to the plates. Lysates were probed with anti-Ras or with anti-ERK1/2 antibodies or used for affinity precipitation using Raf-1 Ras-Binding Domain (RBD) following by anti-Ras immunoblotting. *B*, BIM-treated and control cells from *a* were treated for 15 minutes with PMA before lysis and electrophoresis on 15% Low Bis SDS-PAGE. The immunoblot was probed with anti-ERK1/2.

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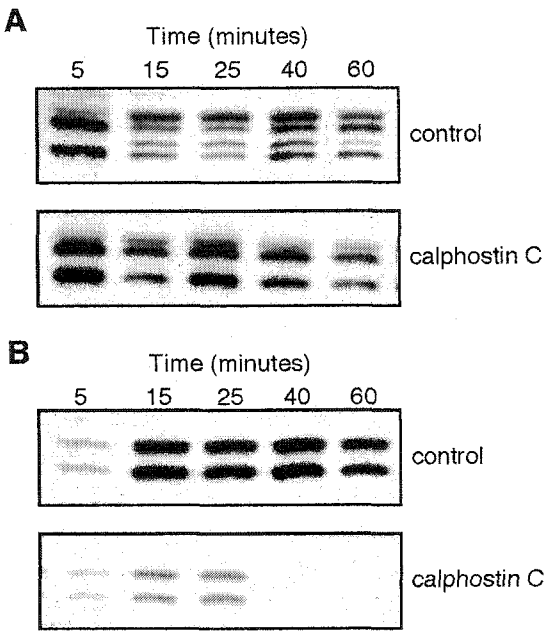

Figure V-6 ERK activation is inhibited by the DAG-binding domain inhibitor calphostin C.
Clone AB.1 cells were added to 145-2C11 coated microtitre wells at $1x10^5$ cells/well in the presence of 1μg/ml calphostin C or ethanol carrier control. *A*, Whole cell lysates were analyzed by p44/42 MAP kinase mobility shift assay. *B*, The same membrane was stripped and reprobed with anti-phospho-p44/42 MAP kinase mAb.

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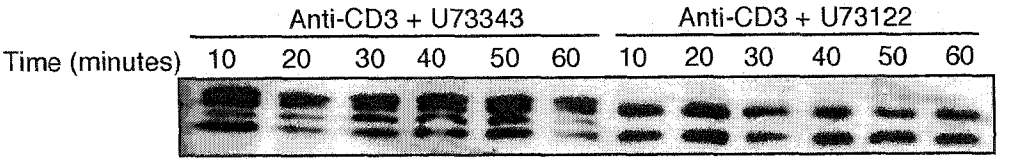

Figure V-7 ERK activation is strongly inhibited by the PLC-γ1 inhibitor U73122.
Clone AB.1 cells were added to 145-2C11 coated microtitre wells at $1x10^5$ cells/well in the presence of 1μM of U73122 or its inert analogue U73343. Whole cell lysates were analyzed by p44/42 MAP kinase mobility shift assay as described in *Materials and Methods*.

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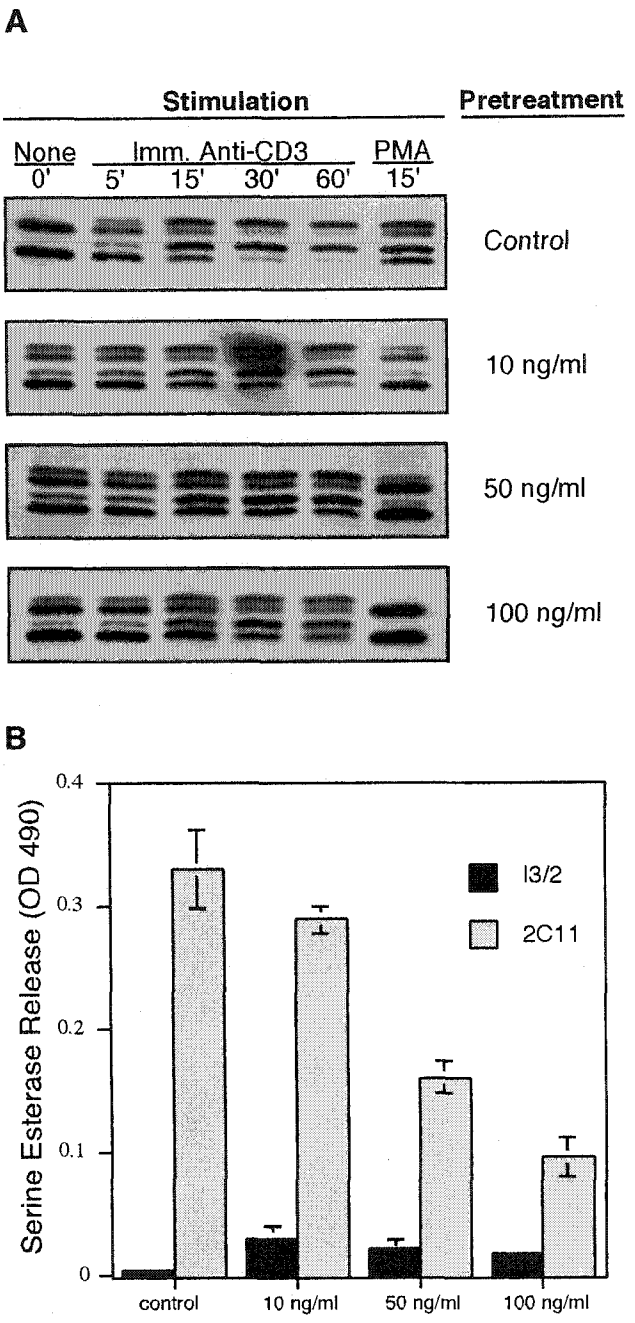

Figure V-8 PKC down-regulation by chronic PMA treatment inhibits acute PMA-stimulated ERK activation but not anti-CD3 stimulated ERK activation. Clone AB.1 cells were treated with 10, 50, or 100 ng/ml PMA or an equivalent volume of ethanol carrier control for 18 hours. A, Cells were stimulated for 15 minutes. MAPK (ERK1 and ERK2) activation was assayed by gel mobility shift assay. B, PMA-pretreated or control AB.1 cells were stimulated with anti-CD3 mAb (145-2C11) or anti-CD45 mAb (I3/2) as a control. After a 5-hour incubation, degranulation was measured by assaying culture supernatant for serine esterase activity. Both ERK1/2 blot and degranulation assay were performed on samples from the same pool of cells.

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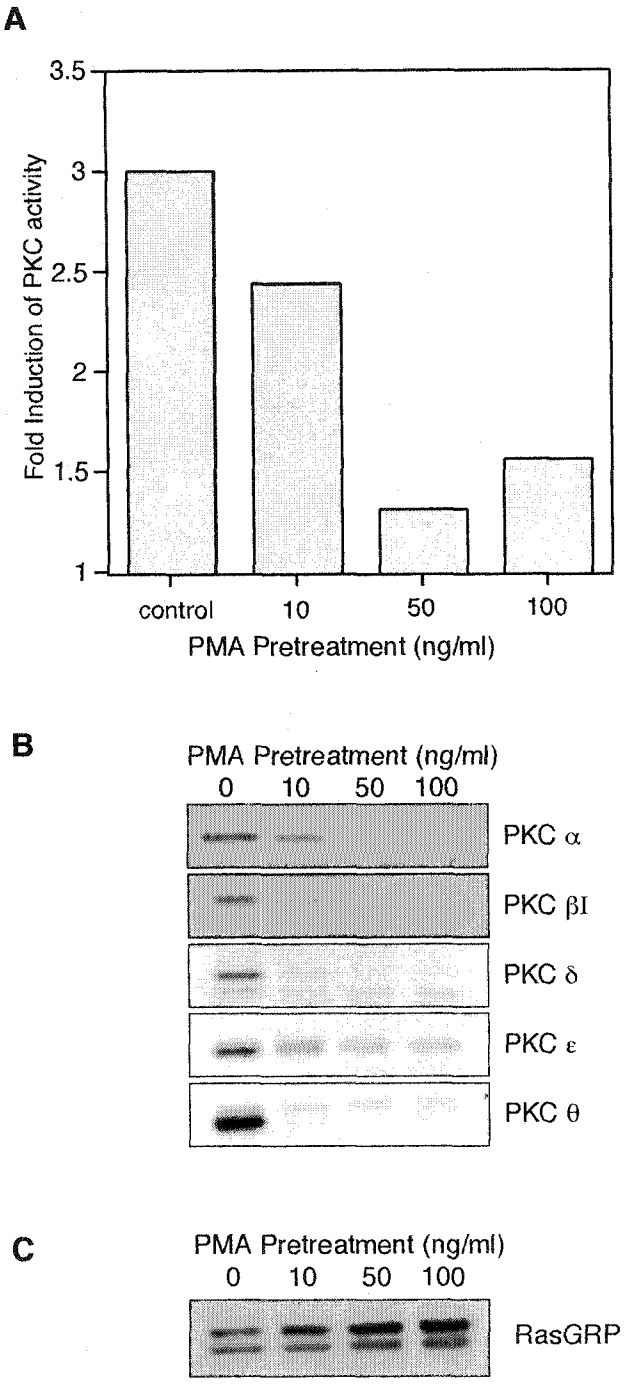

Figure V-9 Chronic PMA treatment down-regulates PKC activity and expression but does not reduce RasGRP expression. AB.1 cells were preincubated with 10, 50 or 100 ng/ml PMA or an equivalent volume of ethanol carrier control for 18 hours. *A,* Cells were assayed for total PKC activity. *B,* Cell lysates were assayed for PKC expression by immunoblotting for the indicated PKC isozyme. *C,* The blot used for PKC-a analysis in B was stripped and reprobed with a mAb to RasGRP. All experiments in this figure were performed on samples from the same pool of cells. The experiment shown in Part A was performed by H. Ostergaard.

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CHAPTER VI Distinct PKC Isoforms Are Required For ERK Activation in Suspended *vs.* Anchored CTL: Role and Regulation of PKC θ

A. Introduction

In the previous chapter, it was demonstrated that adherent cytotoxic T lymphocytes (CTL) require PKC activity to sustain, but not to initiate, TCR-mediated MAP kinase activation (Puente et al., 2000). In contrast, CTL stimulated in suspension display an absolute requirement for PKC in MAP kinase activation. PKC activity comprises a family of isozymes categorized as classical (cPKC α, β, γ), novel (nPKC δ, ε, η, θ), or atypical. Both classical and novel isoforms are activated in response to diacylglycerol, whereas only the classical isoforms require calcium for optimal activity (Nishizuka, 1995). Different isoforms have been associated with distinct functions in various cell types, but the specific PKC isoform or isoforms responsible for MAP kinase regulation in T cells have not been identified.

Novel PKC isoform θ is most highly expressed in lymphocytes (Baier et al., 1993; Baier et al., 1994). Transfection studies demonstrated that PKC θ activity can regulate transcription factors NF-κB (Dienz et al., 2000; Khoshnan et al., 2000) and AP-1 (Baier-Bitterlich et al., 1996). T cells from PKC θ deficient mice fail to activate NF-κB and AP-1 and exhibit reduced proliferation, cytokine production, and CD69 expression in response to stimulation (Sun et al., 2000). Whether PKC θ has a role in the regulation of Ras and ERK is unclear. Induction of AP-1 activity and CD69 expression by constitutively active PKC θ are both blocked by dominant-negative Ras

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(Baier-Bitterlich et al., 1996; Isakov and Altman, 2002), but PKC θ deficient mice appear to have intact JNK and ERK MAP kinase signaling (Sun et al., 2000).

