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UNIVERSITY OF ALBERTA

REQUIREMENTS FOR STIMULATION AND ACTIVATION OF CYTOTOXIC T LYMPHOCYTES

BY

NANCY NADINE BERG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

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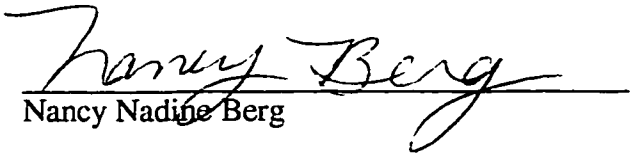

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Requirements for Stimulation and Activation of Cytotoxic T Lymphocytes** submitted by **Nancy Nadine Berg** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Immunology.**

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ABSTRACT

Cytotoxic T lymphocytes (CTLs) are critical to the function of the immune system by eliminating virally infected or altered self cells. Activation of CTLs is a complex process initiated with antigen-specific triggering of the T cell receptor (TCR). Cellular activation, however, involves obligatory contributions from multiple accessory molecules in addition to adhesion and cytoskeleton-mediated events. The CTL must coordinate events at the cell surface with the plethora of intracellular interactions and signalling cascades to generate a response. We examined several aspects of triggering and activation of CTLs.

We investigated the interaction of cell surface integrin LFA-1 with ICAM-1. We demonstrate that ICAM-1 is able to facilitate both degranulation and tyrosine phosphorylation events to a substimulatory TCR/CD3 complex signal. In order to achieve this and to promote conjugate formation between cells and protein-coated latex beads, ICAM-1 and anti-CD3 must be on the same surface. ICAM-1 alone does not generate signalling events such as a Ca^{++} flux and tyrosine phosphorylation. We conclude that the ability of ICAM-1 to lower the threshold of TCR complex stimulation is by enhancing TCR-mediated signalling events through adhesion.

Many proteins contribute to the TCR/CD3-stimulated signal transduction cascades by undergoing tyrosine phosphorylation and engaging in protein-protein interactions. We found that both FAK and Pyk2 were tyrosine phosphorylated and associated with Lck following TCR stimulation. The phosphorylation of FAK and Pyk2 did not appear to rely solely on either Lck or Fyn, however closer examination suggested that there is an undefined role for the Src family of protein tyrosine kinases. Through their association with both cytoskeleton and signalling proteins, FAK and Pyk2 may contribute to T cell activation in an adhesion-dependent manner.

The outcome of triggering the TCR with soluble cross-linked antibody differed from triggering with immobilized antibody. Immobilized antibody was necessary to

generate sustained tyrosine phosphorylation and MAP kinase activation, a mobility shift in Lck, and degranulation. The sustained tyrosine phosphorylation required a functional cytoskeleton.

Together our results further delineate the requirements for TCR triggering and the events necessary for T cell activation and suggest a role for cytoskeleton-mediated adhesion and intracellular signalling complexes.

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I can not think of a way to express my appreciation for the daily comfort, love and support that I have received from my partner, Wojtek. His enduring motivation and assurance have not only helped me finish my Ph.D., but have made the past two years a true pleasure. I am extremely grateful for the unconditional support and encouragement

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ABBREVIATIONS

AA	Amino acid
Ab	Antibody
APC	Antigen presenting cell
APL	Altered peptide ligand
ATCC	American type culture collection
BCR	B cell receptor
BIS	N, N'-Methylene-bis-acrylamide
BSA	Bovine serum albumin
CADTK	Calcium dependent tyrosine kinase
CAKβ	Cell adhesion kinase β
cAMP	Adenosine 3',5'-cyclic monophosphate
CAS	Crk-associated tyrosine kinase substrate
CSK	Carboxy-terminal Src kinase
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytolytic T-lymphocyte-associated antigen
DAG	Diacylglycerol
DNAM-1	DNAX accessory molecule-1
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular regulated kinase
FAK	Focal adhesion kinase
FAT	Focal adhesion targeting
FCS	Fetal calf serum
FN	Fibronectin
FRNK	FAK-related non-kinase

GAH	Goat anti-hamster
GAP	GTPase-activating protein
GRB2	Growth factor receptor-bound protein-2
GST	Glutathione-S-transferase
HEV	High endothelial venules
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IFN	Interferron
Ig	Immunoglobulin
IL	Interleukin
ILK	Integrin-linked kinase
IMM	Immobilized
IP	Immunoprecipitate
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun amino-terminal kinase
LAD	Leukocyte adhesion deficiency
LAT	Linker for activation of T cells
LCK	Lymphocyte-specific cytoplasmic protein-tyrosine kinase
LFA	Leukocyte function-associated antigen
LN	Laminin
mAb	Monoclonal antibody
MAP	Mitogen-activated protein
MEK	Mitogen-activated, ERK-activating kinase
MHC	Major histocompatibility complex
MIN	Minute
MTOC	Microtubule organizing center
MW	Molecular weight
NFAT	Nuclear factor of activated T cells
OD	Optical density

$p70^{S6K}$	p70 ribosomal protein S6 kinase
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PI	Phosphoinositol
PI3K	Phosphatidylinositol 3'-hydroxyl kinase
PLC	Phospholipase C
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PMA	Phorbol-12-myristate acetate
PMSF	Phenylmethylsulfonyl fluoride
PR	Proline rich
PTK	Protein tyrosine kinase
PTPase	Protein tyrosine phosphatase
pTYR	Phosphotyrosine
PYK2	Proline rich tyrosine kinase 2
RAFTK	Related adhesion focal tyrosine kinase
RSB	Reducing sample buffer
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SER	Serine esterase release
SH2	Src homology 2
SH3	Src homology 3
SLP-76	Src homology 2 domain-containing leukocyte protein of 76 kDa
TCR	T cell receptor
Th	T helper cell
TNF	Tumor necrosis factor
VLA	Very late after activation
VN	Vitronectin
XL	Cross-linked

ZAP70 Zeta-associated protein 70

CHAPTER I

Introduction

The immune system is designed to be an exquisitely sensitive system to safeguard against invading microorganisms and alterations to self. The adaptive immune system has the ability to distinguish between self and non-self and can learn from previous experiences, two hallmarks that are critical to its function. In order to protect most efficiently, the immune system consists of two cooperative arms. The humoral or antibody-mediated arm functions to safeguard against extracellular pathogens. Intracellular pathogens are eliminated by the cell-mediated arm which encompasses both T helper (Th) cells and cytotoxic T lymphocytes (CTLs), typically $CD4^+$ and $CD8^+$, respectively. Th cells provide necessary costimulation for B cell antibody production through secreted soluble cytokines.

CTLs function in the immune system to eliminate virally infected cells and cancerous or otherwise altered self cells. Following antigen-specific recognition and conjugation between the CTL and target cell, the CTL is activated and delivers a lethal hit, ultimately resulting in target cell death (Atkinson and Bleackley, 1995).

A. T cell activation

T cells recognize foreign antigens via their cell surface T cell receptor (TCR). During development, the TCR undergoes extensive gene rearrangement to produce a clonally expressed receptor with the overall repertoire of T cells having vast TCR diversity (Jorgensen et al., 1992). The TCR is generated such that it is specific for a small peptide in the context of a major histocompatibility complex (MHC) molecule on antigen presenting cells (APCs) or target cells. The peptides presented by cell surface MHC class I are 8-10 amino acids in length that have been generated from proteolytically cleaved cytosolic proteins (Groettrup et al., 1996). The interaction of MHC/peptide with the TCR is critical

for signal transduction and activation of peripheral T cells in an immune response (Jorgensen et al., 1992). There are specific amino acids within each peptide that are critical contact residues for either the MHC or the TCR (Jorgensen et al., 1992). The TCR/MHC/peptide complex is also essential during selection in the thymus to generate the mature T cell repertoire from immature thymocytes (Jameson et al., 1995).

The TCR is expressed as a complex. It consists of an α and β chain that dictate the specificity of TCR recognition (Garcia et al., 1996; Qian and Weiss, 1997). Importantly, the TCR α/β does not have intrinsic signal transduction capability and hence relies on other complexed subunits for this function. The α/β TCR is associated with the non-polymorphic transmembrane proteins CD3γ/ε, CD3δ/ε, and one dimer of TCR ζ/ζ, ζ/η or ζ/γ, the majority being ζ/ζ (Weiss and Littman, 1994; Qian and Weiss, 1997). Important to future discussions, TCR ζ chains are associated with the cytoskeleton and this requires the integrity of the cytoskeleton (Caplan et al., 1995). Most importantly, each of the γ, ε, and δ chains contain one immunoreceptor tyrosine-based activation motif (ITAM) sequence and each of the TCR ζ chains have three ITAMs. Each ITAM consists of the sequence $YXXLX_{(6-8)}$ YXXL and is the key component of signal transduction from the TCR complex (Weiss and Littman, 1994; Qian and Weiss, 1997).

CD4/CD8

The CD4 and CD8 glycoproteins, typically present on Th and CTLs respectively, are co-receptors that contribute to T cell activation (Weiss and Littman, 1994). Because the specific MHC is engaged by both the TCR and the TCR-associated CD4/CD8, the latter have the dual function of increasing the overall avidity between CTL/target or Th/APC and in signal transduction (Weiss and Littman, 1994; Weil and Veillette, 1996).

CD8 is present on essentially all MHC class I-restricted T cells. The majority of CD8 is a disulfide-linked heterodimer of an α and β chain; the α/α homodimer is a minor species. CD8 is a co-receptor based on its ability to participate in both adhesion and

costimulation. The CD8 cytoplasmic domain binds the protein tyrosine kinase (PTK) Lck which is critical to its co-signalling function (Zamoyska et al., 1989), while CD8-triggered binding to MHC class I contributes to both increased cell-cell adhesion and subsequent signalling (O'Rourke et al., 1993).

Key signalling molecules

One of the earliest events detectable after triggering the TCR/CD3 complex is protein tyrosine phosphorylation. The signal transduction cascade initiated at the TCR complex involves a series of cytoplasmic PTKs including Lck, Fyn, and ZAP70 (Weiss and Littman, 1994; Qian and Weiss, 1997).

p56Lck (Lck) is a Src-family non-receptor PTK associated with the co-receptors CD4 and CD8 (Veillette et al., 1988b). Structurally, Lck has amino-terminal myristylation and palmitylation sites for association with the inner plasma membrane (Figure 1-1A). Lck has a Src homology 2 (SH2) domain, an SH3 domain, and a catalytic domain (Weil and Veillette, 1996). The SH2 and SH3 domains are conserved modules important in numerous signal transduction pathways. They mediate protein-protein interactions with SH2 domains binding peptides containing phosphorylated tyrosine residues and SH3 domains binding poly-proline sequences (Pawson, 1995).

Lck is regulated by two enzymes, Csk (carboxy-terminal Src kinase) and CD45 (Weil and Veillette, 1996). When the tyrosine at position 505 (Y505) is phosphorylated, Lck maintains a catalytically inactive conformation with the Y505 binding to its own SH2 domain (Marth et al., 1988; Gervais et al., 1993). CD45, a transmembrane protein tyrosine phosphatase (PTPase) that is critical for T cell activation (Mustelin, 1994), dephosphorylates Lck Y505 converting Lck to an active enzyme. Lck is negatively regulated by the PTK Csk as it phosphorylates Lck Y505 reverting Lck to an inactive state. Csk also phosphorylates the carboxy-terminal tyrosine in Fyn and other Src-family members (Weil and Veillette, 1996). Another likely mechanism of regulating Lck activity

is phosphorylation at Y394, its autophosphorylation site (Abraham and Veillette, 1990; D'Oro et al., 1996). Additional Lck tyrosine and serine phosphorylation occurs following T cell stimulation, the latter resulting in a decrease in mobility upon SDS-PAGE (Weil and Veillette, 1996). The importance of Lck is underscored by the inability of Lck deficient cell lines to be activated (Straus and Weiss, 1992). In addition, Lck is critical during T cell development as Lck deficient mice have an arrest in early thymocyte development resulting in very few T cells in the periphery (Molina et al., 1992).

A second Src-family non-receptor PTK, p59Fyn (Fyn), has been implicated in T cell activation (Cooke et al., 1991; Weiss, 1993). Lck and Fyn may serve some redundant functions during T cell activation (Weiss and Littman, 1994). Like Lck, Fyn has a myristylation signal, an SH3 and SH2 domain, and a negative regulatory site at Y528 that is regulated by CD45 and Csk. Fyn exists in two isoforms, one highly expressed in brain and one exclusive to lymphoid cells (Rudd et al., 1994). Fyn has been found to associate with the TCR (Samelson et al., 1990), specifically with the CD3ε ITAM sequence (Gauen et al., 1994). Fyn deficient mice have no gross T cell development abnormalities although there are some T cells with reduced signalling responses (Appleby et al., 1992; Stein et al., 1992). Since mice deficient in both Lck and Fyn have a complete arrest of TCR α/β thymocyte development at the double negative ($CD4^-CD8^-$) stage, there is a possible role for Fyn in development (van Oers et al., 1996; Groves et al., 1996).

ZAP70 (zeta-associated protein 70) is a non-receptor PTK that was discovered by its association with the TCR ζ chain (Chan et al., 1992). ZAP70 is a member of the Syk PTK family and contains 2 tandem SH2 domains (Chan et al., 1992; Figure 1-1B). The importance of ZAP70 to the immune system is emphasized by the lack of CD4 and CD8 single positive cells in ZAP70 deficient mice (Negishi et al., 1995).

The TCR-triggered protein tyrosine phosphorylation cascade

Upon TCR triggering, CD45-mediated dephosphorylation and activation of Lck occurs (Weiss and Littman, 1994; Figure 1-2). Importantly, by CD4/CD8 binding to the same MHC as the TCR, Lck is brought into proximity of TCR ζ (Thome et al., 1995). Lck then phosphorylates the TCR ζ ITAM sequences (Chan et al., 1992; Iwashima et al., 1994). CD3γ, CD3ε, and CD3δ also become tyrosine phosphorylated in response to TCR stimulation (Qian et al., 1993). The nature of multiple ITAM sequences within the TCR/CD3 complex may have a signal amplification role (Chan and Shaw, 1995) or each chain may perform a distinct function yet to be defined.

Phosphorylated TCR ζ ITAMs are able to bind to multiple signalling proteins including Shc, Grb2, and the p85 subunit of phosphatidylinositol 3'-hydroxyl kinase (PI3K) (Osman et al., 1996). Most importantly, the phosphorylated TCR ζ chains are able to bind ZAP70 and this is dependent on Lck or Fyn (Chan et al., 1992; Iwashima et al., 1994). There is some fraction of ZAP70 that is constitutively associated with TCR ζ (van Oers et al., 1994). ZAP70 can also bind to phosphorylated CD3γ, CD3δ, and CD3ε ITAM sequences (Osman et al., 1996). An association between Lck and ZAP70 may facilitate Lck to be proximal to its target, the TCR ζ ITAM sequences (Thome et al., 1995). The binding of ZAP70 to TCR ζ involves both tandem SH2 domains of ZAP70 and a doubly phosphorylated ITAM (Iwashima et al., 1994; Hatada et al., 1995).

Following binding to TCR ζ, ZAP70 is itself phosphorylated at multiple residues including Y292, Y492, and Y493 (Watts et al., 1994). ZAP70 Y493 is phosphorylated by Lck and this facilitates ZAP70 activation (Chan et al., 1995). The mechanism by which ZAP70 contributes to T cell activation is undefined. However, the binding of ZAP70 to TCR ζ and the activity of ZAP70 is required for downstream signalling events (Qian et al., 1996). ZAP70 has been shown to bind Lck (Duplay et al., 1994), Fyn (Fusaki et al., 1996), Shc (Milia et al., 1996), Abl, ras-GAP, Vav, SHP, and Cbl (Chan and Shaw, 1995; Qian and Weiss, 1997). These interactions may be important in mediating undefined

downstream signal transduction events. ZAP70 may be negatively regulated by CD45 dephosphorylation (Mustelin et al., 1995) or the ZAP70-associated PTPase SHP-1 (Plas et al., 1996).