PKC θ is unique amongst PKC isozymes studied to date in that only PKC θ is relocalized to the central region of the contact site between the T cell and the antigen presenting cell (APC) during T cell activation by antigen-specific APC (Monks et al., 1997; Monks et al., 1998; Potter et al., 2001). The mechanism for this selective modulation is unknown. Transfection studies in Jurkat suggest that PKC θ and Vav participate in a common pathway (Dienz et al., 2000; Villalba et al., 2000). It has been proposed that Rac-mediated cytoskeletal reorganization downstream of Vav results in recruitment of PKC θ to the cell membrane where it becomes activated (Villalba et al., 2000). Vav contains a PH (pleckstrin homology) domain, which binds 3-phosphatidylinositol lipids generated by phosphatidylinositol-3-kinase (PI3K) (Han et al., 1998), and a PI3K inhibitor was found to block membrane recruitment of PKC θ (Villalba et al., 2002). PI3K inhibition also diminishes the ability of mutant constitutively active PKC θ to activate NF-κB (Bauer et al., 2001).

PKCs are also regulated by phosphorylation (Parekh et al., 2000). Phosphorylation of the kinase loop motif is required for activity of cPKCs and nPKC θ (Liu et al., 2002), but not nPKC δ (Stempka et al., 1997). The PI3K-dependent kinase PDK-1 phosphorylates PKC ζ and δ (Le Good et al., 1998) and is predicted to phosphorylate other isoforms including θ (Liu et al., 2002).

Although the selective relocalization of PKC θ was observed in *ex vivo* T cells (Monks et al., 1997; Monks et al., 1998), including CTL (Potter et al., 2001; Stinchcombe et al., 2001), most data regarding the regulation of PKC θ has been

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generated by overexpression of PKC θ mutants in Jurkat T cells. Jurkat T cells are a spontaneously proliferating human T cell leukemia cell line that is deficient in the D3 phosphoinositide phosphatase PTEN (Shan et al., 2000). Consequently, Jurkat cells have elevated levels of PI3K products, elevated Itk and PLC-γ activity, and hyperstimulatable ERK activity. Jurkat cells are stimulated experimentally in suspension culture by antibody-mediated crosslinking of TCR/CD3 and/or CD28, but suspension stimulation does not induce relocalization of PKC θ (Monks et al., 1997) and does not trigger sustained activation of MAP kinases or cytotoxic effector function in CTL (Berg et al., 1998). Therefore, Jurkat cells may not represent an ideal model for the study of PKC θ. In the present study, I examined PKC θ regulation in non-transformed cytotoxic T cell clones which respond differentially to stimulation in suspended or adherent states and require PKC activity, MAP kinase activity, and an intact cytoskeleton for cytotoxic function. These studies were conducted to determine both how PKC θ activity is regulated and whether PKC θ is specifically involved in PKC-dependent ERK activation in CTL.

B. Results

Immobilized anti-CD3 but not soluble anti-CD3 stimulation activates PKC θ

To examine the requirements for PKC θ activation in cytotoxic T lymphocytes (CTL), AB.1 T cell clones were stimulated by plate-bound immobilized anti-CD3 mAb, soluble crosslinked anti-CD3, or PMA (Figure VI-1). Kinase activity was readily detected in PKC θ immunoprecipitated from immobilized-anti-CD3 stimulated cells (Figure VI-1A). To ensure that *in vitro* kinase activity was not due to an irrelevant co-precipitated kinase, a control immunoprecipitation was performed

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with protein A-sepharose beads alone (Figure VI-1A) and a control kinase assay was performed with PKC θ or α pseudosubstrate peptide inhibitor present (Figure VI-1B). In addition, Western blot of PKC θ IP using a pan-isoform anti-phospho-PKC antibody detected no additional bands besides PKC θ (not shown). No kinase activity was recovered from unstimulated cells.

Previous comparison of signaling outcomes in CTL stimulated by immobilized or soluble anti-CD3 suggested that suspension stimulation activates largely the same pathways as immobilized stimulation but for shorter durations (Berg et al., 1998). In contrast, PKC θ was not active following soluble anti-CD3 stimulation at any of the time points examined (Figure VI-1A). Therefore, in mature antigen-experienced CTL, the mode of stimulation (suspension *vs.* anchored) is critical for the regulation of PKC θ. PKC θ appears to represent a strictly anchorage-dependent point in TCR signal transduction.

Surprisingly, no kinase activity was recovered from cells stimulated by PMA. PKC θ exhibits *in vitro* kinase activity when phosphatidylserine and diacylglycerol are added directly to the reaction mix (Figure VI-6). This suggests that PKC θ in cells might lack access to required co-factors for PMA-mediated activation, possibly by exclusion from phosphatidylserine-containing membrane domains. PKC θ *in vitro* kinase activity requires phosphatidylserine or phosphatidylglycerol and is not induced by diacylglycerol alone (Pietromonaco et al., 1998). Alternatively, PMA might activate PKC θ in a manner that is not preserved in this assay.

Aliquots of pre-IP cell lysate were analyzed by Western blotting using a 7.5% SDS-PAGE gel. Under these conditions, a reduced mobility form of PKC θ could be

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detected in all stimulated cells (Figure VI-1A, lower panels). This reduced mobility band was due to phosphorylation (Figure VI-1C). In suspension-stimulated cells, this phosphorylated form of PKC θ was less abundant and did not appear for as long, as compared to immobilized anti-CD3 conditions. Therefore, PKC θ may be phosphorylated independently of increased activity, but greater phosphorylation is associated with greater activity. As described previously (Figure III-2), appearance of the active, dual-phosphorylated form of ERK1 and ERK2 MAP kinases was weak and transient under suspension conditions but strong and sustained under immobilized conditions (Figure VI-1A). Although activation of ERK1 and ERK2 under suspension conditions is sensitive to inhibitors of PKC, this can not be attributed to the θ isoform as it was found to be inactive under those conditions. Immobilized conditions are required for both PKC θ activity and sustained MAP kinase activation.

Membrane association is not sufficient for PKC θ activation

PKC θ activation is thought to be concomitant with membrane relocalization (Villalba et al., 2000). Therefore, I examined whether CD3-crosslinking or PMA treatment was sufficient to induce association of PKC θ with membranes. Surprisingly, a significant amount of PKC θ was constitutively associated with membranes in unstimulated cells (Figure VI-2). The proportion of PKC θ associated with membranes, relative to the amount in the cytosol, increased slightly following anti-CD3 crosslinking or TPA treatment. To ensure that membrane-associated PKC θ did not reflect contamination of the membrane preparation with cytosolic material, samples were also probed for paxillin (Figure VI-2, lower panel). Since a significant proportion of PKC θ was membrane associated following CD3 crosslinking or PMA

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treatment, lack of kinase activity can not be attributed to a failure to become recruited to membranes. Conversely, increased membrane association is not sufficient for PKC θ activation (compare Figure VI-1 and Figure VI-2). Recovery of PKC θ from the membrane fraction does not necessarily indicate localization to the plasma membrane and may reflect association with other membrane compartments.

PKC θ in vitro kinase activity is sensitive to bisindolylmaleimide but resistant to rottlerin

I next wished to determine the consequences of PKC θ inhibition in CTL. Rottlerin has been reported to be a highly selective inhibitor of PKC θ (Villalba et al., 1999) and δ (IC_{50} 3-6 μM) (Gschwendt et al., 1994). Rottlerin inhibited TCR-mediated MAP kinase activation (Figure VI-3), PKC θ phosphorylation (Figure VI-4) and CTL degranulation (not shown) in a dose-dependent manner. However, rottlerin only partially inhibited PKC θ *in vitro* kinase activity at a concentration of 25 μM (Figure VI-6). Therefore the effects of rottlerin can not be wholly attributed to specific inhibition of PKC. Similarly, Soltoff found no direct inhibition of PKC δ *in vitro* kinase activity by rottlerin (Soltoff, 2001). However, while Soltoff reported a global decrease in cellular phosphorylation, western blotting of rottlerin-treated AB.1 T cells using anti-phosphotyrosine mAb showed decreased phosphorylation in only a small subset of tyrosine phosphorylated proteins under both TCR-stimulated and unstimulated conditions (Figure VI-5). All rottlerin experiments were performed in the presence of 1%-2% defined calf serum since rottlerin was found to induce rapid CTL death when applied in serum-free media.

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Since the effects of rottlerin appeared to be largely nonspecific, I determined whether a panel of known PKC inhibitors affected PKC θ kinase activity *in vitro* (Figure VI-6A). Bisindolylmaleimide, Ro-8220 and Gö-6983 significantly reduced PKC θ kinase activity *in vitro* while Gö-6976 and DMSO carrier control did not (Figure VI-6A, Table VI-1). This is consistent with reports that Gö-6976 selectively inhibits classical PKCs (Martiny-Baron et al., 1993). I also attempted to use PKC isoform-specific myristoylated pseudosubstrate peptides as inhibitors but surprisingly, these were highly toxic to CTL.

A novel isoform PKC is required for sustained ERK activation

To examine whether specific PKC isoforms were required for MAP kinase activation downstream of TCR/CD3 stimulation, AB.1 T cells were stimulated with either immobilized or soluble anti-CD3 mAb in the presence of the inhibitors characterized above. Late phase MAP kinase activation in immobilized-stimulated cells was sensitive to Gö-6983 and Ro-8220, but not to Gö-6976 (Figure VI-6B). In contrast, all three inhibitors blocked MAP kinase activation in suspension-stimulated cells (Figure VI-6C). Therefore, different PKC isozymes are required for MAP kinase activation depending on the mode of stimulation. CTL stimulated in suspension activate MAP kinase through a mechanism that requires classical isoform PKCs which are sensitive to all three inhibitors whereas the sustained MAPK activity in immobilized-stimulated CTL requires one or more novel isoform PKCs, potentially including PKC θ. Consistent with this result, the novel isoform PKC θ is activated in immobilized but not suspension stimulated cells (Figure VI-1). Ro-31-8220, Gö-6976 and BIM may inhibit the MAPKAP-K1b, MSK1, S6K1, and GSK3β kinases to

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various extents when used at high concentration in cell-free assays (Davies et al., 2000), however only Ro-31-8220 and BIM inhibited ERK activation in adherent cells supporting the conclusion that it is the nPKC inhibitory function of these drugs that is responsible for blocking late-stage ERK.

cPKC-mediated ERK activation is downstream or independent of Ras

In immobilized cells, PKC appears to act upstream of Ras (Figure V-5). Since stimulation of non-immobilized cells activated MAPK through cPKCs rather than nPKCs (Figure VI-6), I examined whether cPKCs also acted upstream of Ras. CTL were stimulated in suspension by soluble, crosslinked anti-CD3 mAb in the presence or absence of the PKC inhibitor BIM. In this case, BIM had no effect on the activation of Ras during the time that ERK was inhibited (Figure VI-7), indicating that in the cPKC pathway of MAPK activation, cPKCs act downstream of Ras or possibly bypass Ras altogether.

PLC inhibition does not prevent PKC θ activation

Sustained activation of MAP kinases in CTL requires nPKC activity (Figure VI-6). Since PKCs are activated by diacylglycerol, this is consistent with the observation that sustained activation of MAP kinases requires PLC activity (Figure V-7). Diacylglycerol is thought to act in part by recruiting PKC to the plasma membrane resulting in its activation. However, it was recently reported that relocalization of PKC θ can occur in the absence of PLC activity (Villalba et al., 2002). To examine whether this impacted PKC kinase activity, PKC θ kinase assays were performed on PKC θ recovered from cells treated with PLC inhibitor U73122 or the non-inhibiting analogue U73343. Interestingly, PLC inhibition did not diminish PKC θ activity

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(Figure VI-8). This result is consistent with the observation that PMA did not stimulate PKC θ activity (Figure VI-1). Lack of PKC θ inhibition by U73122 has two possible implications for the mechanism of sustained Erk activation in CTL, which is U73122 sensitive (Figure V-7). Firstly, this could indicate that a nPKC other than PKC θ is required for sustaining Erk activation. Alternatively, this could indicate that a PLC-dependent enzyme acts between nPKC θ and Ras, upstream of Erk regulation. The possibility that RasGRP is regulated by PKC θ-mediated phosphorylation is presently being examined by other investigators (J. Stone, personal communication).