More recently, studies with proteins Vav, a hematopoietic GTP-exchange factor (Gulbins et al., 1993) and SLP-76 (Src homology 2 domain-containing leukocyte protein of 76 kDa; Qian and Weiss, 1997) indicate that they are also involved in T cell activation. Both proteins are tyrosine phosphorylated upon TCR stimulation and contain motifs common to signal transduction molecules suggesting that they may be involved in protein-protein interactions during the signal transduction cascades. SLP-76 and Vav have been shown to associate with one another and synergize in mediating NF-AT activity and IL-2 gene transcription (Wu et al., 1996). Additionally, substrates for ZAP70 may include SLP-76 (Wardenburg et al., 1996) and LAT (linker for activation of T cells) (Zhang et al., 1998).

TCR-triggered inositol lipid metabolism

Inositol lipid metabolism results from TCR-triggered activation of phospholipase C γ (PLCγ) and PI3K. TCR stimulation results in the phosphorylation and activation of PLCγ (Weiss and Littman, 1994), possibly by Fyn (Hall et al., 1993) or Lck (Weil and Veillette, 1996). PLCγ catalyzes the breakdown of phosphoinositol 4,5-bisphosphate (PIP_2) to two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is responsible for the increase in intracellular Ca^{++} and DAG for the activation of protein kinase C (PKC), a family of serine/threonine kinases. Both IP_3 and DAG are believed to lead to IL-2 gene transcription. One result of the increased Ca^{++} is activation of Ca^{++}/calmodulin-dependent enzymes such as the phosphatase calcineurin which has also been linked with IL-2 gene transcription (Weiss and Littman, 1994). Activated PI3K phosphorylates phosphoinositols (PI) generating PI_3P, $PI_{3,4}P_2$, and $PI_{(3,4,5)}P_3$, the functions of which are largely unknown. Recent work has started to delineate the

mechanisms by which $PI(_{3,4,5})P_3$ acts in signal transduction via the p70 ribosomal protein S6 kinase ($p70^{S6K}$) and PKB (Downward, 1998). Although activation of PI3K occurs through TCR stimulation, the CD28/B7 interaction is likely the major pathway to PI3K activation (Cantrell, 1996).

T cells require costimulation for proliferation and differentiation (Janeway and Bottomley, 1994). CD28 on T cells interacts with B7-1 and B7-2 on APCs and target cells to generate costimulatory signals that are distinct from those generated by TCR engagement (June et al., 1994). Ligation of CD28 results in its phosphorylation and subsequent association with PI3K leading to phosphoinositol phosphorylation. Signals generated by engagement of both the TCR and CD28 are necessary for optimal PI3K activity and T cell activation (Cantrell, 1996). Cytolytic T-lymphocyte-associated antigen-4 (CTLA-4) is a second CD28 family member that associates with B7-1/B7-2 (June et al., 1994). CTLA-4 also has a PI3K consensus binding site (June et al., 1994), but there is evidence to suggest that CD28 and CTLA-4 having opposing roles in regulating T cell activation with CTLA-4 acting as a negative regulator (Green et al., 1994; Walunas et al., 1994).

MAP kinase pathway

Growth factor receptor and TCR stimulation leads to the activation of Ras, a guanine nucleotide binding protein. Two ways that TCR stimulation could regulate Ras is by inhibition of Ras GTP-ase activating proteins (GAPs) or by recruitment of Sos, a guanine nucleotide exchange protein, to Ras. Upon TCR ligation, Sos associates via its proline rich (PR) region with the SH3 domain of Grb2, an adaptor protein, and this correlates with the activation of Ras. Another guanine nucleotide exchange protein, C3G, interacts with the adaptor protein Crk and may regulate Ras via a C3G/Crk complex. Whatever the mechanism, TCR stimulation of Ras activity is linked to activation of transcription factors, leading to IL-2 and other cytokine gene transcription (Seger and Krebs, 1995; Cantrell, 1996). Adding complexity to the possible regulation of Ras, Shc

and SLP-76 become associated with the Grb2-Sos complex after T cell stimulation (Qian and Weiss, 1997).

TCR activation of Ras leads to the serine/threonine kinase signalling cascade of MAP (mitogen-activated protein) kinases. Activated Ras binds Raf-1 (MAP kinase kinase kinase), which in turn phosphorylates MEK1 and MEK2 (mitogen-activated, ERK-activating kinase; MAP kinase kinase) which then go on to phosphorylate the MAP kinases, ERK1 and ERK2 (extracellular regulated kinase). ERK2 can then translocate to the nucleus and phosphorylate transcription factors such as Elk1 (Cantrell, 1996). This pathway is therefore key to TCR-stimulated proliferation and differentiation.

TCR triggering models

In addition to deciphering the mechanics of the signalling cascades that occur following TCR triggering, recent work has begun to define the thresholds for TCR engagement to achieve activation. It has been suggested that a T cell can be stimulated by very few MHC/peptide complexes with each complex serially engaging multiple TCRs (Valitutti et al., 1995b). In this manner, the T cell may have the capacity to "count" the number of triggered TCRs. What is clear is that a threshold of TCR occupancy is necessary for activation and effector function (Viola and Lanzavecchia, 1996). Overall, TCR occupancy is necessary for sustained signalling and subsequent activation (Valitutti et al., 1995a; Valitutti et al., 1996).

The T cell must coordinate multiple antigen specific TCR-triggered signalling cascades with activatory signals from other cell surface proteins such as CD4/CD8 and CD28. In addition, the T cell must integrate adhesion and signalling contributions from other cell surface molecules, such as integrins.

B. Adhesion

Adhesion is fundamental to the function of the immune system. Lymphocytes require cell-cell and cell-extracellular matrix (ECM) adhesion to circulate and emigrate to lymph nodes which is essential for maintenance of the immune system, immune responsiveness, and for immune surveillance (Springer, 1990). For the same reasons that adhesion is important, adhesion must be regulated; the expression and affinity of both adhesion molecules and their ligands are regulated (Shimizu et al., 1990). Correspondingly, progression of cells from naive to activated memory states correlates with alterations of cell surface levels of adhesion receptors. Expression often increases 2-3 fold after activation and this may be important for localization and reactivation of memory cells (Springer, 1990; Shimizu et al., 1990).

Adhesion and accessory molecules

Adhesion receptor/co-receptor molecules participate in T cell activation in three ways. Firstly, adhesion molecules can contribute to cellular activation by simply increasing adhesion between the two opposing cells allowing more cell surface area to be conjugated and thereby increasing TCR occupancy. Secondly, adhesion molecules can generate costimulatory signals that are distinct from the TCR generated signals. The ligation of CD28 with B7-1/B7-2 is an example. Thirdly, ligation of adhesion molecules can trigger signals that contribute to the same pathways generated through the TCR thereby lowering the threshold for activation. These modes are not mutually exclusive as some adhesion molecules contribute to both adhesion and signalling functions.

Adhesion is mediated by multiple receptor/co-receptor pairs from the integrin family, the immunoglobulin (Ig) superfamily, and the selectin family (Springer, 1990). Briefly, L, P, and E-selectin are adhesion receptors for carbohydrate-containing ligands that regulate leukocyte binding to endothelium at inflammatory sites (Springer, 1990; Albelda et al., 1994). CD2 is present on T cells and its ligand is leukocyte function-

associated antigen-3 (LFA-3); both are members of the Ig superfamily and mediate adhesion that is increased with T cell activation (Springer, 1990). CD2 may also transduce a signal which augments the signal generated via the TCR (Collins et al., 1994). Therefore CD2/LFA-3 contributes both adhesion and augmentation of TCR-specific signals. More recently cloned accessory molecules from the Ig superfamily include DNAM-1, implicated in adhesion during CTL-mediated cytotoxicity (Shibuya et al., 1996), and SLAM, implicated in enhancing antigen-specific proliferation and cytokine production in a CD28-independent manner (Cocks et al., 1995).

Cell adhesion molecules mediate the events of the adhesion cascade responsible for inflammation. Neutrophil emigration from circulation to sites within interstitial tissues involves the sequential steps of rolling mediated by the selectin family, firm adhesion mediated by the β2 integrins, and transendothelial cell migration mediated by molecules such as PECAM-1, the platelet-endothelial cell adhesion molecule-1 (Albelda et al., 1994). Lymphocytes bind and undergo transendothelial migration at specialized postcapillary vascular sites called high endothelial venules (HEV). This is important for lymphocyte-specific migration within lymphoid organs and recirculation. Like neutrophils, this involves sequential L-selectin and integrin-mediated events (Girard and Springer, 1995).

Cell adhesion molecules are critical for mediating cell-cell conjugation, specifically between CTLs and target cells. Initially there is antigen-independent adhesion between a CTL and a potential target during immune surveillance and this adhesion is not sufficient to activate the CTL. In the presence of specific antigen and TCR cross-linking, tight adhesion and co-signalling mediated by adhesion molecules leads to T cell activation (Shimizu et al., 1990).

Cell adhesion and the cytoskeleton are intimately linked. A functioning cytoskeleton is required for cell adhesion, shape, movement, and maintenance of cell polarity. Not only does the cytoskeleton influence adhesion, but there is evidence that receptor-triggered signalling regulates the state of the cytoskeleton. The sites within the cell

where the cytoskeletal filaments anchor are at the cell-cell or cell-ECM contact regions which will be discussed in the context of focal adhesions (Pavalko and Otey, 1994).

It is clear that multiple receptor/co-receptor pairs are needed to generate the threshold of necessary activation signals that the cell requires. Not only does adhesion influence T cell activation, but activation influences adhesion (Dustin and Springer, 1989). One way in which adhesion can link the outside and inside of the cell is via transmembrane integrins (Pavalko and Otey, 1994).

C. Integrins

The integrins are a key family of adhesion molecules. They are glycoprotein heterodimers composed of transmembrane α and β subunits, noncovalently associated through their extracellular domains with short cytoplasmic tails (Springer, 1990; Hynes, 1992). There are at least 8 β and 16 α subunits paired in various combinations and divided into three families based on the β subunit expressed: β1 (CD29), β2 (CD18), and β3 (CD61) (Springer, 1990; Pavalko and Otey, 1994). All β subunits have a 4-fold repeat of a cysteine-rich segment thought to be internally disulfide linked and all α subunits have a 7-fold repeat (Asp-X-Asp-X-Asp-Gly-X-X-Asp or related) thought to participate in divalent cation binding necessary for integrin function. Some α subunits have an extra segment of 180 amino acids called the I (inserted) domain. Cumulative data suggests that both integrin subunits are involved in ligand binding. Some integrin molecules recognize only one ligand, whereas other integrins bind multiple ligands. In addition, one ligand may bind multiple integrins and one cell may express multiple integrins (Hynes, 1992). As with other adhesion molecules, integrin expression is highly regulated (Springer, 1990).

Integrins mediate cell-cell and cell-ECM adhesion (Hynes, 1992). Integrins can localize to focal contacts which are areas of interaction between the cell membrane and the ECM in tissue culture (Springer, 1990). Binding to ECM regulates many cellular responses including anchorage-dependent cell growth, cell differentiation, cell activation,

and gene expression (Schwartz, 1992). The integrins also play a role in tissue organization by binding to the ECM in tissues and basement membranes and in leukocyte localization during inflammation (Springer, 1990). A process that is imperative for survival is the control of apoptosis and it appears that integrin-induced cell spreading may regulate apoptosis of epithelial and endothelial cells (Ruoslahti and Reed, 1994).

The β1 and β3 integrin families are the receptors for ECM proteins. The more extensively characterized β1 integrins are also called the VLA (very late after activation) molecules (Springer, 1990). For example, VLA-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$)bind fibronectin (FN) and VLA-6 ($\alpha_6\beta_1$)binds laminin (LN) (Shimizu et al., 1990). VLA-4 can also bind cell surface VCAM-1 to mediate lymphocyte-endothelium interactions, such as during recirculation (Springer, 1990).

The β2 integrins include LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18). LFA-1 is necessary for T cell recognition of target cells or APCs while Mac-1 and p150,95 are important in adhesion of myeloid cells either to other cells or to ligands during activation of complement and clotting factors (Springer, 1990). The importance of the β2 integrins to the function of the immune system is realized in patients with LAD (leukocyte adhesion deficiency). LAD patients are characterized by a mutation in the β2 integrin subunit that results in recurring infections that are often fatal (Springer, 1990; Shimizu et al., 1990).

Integrin avidity is regulated by 'inside-outside signalling'. This refers to the changes that occur intracellularly following TCR stimulation that translates to a change in the affinity of the integrin for its extracellular ligand, possibly due to a conformational change (Springer, 1990). The mechanism for this is unknown. This was originally shown for LFA-1 binding to ICAM-1 (Dustin and Springer, 1989), but is also true of VLA-4 and VLA-5 for FN and VLA-6 for LN (Springer, 1990). CD8/class I MHC (O'Rourke et al., 1993) and CD2/LFA-3 (Springer, 1990) adhesion appears to be similarly triggered.

Integrins may be the essential link between the adhesion-mediated functions in the immune system and the cytoskeleton. Cytoskeleton proteins talin, α-actinin, and vinculin have been shown to interact with integrin cytoplasmic tails (Pardi et al., 1992; Pavalko and Otey, 1994) and antibodies against integrins induce cell aggregation resulting in the reorganization of the cytoskeleton to areas of cell-cell contact (Sanchez-Mateos et al., 1993). Collins et al. (1994) suggest that the integrin/cytoskeleton association could influence integrin avidity and therefore adhesion by localizing integrins into a stable network, by recruiting additional integrins, or by stabilizing integrins in the high-avidity state.

T cells binding to FN or LN are able to proliferate in response to an otherwise non-stimulatory anti-CD3 signal (Shimizu et al., 1990). Similar results were found for ICAM-1 (van Seventer et al., 1990). The different integrins appear to have distinct capacities for signal transduction. Events that have been demonstrated to occur upon ligand engagement by different integrins include elevation in intracellular pH and Ca^{++}, enhanced tyrosine phosphorylation, activation of protein kinases, phospholipid metabolism, gene expression, and activation of MAP kinase and JNK (c-Jun amino-terminal kinase) cascades (Richardson and Parsons, 1995; Schlaepfer and Hunter, 1996a). Integrins may facilitate these signalling events by organizing the cytoskeleton and associated proteins at nucleation sites during ECM binding as discussed above or as *bona fide* signalling receptors (Juliano and Haskill, 1993). Integrins have been linked to the formation of focal adhesions and to the activation of the Ras/MAP kinase signalling pathway, as will be discussed.

D. LFA-1/ICAM-1

LFA-1 ($\alpha_L\beta 2$) is a member of the integrin family and is found on all leukocytes (Springer, 1990). The intercellular adhesion molecule (ICAM) family of transmembrane glycoproteins are the LFA-1 receptors; ICAM-1 (Rothlein et al., 1986), ICAM-2 (Staunton et al., 1989b), and ICAM-3 (de Fougerolles and Springer, 1992) are found on

hematopoietic and non-hematopoietic cells. Interestingly, ICAM-1 has also been shown to be the receptor for rhinoviruses (Staunton et al., 1989a). As a member of the Ig superfamily, ICAM-1 has 5 Ig domains each consisting of 90-100 amino acids stabilized with a disulfide bond (Springer, 1990). The LFA-1/ICAM-1 interaction mediates adhesion of multiple leukocytes (Makgoba et al., 1988a), enhances proliferation of T cells (van Seventer et al., 1990; Kuhlman et al., 1991), localizes T cells to sites of active immune responses, and is necessary for CTL-mediated killing (Springer, 1990). Important to its function in the immune system, ICAM-1 expression is upregulated during inflammation in response to lipopolysaccharide, interferon-γ (IFNγ), IL-2 and tumor necrosis factor (TNF) (Springer, 1990). Inhibition of T cell activation using antibodies against either LFA-1 or ICAM-1 emphasizes the importance of the LFA-1/ICAM-1 interaction (Dougherty and Hogg, 1987; Dougherty et al., 1988; Makgoba et al., 1988b).

ICAM-2 and ICAM-3 have been less well studied. ICAM-2 also binds LFA-1, but its expression levels do not change with inflammatory mediators (de Fougerolles et al., 1991). However, like ICAM-1, there is ICAM-3-mediated adhesion following cell activation and ICAM-3 can costimulate for proliferation (de Fougerolles et al., 1994). A combination of antibodies against ICAM-1, -2, and -3 results in complete inhibition of binding to purified LFA-1 (de Fougerolles et al., 1994).