PI3K activity is required for PKC θ kinase activity

Several pieces of evidence link PKC θ and PI3K. PI3K might be required for Vav-mediated PKC θ recruitment to the plasma membrane (Villalba et al., 2000; Villalba et al., 2002) or for PDK-1-mediated PKC θ phosphorylation (Le Good et al., 1998; Parekh et al., 2000). Alternatively, PI3K-dependent pathways may converge with PKC θ signaling to regulate NF-κB (Bauer JBC 276,31627). Therefore, I examined whether PI3K activity was required for the induction of PKC θ *in vitro* kinase activity.

Initially, PKC θ *in vitro* kinase activity appeared to be unchanged or even enhanced by treatment with the PI3K inhibitor wortmannin (Figure VI-9A, upper panel). However, closer inspection revealed that PKC θ was recovered more efficiently from wortmannin treated cells (Figure VI-9A, lower panel). This effect was consistently observed in multiple experiments. Therefore, PI3K inhibition may decrease PKC protein turnover, or may alter PKC θ in a way (*e.g.* phosphorylation)

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that favours recognition and recovery by the immunoprecipitating antibody. The results of autoradiography and anti-PKC θ western blotting (Figure VI-9A) were quantitated by scanning optical densitometry and the kinase activity relative to PKC θ amount was estimated (Figure VI-9C). This data indicates that PKC θ recovered from cells pretreated with PI3K inhibitor has significantly decreased kinase activity compared to controls. Wortmannin treatment also diminished PKC θ phosphorylation as indicated by mobility retardation on SDS-PAGE (Figure VI-9B).

Sustained MAP kinase activation requires PI3K activity and actin polymerization

The preceding results show that the duration of MAPK activation in CTL correlates with PKC θ activation and with the presence of a phosphorylated form of PKC θ (Figure VI-1). Since PKC θ activation required PI3K activity (Figure VI-9) and PKC θ phosphorylation required both PI3K activity and actin polymerization (L. Mireau and H. Ostergaard, unpublished observations), I examined whether these factors were also requirements for MAP kinase activation. AB.1 T cells were stimulated by immobilized anti-CD3 mAb in the presence of cytochalasin E, PI3K inhibitors, or solvent controls (Figure VI-10). Since cytochalasin treatment disrupts early tyrosine phosphorylation signals (Berg et al., 1998), cells were allowed to interact with plate-bound 145-2C11 anti-CD3 mAb for 15 minutes before the addition of cytochalasin E to disrupt actin polymerization. This treatment reversed the activation of both ERK1 and ERK2 MAP kinases (Figure VI-10A). When CTL were pretreated with LY-294002 or wortmannin to inhibit PI3K activity, MAP kinase activation in response to plate-bound anti-CD3 was not sustained (Figure VI-10B). This result is strikingly similar to the effect of PKC inhibitors (Figure V-2). LY-

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294002 and wortmannin did not inhibit TPA-induced Erk phosphorylation (Figure VI-10C) which may proceed primarily through cPKCs. These data show that sustained activation of MAP kinases in CTL not only requires nPKC (Figure VI-6), but also requires PI3K activity and actin polymerization (Figure VI-10).

In PKC θ deficient mice, cPKC activity replaces nPKC activity as a requirement for ERK activation

Sustained ERK activation in anchored CTL clones correlated with PKC θ activity and was blocked by treatments that inhibited nPKCs. Previous studies found that some effects of constitutively active PKC θ expression were blocked by expression of dominant negative Ras, suggesting that PKC θ may act upstream of Ras (Baier-Bitterlich et al., 1996; Isakov and Altman, 2002). These data suggest that the θ isoform of PKC might be responsible for nPKC-mediated ERK activation. However, T cells from PKC θ deficient mice do not appear to be defective in TCR-triggered ERK or JNK signaling (Sun et al., 2000). We hypothesized that in the absence of PKC θ, the cPKC pathway utilized by suspension-stimulated CTL might provide for the observed ERK activation. To test this hypothesis, splenic T cells were prepared from PKC $\theta^{-/-}$ mice that had been backcrossed on to a C57BL/6 background. These splenic T cells were treated with DMSO control or with Gö-6976 cPKC inhibitor, then stimulated by immobilized 145-2C11 anti-CD3 mAb. PKC $\theta^{+/+}$ and PKC $\theta^{-/-}$ mice had similar levels of cell-surface TCR as judged by FACS analysis (H. Ostergaard, data not shown). In agreement with published results (Sun et al., 2000), control-treated T cells from PKC $\theta^{-/-}$ mice were only moderately impaired in their ability to activate ERK1 and ERK2 in response to TCR/CD3 stimulation (Figure

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VI-11A). However, consistent with our hypothesis, Gö-6976 treatment strongly inhibited ERK activation in PKC $\theta^{-/-}$ mice and not in PKC θ sufficient mice (Figure VI-11B). This data implies that PKC θ is the nPKC normally involved in the activation of ERK MAP kinases in T cells since in the absence of PKC θ, ERK activation became dependent on cPKC activity.

C. Discussion

As discussed in CHAPTER V PKC activity plays specific roles in TCR-mediated MAP kinase activation in both anchored and suspended CTL. PKC activity comprises a family of isozymes that can have distinct cellular functions. PKC θ is known to be a crucial isoform for T cell activation. Various pharmacological agents were assayed for their ability to inhibit PKC θ activity and tested for their effects on MAP kinase activation. MAP kinase activation in PKC θ deficient mice was also examined. The behaviour of PKC θ in cytotoxic T cells has not been extensively characterized. Therefore, requirements for PKC θ relocalization, phosphorylation and activity were examined in CTL in parallel with measurements of ERK1 and ERK2 MAP kinase signaling.

Regulation of PKC θ by phosphorylation

PKC θ activity is regulated by lipid cofactors (Nishizuka, 1984; Nishizuka, 1995) and by phosphorylation (Liu et al., 2002). Stimulation of CTL by PMA or anti-CD3 mAb resulted in phosphorylation of PKC θ but only immobilized mAb stimulation induced kinase activity. This indicates that PKC θ can be phosphorylated at certain sites without measurably increasing its activity. PKCs contain three key phosphorylation sites termed the activation loop, the autophosphorylation or turn

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motif, and the hydrophobic motif (Parekh et al., 2000). For PKC θ, activation loop phosphorylation results in significant kinase activity while phosphorylation of the other two sites can modulate activity of the activation loop phosphorylated form (Liu et al., 2002). Therefore, the mobility-shifted form of PKC θ seen in these assays may represent phosphorylation of the turn motif or the hydrophobic motif. Hydrophobic motif phosphorylation is insufficient to impart PKC θ kinase activity but enhances activity when the activation loop is phosphorylated (Liu et al., 2002). Therefore, the mobility-retarded form of PKC θ likely represents a form of PKC θ that is competent to become fully activated.

PKC θ subcellular localization

A significant proportion of PKC θ was constitutively associated with membranes. The proportion of membrane-associated θ increased somewhat following CD3 crosslinking (Figure VI-2) or interaction with target cells (L. Mireau, personal communication) but this did not necessarily result in increased PKC θ kinase activity. Cells stimulated by anti-CD3 mAb in suspension exhibited reduced-mobility PKC θ that was membrane associated (Figure VI-2), but not active (Figure VI-1). Consistent with these observations, confocal microscopy data appearing in a recent study appears to show PKC θ localized to intracellular compartments in unstimulated cells (Egen and Allison, 2002).

PKC θ kinase activity

PKC θ activity appears to be strictly dependent on cell anchorage (Figure VI-1). Anchorage-dependence might indicate input from signaling pathways that sense cell shape or cytoskeletal stress, as observed for anchorage-dependent

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proliferation in other cell types (Folkman and Moscona, 1978). In CTL, anchorage-dependence likely serves to co-ordinate cytotoxic granule release with tight adhesion to targets. In contrast to PKC θ activity, most tyrosine kinase signaling is intact but transient in suspension-stimulated CTL, suggesting that anchorage-dependent regulation occurs close to the point of PKC θ activation.

It has been proposed that PKC is regulated by PI3K either via PI3K-dependent phosphorylation or PI3K-dependent relocalization (Isakov and Altman, 2002). The present study confirms that PKC θ activity is PI3K-dependent. Although PKC activation is generally thought to be concomitant with membrane recruitment, membrane association was not sufficient to induce kinase activity. Acquisition of the reduced mobility phosphorylated form was PI3K-dependent, but not sufficient for PKC θ *in vitro* kinase activity. Therefore, PI3K-dependent relocalization and phosphorylation may be necessary but not sufficient for kinase activity. What further steps might be required? Full activation of PKC θ may require additional PI3K-dependent phosphorylation at sites that do not affect mobility on SDS-PAGE.

A role for PKC θ activity in MAP kinase activation

Several previous lines of evidence suggested that PKC θ might act upstream of Ras (Isakov and Altman, 2002). Expression of a dominant negative PKC θ mutant prevented upregulation of the T cell activation marker CD69 (Villalba et al., 2000), a process which is thought to be strictly Ras dependent (D'Ambrosio et al., 1994). Reciprocally, induction of AP-1 activity and CD69 expression by constitutively active PKC θ are both blocked by dominant-negative Ras (Baier-Bitterlich et al., 1996;

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Isakov and Altman, 2002). However, it had been shown that PKC θ deficient mice have intact MAP kinase signaling (Sun et al., 2000).

The present study found that sustained MAP kinase activity in anchored CTL correlates with PKC θ activation and is sensitive to inhibitors of nPKCs. Late phase MAP kinase activity was also found to be sensitive to inhibitors of PI3K and PI3K activity was found to be a requirement for PKC θ phosphorylation and activity. Therefore we hypothesized that intact MAPK signaling in PKC $\theta^{-/-}$ mice might reflect the operation of an alternate or compensatory pathway replacing the activity of a PKC θ-dependent pathway present in wild-type cells. CTL stimulated in suspension were found to activate ERK1/ERK2 MAP kinases via a cPKC-dependent pathway (Figure VI-6). We hypothesized that this pathway might also mediate ERK activation in anchored cells in the absence of PKC θ. Indeed, the genetic absence of PKC θ changed the phenotype of ERK activation in anchored cells from Gö-6976 insensitive to Gö-6976 sensitive implying that loss of PKC θ caused loss of the nPKC pathway and reliance on the alternate cPKC pathway. This data implicates PKC θ in the normal regulation of the ERK1 and ERK2 MAP kinases in adherent CTL.

Summary

I previously demonstrated two modes of TCR-mediated MAP kinase activation in CTL, a wholly PKC-dependent mechanism operating in suspended cells and a partially PKC-dependent mechanism operating in anchored cells. The present study demonstrates that different PKC isoforms are involved in each mechanism. Suspended cells activate MAP kinase through one or more members of the conventional PKC family, whereas anchored cells require a novel family PKC for the

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PKC-dependent phase of MAP kinase activation. In mice lacking expression of the θ isoform of PKC, MAP kinase activation in anchored cells occurred via cPKCs implying that PKC θ is required for the nPKC pathway to function. Sustained activation of ERK1 and ERK2 MAP kinases in anchored cells required actin polymerization and required PI3K activity for the late (PKC-dependent) period of MAP kinase activity. PKC θ activity was found to be dependent on cell anchorage and on PI3K activity but unaffected by an inhibitor of PLC. The strict requirement for cell anchorage suggests that PKC θ could participate in coordinating CTL activation with adhesion to target cells. Given its relatively limited tissue expression, PKC θ represents an attractive target for disrupting unwanted lymphocyte activation. Greater understanding of PKC θ regulation and signaling will assist efforts to create therapies in this area.