The regulation of binding of LFA-1 to ICAM-1 is very interesting. Although resting cells exhibit little adhesion to ICAM-1, cross-linking the TCR results in increased adhesiveness of LFA-1 for ICAM-1 (Dustin and Springer, 1989). This is referred to as 'inside-outside signalling' or avidity regulation, as discussed above. This has been accepted as a conversion of LFA-1 to a high avidity state (Dustin and Springer, 1989). Through affinity measurements, an actual increase in affinity of LFA-1 for ICAM-1 following PMA stimulation has since been shown (Lollo et al., 1993). ICAM-1 appears to be constitutively avid for LFA-1 (Dustin and Springer, 1989).

There is additional complexity to the regulation of LFA-1 adhesiveness (Springer, 1990). One possibility is that avidity regulation may be a result of integrin conformational change following binding of divalent cations (Lub et al., 1995). It was found that both Mn^{++} and Mg^{++} promoted the binding of T cell LFA-1 to ICAM-1. The removal of Ca^{++} was also necessary for increased binding suggesting that Ca^{++} may serve to maintain LFA-1 in an inactive state (Dransfield et al., 1992). Additionally, the I domain of the LFA-1 α subunit, amino-terminal to the divalent cation binding domain, is necessary and sufficient for binding to ICAM-1 (Randi and Hogg, 1994; Edwards et al., 1995). Most recent results demonstrate that the LFA-1 I domain binds to ICAM-1 domain 1 at Glu-34 (Stanley and Hogg, 1998). Furthermore, the cytoplasmic domain of the LFA-1 β subunit, specifically the sequence SATTT (AA756-760), is also necessary for binding to ICAM-1 (Hibbs et al., 1991a; Hibbs et al., 1991b). S756 is phosphorylated in response to PMA stimulation, but this is not the mechanism of avidity regulation (Hibbs et al., 1991b). Although the TTT sequence is needed for the LFA-1 extracellular domain to bind to ICAM-1, it is not clear whether this is the mechanism of avidity regulation (Hibbs et al., 1991b).

LFA-1 co-localizes with talin at sites of antigen-specific cell-cell contact (Kupfer and Singer, 1989) and is associated with α-actinin (Pardi et al., 1992). The β2 tail also associates with vinculin (Pardi et al., 1992; Pardi et al., 1995). Not surprisingly, the ability of LFA-1 to bind ICAM-1 and the subsequent adhesion appears to depend on the association of LFA-1 with the cytoskeleton. By using chimeric transfectants of LFA-1 cytoplasmic domains, a link was found between the cytoplasmic domain of β2 required for cell spreading and focal adhesion formation to the adhesiveness of LFA-1 (Peter and O'Toole, 1995).

LFA-1 has been proposed to have a signalling function in T cells, stimulating a Ca^{++} flux and the phosphorylation of PLCγ1 (Kanner et al., 1993) and in neutrophils, stimulating increases in intracellular Ca^{++} levels, LFA-1 expression, and F-actin content (Walzog et al., 1994). However, data comparing the ability of ICAM-1, FN, and class I

MHC to contribute to signalling suggests that ICAM-1 is acting primarily as an adhesion molecule and this is distinct from the contribution to T cell activation mediated by FN and MHC class I (Ybarrondo et al., 1994).

E. Focal adhesions and FAK

Integrins interact with ECM proteins such as FN, LN, collagen, and vitronectin (VN) at sites called focal adhesions. These are the sites where actin filaments interact with the plasma membrane. The focal adhesion provides a link between the exterior of the cell and the intracellular actin cytoskeleton. Linkage is mediated by integrin cytoplasmic domains interacting with proteins such as talin and α-actinin. These proteins then associate with other cytoskeletal proteins such as vinculin, tensin, paxillin, zyxin, and actin resulting in their localization to the cell/ECM interface (Schaller and Parsons, 1993; Pavalko and Otey, 1994; Richardson and Parsons, 1995; Schlaepfer and Hunter 1996a).

The formation of focal adhesions within fibroblasts leads to changes in intracellular Ca^{++} levels and intracellular pH, tyrosine phosphorylation, and gene expression (Schaller and Parsons, 1993). Two substrates for this integrin-induced phosphorylation are paxillin (Schaller and Parsons, 1994) and tensin (Bockholt and Burridge, 1993). In addition to structural proteins, signalling proteins co-localize with integrins in the focal adhesion. One such protein is the focal adhesion kinase, FAK (Schaller et al., 1992). FAK has been primarily characterized in fibroblasts and platelets.

FAK is a 125 kDa non-receptor PTK expressed in most tissues, erythroid cells, platelets, and in B and T cell populations (Schaller and Parsons, 1993). FAK has a central catalytic domain and amino- and carboxy-terminal non-catalytic regions (Figure 1-3A). It does not contain SH2 or SH3 domains, but does contain regions for interaction with SH2/SH3 domain containing proteins such as two proline rich regions (PR1 and PR2) (Hanks and Polte, 1997). The amino-terminal region of FAK can bind peptides of integrin cytoplasmic subunits providing a link between the cell exterior and the signalling machinery

inside (Schaller et al., 1995). The carboxy-terminal region contains the focal adhesion targeting (FAT) region that is required for the recruitment of FAK to focal adhesions (Richardson and Parsons, 1995). Through the FAT domain, FAK interacts with tyrosine phosphorylated paxillin (Tachibana et al., 1995) and talin (Chen et al., 1995), providing another link between signalling and structural proteins. Interestingly, despite the association of FAK with paxillin and FN-induced phosphorylation of paxillin, targeting of FAK to focal adhesions is not due to its association with paxillin (Burridge et al., 1992).

FAK is tyrosine phosphorylated following integrin engagement by ECM or antibody cross-linking (Burridge et al., 1992; Kornberg et al., 1992; Maguire et al., 1995). Although the mechanism by which this occurs is undefined, it has been proposed to be a result of conformational changes in FAK or of integrin oligomerization and clustering (Schaller and Parsons, 1994; Richardson and Parsons, 1995). FAK is also tyrosine phosphorylated following stimulation with lysophosphatidic acid, mitogenic peptides, growth factors, phorbol esters, and through the small GTPase Rho (Hanks and Polte, 1997). Stimulation of G-protein-coupled receptors and PTK receptors (Schaller, 1996) as well as Src-transformation (Guan and Shalloway, 1992) results in FAK tyrosine phosphorylation. In T cells, β_1 integrin- and TCR-mediated signals lead to FAK tyrosine phosphorylation (Maguire et al., 1995). Accumulated data suggests that the actin cytoskeleton is required for FAK phosphorylation (Schaller and Parsons, 1994).

Y397 (YAEI motif) is the FAK autophosphorylation site that becomes phosphorylated following stimulation (Schaller et al., 1994). Whether this phosphorylation is through inter- or intra-molecular autophosphorylation is not clear (Schlaepfer and Hunter, 1996a; Hanks and Polte, 1997). Phosphorylation of FAK correlates with its activation (Richardson and Parsons, 1995), although it appears that the autophosphorylation does not impact the kinase activity (Schaller and Parsons, 1994). Src can bind FAK at phosphorylated Y397 (Schaller et al., 1994). FAK Y576 and Y577 within the kinase domain are also phosphorylated, possibly by Src, and these

phosphorylation events are required for maximum FAK catalytic activity (Calalb et al., 1995). Although it is still unclear, paxillin, tensin (Schaller and Parsons, 1993) and Cas (Tachibana et al., 1997) may be substrates of FAK. A site upstream of FAK Y397 also binds Src and this may serve to regulate the kinase activity of Src as well (Thomas et al., 1998).

FAK is phosphorylated at a number of sites in addition to the sites discussed above including Y407 (Calalb et al., 1995), Y861 (Calalb et al., 1996), and Y925 within the FAT domain (Schlaepfer et al., 1994). Following integrin binding to FN, phosphorylated FAK at Y925 binds to the SH2 domain of Grb2 and MAP kinase is activated (Schlaepfer et al., 1994; Chen et al., 1994). Based on data showing that Src phosphorylates FAK at Y925, Schlaepfer and Hunter (1996b) propose that following integrin stimulation Src binds to phosphorylated FAK at Y397 and Src subsequently phosphorylates FAK at Y925 resulting in Grb2 binding and MAP kinase activation. It is suggested that the small GTPase Rho is involved in the integrin stimulation of MAP kinase (Renshaw et al., 1996). Overexpression of FAK also leads to an increase in FN-induced activation of Src and of MAP kinase, which requires the association of Src and FAK (Schlaepfer and Hunter, 1997). There is evidence, however, that FAK phosphorylation and activation of the MAP kinase pathway are independent integrin-stimulated events (Lin et al., 1997a).

One recently proposed mechanism to regulate FAK activity is by FRNK (FAK-related non-kinase), the carboxy-terminal domain of FAK that is expressed as a separate protein. Overexpression of FRNK results in decreased tyrosine phosphorylation of FAK, paxillin, and tensin. Interestingly, the decrease in FAK phosphorylation does not alter the number of focal adhesions (Richardson and Parsons, 1996). These data, together with evidence that FAK can be phosphorylated in the absence of focal adhesion formation (Lyman et al., 1997), suggests that FAK is not required for the maintenance of focal adhesions. Cultured cells from FAK deficient mice showed reduced motility and increased

focal adhesion formation suggesting that FAK is required for focal adhesion turnover (Ilic et al., 1995).

FAK is implicated in cell spreading and migration (Schaller, 1996) as well as cell survival and proliferation (Hanks and Polte, 1997). Overexpression of FAK leads to increased migration on FN (Cary et al., 1996) and inhibition of FAK phosphorylation is associated with decreased focal adhesion formation and stress fibers (Burridge et al., 1992). Recent evidence suggests that FAK-associated $p130^{Cas}$ (Crk-associated tyrosine kinase substrate; Cas) may be required for FAK-mediated migration on FN (Cary et al., 1998).

Since its initial characterization, FAK has been found in various cell types, is phosphorylated following various stimulation conditions, and binds to a plethora of signalling and cytoskeleton proteins. There is much speculation about the importance of FAK. The protein-protein interactions that occur may be important to cell activation by allowing enzymes and substrates to interact or for the formation of specific protein complexes necessary for downstream signalling events. Some of the interactions are summarized in Figure 1-4.

F. Pyk2

Proline-rich tyrosine kinase 2 (Pyk2) was cloned as a Grb2-associated protein from a human brain cDNA library (Lev et al., 1995). Identical proteins were cloned independently and named cell adhesion kinase β (CAK β; Sasaki et al., 1995), related adhesion focal tyrosine kinase (RAFTK; Avraham et al., 1995), and calcium-dependent tyrosine kinase (CADTK; Yu et al., 1996). Pyk2 is approximately 116 kDa in neuronal cells and has 48% amino acid identity to FAK (60% in catalytic region) (Lev et al., 1995; Sasaki et al., 1995; Avraham et al., 1995). Like FAK, Pyk2 has no SH2 or SH3 domains or myristylation site, but does contain two carboxy-terminal proline-rich regions (Sasaki et al., 1995; Avraham et al., 1995; Figure 1-3B). Pyk2 is expressed in human adult tissues

including spleen and thymus (Avraham et al., 1995), in rat brain and spleen, and in Jurkat T cells (Sasaki et al., 1995). Y402 (YAEI motif) is likely to be the Pyk2 autophosphorylation site (Dikic et al., 1996).

In transfected Cos cells, Pyk2 was found localized to sites of cell-to-cell contact (Saskai et al., 1995). Pyk2 exhibits a diffuse staining pattern in non-transfected cells (Schaller and Sasaki, 1997). Data suggests that the carboxy-terminal domain of Pyk2 may be responsible for the differential localization of FAK and Pyk2 (Zheng et al., 1998). Given the location of FAK in focal adhesions, this implies a key difference in the roles of FAK and Pyk2 in cells. The extreme 88 amino-terminal amino acids of Pyk2 are entirely different from those in FAK (Sasaki et al., 1995). This may also prove to be an important difference between FAK and Pyk2 as it is through this region that FAK is suggested to interact with integrin cytoplasmic tails (Schaller et al., 1995).

Pyk2 is phosphorylated and activated in neuronal cells in response to nicotinic acetylcholine receptor stimulation, membrane depolarization, and bradykinin activation of G-protein-coupled receptors (Lev et al., 1995). Additionally, lysophosphatidic acid stimulates Pyk2 phosphorylation and association between Pyk2 and the SH2 domain of Src (Dikic et al., 1996). Both B cell receptor (BCR) and β1 integrin cross-linking on B cells induces Pyk2 tyrosine phosphorylation (Astier et al., 1997a). Key tyrosine residues are conserved between FAK and Pyk2. Y397 and Y925 of FAK are homologous to Y402 and Y881 of Pyk2 (Schlaepfer and Hunter 1996a).

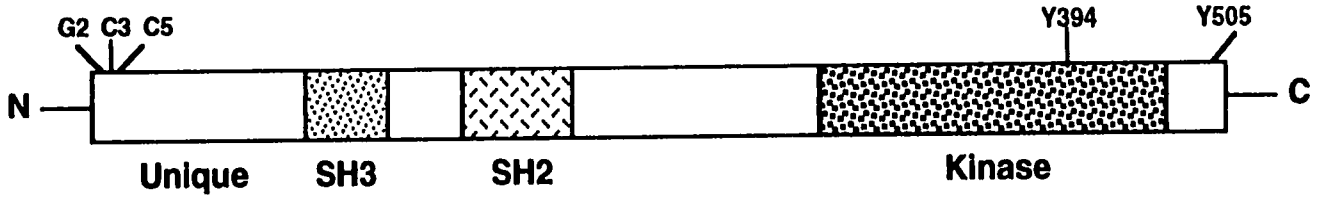
The phosphorylation of Pyk2, like FAK, seems to be dependent on cytoskeleton integrity (Astier et al., 1997a; Ganju et al., 1997). Pyk2 is suggested to be a Ca^{++} regulated enzyme in neuronal cells as these phosphorylation events are dependent on Ca^{++} while a Ca^{++} ionophore can trigger the phosphorylation (Lev et al., 1995). Cas has been identified as a possible substrate of Pyk2 that may require cooperation of associated Src (Astier et al., 1997b).

Analogous to FAK, Pyk2 has been examined for its involvement in the MAP kinase pathway. In neuronal cells, Pyk2 and Grb2 co-immunoprecipitate. In Pyk2 overexpressing cells, Pyk2 co-immunoprecipitates with Sos and leads to an increase in MAP kinase activity (Lev et al., 1995). Furthermore, dominant negative Pyk2 leads to a reduction in MAP kinase activation (Dikic et al., 1996). This leads to the model that Pyk2 is involved in MAP kinase activation via the Grb2/Sos association (Dikic et al., 1996). Finally, overexpression of Pyk2 leads to an increase in JNK activation and stimulation of JNK activation was reduced with a dominant negative Pyk2 (Tokiwa et al., 1996).

Pyk2-associated proteins are beginning to be identified. Pyk2 associates with Cas constitutively (Astier et al., 1997a) and Pyk2 Y402 associates with Src (Astier et al., 1997b). Hic-5, a protein with regions homologous to paxillin, binds the carboxy-terminal domain of Pyk2 (Matsuya et al., 1998). Pyk2 associates with paxillin, but unlike FAK, Pyk2 does not associate with talin (Schaller and Sasaki, 1997; Zheng et al., 1998). A difference in associated proteins may define a unique function for FAK and Pyk2.

Many questions remain as to how triggering the CTL leads to the generation of intracellular signals and a functional response. Understanding both the requirements for achieving a threshold of cell triggering and the intracellular signal transduction cascades is key to understanding CTL function in the immune system. I examined three aspects of CTL stimulation and activation: i) the contribution to CTL signalling and activation of the LFA-1 interaction with ICAM-1, ii) the potential involvement of FAK and Pyk2 during CTL activation, and iii) the requirements for triggering the TCR/CD3 complex with antibody.

A. Lck



B. ZAP70

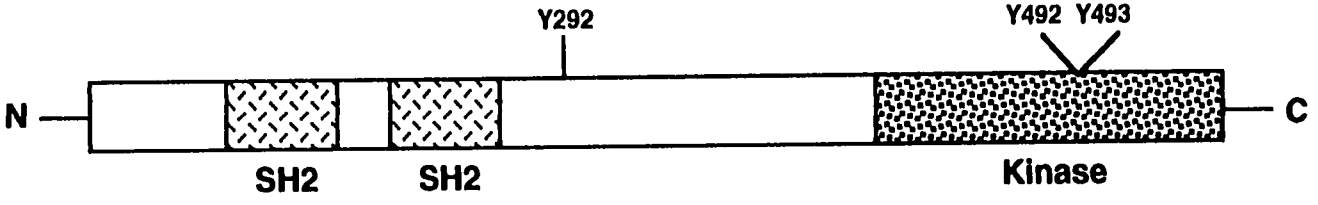
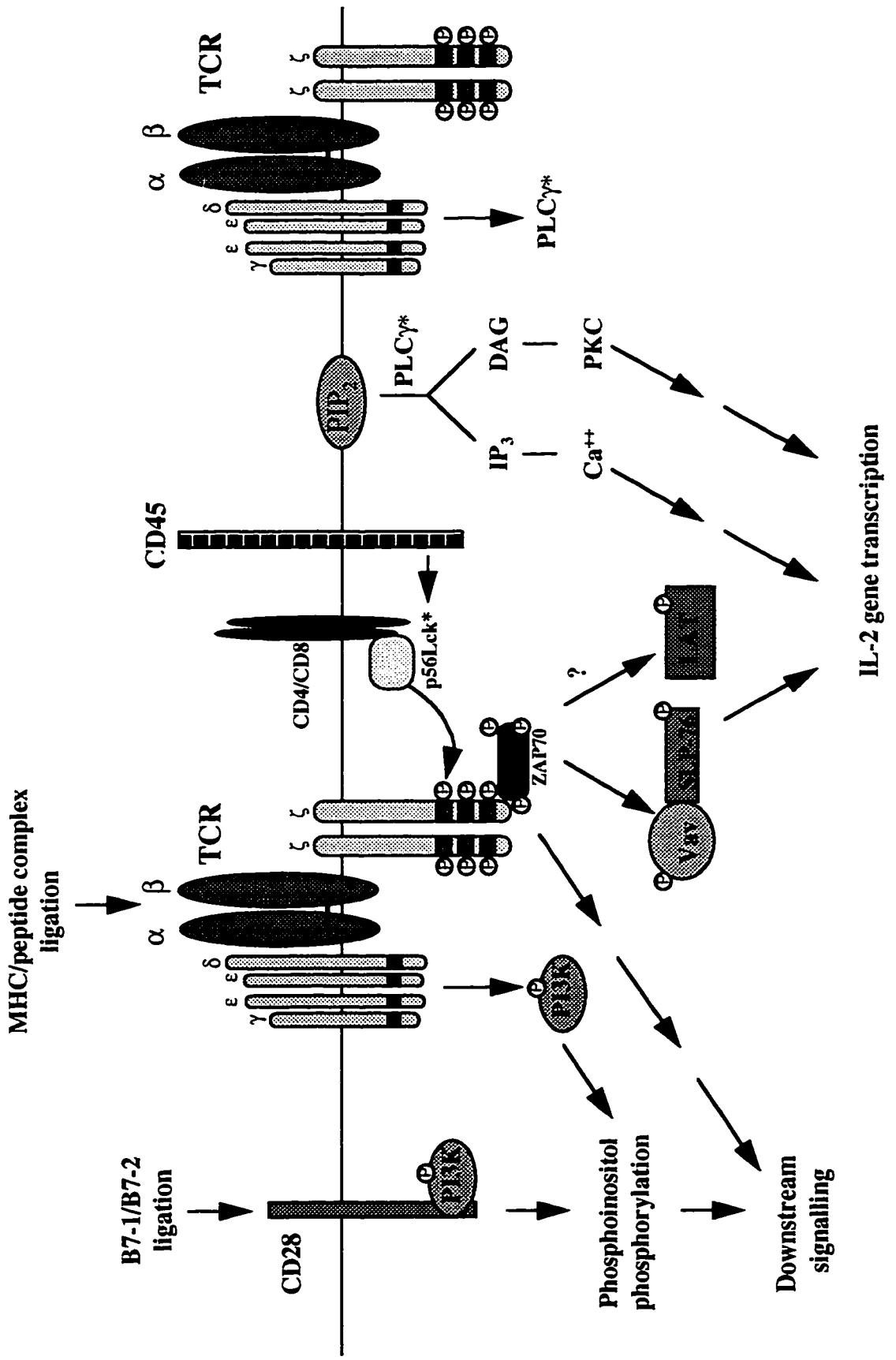
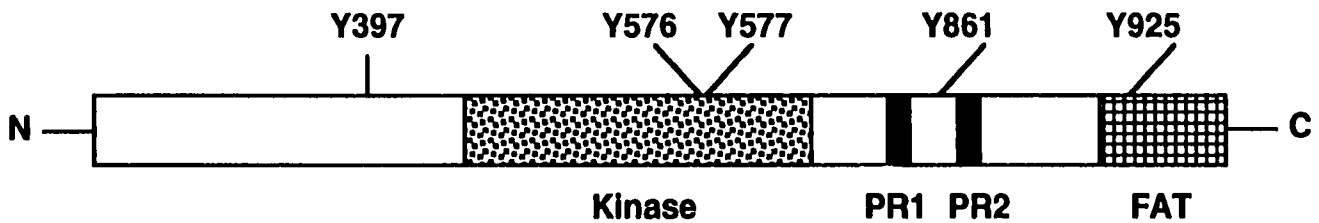


Figure 1-1. Lck and ZAP70 non-receptor protein tyrosine kinases. **A.** The Lck myristylation site is G2 and the palmitylation sites are C3 and C5. Lck binding to CD4 and CD8 is mediated through the unique region. The SH3, SH2, and kinase domains are shown. Lck Y394 is the autophosphorylation site and Y505 is the negative regulatory site. **B.** The ZAP70 SH2 and kinase domains are shown with key tyrosine residues Y292, Y492, and Y493 indicated.

Figure 1-2. T cell signal transduction. Antigen-specific triggering of the TCR results in Lck-mediated tyrosine phosphorylation of the TCR ζ ITAM sequences and subsequent association, phosphorylation, and activation of ZAP70. LAT and SLP-76 are candidate substrates of ZAP70. TCR stimulation also results in the activation (*) of PLCγ which catalyses the breakdown of PIP_2 to IP_3 and DAG leading to Ca^{++} flux and activation of PKC, respectively. The pathways downstream of Ca^{++} and PKC lead to IL-2 gene transcription. Vav and SLP-76 are phosphorylated following TCR stimulation and may also be involved in IL-2 gene transcription. Ligation of both the TCR/CD3 complex and CD28 result in activation of PI3K resulting in phosphoinositol phosphorylation.


MHC/peptide complex ligation
B7-1/B7-2 ligation
CD28
TCR
CD45
CD4/CD8
p56Lck*
ZAP70
PIP2
PLCγ*
DAG
PKC
IP3
Ca++
?
PI3K
Phosphoinositol phosphorylation
Downstream signalling
IL-2 gene transcription

A. FAK



B. Pyk2

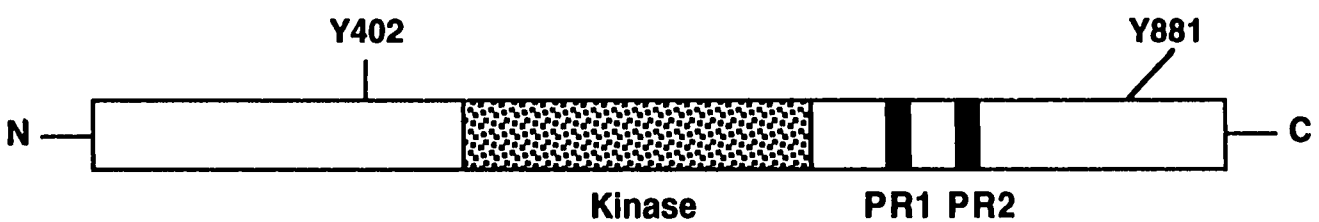
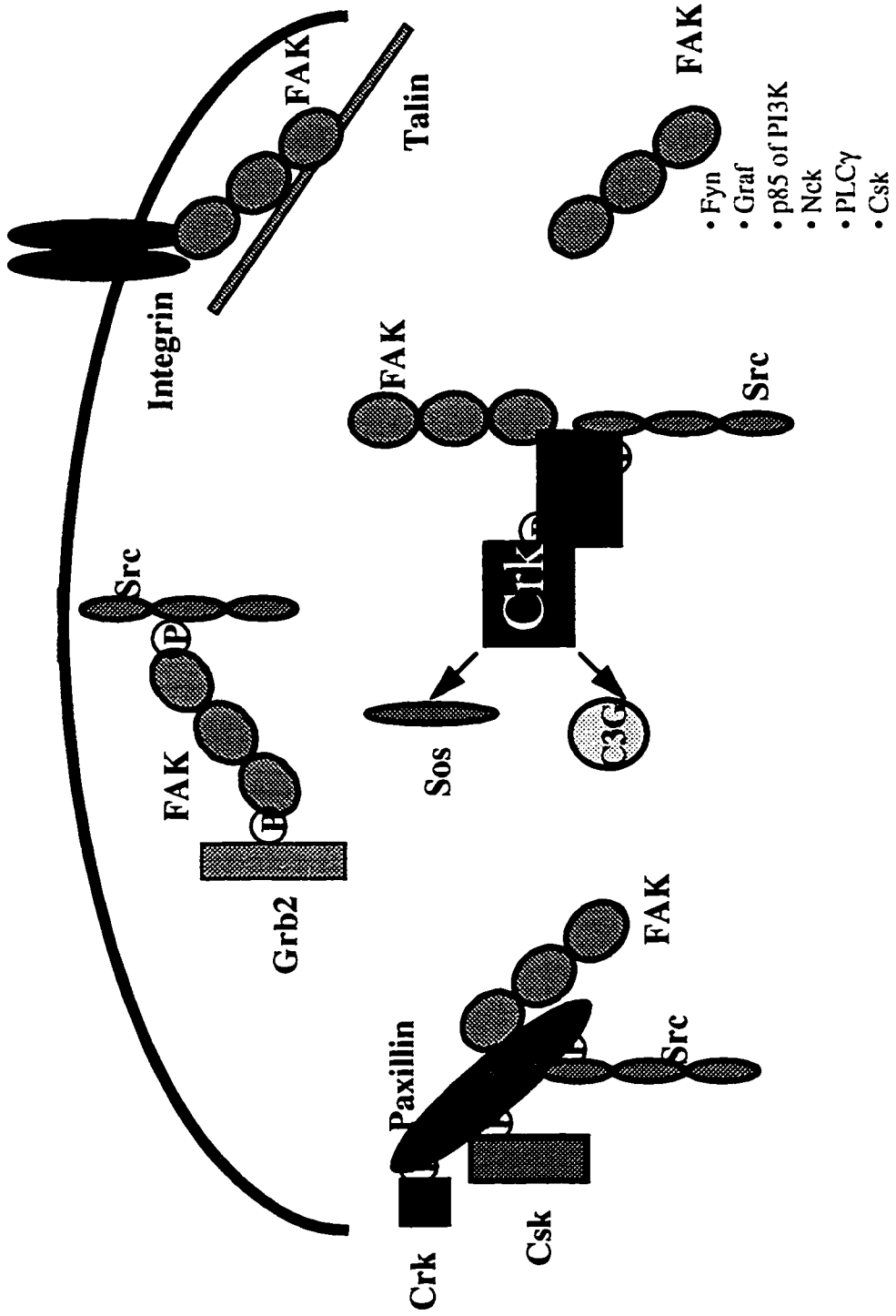


Figure 1-3. FAK and Pyk2 non-receptor protein tyrosine kinases. **A.** The kinase domain, the PR1 and PR2 regions and the FAT domain are shown. FAK Y397 is the major site of autophosphorylation. Other key tyrosine residues are Y576, Y577, Y861, and Y925. **B.** The kinase and PR1 and PR2 domains are shown. Pyk2 Y402 and Y881 are conserved with FAK Y397 and Y925.

Figure 1-4. Association of FAK with signalling and structural proteins. This represents a compilation of data describing interactions in multiple cell types following many stimulation conditions. FAK can bind peptides of integrin cytoplasmic subunits (Schaller et al., 1995) and co-localizes with vinculin in focal adhesions (Schaller and Sasaki, 1997; Zheng et al., 1998). Through its FAT domain, FAK interacts with tyrosine phosphorylated paxillin (Tachibana et al., 1995) and talin (Chen et al., 1995). Paxillin can bind to the SH2 domains of Crk, Src and Csk and the SH3 domain of Src (Schaller, 1996; Hanks and Polte, 1997). Following phosphorylation, FAK Y397 binds the SH2 domain of Src (Cobb et al., 1994) and FAK Y925 binds to the SH2 domain of Grb2 (Schlaepfer et al., 1994; Chen et al., 1994). FAK PR1 and PR2 regions bind the SH3 domain of Cas (Polte and Hanks, 1995; Harte et al., 1996). Phosphorylated Cas, like paxillin, can then bind to the SH2 domain of Crk and to the SH2 and SH3 domains of Src. Crk in turn binds to Sos and C3G, two guanine nucleotide exchange factors (Hanks and Polte, 1997). FAK has also been demonstrated to bind Fyn (Cobb et al., 1994), the SH3 domain of Graf (a GAP for Cdc42 and Rho) (Hildebrand et al., 1996), the SH3 domain of the p85 subunit of PI3K (Chen et al., 1996; Schlaepfer and Hunter 1996a), and the SH2 domains of Nck, PLCγ, and Csk (Schlaepfer et al., 1994).


Integrin
FAK
Talin
Src
FAK
Grb2
FAK
Src
Crk
Sos
C3G
FAK
• Fyn
• Graf
• p85 of PI3K
• Nck
• PLCγ
• Csk
Paxillin
FAK
Src
Crk
Csk

CHAPTER II

Materials and Methods

Cells

Murine $CD8^+$ CTL clones, clone 10/1 (H-2^b anti-H-2^d) and clone 11 and clone AB.1 (H-2^d anti-H-2^b), have been described previously (Kane et al., 1989a; Kane and Mescher, 1993). Murine $CD8^+$ CTL clone IF10 (H-2^b anti-H-2^d) has been described (Atkinson et. al., 1996). Clone IF10 was derived from alloantigen stimulated spleen cells from a Fyn deficient mouse (gift of Dr. Roger Perlmutter, University of Washington). Clones were grown in HEPES-buffered RPMI (Gibco-BRL, Burlington, ON) supplemented with 10% heat inactivated fetal bovine serum (HyClone Laboratories, Logan, Utah), sodium pyruvate, non-essential amino acids, L-glutamine, penicillin-streptomycin, and ß-mercaptoethanol, all from Gibco-BRL. All the clones were stimulated weekly with irradiated spleen cells in media supplemented with IL-2. The spleen cells were from C57BL/6 mice for clone 11 and clone AB.1 and from Balb/c mice for clone IF10 and clone 10/1 (The Jackson Laboratory, Bar Harbor, ME). Experiments were performed 4 to 6 days after stimulation.

The T leukemic cell line Jurkat and the Lck-deficient variant JCaM1.6 were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and were grown in HEPES-buffered RPMI (Gibco-BRL) supplemented with 10% heat inactivated fetal bovine serum (Immunocorp, Montreal, QC).

Antibodies

The hybridomas producing monoclonal antibodies 145-2C11 (anti-CD3ε), H57-597 (anti-TCR), OKT3 (anti-CD3), M17/5.2 (anti-LFA-1), and YN1/1.7 (anti-ICAM-1) were obtained from ATCC (Rockville, MD) and M1/42 (anti-MHC class I, all haplotypes) from Dr. Kevin Kane. I3/2 (anti-CD45) and PY-72 (anti-phosphotyrosine) were obtained

from Dr. I. Trowbridge and Dr. B. Sefton, respectively (The Salk Institute, La Jolla, CA). All hybridomas were grown in Protein Free Hybridoma Medium-II (Gibco-BRL) and the monoclonal antibodies were purified by ammonium sulfate precipitation followed by either protein A or protein G chromatography, if required.

Horseradish peroxidase (HRP)-coupled antibodies, goat anti-hamster IgG, and rabbit anti-hamster IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-MAPK2, anti-p56Lck, and anti-paxillin mAbs were purchased from Transduction Laboratories (Lexington, KY). Anti-GST antisera was purchased from Sigma Chemical Company (Mississauga, ON).

Antiserum to the carboxyl terminus of Lck was generated in our laboratory using a bovine serum albumin (BSA)-coupled peptide based on amino acids 476-509 of human Lck (Arendt et al., 1995).