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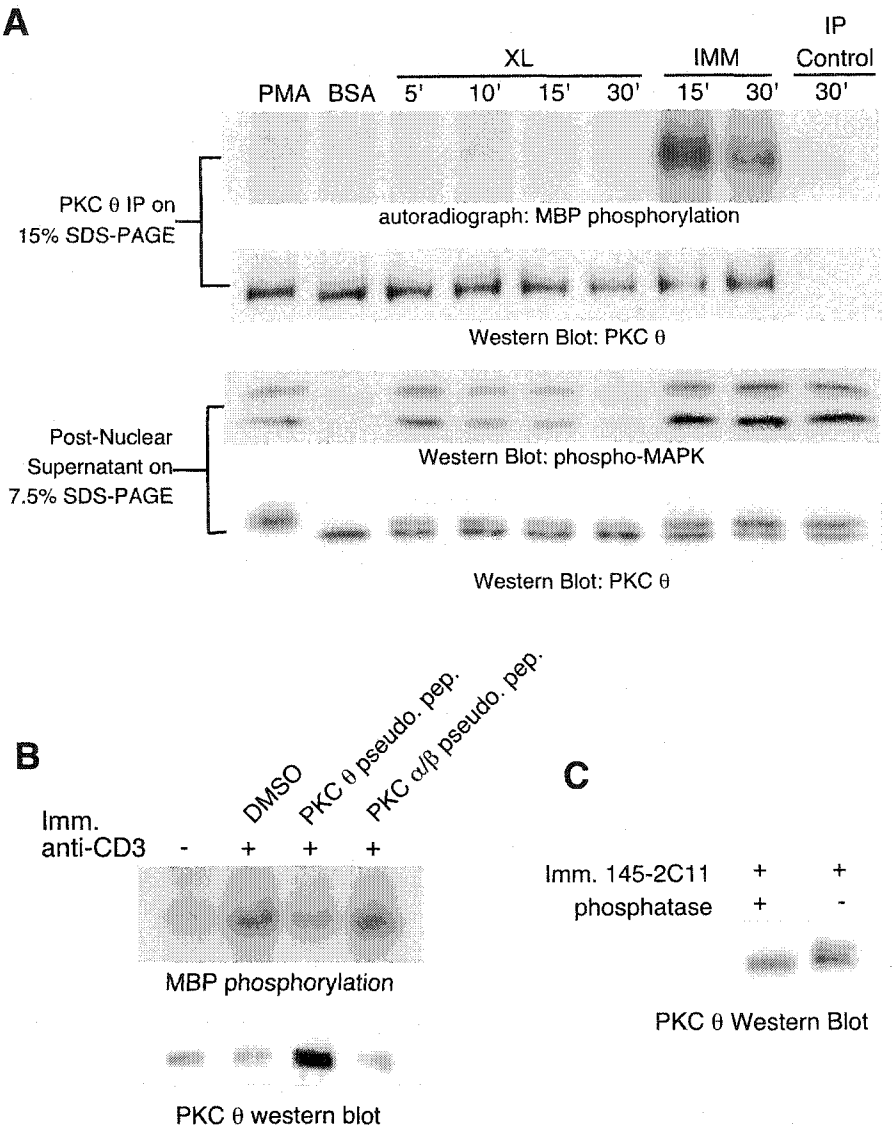

Figure VI-1 Immobilized, but not soluble anti-CD3 stimulation induces PKC θ activity.

(A), Clone AB.1 cells were stimulated by immobilized (IMM) or soluble cross-linked (XL) 145-2C11 or by PMA. PKC θ was immunoprecipitated and kinase activity assessed by ^{32}P incorporation into the exogenous substrate MBP (top panel). Western blot for PKC θ was performed to show similar recovery of PKC θ in all lanes (second panel down). As a control, a sample with no immunoprecipitating antibody was included (IP Control). Aliquots of post-nuclear supernatant from the same set of samples were analyzed on a 7.5% SDS-PAGE gel (lower two panels) and immunoblotted for the phosphorylated, catalytically active form of MAP kinases p44ERK1 and p42ERK2, and for PKC θ. *(B)*, Kinase reactions were performed in the presence of 5 μM of PKC θ or PKC α/β pseudosubstrate peptide as a competitive inhibitor. *(C)*, Cloned AB.1 cells were stimulated by immobilized 145-2C11 and PKC θ immunoprecipitates were treated with alkaline phosphatase or carrier control. PKC θ was visualized by Western blotting following 7.5% SDS-PAGE. The experiment shown in part C was performed by Laura Mireau.

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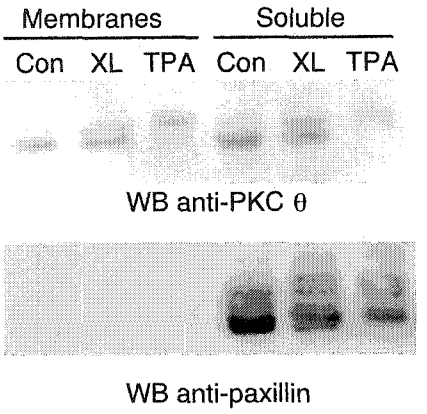

Figure VI-2 PKC θ is constitutively associated with membranes. Cloned AB.1 T cells were simulated by PMA (TPA), by CD3 crosslinking (XL) or by secondary antibody alone as control (Con). Cells were fractionated into insoluble membrane and soluble cytosolic fractions. Samples were analyzed by Western blotting for the presence of PKC θ (upper panel) and paxillin as a cytosol-specific control (lower panel).

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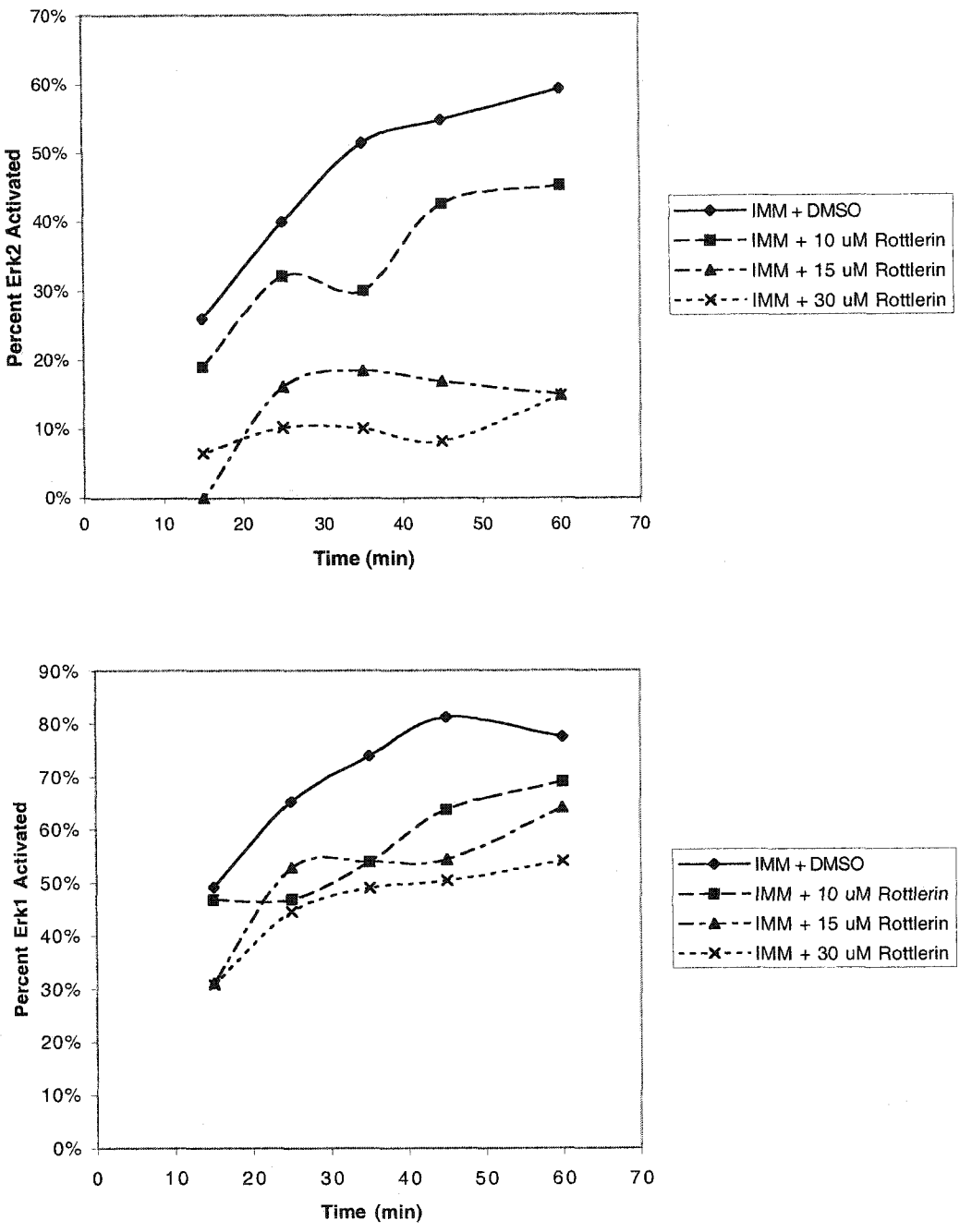

Figure VI-3 Rottlerin inhibits MAPK activation.
AB.1 cells were pretreated with the indicated concentrations of rottlerin and stimulated by plating on immobilized 145-2C11 mAb (IMM). MAPK activation was measured by mobility shift assay. Optical density of the resulting bands was measured using NIH Image software. Results are expressed as percent upper band (active MAPK) out of total MAPK detected.

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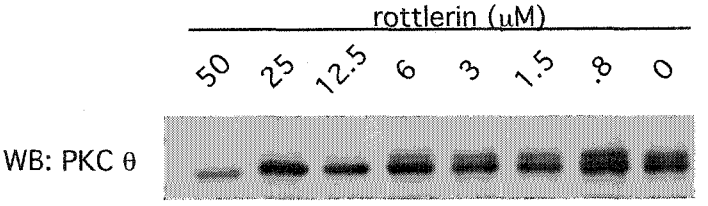

Figure VI-4 Rottlerin inhibits phosphorylation of PKC θ.
Cells were treated with the indicated concentration of rottlerin, then stimulated by plating on immobilized 145-2C11 mAb. PKC θ was visualized by Western Blot (WB) on 10% SDS-PAGE. Mobility shift of the PKC θ band reflects phosphorylation (see Figure VI-1).

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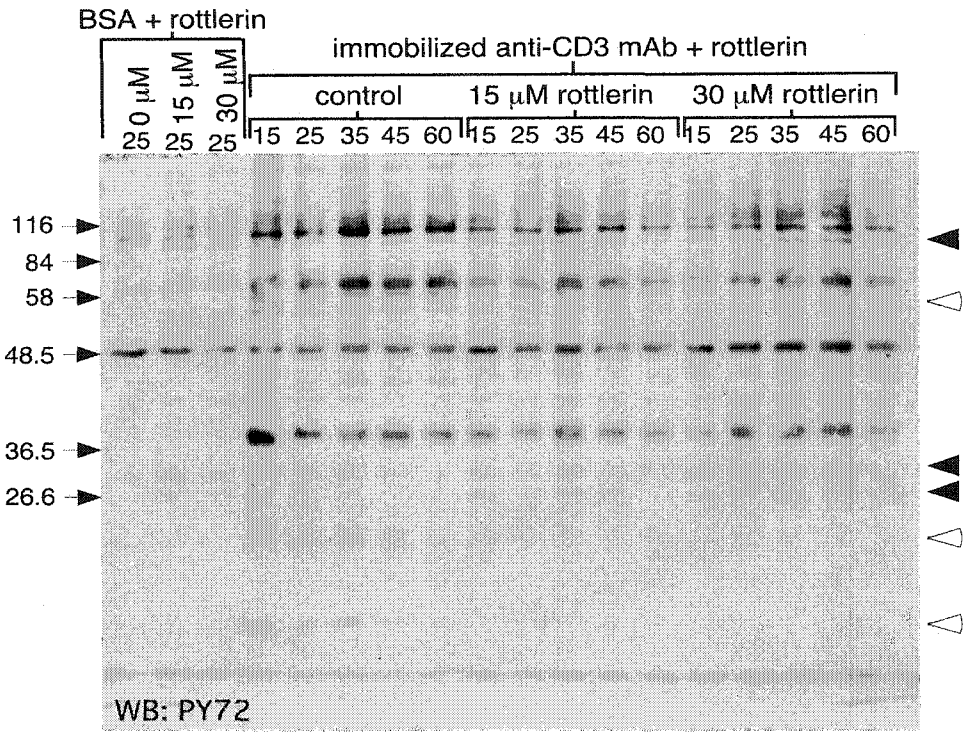

Figure VI-5 Rottlerin treatment alters tyrosine phosphorylation of a subset of CTL proteins.
Clone AB.1 CTL were treated with the indicated concentrations of rottlerin or DMSO (control), then plated on anti-CD3 mAb or BSA alone. Proteins whose tyrosine phosphorylation increased with rottlerin treatment are indicated by closed arrowheads whereas proteins whose tyrosine phosphorylation decreased with rottlerin treatment are indicated by open arrowheads. The position of molecular weight markers is indicated at left.