Reagents

Genistein and herbimycin A were purchased from Gibco-BRL (Burlington, ON). PMA and cytochalasin E were purchased from Sigma. A23187, BAPTA, and PP1 inhibitor were purchased from Calbiochem (San Diego, CA). EDTA was purchased from Fischer Scientific (Nepean, ON) and cellular FN (human) was purchased from UBI (Lake Placid, NY).

ICAM-1 purification

NS-1 B lymphoma cells (ATCC) were grown in DMEM media (Gibco-BRL) with HEPES supplemented with 8% bovine calf serum (HyClone Laboratories) and penicillin-streptomycin (Gibco-BRL). ICAM-1 was purified using immunoaffinity chromatography as described (Kane et al., 1989b). Briefly, approximately 5 X 10^9 cells were harvested and disrupted by nitrogen cavitation. Membranes were isolated by a low speed spin to remove the nuclei followed by a high speed spin to pellet the membranes. The membranes were

detergent solubilized by resuspension in 20 mM Tris / 0.5% Triton X-100 / 2 mM PMSF. Following an ultra-centrifuge spin, ICAM-1 was purified from the supernatants by chromatography as previously described (Welder et al., 1993). Briefly, lysates were passed over a sepharose 4B guard column followed by a YN1/1.7 affinity column. The column was washed with 0.1% Triton X-100 / 0.15 M NaCl / 10 mM Tris, pH 7.5 and then eluted with 0.1% Triton X-100 / 0.15 M NaCl / 50 mM diethylamine, pH 11.0. Fractions were collected into tubes containing 1 M Tris, pH 8.0 to neutralize the samples. Isolated ICAM-1 was subjected to an enzyme-linked immunosorbent assay (ELISA) and fractions showing activity were pooled and stored at -70°C.

ELISA

Wells of 96-well plates were coated with ICAM-1 as described below. YN1/1.7 antibody (1 μg) in 100 μl was added to each well and incubated for 45 minutes at 4°C. Wells were washed 3 times with blocking solution and 100 μl of goat anti-rat^{HRP} (1:1000) was added and incubated for 45 minutes at 4°C. Wells were washed 6 times with blocking solution and 75 μl development solution (0.1 M citric acid / 0.2 M Na_2HPO_4, pH 7.5 / hydrogen peroxide / O-phenyl diamine dihydrochloride) added. Reaction was stopped with 4 N H_2SO_4 and optical density (OD) read at 490 nm in a kinetic microplate reader (Molecular Devices, Palo Alto, CA).

Silver staining

Purified ICAM-1 (20 μl) was run on a standard 7.5% SDS-PAGE. The gel was treated with sequential washes: 1 wash of 5 gel volumes 30% ethanol / 10% acetic acid overnight, 2 washes of 5 volumes of 30% ethanol for 30 minutes each, and 3 washes of 10 volumes of deionized water. The gel was stained with 5 volumes 0.1% $AgNO_3$ for 30 minutes, washed, and visualized after incubation with 2.5% sodium carbonate / 0.02% formaldehyde. Reaction was stopped with 1% acetic acid.

Protein immobilization

96-well flat-bottom plastic microtiter plates (Becton Dickinson, Oxnard, CA) were incubated with 40 µl protein diluted in phosphate buffered saline (PBS; 0.15 M NaCl / 0.5 M $NaPO_4$, pH 7.4). Wells were coated with 145-2C11 or H57-597 at the indicated concentration alone or ICAM-1 was co-immobilized. Plates were incubated at 4°C overnight or at 37°C for 90 minutes. Each well was washed twice with PBS (100 µl/well) and the wells were blocked with 2% BSA in PBS (100 µl/well) at 37°C for 30 minutes. Wells were washed twice as above and were immediately used for assay. Degranulation assays and stimulation of cells for immunoblotting whole cell lysate proteins utilized these plates.

For immunoprecipitation experiments, 60 mm dishes (Fischer Scientific) were coated with 12.5 µg/ml 145-2C11, washed, and blocked with 2% BSA in PBS. Plates coated with BSA alone were used as controls.

Polystyrene latex beads (5 µm) or fluor.sulfate polystyrene latex orange beads (4.07 µm, Interfacial Dynamics Corp., Portland, OR) were coated by adding either 8 µg of antibody, 0.5 µg ICAM-1, or both to 5 X 10^7 beads in 1 ml of PBS. Beads were gently rotated at 4°C overnight then blocked with 500 µl 2% BSA in PBS at room temperature for 30 minutes with rotation. Beads were pelleted by centrifugation and washed 3 times in RPMI containing 2% newborn calf serum. Beads were counted and dilutions made in RPMI with 2% newborn calf serum.

Degranulation

Degranulation, as measured by release of serine esterase, was assayed as previously described (Kane et al., 1989a). Clone AB.1 or clone 11 cells were washed three times by centrifugation in RPMI 1640 with 2% fetal calf serum (FCS). For stimulation with immobilized antibody or protein, cells were added directly to the protein-coated wells at 1.5 X 10^5 cells/well in 150 µl. For antibody blocking experiments,

antibodies were added to cells at a final concentration of 20 μg/ml and incubated on ice for 15 minutes prior to addition to the wells.

For the soluble cross-linked antibody stimulation, cells were incubated with various concentrations of 145-2C11 for 30 minutes on ice, washed, and resuspended in fresh RPMI with 2% FCS. The cells were added to the wells immediately after addition of either 1.0 or 10.0 μg/ml goat anti-hamster IgG. For the assays testing PMA (125 ng/ml) and/or A23187 (50 μM), reagents were added directly to cells or immediately after adding the cross-linking antibody. Cells were then added to BSA-coated wells at 1.5 X 10^5 cells/well in 150 μl.

For experiments utilizing protein-coated beads, the indicated number of beads and cells were added to BSA-coated wells. For the conditions stimulating with ICAM-1-coated and 2C11-coated beads (separate), the same number of ICAM-1-coated beads were present as in ICAM-1-coated bead only stimulation.

In all experiments cells were allowed to incubate at 37°C for 4-5 hours. After incubation, 25 μl supernatant was assayed for benzyloxycarbonil-L-lysine thiobenzyl ester (BLT)-esterase activity (Kane et al., 1989a). Results were read at 405 nm using a kinetic microplate reader (Molecular Devices). All samples were done in triplicate or quadruplicate and the standard deviation shown. All degranulation experiments were repeated at least three times with identical results.

Cell stimulation and Immunoprecipitation

CTL clones were harvested and washed in serum-free D-PBS (Life Technologies, Burlington, ON). For Western blotting analysis of whole cell lysate proteins, clone 11 or clone AB.1 (1.5 X 10^5) cells were added to protein-coated wells in microtiter plates. For experiments testing PP1 inhibition, cells were pretreated with PP1 (10 μM) for 30 minutes on ice before adding to wells. For the soluble cross-linked antibody stimulation, cells were treated as described for the degranulation assay. Cells were allowed to incubate at 37°C for

the indicated times. The cells were lysed with 40 µl 2X Laemmli reducing sample buffer (RSB) and boiled for 2 minutes.

For immunoprecipitation experiments, clone 11 or clone AB.1 (10^7) cells in D-PBS were plated on 60 mm protein-coated dishes. For experiments testing PP1 (10 µM), cells were pretreated for 30 minutes on ice before adding to dishes. After the indicated time, the cells were lysed by the addition of lysis buffer (1.5% NP-40 / 10 mM Tris, pH 7.6 / 150 mM NaCl / 1 mM vanadate / 5 mM EDTA) and proteins from post-nuclear extracts were immunoprecipitated with 10 µl of antisera and Protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden). Beads were washed 5 times with RIPA buffer (50 mM Tris, pH 7.2 / 150 mM NaCl / 1% Nonidet P-40 / 1% deoxycholate / 0.1% SDS) and resuspended in 2X RSB and boiled for 2 minutes. For experiments examining Ca^{++} requirements, cells were pre-incubated with EGTA (4 mM) and $MgCl_2$ (8 mM) on ice for 30 minutes before stimulation or were stimulated in microfuge tubes with A23187 (50 µM) for 2 minutes at 37°C.

Jurkat and JCaM1.6 cells (3 $X10^6$) were stimulated in microfuge tubes with 20 µg/ml OKT3. Post-nuclear lysate (40 µl) was used for the whole cell lysate sample. Immunoprecipitations were performed as above.

For experiments testing the association between FAK and/or Pyk2 and Lck immunoprecipitation was performed using anti-Lck antisera from twice the number of cell equivalents (2 X 10^7). Beads were washed 4 times in RIPA buffer and incubated at 4°C for 1 hour in 100 mM phenyl phosphate (Sigma) in RIPA buffer. Beads were centrifuged and FAK and Pyk2 re-immunoprecipitated from the eluates.

SDS-PAGE and Western blotting

The total cell lysates or immunoprecipitated proteins were loaded on either 7.5% standard (37.5:1 acrylamide:bis) or 8.5% low bis (56.6:1 acrylamide:bis) SDS-PAGE.

For the MAP kinase mobility shift assay, cell lysates were subjected to SDS-PAGE on a 15% low bis (175:1 acrylamide:bis) SDS-PAGE.

The proteins were electrophoresed and transferred to Immobilon-P (Millipore Corp., Bedford, MA) in transfer buffer (20% methanol / 20 mM Tris base / 96 mM glycine) for 4 hours to overnight at 75V. Immobilons were blocked with 4% BSA overnight at 4°C.

Immunoblotting was done using PY-72 (anti-phosphotyrosine), anti-p56Lck mAb, or anti-MAPK2 mAb as the primary antibody and rabbit anti-mouse^{HRP}. Immunoblotting with anti-Pyk2 antisera was followed by protein A^{HRP}. Far Western blotting was performed using the GST-Lck-SH2 fusion protein (Lck AA 121-225) generated as described below for FAK and Pyk2. Following extensive washing, the immobilon was incubated with anti-GST antisera and protein A^{HRP}. The development system used to detect bound antibody was Enhanced Chemiluminescence (ECL) (Amersham, Buckinghamshire, England and NEN, Life Science Products, Boston, MA) as described in the product information. Molecular size standards (in kilodaltons) are shown.

For some experiments the immobilons were stripped for 30 minutes at 56°C in stripping buffer (10 mM β-mercaptoethanol / 2% SDS / 62.5 mM Tris, pH 6.7), blocked overnight with 4% BSA and Western blotting repeated.

Kinase assay

Clone AB.1 cells were stimulated as described for immunoprecipitation experiments followed by cell lysis in kinase lysis buffer (50 mM HEPES, pH 7.5 / 150 mM NaCl / 10% glycerol / 0.1% Triton X-100 / 1.5 mM $MgCl_2$ / 1 mM EDTA / 1 mM PMSF / 200 μM vanadate). Immunoprecipitates, prepared as described above, were washed twice in kinase lysis buffer, once in Tris buffer (50 mM Tris-HCl, pH 7.5 / 10 mM $MnCl_2$), and resuspended in 35 μl Tris buffer. Immunoprecipitated proteins were incubated for 30

minutes at 37°C with 10 μCi[g-^{32}P]ATP, run on 7.5% SDS-PAGE, and visualized by autoradiography.

For kinase assays testing PP1, cells were pretreated with PP1 (10 μM) on ice for 30 minutes before stimulation. In the appropriate kinase assays, phenyl phosphate (100 mM) was added either during the primary antibody or the protein A Sepharose bead incubation.

Measurement of intracellular free Ca^{++} concentration

Wells of 96-well microtiter plates were coated with protein as described above. Clone 11 cells were harvested and washed 3 times in RPMI containing 10% newborn calf serum and diluted to 1 X 10^6 cells/ml. FLUO-3, AM (Molecular Probes, Eugene, OR), dissolved in DMSO, was added to the cells at 5 mM and incubated at 37°C for 45 minutes. Cells were washed 3 times in RPMI/10% serum and resuspended to 5 X 10^6 cells/ml and added to the coated wells at 100 ml/well. At the indicated times the plates were read in a CytoFluor 2350 plate reader (Millipore) at 530 nm with an excitation wavelength of 485 nm. $[Ca^{++}]_i$ was calculated as $[F - F_{min}] / [F_{max} - F]$.

Bead/cell conjugates

Latex beads immobilized with the appropriate protein(s) and clone 11 cells were aliquotted to microfuge tubes and were incubated at 37°C with rotation for the indicated times. The conjugates were then gently loaded onto a hemocytometer using a blunt end pipette tip and conjugates between cells and beads were counted using a fluorescent microscope (Zeiss, West Germany).

Generation of GST fusion proteins and specific antisera to FAK and Pyk2

A GST fusion protein was made of a sequence of Pyk2 (AA 720-862) and a sequence of FAK (AA 699-845). For Pyk2 the primers were 5' AAG GAT CCC CCC CAC CCA AGC CCA GC 3' and 5'AAG AAT TCG CTG GAT GGA CTA TGC GCC 3' which introduced a BamH1 and an EcoR1 site into the amplified sequence. The sequence was amplified by RT-PCR from Jurkat cell poly A RNA. For FAK the primers were 5' GAG GAT GGG ATC CAG AAG 3' and 5' CCC GTC TTC CCT G 3' which introduced a BamH1 site. The sequence was amplified by RT-PCR from CTL clone AB.1 RNA.

The DNA was isolated and Genecleaned (Geneclean III Kit, BIO 101 Inc., Vista, CA). The pGEX vector (Pharmacia, Piscataway, NJ) and the Pyk2 DNA were digested with restriction enzymes BamH1 and EcoR1 overnight whereas the vector and FAK DNA were digested with BamH1 and Xho1. The DNA was Genecleaned and the pGEX vector dephosphorylated using alkaline phosphatase (Promega, Madison, WI). The pGEX vector and the Pyk2 or FAK DNA were ligated overnight using T4 ligase (Gibco-BRL) with the Pyk2 or FAK sequence in frame with the GST coding sequence. JM105 competent cells were transformed with ligated vector and transformed bacteria grown under conditions of ampicillin. Confirmation of Pyk2 or FAK sequence being present was done in 2 ways. First, DNA was isolated using the Miniprep kit (Qiagen, Mississauga, ON) and digested with BamH1 and EcoR1 (Pyk2) or BamH1 and Xho1 (FAK) to confirm that a product of approximately 466 (Pyk2) or 474 (FAK) bases was present. Secondly, Miniprep-isolated DNA was digested using enzyme Pst1 for which there is a unique site in the pGEX vector and in the Pyk2 DNA insert confirming the correct DNA size. Similarly, Miniprep-isolated DNA was digested using enzyme Sma1 for the unique site in the FAK insert and Pst1 for the unique site in the vector. GST fusion proteins were expressed and purified according to the literature accompanying the vector. Purified GST fusion proteins were the only proteins detected on a Coomassie-stained gel. For generation of antisera, the purified GST fusion proteins were injected

subcutaneously into New Zealand White rabbits 6 (Pyk2) and 8 (FAK) times at 2 week intervals and terminal bleeds collected.

CHAPTER III

Characterization of Intercellular Adhesion Molecule-1 (ICAM-1)-Augmented Degranulation by Cytotoxic T Cells: ICAM-1 and Anti-CD3 must be Co-localized for Optimal Adhesion and Stimulation

(Most of the data presented in this chapter is reproduced, with permission, from **The Journal of Immunology (1995), 155:1694-1702.**

A. Introduction

T cell activation is a complex multi-step cascade of events that involves the interactions of a number of accessory molecules on a T cell with their respective ligands on the APC to provide costimulatory signals for activation. Integrins, a family of heterodimeric glycoproteins found on many cell types, appear to facilitate T cell activation events (Collins et al., 1994). Integrins may enhance activation by increasing the overall avidity between the T cell and the APC or by mediating signalling events.

LFA-1 (CD11a/CD18) is a β2 integrin found on nearly all leukocytes (Springer et al., 1987). The first ligand for LFA-1 was identified as the intercellular adhesion molecule-1 (ICAM-1; CD54) which is present on hematopoietic and non-hematopoietic cells (Rothlein et al., 1986). Subsequently identified ligands include ICAM-2 (Staunton et al., 1989b) and ICAM-3 (de Fougerolles and Springer, 1992). LFA-1/ICAM-1 interactions mediate homotypic and heterotypic adhesion in a number of different cell types and appear to be the primary adhesive interaction for CTL/target cell conjugation (Martz, 1987). The importance of LFA-1 is demonstrated by observations that antibodies against either ICAM-1 (Dougherty et al., 1988; Makgoba et al., 1988b) or LFA-1 (Dougherty and Hogg, 1987) block T cell activation. Furthermore, purified ICAM-1 costimulates proliferation of human

T cells in the presence of anti-CD3 antibodies (van Seventer et al., 1990) and enhances anti-CD3 induced proliferative responses by murine splenic T cells (Kuhlman et al., 1991).

The contribution of LFA-1/ICAM-1 is widely perceived as being primarily adhesive, bringing more surface area of the interacting cells in contact to augment the TCR-specific activation process. However, some evidence suggests a signalling function for LFA-1/ICAM-1. Antibodies to LFA-1 induce a Ca^{++} flux in T cells (Pardi et al., 1989; Kanner et al., 1993), although other experiments suggest that anti-LFA-1 does not itself induce a Ca^{++} flux, but rather enhances anti-CD3 stimulated fluxes (Wacholtz et al., 1989). It has also been shown that anti-LFA-1 increases inositol phosphate production (Pardi et al., 1989) and induces phosphorylation of PLCγ (Kanner et al., 1993) consistent with a role in signalling, however, it is not clear if these signalling events are stimulated with natural LFA-1 ligands. Purified ICAM-1 was shown to increase and prolong the level of anti-CD3-induced inositol phosphate production, but ICAM-1 alone had no effect on the production of these metabolites (van Seventer et al., 1992).

The question of whether engagement of LFA-1 by ICAM-1 induces signalling in T cells remains largely unanswered. Evidence suggests that the contribution of ICAM-1 to the activation of CTL is distinct from that of either fibronectin or class I MHC (Ybarrondo et al., 1994). In this study, we further define the role of ICAM-1 in the initiation of adhesion and signalling events in triggering degranulation by CTL using purified ICAM-1.

B. Results

Purification of ICAM-1

ICAM-1 was purified from isolated membranes by a single antibody affinity chromatography step using a YN1/1.7 column as described in the Materials and Methods (Welder et al., 1993). Isolated fractions were tested for reactivity to the YN1/1.7 antibody by ELISA. The ELISA results of one such preparation is shown (Figure 3-1A); fractions 6-10 were pooled as peak fractions of serologically active protein. This method provided

pure material as determined by silver staining of a sample subjected to SDS-PAGE (Figure 3-1B). A protein of the predicted molecular weight (95-110 kDa) is visible with no other proteins detected on the gel. ICAM-1 appears as a faint smear, most likely due to its high level of glycosylation. An ELISA assay of the pooled ICAM-1 using YN1/1.7 verified that serologically active protein is present and that the reactivity increases with increasing protein concentration (Figure 3-1C). Protein is detected at levels as low as 2 ng/well and begins to plateau at about 30 ng/well.

CTL clone degranulation

We used the non-transformed, antigen driven CTL clone 11 to study the contribution of ICAM-1 engagement to T cell activation. Degranulation, as measured by the release of serine esterase (SER) (Kane et al., 1989a), is a measure of CTL activation. The clones were stimulated with immobilized antibody directed against CD3ε, 145-2C11 (2C11), or the TCR, H57-597 (H57). BSA was used as a control. As shown in Figure 3-2, stimulation of clone 11 with 2C11 or H57 results in a strong dose-dependent degranulation response.

Immobilized ICAM-1 enhances degranulation by CTL clones

The data presented in Figure 3-3A demonstrates that ICAM-1 alone does not induce degranulation by CTL clone 11, but does synergize with substimulatory amounts of 2C11 (anti-CD3) to induce degranulation. This experiment was done with a concentration of 2C11 that is limiting (40 ng/well) and is not sufficient to lead to significant degranulation in the absence of additional costimulation. The extent of costimulation increases with increasing densities of purified ICAM-1 from 1 ng/well and plateaus at about 30 ng/well (Figure 3-3A). When the reciprocal experiment was performed, that is to vary the density of 2C11 in the presence of a near saturating density of immobilized ICAM-1 (20 ng/well), it is clear that ICAM-1 lowers the amount of antibody required to stimulate a response by

more than 10-fold (Figure 3-3B). The degranulation is significantly and reproducibly enhanced relative to that seen with the substimulatory antibody alone.

Essentially identical results were observed with antibodies to the TCR. CTL clone 11 were stimulated with anti-TCR (H57) at three different concentrations in the absence and presence of co-immobilized ICAM-1 (Figure 3-4). It is evident that ICAM-1 can enhance the degranulation response to H57 stimulation, although the effect is not as dramatic as that seen with 2C11. H57 alone may have a higher affinity for the TCR or may aggregate. We continued our studies primarily with 2C11 stimulation.

To confirm that the augmented degranulation is dependent on the immobilized ICAM-1, and the interaction with cell surface LFA-1, antibody blocking experiments were performed. Figure 3-5 shows that antibodies to ICAM-1 or LFA-1 significantly inhibit degranulation implicating both LFA-1 and ICAM-1 in the ICAM-1-augmented response. An antibody to CD45 was used as a negative control and clearly has no effect on the ICAM-1-facilitated degranulation response.

Enhanced degranulation by CTL clones with anti-class I MHC and FN

We investigated whether the enhanced degranulation by CTL clones could be induced by engaging other cell surface molecules. Figure 3-6 shows that immobilized anti-class I MHC (M1/42) and fibronectin (FN), the ligand for VLA-4 and VLA-5 integrins (Collins et al., 1994), enhances degranulation of CTL clone 11 in response to substimulatory 2C11 similarly to ICAM-1. Augmenting degranulation is, therefore, not unique to ICAM-1.

ICAM-1 does not augment degranulation if it is spatially removed from anti-CD3

We demonstrated above that ICAM-1 lowers the amount of anti-CD3 antibody required for triggering degranulation by about 10-fold. The role of the LFA-1/ICAM-1

interaction in enhancing degranulation could be either adhesion or signalling. If ICAM-1 enhances activation by increasing the avidity between the CTL and the antibody-bearing surface, then no enhanced response should occur when ICAM-1 and anti-CD3 are presented on two different surfaces. If, however, ICAM-1 transmits a global signal into the cell, such as an increase in intracellular Ca^{++}, ICAM-1 should still facilitate the response even when it is spatially removed from the anti-CD3.

We addressed this question in two ways. First, we stimulated CTLs with ICAM-1 immobilized on plastic wells and anti-CD3 (2C11) immobilized on 5.0 μm latex beads, spatially removed from the ICAM-1. Figure 3-7 shows dose-dependent degranulation in response to the anti-CD3-coated latex beads, irrespective of the immobilized ICAM-1.

Second, we stimulated CTLs with ICAM-1-coated beads and 2C11-coated beads either individually or together or with ICAM-1 and 2C11 co-immobilized on the same beads. When ICAM-1 and 2C11 are co-immobilized on latex beads, there is a significant enhancement of degranulation over that seen with 2C11-coated beads alone (Figure 3-8). However, when ICAM-1-coated beads are mixed with 2C11-coated beads, the response was identical to that observed with 2C11-coated beads alone. This result is observed when the latex beads are immobilized with either 1 μg/ml (Figure 3-8A) or 5 μg/ml (Figure 3-8B) 2C11. There is also no significant difference when 0.5 μg/ml or 0.9 μg/ml ICAM-1 is used. Overall, the degranulation response is greater when 5 μg/ml 2C11 is used, but the effect of ICAM-1 is relative between the two experiments. Therefore, ICAM-1, when spatially removed from anti-CD3, does not augment the degranulation response.

ICAM-1 does not induce tyrosine phosphorylation in CTL

Tyrosine phosphorylation of cellular proteins is one of the earliest events to occur upon T cell stimulation. To determine if ICAM-1 is capable of triggering signalling events in T cells, we examined the ability of ICAM-1 to stimulate tyrosine phosphorylation of cellular proteins and to influence anti-CD3-triggered tyrosine phosphorylation. We

demonstrate by anti-phosphotyrosine immunoblotting that phosphorylation of cellular proteins in response to ICAM-1 stimulation (Figure 3-9, lanes 1-4) is not enhanced compared to that seen with substimulatory amounts of 2C11 (Figure 3-9, lanes 13-16). However, when a substimulatory amount of 2C11 was co-immobilized with ICAM-1, there is an increase in the tyrosine phosphorylation of a number of cellular proteins 10, 20 and 40 minutes after stimulation (Figure 3-9, lanes 6-8). These are the same proteins that normally become phosphorylated in the presence of maximally stimulatory 2C11 (800 ng/well; Figure 3-9, lanes 9-12). Therefore, co-immobilization of ICAM-1 with substimulatory amounts of anti-CD3 does not induce the tyrosine phosphorylation of novel proteins, but does provide a synergistic signal that induces the phosphorylation of proteins normally stimulated with optimal amounts of anti-CD3.

ICAM-1 does not induce an increase in intracellular Ca^{++} in CTL

In addition to cellular tyrosine phosphorylation, there is an increase in intracellular Ca^{++} in T cells upon T cell triggering. We examined the ability of ICAM-1 to induce changes in intracellular Ca^{++} since it has been reported that antibodies to LFA-1 induce a Ca^{++} flux (Pardi et al., 1989; Kanner et al., 1993). Surprisingly, we found that immobilized ICAM-1, the natural ligand for LFA-1, does not induce changes in intracellular Ca^{++} concentrations (Figure 3-10). However, if the ICAM-1 is co-immobilized with substimulatory amounts of anti-CD3, a substantial Ca^{++} flux is observed (Figure 3-10). Consistent with previous data (Pardi et al., 1989; Kanner et al., 1993), we also find that anti-LFA-1 induces a substantial increase in the concentration of intracellular Ca^{++} even though ICAM-1 is unable to do so (Figure 3-10).

ICAM-1 must be co-immobilized with anti-CD3 for stable adhesion of CTL to ICAM-1

It has been previously demonstrated that T cells will not adhere to immobilized ICAM-1 unless they are first triggered with either PMA or cross-linked anti-TCR (Dustin and Springer, 1989). Our results presented in Figure 3-8 suggest that ICAM-1 can augment anti-CD3-stimulated degranulation only when the ICAM-1 and anti-CD3 are immobilized on the same surface. It is possible that ICAM-1 adheres to CTL only when ICAM-1 is co-immobilized with anti-CD3 on the same surface and that the anti-CD3 transmits a localized signal that leads to adhesion to ICAM-1. To test the hypothesis that the ICAM-1 and anti-CD3 must be immobilized on the same surface to generate stable adhesion of CTL to ICAM-1, we examined conjugate formation between CTL and ICAM-1 presented either on the same bead with anti-CD3 or on separate beads as in Figure 3-8. Surprisingly, we found that ICAM-1 beads form stable conjugates with CTL only when anti-CD3 is immobilized on the same bead (Figure 3-11). Low level, transient adhesion occurs between CTL and ICAM-1-coated beads alone, or when ICAM-1-coated beads are mixed with anti-CD3-coated beads (Figure 3-11). In contrast, a large number of conjugates are formed between the CTL and beads coated with both ICAM-1 and anti-CD3 that are stable for up to 4 hours. These results demonstrate that anti-CD3 transmits a localized signal into the cell resulting in increased adhesion to ICAM-1 since ICAM-1 and anti-CD3 must be spatially juxtaposed for stable adhesion. As expected, conjugate formation is inhibited in the presence of anti-ICAM-1 or anti-LFA-1 (Figure 3-12).

One could argue that when the 2C11-coated beads and ICAM-1 coated beads were presented to the cells together, the inability of the cells to adhere to the ICAM-1 beads was because the cells could only bind to one bead at a time and the 2C11-coated beads were blocking the adhesion. Figure 3-13 indicates the number of beads binding to individual CTLs under the different bead-coated stimulation conditions. Mixing 2C11-coated and ICAM-1-coated beads (separate) or ICAM-1 beads alone not only have low and transient

conjugate formation (Figure 3-11), but of the CTL that are engaged in conjugates, the vast majority of cells are binding to 1 bead only (Figure 3-13). However, in addition to the large number of cells that are in stable conjugates with (2C11 and ICAM-1)-coated beads (same) (Figure 3-11), over 50% of the CTL that are engaged in conjugates are conjugated with 2 or more beads (Figure 3-13). This indicates that the CTL are engaging in even more adhesive events in addition to demonstrating that there is not a constraint on binding to ICAM-1 beads alone.

Requirements for stable adhesion to ICAM-1 and anti-CD3-coated latex beads

To determine if the activation of tyrosine kinases is required to generate the stable adhesion we carried out the conjugation experiment in the presence of tyrosine kinase inhibitors. The conjugation of the CTL with the ICAM-1 and anti-CD3-coated beads is not significantly inhibited with the tyrosine kinase inhibitors herbimycin A (6 μg/ml) or genistein (50 μg/ml) (Figure 3-14A) in three independent experiments. Figure 3-14B shows that both herbimycin A (lane 3) and genistein (lane 4) were used at concentrations that clearly inhibited anti-CD3-induced tyrosine phosphorylation events (lane 2) in the CTL, bringing the level of phosphorylation to near basal levels (lane 1). Taken together, these data suggest that tyrosine phosphorylation is not required to generate stable adhesion between CTL and immobilized ICAM-1.

When a Th cell binds to an APC or a CTL to a target cell, the T cell undergoes re-orientation of many intracellular proteins towards the interacting cell (Kupfer and Singer, 1989) and anti-CD3 stimulation has been shown to cause actin polymerization in T lymphocytes (Phatak and Packman, 1994). In an effort to understand if our CTL clones were undergoing similar intracellular changes when binding to the ICAM-1 and 2C11-coated beads, we treated cells with the cytoskeleton disrupting drug, cytochalasin E, and examined the effect on conjugate formation. Figure 3-15 indicates that cytochalasin E

inhibited the formation of bead/cell conjugates. It is clear that in the process of engaging the beads the CTLs are undergoing cytoskeletal rearrangements that are necessary for the stable adhesion.

C. Discussion

We have shown that ICAM-1 is able to potentiate degranulation by murine $CD8^+$ CTL clones stimulated with antibodies to either CD3 or TCR. This is consistent with observations that purified, immobilized ICAM-1 can facilitate proliferation of either $CD4^+$ human PBL or murine splenic T cells (van Seventer et al., 1990; Kuhlman et al., 1991). We further demonstrate that for ICAM-1-mediated costimulation to work, the anti-CD3 antibody must be co-immobilized on the same surface as the ICAM-1, suggesting that ICAM-1 does not transmit global signals through LFA-1 into the cell, but acts by either increasing the avidity between the cell and the antibody-presenting surface or transmits a very localized signal. Furthermore, we find that for ICAM-1-coated beads to form stable conjugates with CTL, they must have anti-CD3 co-immobilized on the same bead as the ICAM-1; it is not sufficient for anti-CD3-coated beads to be mixed with ICAM-1-coated beads. This is true even if stimulatory amounts of anti-CD3-coated beads are used to trigger the CTL suggesting that activation of the CTL with anti-CD3 is not sufficient for stable adhesion to ICAM-1. These results are in apparent conflict with those presented by Ybarrondo et al. (1994) where they find that cross-linking anti-TCR induces dramatic adhesion to immobilized ICAM-1. It is possible that these cross-linked anti-TCR conditions provide sufficient overall stimulation to the cell to induce integrin binding to ICAM-1 resulting in augmented adhesion. Using two different surfaces, the anti-TCR will bind to only delineated portions of the cell in contact with the beads, as would be the case during CTL/target conjugation, and therefore co-immobilization on beads is necessary to achieve this level of adhesion.

Our results suggest that the interaction between CTL and ICAM-1 is a very stable interaction when ICAM-1 and anti-CD3 are co-immobilized. In contrast, Dustin and Springer (1989) have shown that when cross-linked antibodies to the TCR complex are used, the adhesion to immobilized, purified ICAM-1 is very transient. One possible explanation for these differing results is that when soluble cross-linked antibody is used to stimulate binding to ICAM-1, the TCR complex becomes internalized whereas when the antibody is immobilized as in our system, no internalization is likely to occur. This would suggest that not only is localized triggering through the TCR complex required for adhesion to ICAM-1, but that ongoing engagement of the TCR is required to maintain the adhesion to ICAM-1.