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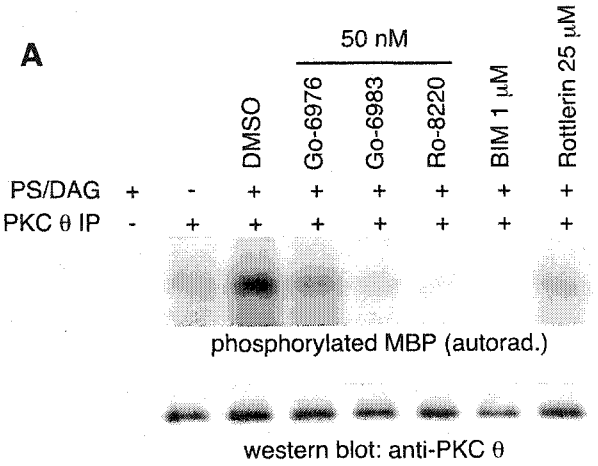

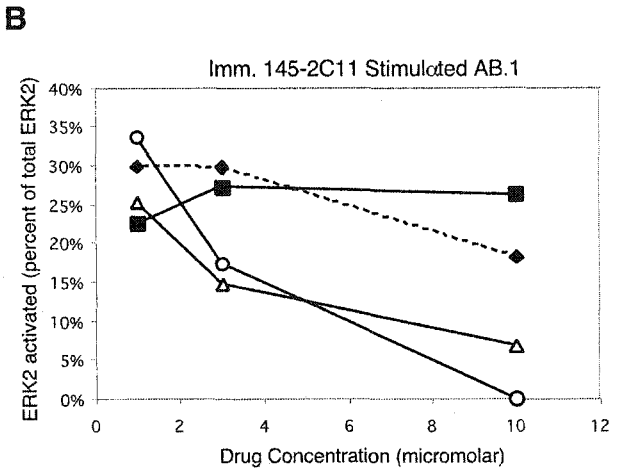

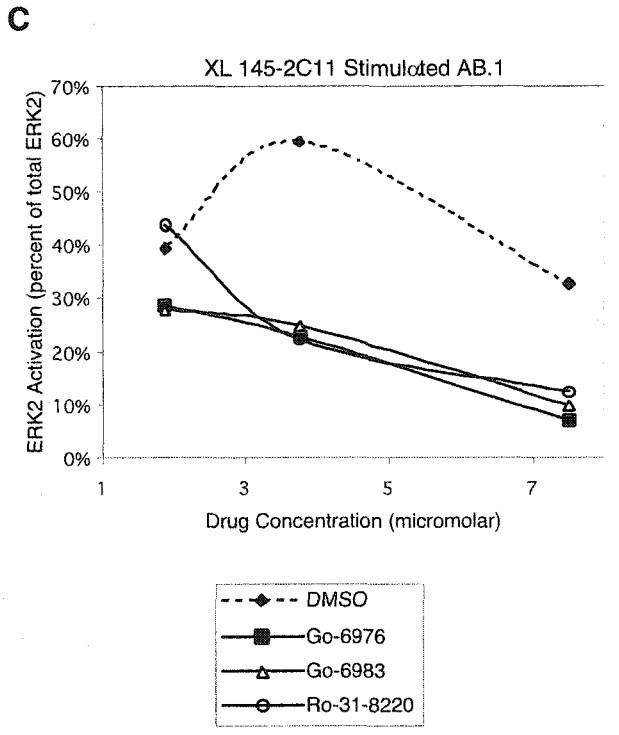

Figure VI-6 Effect of PKC inhibitors on PKC θ and MAPK activity.
(A), PKC θ was immunoprecipitated from AB.1 T cells and activated *in vitro* by the addition of phosphatidylserine and diacylglycerol (PS/DAG). *In vitro* kinase reactions were performed in the presence or absence of potential inhibitors, with MBP as an exogenous substrate. *(B)* AB.1 cells were stimulated by immobilized 145-2C11 mAb for 40 minutes in the presence of DMSO (dashed line), Go-6976 (filled squares), Go-6983 (open triangles), or Ro-31-8220 (open circles). Activation of ERK2 was determined by mobility shift assay. Results were scanned and optical density determined using NIH Image software. The optical density of shifted and unshifted ERK2 bands was compared and plotted as percent active ERK2 out of total ERK2. *(C)* The same experiment was performed except that cells were stimulated by soluble, cross-linked 145-2C11 for 10 minutes.

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	Bisindolylmaleimide-1	Go-6976
cPKC family		
PKC α	8.1 nM	2.3 nM
PKC β	18 nM	6.2 nM
nPKC family		
PKC δ	210 nM	-
PKC ε	132 nM	-
PKC θ	+	-

Table VI-1 Isoform selectivity of PKC inhibitors.
IC_{50} for inhibition of PKC *in vitro* kinase activity is shown (Martiny-Baron et al., 1993). For cases where precise IC_{50} is not known, the relative efficacy *vs.* control is indicated by + (inhibition) or – (no inhibition). Capacity for inhibiting PKC θ is based upon the data shown in Figure VI-6.

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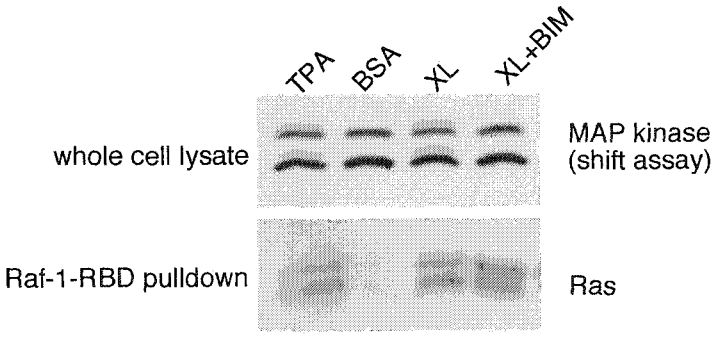

Figure VI-7 BIM does not block Ras activation in suspension-stimulated cells.
Cells were pretreated with bisindolylmaleimide (BIM) or DMSO carrier control then stimulated with soluble crosslinked 145-2C11 mAb (XL), TPA, or left untreated in BSA-blocked wells, for 5 min. MAP kinase activation was assayed by mobility shift (top panel). Active Ras was recovered by interaction with Raf-1-RBD-GST bound to agarose beads and detected by anti-Ras Western blot.

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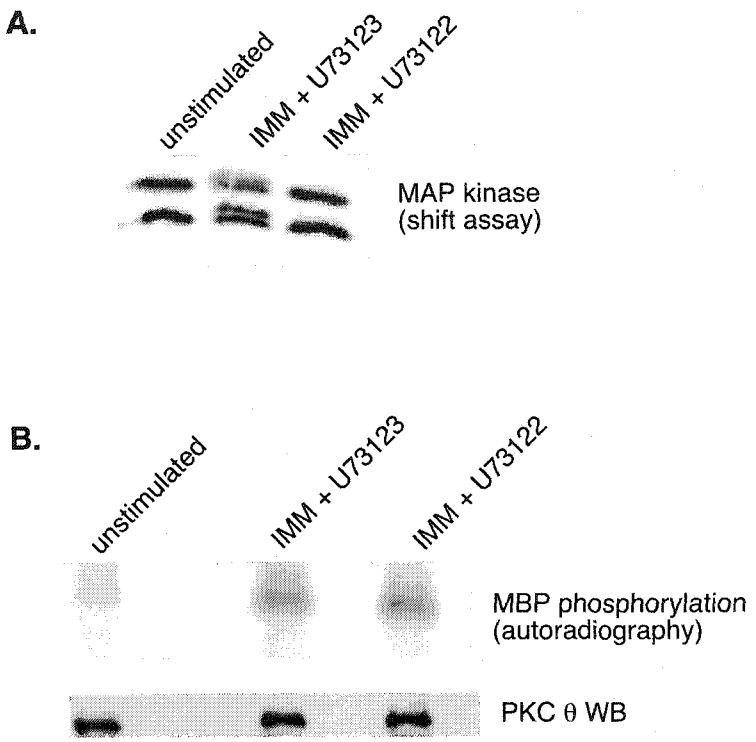

Figure VI-8 A PLC inhibitor does not block PKC θ activity.
Cells were stimulated on plastic-immobilized 145-2C11 (IMM) in the presence of U73122 PLC-γ inhibitor or U73343 non-inhibitory analogue control. *(A)* Whole cell lysates were assayed for MAP kinase activity by gel mobility shift assay. *(B)* From the same pool of cells, PKC θ *in vitro* kinase activity was assayed. The same membrane was used for PKC θ western blot (WB) to verify that similar amounts of PKC θ were present in all reactions.

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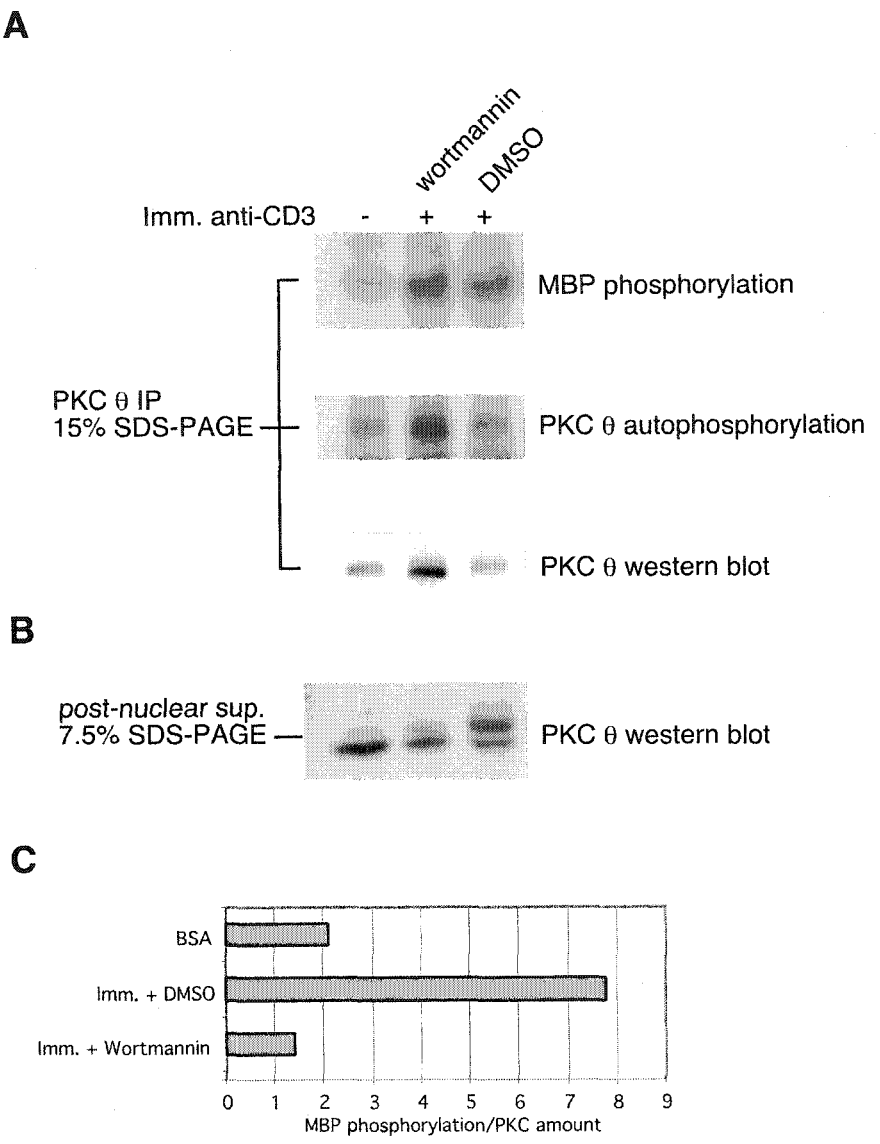