There have been reports in the literature to suggest that antibodies to LFA-1 induce a Ca^{++} flux (Pardi et al., 1989; Kanner et al., 1993), phosphatidylinositol metabolism (Pardi et al, 1989) and phosphorylation of PLCγ (Kanner et al., 1993). However, all of these studies were performed with cross-linked antibodies to LFA-1 and not with a ligand for LFA-1. It is conceivable that the natural LFA-1 ligand, which presumably has a much lower affinity for LFA-1 than antibody, is unlikely to oligomerize LFA-1 as efficiently as antibody and would therefore not normally induce these events in cells. Our results suggest that ICAM-1 does not stimulate tyrosine phosphorylation of cellular proteins or a Ca^{++} flux, but does synergize with substimulatory amounts of anti-CD3 to stimulate these signals likely from increased CD3 occupancy due to increased ICAM-1 adhesion. It is still conceivable that anti-CD3 triggers adhesion to ICAM-1 which in turn stimulates unique tyrosine phosphorylation events and a Ca^{++} flux. Although this possibility cannot be eliminated, we think it is unlikely because the pattern of tyrosine phosphorylation is identical to that observed with stimulatory anti-CD3 alone suggesting that the ICAM-1 is enhancing an anti-CD3-stimulated response.

Our data suggest that ICAM-1 does not induce signalling events in the CTL leading to increased responsiveness, but acts primarily as an adhesion molecule bringing more of

the TCR complex in contact with the immobilized antibody thereby increasing the avidity of the interaction. If this is true then one would predict that engagement of other cell surface molecules would also enhance CTL degranulation in response to substimulatory amounts of anti-CD3. Kane and Mescher (1990) have demonstrated that antibodies to a number of cell surface molecules can facilitate the response to suboptimal amounts of alloantigen to different degrees. We have also found that fibronectin and antibody to class I MHC lower the threshold requirement for anti-CD3 antibody. These observations suggest that there is nothing special about the interaction of ICAM-1 with LFA-1. What is unique about the LFA-1/ICAM-1 interaction is its regulation. It does not occur unless neighboring TCR are engaged. Fibronectin, however, effectively facilitates T cell responses when presented on a different surface than the TCR-triggering antibody (Davis et al., 1990; Ostergaard and Ma, 1995). These previous studies, along with our data presented in this study, suggest that although both fibronectin and ICAM-1 are able to facilitate CTL degranulation, they appear to do so through distinct mechanisms and therefore may play distinct roles during T cell activation in response to antigen presented on APC.

A previous report has demonstrated that immobilized ICAM-1 does not lower the amount of anti-CD3 antibody required to stimulate proliferation of naive murine spleen cells, but rather enhances the extent of the overall response induced with anti-CD3 (Kuhlman et al., 1991). In contrast, we show that ICAM-1 lowers the amount of antibody required to induce the response by about 10-fold. This could reflect differences in the state of maturation/activation of the cells used in that we are examining activation of mature CTL.

Evidence presented in this chapter suggests that ICAM-1 facilitates activation of CTL by either increasing the avidity between the cells and the antibody-bearing surface or by stimulating only very localized signals that do not lead to protein phosphorylation or Ca^{++} fluxes. One such localized signal involves cytoskeletal rearrangements. We show that disruption of actin polymerization, by treating cells with cytochalasin E, prevented

conjugate formation. It is possible that anti-CD3 activates local cytoskeletal rearrangements and that LFA-1 serves as a nucleation site for these rearrangements as has been demonstrated with a number of integrins (Schwartz, 1992; Juliano and Haskill, 1993). Consistent with this model, Pardi et al. (1992) have demonstrated that cross-linking the TCR complex with anti-CD3 results in actin polymerization and colocalization with LFA-1. Additionally, it has been demonstrated that PMA induces talin association with LFA-1 (Kupfer et al., 1990). These results suggest that triggering the T cell results in cytoskeletal linkage with LFA-1 and that these changes may stabilize the interaction between the triggering surface (antigen on APC or immobilized antibody) and the T cell resulting in enhanced activation.

Finally, the regulation of adhesion between LFA-1 and ICAM-1 by localized engagement through the TCR has important implications for the interaction of CTL with potential target cells *in vivo*. For example, when a CTL encounters a virally infected target cell, the CTL would undergo stable adhesion with only the appropriate target cell ultimately leading to cell lysis. This localized TCR-regulated LFA-1-mediated adhesion would prevent CTL from tightly adhering to surrounding cells that do not bear the appropriate antigen thus preventing the CTL from being engaged in non-productive interactions.

In summary, we demonstrate that the LFA-1/ICAM-1 interaction serves an adhesive role, likely by increasing cell-cell avidity, in potentiating phosphorylation events and increases in Ca^{++} concentrations, in formation of stable conjugates, and in augmenting CTL degranulation.

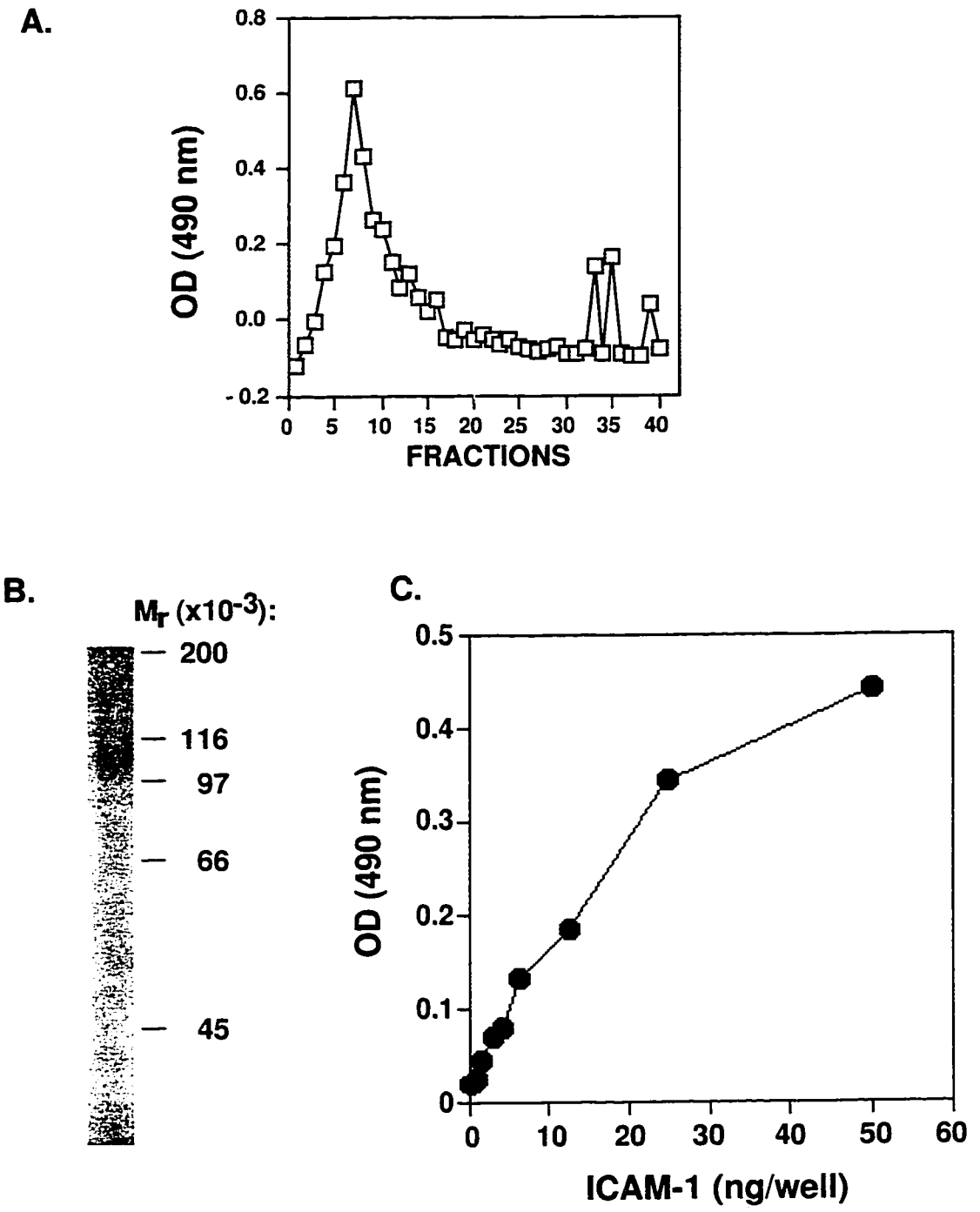


Figure 3-1. Purification of ICAM-1. **A.** ELISA of ICAM-1 fractions from YN1/1.7 column. YN1/1.7 Ab was added to wells of a 96-well plate coated with ICAM-1 column fractions. Ab binding was determined after binding of HRP-conjugated second Ab. Fractions 6 to 10 were pooled. **B.** Silver stained gel of purified, pooled ICAM-1. **C.** ELISA reactivity of purified ICAM-1 coated at various concentrations.

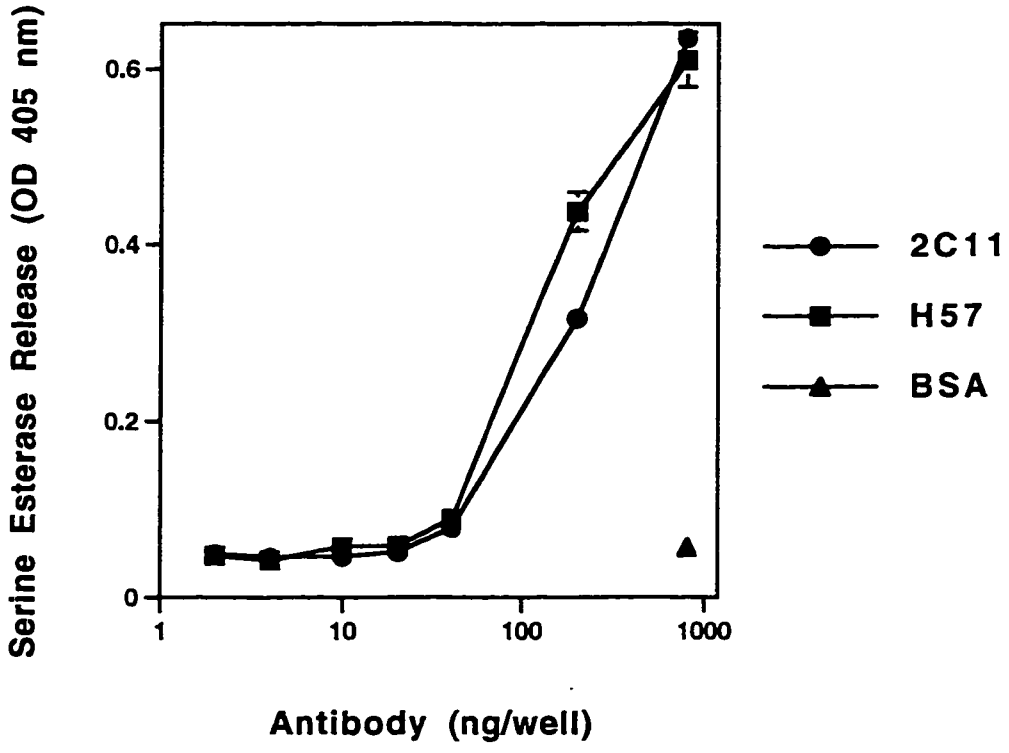


Figure 3-2. Titration of immobilized 2C11 and H57 in a serine esterase release assay. Clone 11 cells (1.5 X 10^5) were added to triplicate wells of a 96-well plate coated with 2C11 or H57 at various concentrations. Control wells were BSA-coated. Cells were incubated for 4 hours at 37°C and cell supernatants were assayed for serine esterase release as a measure of CTL degranulation.

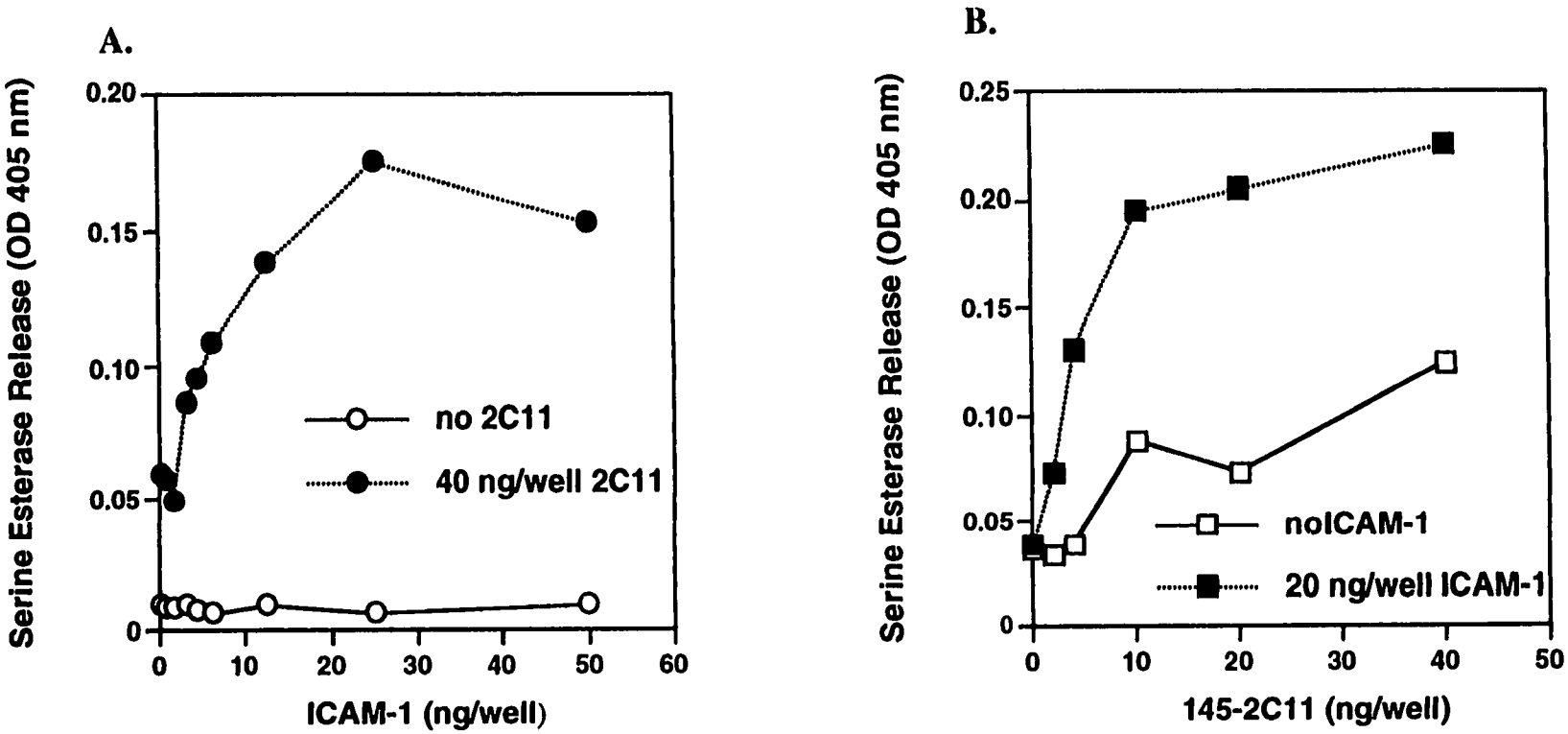


Figure 3-3. Immobilized ICAM-1 enhances degranulation in response to immobilized 2C11 (anti-CD3). **A.** Titration of ICAM-1 in the presence or absence of substimulatory (40 ng/well) 2C11. **B.** Degranulation of CTL after stimulation with various concentrations of 2C11 alone or co-immobilized with 20 ng/well of purified ICAM-1. Clone 11 (1.5 X 10^5) cells were added to each of the triplicate wells and incubated for 4 hours at 37°C. Cell supernatants were assayed for serine esterase release.