Figure VI-9 Induction of PKC θ kinase activity is PI3K-dependent. *(A),* AB.1 cells were pretreated for 25 minutes with 50 nM wortmannin or an equivalent volume of DMSO, then stimulated by immobilized 145-2C11 anti-CD3 mAb. PKC θ was immunoprecipitated and kinase reactions performed with MBP as an *in vitro* substrate. Incorporation of ^{32}P was detected by autoradiography. The same membrane was immunoblotted to determine the amount of PKC θ recovered in each immunoprecipitate. *(B),* Aliquots of pre-immunoprecipitation sample were analyzed by 7.5% SDS-PAGE and anti-PKC θ immunoblot. *(C),* Bands from autoradiography and anti-PKC θ Western blot were quantitated by scanning optical densitometry and the amount of ^{32}P incorporation relative to the amount of PKC θ detected was calculated.

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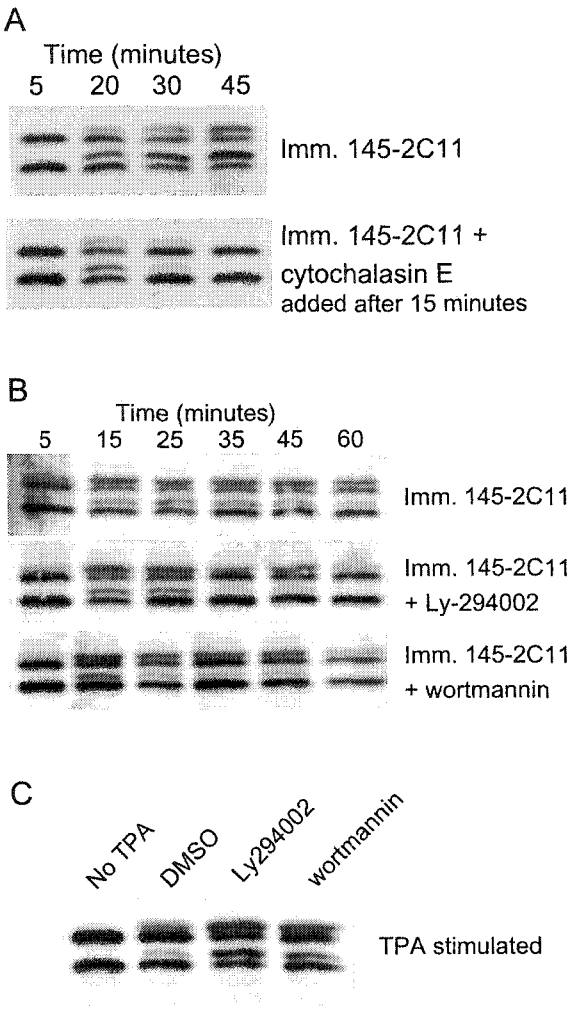

Figure VI-10 Sustained MAPK activation requires an intact cytoskeleton and PI3-kinase activity.
Activation of the MAP kinases ERK1 and ERK2 was determined by gel mobility shift assay. *(A),* AB.1 cells were stimulated on plate-immobilized 145-2C11 (anti-CD3) mAb for the indicated times with or without the addition of 10 μM cytochalasin E. *(B),* AB.1 cells were pretreated with 10 μM Ly-294002 or 50 nM wortmannin, then stimulated by plate-immobilized 145-2C11. *(C),* AB.1 cells were pretreated with 10 μM Ly-294002, 50 nM wortmannin, or DMSO carrier control then stimulated by the addition of TPA. The samples analyzed in part *A* were provided by Laura Mireau.

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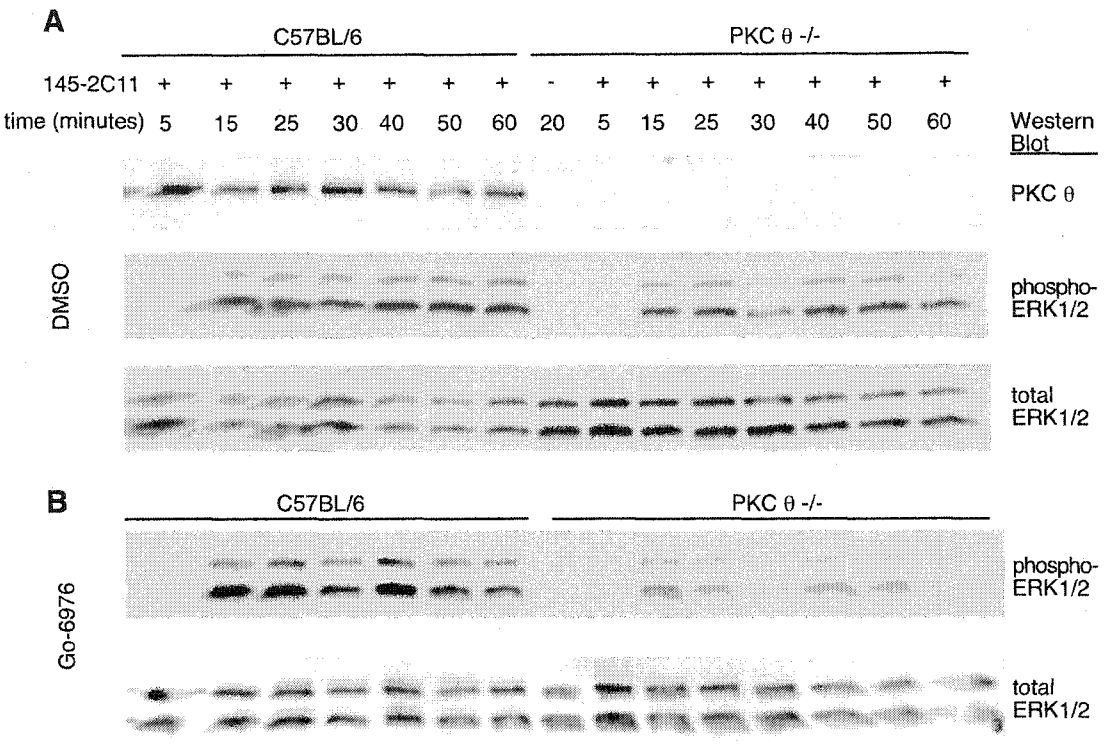

Figure VI-11 ERK activation in splenic T cells is sensitive to an inhibitor of cPKC activity in PKC $\theta^{-/-}$ but not in PKC $\theta^{+/+}$ mice.
Splenic T cells were prepared from PKC $\theta^{-/-}$ mice and from C57BL/6J PKC $\theta^{+/+}$ mice. Cells were pretreated with (A) DMSO carrier control or (B) cPKC inhibitor Gö-6976 prior to stimulation on plastic-immobilized 145-2C11 anti-CD3 mAb. Active ERK was detected by anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) mAb 9106.

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CHAPTER VII General Discussion

A. Summary of Results

The MAP kinases are an ubiquitous and important family of signaling proteins. The present study examined the regulation of the MAPKs ERK1 and ERK2 in the context of CTL activation and function. ERK activation was strongly correlated with activation of CTL effector function. ERK activation was sustained under all conditions that resulted in CTL degranulation and was transient or absent under all conditions that failed to induce degranulation. Treatment of CTL with a compound that inhibits the MAP kinase kinase MEK inhibited CTL degranulation. PMA treatment triggers ERK activation but not degranulation (Berg et al., 1998). This data indicates that MAPK activation is necessary but not sufficient for CTL degranulation.

The molecular mechanism of Ras and ERK activation in T cells has been controversial ever since the first demonstration that these signaling proteins are activated downstream of the TCR. This question is of more than purely academic significance, as the existence of T cell specific mechanisms of Ras/ERK activation might allow for the creation of immunosuppressive or immune-enhancing therapies with minimal side-effects on non-immune cells. At least five mechanisms of TCR-mediated Ras activation have been proposed (Figure VII-1). In the present study, using anti-CD3 mAb coated plastic surfaces as a means of TCR stimulation, ERK activation was found to be completely ablated by compounds which inhibited diacylglycerol (DAG) production or function. This suggests that in CTL, Grb2-mediated pathways to Ras (Figure VII-1A-C) are less important than DAG-mediated

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pathways to Ras (Figure VII-1D-E). Previous studies have attributed the effects of DAG and PMA to PKC. I found that the PKC inhibitor BIM selectively diminished the late phase of ERK activation (Figure VII-2) in adherent CTL. This indicates that PKC is important for the sustained phase of ERK activation but also indicates that a non-PKC activity that is PLC-dependent and DAG-dependent, is involved in early ERK activation. This data is consistent with a role for the novel DAG-responsive Ras regulator, RasGRP (Ebinu et al., 1998; Dower et al., 2000; Ebinu et al., 2000). Some previous studies had suggested that the role of PKC in TCR-mediated ERK activation is minor (Izquierdo et al., 1992; Izquierdo et al., 1994). However the present evidence demonstrates that PKC function is essential for late stage ERK activation in adherent CTL.

In suspension-stimulated cells, a distinct PKC-dependent pathway was found to be required for TCR-mediated ERK activation. In suspended CTL, treatment with PKC inhibitor blocked the entire time course of ERK activation (Figure VII-2). PKC inhibitor blocked ERK activation in suspended CTL without inhibition of Ras activity. In contrast, Ras activation was affected by PKC inhibitor treatment in anchored cells. Furthermore, different PKC isoforms were required for ERK activation in suspended *vs.* anchored CTL. ERK activation in suspended CTL was sensitive to inhibitors of cPKC, whereas PKC-dependent ERK activation in anchored cells was only sensitive to compounds that also blocked nPKC (Figure VII-2).

Several studies have demonstrated that the θ isoform of PKC plays specific roles in T cell activation. However, PKC θ had not been extensively characterized in non-transformed T cells. Examination of PKC θ in CTL clones revealed several

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interesting aspects of PKC θ regulation. PKC θ activity was only recovered from cells exposed to an immobilized TCR/CD3 stimulus and not from cells stimulated in suspension. Suspension stimulated CTL contained PKC θ that was membrane-associated and PKC θ that was phosphorylated but these attributes were apparently insufficient for enzymatic activity. PKC θ kinase activity was diminished by inhibitors of PI3K but surprisingly, appeared unaffected by an inhibitor of PLC. PKC θ activity was shown to be sensitive to known inhibitors of nPKC activity and insensitive to an inhibitor of cPKC activity. In anchored T cells, sustained TCR-mediated ERK activation was found to be sensitive to inhibitors of nPKC whereas in suspended T cells, ERK activation was sensitive to inhibitors of cPKC. Given that sustained ERK activation appears to be due to a nPKC activity not present in suspension stimulated cells, and that inhibitors of PI3K or nPKC were found to inhibit both PKC θ activity and late phase ERK activity, sustained ERK activation may be linked to PKC θ activity. In direct support of this model, it was found that ERK activation in PKC $\theta^{-/-}$ mice but not PKC $\theta^{+/+}$ mice was sensitive to a cPKC inhibitor indicating that the nPKC-dependent pathway of ERK activation was lost in the absence of θ.

MAP kinase and Integrins

Integrins promote or enhance T cell activation through an unknown mechanism. In non-lymphocytes, there is evidence that β1 type integrins can trigger ERK activation, suggesting that integrin-mediated ERK activation might contribute to the enhanced stimulation of CTL in the presence of integrin ligands. However, the present study found no evidence for integrin-mediated ERK activation in CTL clones.

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A mechanism of Grb2 recruitment to the cytoplasmic kinase Pyk2 has been proposed to link TCR ligation to Ras/ERK activation in T cells (Ganju et al., 1997), but the present study found no evidence for Grb2 association with Pyk2 following either TCR or integrin ligation.

B. Points of Special Interest

ERK Regulation by Integrins

In contrast to fibroblasts, plating of CTL onto fibronectin or other ECM proteins did not activate ERKs to any detectable extent, even when β1 integrins were directly ligated by mAb. In fibroblasts, integrin-mediated adhesion causes translocation of MAP kinases to the nucleus (Chen et al., 1994; Aplin et al., 2001). Therefore, MAPK activation in this context may induce changes in gene expression reflecting the change from suspension to an anchored state. CTL are highly motile and can carry out multiple rounds of target cell binding, killing, and release without new protein synthesis. Therefore integrin-induced changes in gene expression may not be important for mature CTL.

Recently it was reported that one of the splice variants of β1 integrin, β1c stimulates FAK phosphorylation but suppresses ERK activation (Fornaro et al., 2000). β1c is normally expressed in non-proliferating, fully differentiated tissues and is downregulated in some carcinomas. Expression of β1c would not completely explain the lack of integrin-mediated ERK activation in CTL since adhesion to fibronectin is mediated by β3 integrins in CTL and anti-β3 stimulation also failed to activate ERKs. Nevertheless, it would be interesting to determine which β1 isoforms are expressed in CTL and whether similar isoform variation exists in β3 integrins.

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Importance of PKC in TCR-mediated Ras-ERK activation

TCR-mediated Ras activation was originally proposed to occur as a result of PKC-mediated GAP inhibition (Downward et al., 1990). However, subsequent studies found only modestly reduced Ras and ERK activation under conditions of PKC inhibition (Izquierdo et al., 1992; Izquierdo et al., 1994). The PKC-mediated GAP inhibition model also failed to explain why PMA treatment did not activate Ras in other cell types (Burgering et al., 1993; Szamel and Resch, 1995). Thus, the role of PKC was considered to be "potential but not predominant" (Izquierdo et al., 1994) or to form a secondary pathway to Ras that ran co-linear to an unknown PKC-independent mechanism (Szamel and Resch, 1995).

These results can be reexamined in the light of the present data and the discovery of RasGRP. PMA-mediated activation of Ras was previously attributed to PKC. However, this activity is likely mediated by RasGRP as RasGRP$^{-/-}$ mice display defective Ras activation in response to PMA stimulation (Dower et al., 2000). Since RasGRP has a relatively restricted tissue expression pattern, this could explain why PMA-mediated Ras activation is not seen in all cell types. To demonstrate DAG-independent Ras activation, Izquierdo *et. al.* utilized buffer conditions that were thought to inhibit PLC. As evidence for PLC inhibition, they demonstrated reduced phosphorylation of an artificial PKC substrate (Izquierdo et al., 1992). However, these investigators had no way of knowing whether these conditions also prevented RasGRP activity.

The present data demonstrates that PKC plays an essential role in initiating TCR-mediated ERK activation in suspended cells and sustaining TCR-mediated ERK

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activation in anchored cells. Why were these roles not detected in the studies of Izquierdo *et. al.*? To investigate whether PKC was important for the regulation of Ras, they stimulated lymphoblasts in suspension culture for 5 minutes in the presence or absence of an inhibitory PKC pseudosubstrate (Izquierdo et al., 1992). My data shows that Ras activation is not affected by PKC inhibition in suspended T cells, but only in anchored cells and only after >20 minutes of stimulation. To examine ERK, Izquierdo *et. al.* stimulated Jurkat T leukemia cells in suspension in the presence or absence of 100 nM Ro-31-8425 (which preferentially inhibits PKC α/β) (Izquierdo et al., 1994). My experiments found that TCR-mediated ERK activation in suspended cells is sensitive to cPKC inhibitors. The lack of inhibition in Jurkat might be attributable to differences between leukemia and non-transformed cells. In the above studies, Jurkat were stimulated by soluble anti-CD3 mAb alone whereas CTL clones require further crosslinking or clustering of soluble anti-CD3 mAb to induce signaling. Jurkat have elevated PI3K activity as a result of PTEN deficiency and exhibit hyper-responsive ERK activation in response to TCR ligation as compared to PTEN sufficient cells (Shan et al., 2000).

The importance of PKC in sustaining Ras-ERK activation is not limited to CTL clones. In addition to operating in *ex-vivo* T cells, PKC has been found to be important in the sustained phase of integrin-mediated ERK activation in fibroblasts (Howe and Juliano, 1998).

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Immobilized vs. Crosslinked: Why is there a difference in the duration of MAPK activation?

Clearly the immobilized and soluble, crosslinked methods of CTL stimulation result in markedly different outcomes with respect to MAPK signaling. What mechanisms may account for this?

A simple explanation for the shorter duration of MAPK signaling with soluble *vs.* immobilized antibody stimulation is that TCR/CD3 receptor downmodulation may occur more rapidly with non-immobilized antibody. In PC12 cells, differences in the rate of receptor downmodulation are proposed to explain the difference in duration of MAPK signaling in NGF *vs.* EGF signaling, which in turn determines whether the cellular response is differentiation or proliferation (Marshall, 1995). This interpretation would explain why XL stimulation cannot be enhanced by immobilized fibronectin. However, some receptors may continue to signal after internalization. One model of T cell activation proposes that activation is directly linked to receptor downmodulation (Valitutti et al., 1995; Viola and Lanzavecchia, 1996).

A second explanation for the difference in MAPK signaling duration is that, by analogy to serum-induced ERK activation in fibroblasts, MAPK signaling in CTL is regulated by cell spreading. Permissive signals indicating successful cell anchorage and spreading may come directly from adhesive receptors or indirectly through sensors of mechanical stress or cytoskeletal tension. Consistent with this model, ERK activation and protein tyrosine phosphorylation signaling are terminated by cytochalasin treatment of anchored cells (Figure VI-10), whereas protein tyrosine phosphorylation in XL stimulated cells is not affected by cytochalasin (Berg et al.,

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1998). This model would also explain the strong ability of fibronectin to facilitate ERK activation in response to very low levels of co-immobilized anti-CD3. Simple observation shows that CTL spread extensively when in contact with target cells or artificial targets and it seems plausible that regulation of cytotoxic activity would be coupled to tight anchorage to targets. However, the data in CHAPTER III demonstrates that providing an adhesive substrate in addition to TCR crosslinking is not sufficient to increase the duration of XL-triggered MAPK activation.

A third possibility is that the localized and directional nature of immobilized as opposed to soluble antibody may be extremely important. The process of T cell activation is accompanied by the formation of a very specific arrangement of cell surface receptors, termed a SMAC (supramolecular activation cluster) (Monks et al., 1998) or an 'immunological synapse' (Grakoui et al., 1999) (the latter term is often used when describing the combined relocalization of both the T cell receptors and their counter-receptors on the target cell). A mature SMAC is characterized by a central zone of TCR and PKC θ surrounded by a ring of LFA-1 and talin. This structure does not form under XL stimulation (Monks et al., 1997), which may explain both why XL does not result in sustained ERK activation and why XL signaling can not be complemented or improved by integrin signaling. However, some controversy remains as to whether the SMAC is a cause or consequence of T cell activation (Lee et al., 2002).

The three possibilities just discussed are not mutually exclusive and all three phenomena may contribute to the results observed.

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Mechanisms of MAPK Activation in Immobilized vs. Crosslinked Stimulated CTL

Although the overall cellular "rationale" for differences in the duration of MAPK signaling remain speculative, my study reveals that two distinct mechanisms of MAPK activation occur in CTL and the mechanism employed depends on the mode of stimulation. IMM stimulation results in sustained ERK activation comprised of a PKC-independent but DAG-dependent phase followed by a DAG-dependent, PKC-dependent phase. The PKC dependent phase requires novel family PKC activity and appears to operate upstream of Ras. In contrast, XL-stimulated ERK activation is dependent on conventional PKC and appears to operate downstream or independently of Ras.

One model of the relationship between MAPK and PKC signaling predicts that MAPK activity should indirectly promote PKC activation, resulting in a feedback loop (Bhalla and Iyengar, 1999) Figure VII-3. This system is predicted to be bistable, meaning that an initial input signal of sufficient strength will generate sufficient feedback to maintain the signaling pathway in its active state. Although this model was produced to study long-term MAPK signaling in neurons, it offers striking similarities to our observations of T cells, where it was found that MAPK activity was not sustained in the presence of PKC inhibitors. In my experiments, PKC inhibition did not prevent initial Ras activation but caused decreased Ras activation at later times after stimulation. Consistent with this, the Bhalla & Iyengar model assumes that PKC-mediated feedback occurs at the level of Ras and Raf. XL-stimulated cells exhibited only very transitory activation of Ras, which was not inhibited by BIM.

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Therefore, XL-mediated ERK activation may be of shorter duration in part because it does not utilize this postulated feedback pathway.

Is the XL-mediated ERK activation pathway a *bone fide* signaling pathway or an artifact of the experimental model? Ras-independent ERK activation has been observed in other experimental systems. For instance, EGF-mediated ERK2 activation in Swiss 3T3 was blocked by dominant negative Ras in combination with PMA-mediated PKC downregulation but not by dominant negative Ras expression alone (Burgering et al., 1993). In contrast, PDGF-mediated ERK2 activation in rat-1 fibroblasts was Ras-dependent (de Vries-Smits et al., 1992). Therefore, even though multiple extracellular stimuli can activate ERKs, multiple mechanisms of ERK activation may allow the cell to mount different responses to different initiators of ERK signaling.

What mechanism accounts for differential PKC isozyme usage under IMM *vs.* XL stimulatory conditions and which isozymes are involved? Since calcium flux and DAG production should activate all PKCs it seems likely that PKC function is limited or determined by subcellular localization of the various isoforms. In T cells, there is a striking relocalization of PKC θ to the same region as engaged TCR. I found that PKC θ was only active following IMM and not XL stimulation. Furthermore, activation of ERK in immobilized anti-CD3 stimulated CTL, which was found to be cPKC inhibitor insensitive in wild type mice, was cPKC inhibitor sensitive in PKC $\theta^{-/-}$ mice, implying that the normal nPKC-dependent pathway of ERK activation requires PKC θ.

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The regulation of PKC θ was also examined. PKC θ phosphorylation was dependent on PI3K (Figure VI-9) and was ablated by cytochalasin treatment (L. Mireau, unpublished results). PKC θ kinase activity was also PI3K dependent. This is consistent with a proposed model which holds that PI3K-mediated actin polymerization is required for the relocalization of PKC θ (Villalba et al., 2000; Villalba et al., 2002). If PI3K activity regulates PKC and PKC is involved in a feedback loop that sustains ERK activation, this would explain why wortmannin treatment of immobilized-stimulated CTL has virtually an identical effect on ERK activation as treatment with the PKC inhibitor BIM. These data are therefore consistent with a model in which PKC θ is specifically required to sustain ERK activation in CTL which in turn is required for full cellular activation and effector function.

C. Future Directions

PKC θ

Although PI3K is thought to regulate the subcellular localization of PKC θ, we were unable to detect large changes in the amount of PKC θ associated with membranes as described in transformed lymphocytes. It is likely that finer techniques such as confocal immunofluorescent microscopy will be required to determine whether perturbations in PI3K activity affect the precise cellular localization of PKC θ.

Although experiments with PKC θ deficient mice provided strong evidence that PKC θ is involved in regulation of ERK in CTL, it would be desirable to develop a system to block PKC θ function in T cells that had developed with PKC θ

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sufficiency. Possibly, the inability to use PKC pseudosubstrate peptides in CTL could be overcome, or an inducible PKC θ knock-out could be constructed.

Integrins, FAK, Pyk2 & ERK

In various cell types, phosphorylation of FAK or Pyk2 appears to trigger ERK activation via Grb2 recruitment. However in CTL stimulation of integrins results in phosphorylation of FAK and Pyk2 without association with Grb2 or ERK activation. Since expression of active FAK permits ERK responses in suspended fibroblasts it has also been proposed that FAK mediates an anchorage signal that enables anchorage-dependent ERK responses. In CTL, CD3 crosslinking in the presence of integrin ligand gave FAK/Pyk2 phosphorylation but no increase in ERK signaling. Future studies might examine site-specific phosphorylation of FAK and Pyk2 to reconcile these observations. Examination of integrin β chain splicing and isoform usage in lymphocytes may be important, since specific integrin isoforms may uncouple FAK phosphorylation from ERK activation (Fornaro et al., 2000). The precise role of FAK and Pyk2 in CTL remains unknown. Identification of FAK or Pyk2 substrates or binding partners should prove enlightening.

PKC & RasGRP

The relationship between RasGRP and PKC, particularly PKC θ, may be interesting especially with respect to the two-phase ERK activation seen in immobilized-stimulated CTL clones and blasting T cells. Due to their relatively restricted tissue expression, RasGRP and PKC θ may provide useful targets for experimental or clinical intervention.

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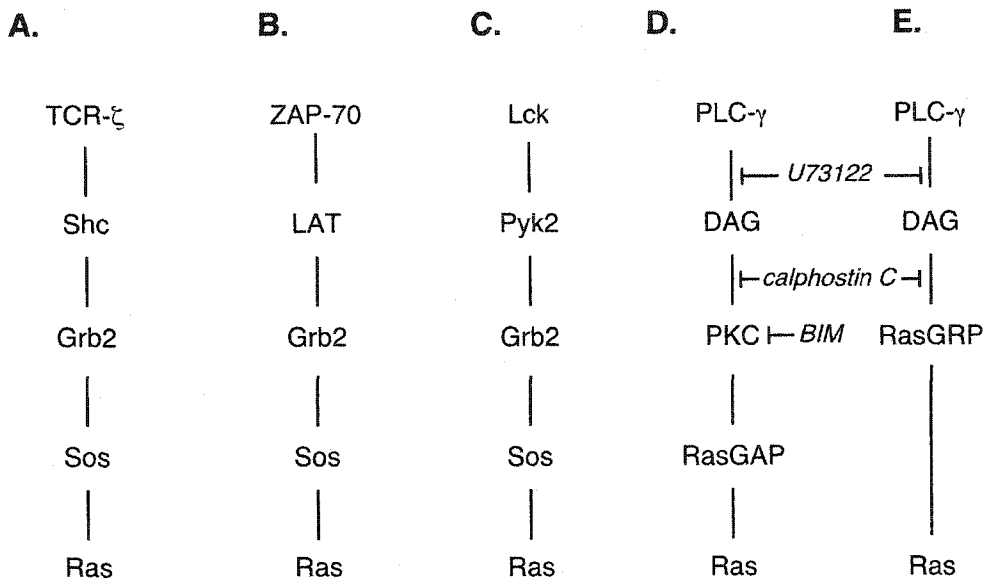

Figure VII-1 Potential mechanisms of TCR mediated Ras activation. This figure is identical to Figure I-3 but additionally indicates some of the inhibitory compounds that were used (shown in italics) and the pathways which they affect. Both U73122 and calphostin C strongly inhibited TCR-mediated ERK activation suggesting that pathways *A* through *C* play a relatively minor role or no role in ERK activation in mature effector CTL. The association between Pyk2 and Grb2 proposed in model *C* could not be detected in CTL clones.

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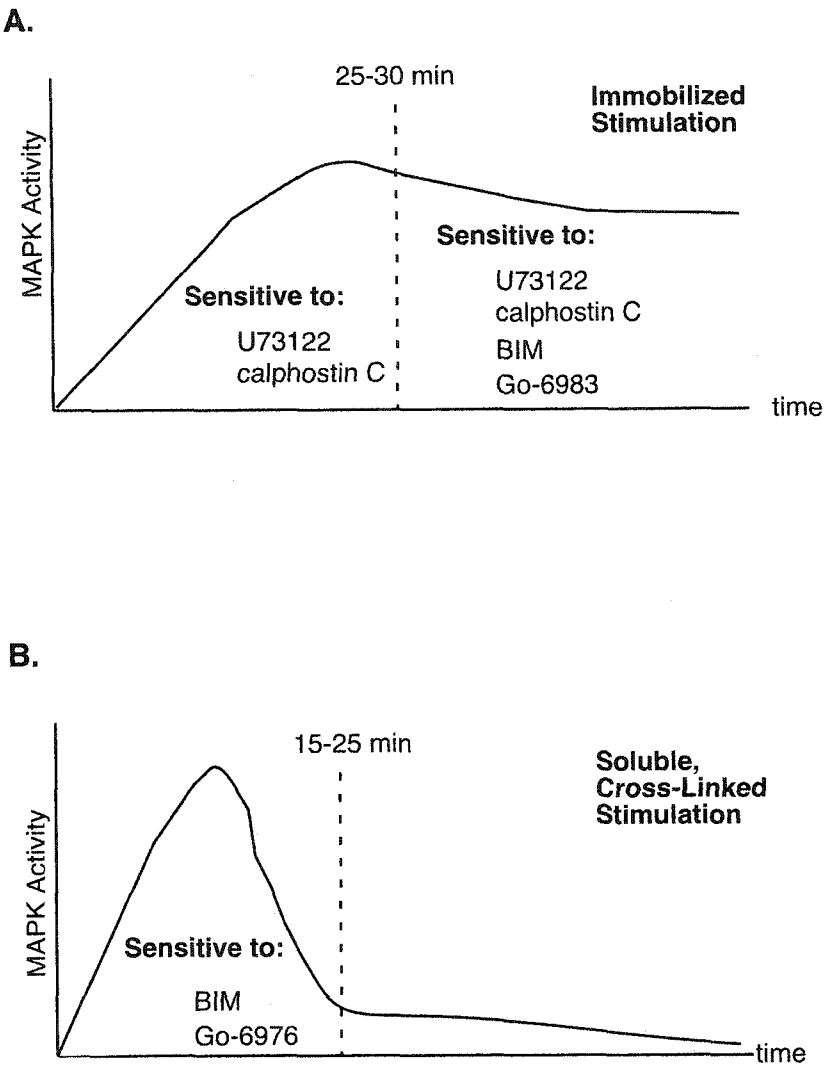

Figure VII-2 Patterns of MAPK activation and inhibitor sensitivity in immobilized *vs.* soluble, cross-linked anti-CD3 stimulated CTL. Diagrams represent the typical pattern of ERK1 and ERK2 MAPK activation seen in CTL under conditions of (A) immobilized and (B) soluble, cross-linked TCR/CD3 stimulation via anti-CD3 mAb. Dashed line indicates division between early and late phases which are distinguished by degree of MAPK activation and by inhibitor sensitivity. U73122 is an inhibitor of PLC function. Calphostin C is an inhibitor of DAG-binding C1 domains. BIM (bisindolylmaleimide) and Go-6983 are inhibitors of both cPKC and nPKC. Go-6976 is an inhibitor of cPKC but not of nPKC.

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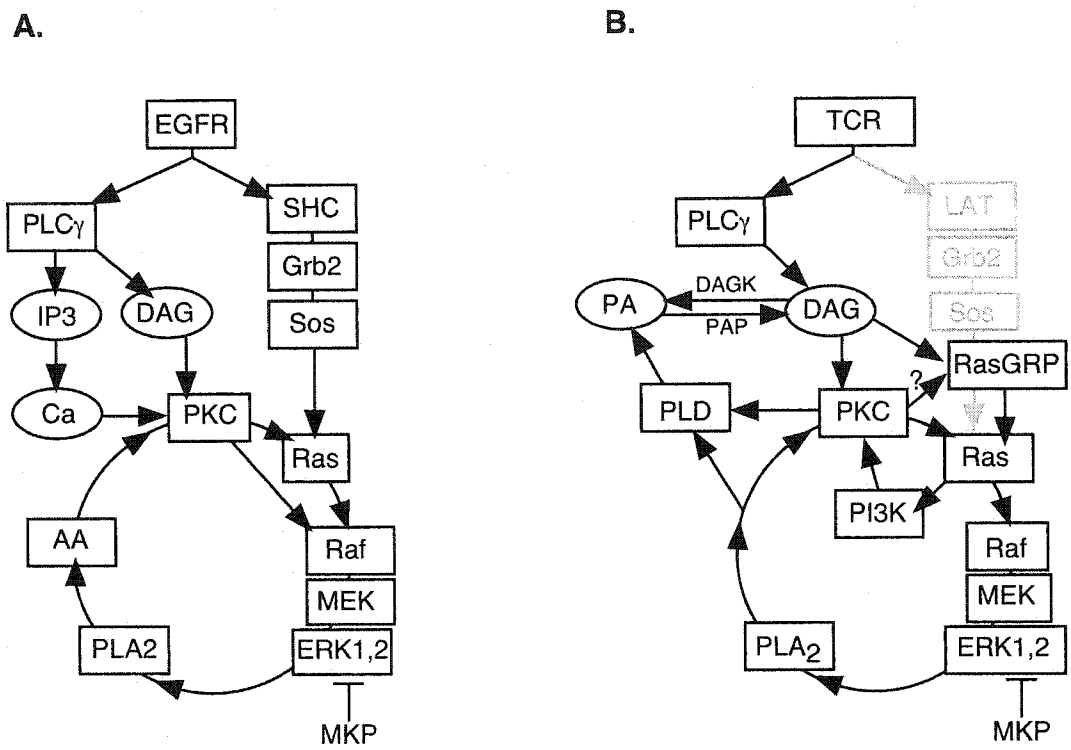

Figure VII-3 Possible feedback loop in EGFR and TCR signaling pathways. *A,* This diagram adapted from Bhalla and Iyengar (1999) depicts the standard model of EGFR-mediated MAPK signaling (Bhalla and Iyengar, 1999). *B,* A model for TCR-mediated Ras and ERK activation. A contribution of the LAT-Grb2-Sos pathway (depicted in gray) was not detected in our system but may contribute to Ras activation under some conditions. The exact mechanism by which PKC acts upstream of Ras is unknown, but might occur through RasGRP (J. Stone, per. comm.), RasGAP (Downward et al., 1990), or an unknown intermediate. PI3K is required for PKC θ activity and may modulate the activity of other PKCs either through PI3K-dependent phosphorylation or through PI3K effects on cytoskeletal architecture which may affect PKC localization. ERK and/or PKC activity may increase activation of PLD which could contribute to continuing production of DAG (Szamel and Resch, 1995) which could in turn prolong PKC or RasGRP-mediated ERK activation. PLD activation can also lead to increased production of phosphatidylglycerol which is a strong activator of PKC θ (Pietromonaco et al., 1998).
Abbreviations: AA, arachadonic acid; DAG, diacylglycerol kinase; DAGK, diacylglycerol kinase; MKP, MAP kinase phosphatase; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PLA2, phospholipase A2; PLD, phospholipase D;

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