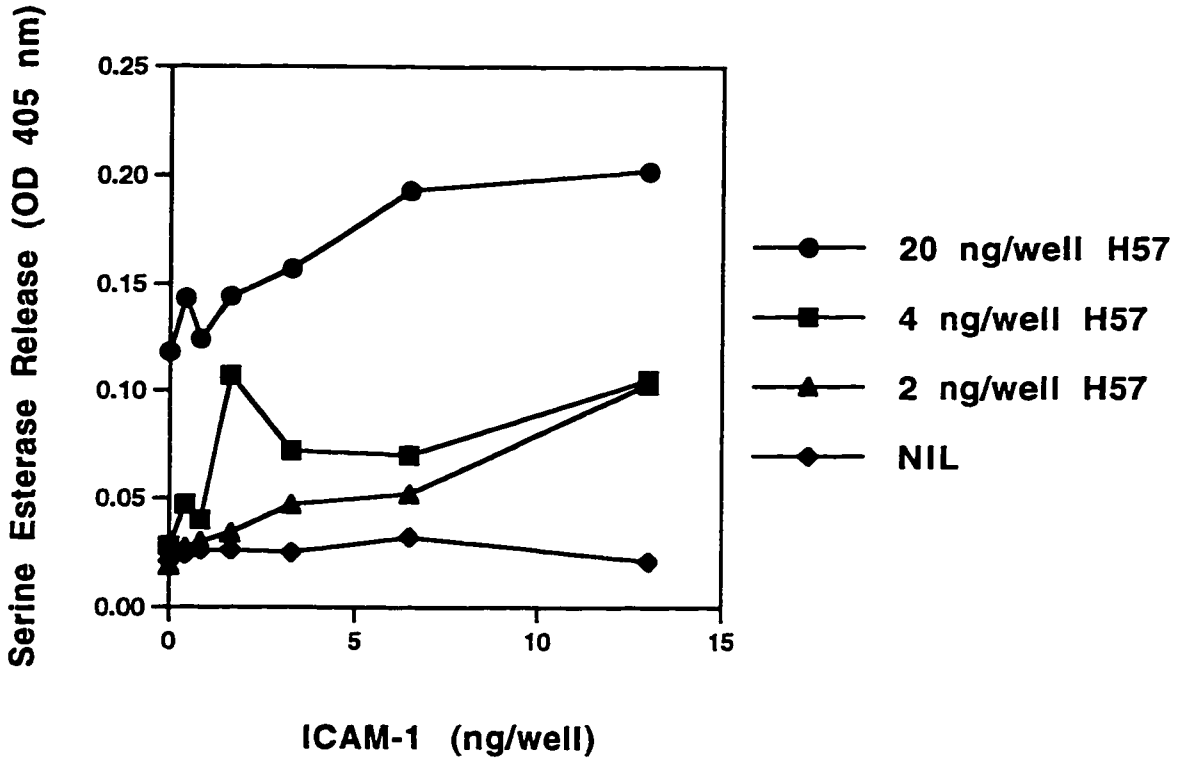


Figure 3-4. Immobilized ICAM-1 enhances degranulation in response to immobilized H57 (anti-TCR). Titration of immobilized ICAM-1 alone or co-immobilized with 20, 4, or 2 ng/well H57. Clone 11 (1.5 X 105) cells were added to triplicate protein-coated wells. Cells were incubated for 4 hours at 37°C and cell supernatants were assayed for serine esterase release.

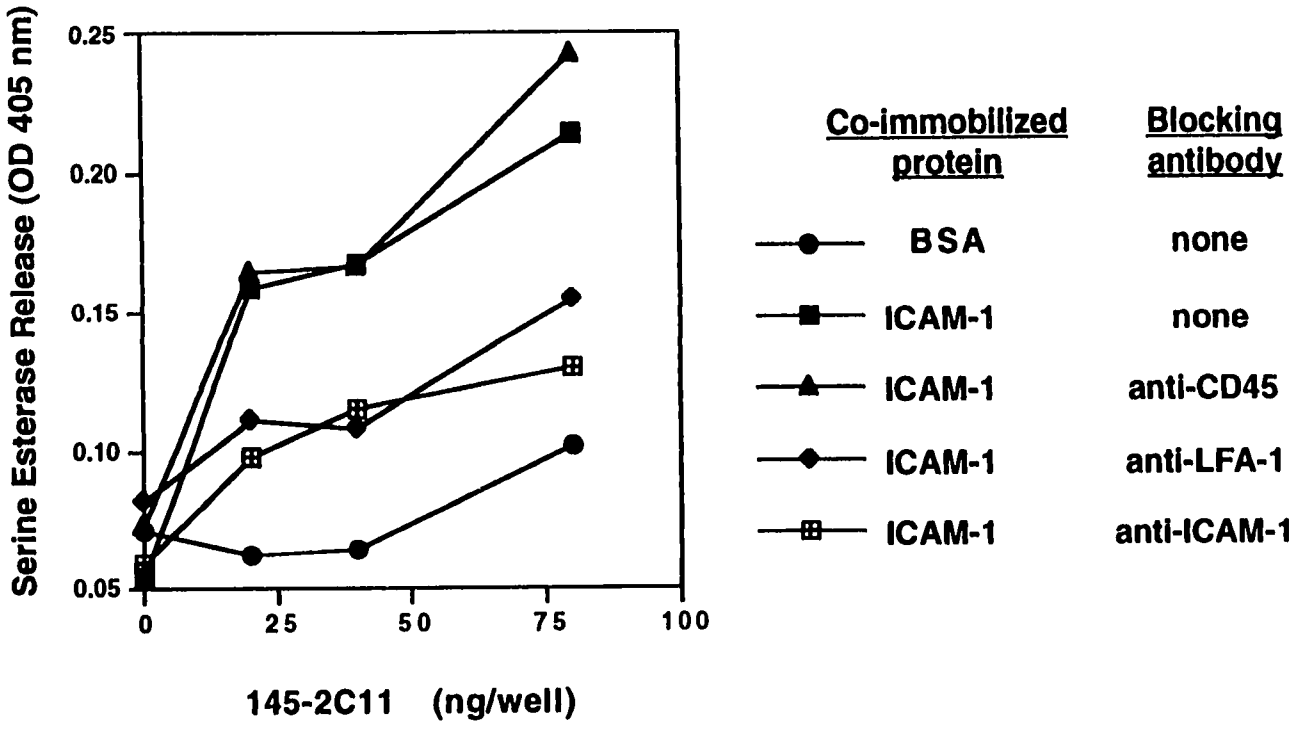


Figure 3-5. ICAM-1-enhanced degranulation is inhibited with anti-ICAM-1 and anti-LFA-1, but not with anti-CD45. Wells of a 96-well plate were coated with various concentrations of 145-2C11 in the absence or presence of 20 ng/well of ICAM-1. Cells were incubated without or with blocking antibodies YN1/1.7 (anti-ICAM-1), M17/5.2 (anti-LFA-1), or I3/2 (anti-CD45), for 15 minutes on ice at a final concentration of 20 mg/ml before addition of the cells to the protein-coated wells. Cells were incubated at 37°C for 4 hours and supernatants assayed for serine esterase release.

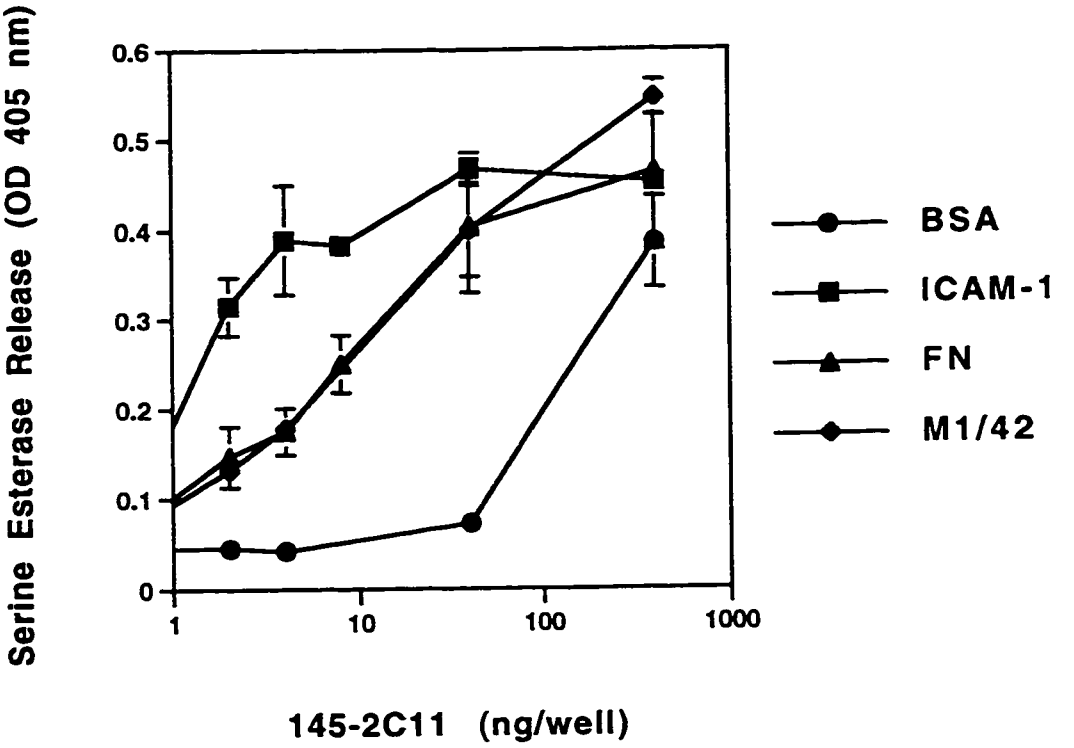


Figure 3-6. Immobilized FN and anti-MHC class I enhance 2C11-stimulated degranulation. Clone 11 cells (1.5 X 105) were added to triplicate wells coated with various concentrations of 145-2C11 alone or co-immobilized with 20 ng/well of purifed ICAM-1, 400 ng/well FN, or 400 ng/well M1/42 (anti-MHC class I). Control wells were BSA-coated. Cells were incubated for 4 hours at 37°C and cell supernatants were assayed for serine esterase release.

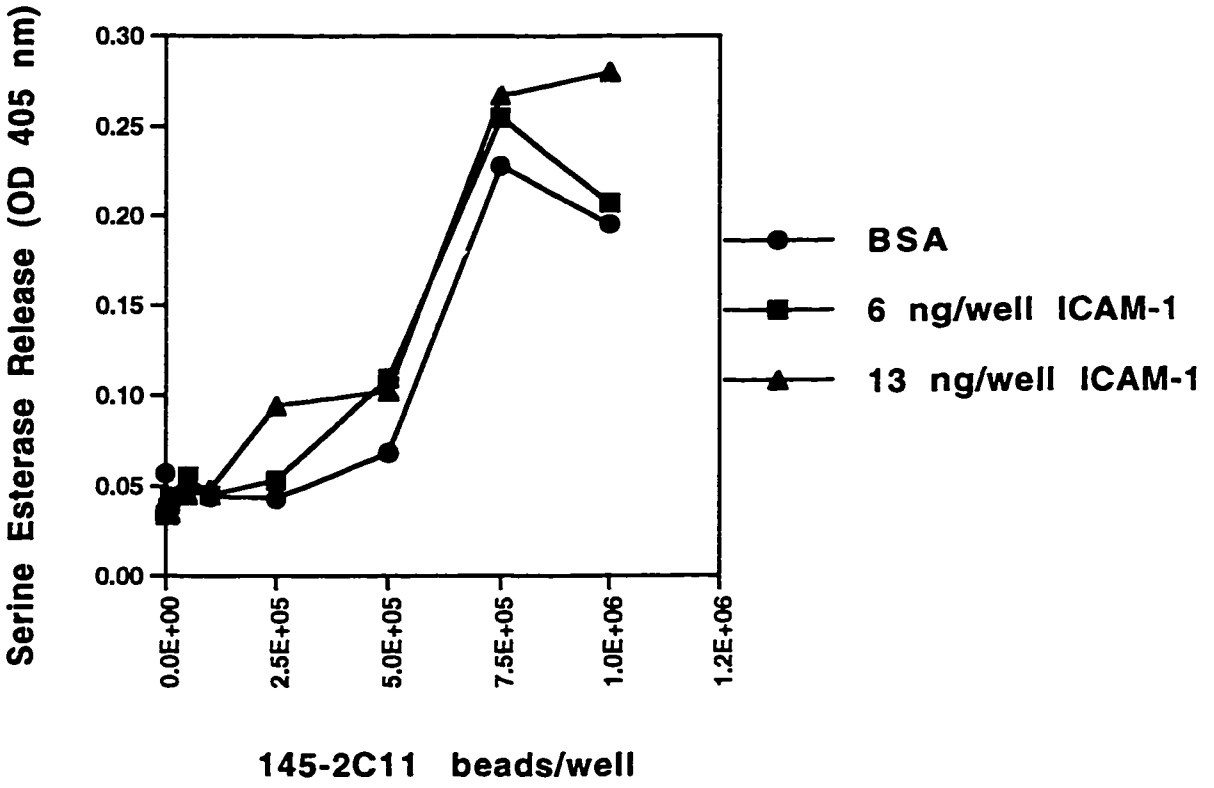


Figure 3-7. ICAM-1 does not enhance degranulation when spatially removed from anti-CD3. Wells of a 96-well plate were coated with BSA (control) or ICAM-1 at 6 or 13 ng/well. Latex beads were coated with 145-2C11 at 10 μg/ml. Clone 11 (1.5 X 105) cells and various concentrations of beads were added to protein-coated wells. Cells were incubated for 4 hours at 37°C and cell supernatants were assayed for serine esterase release.

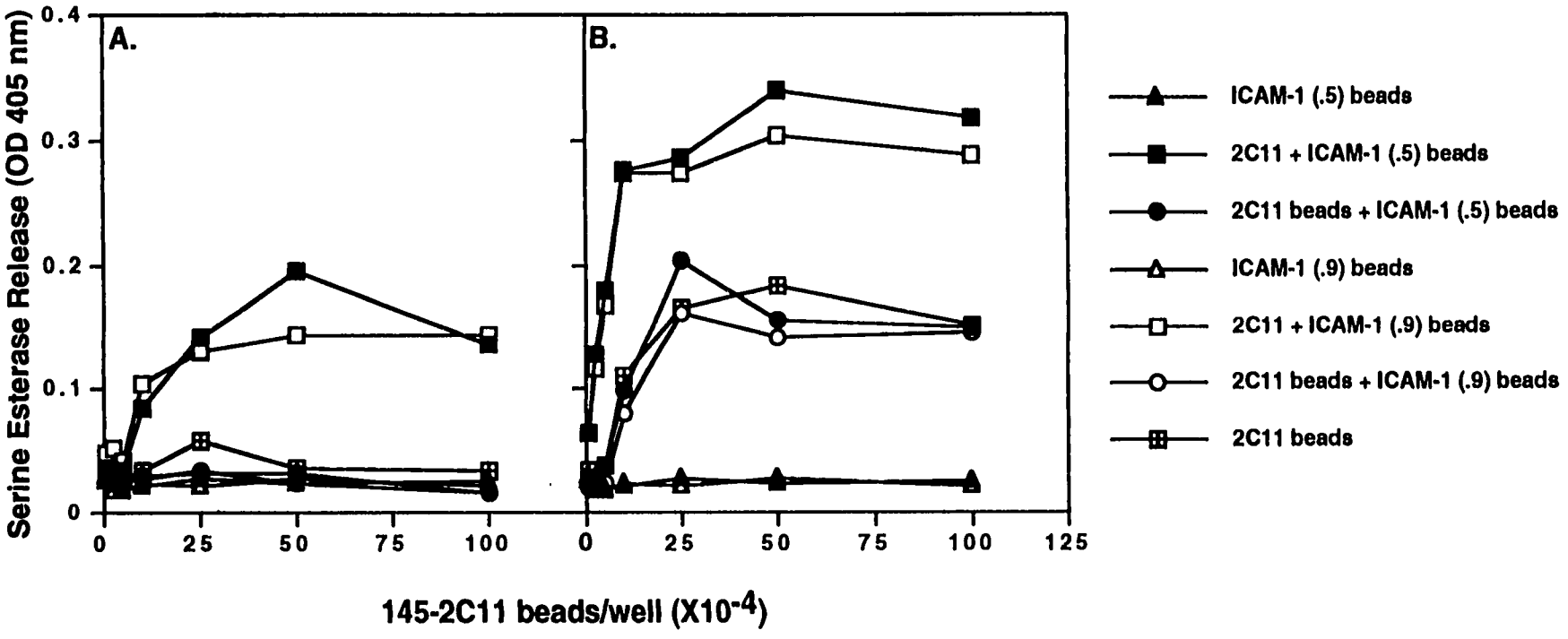
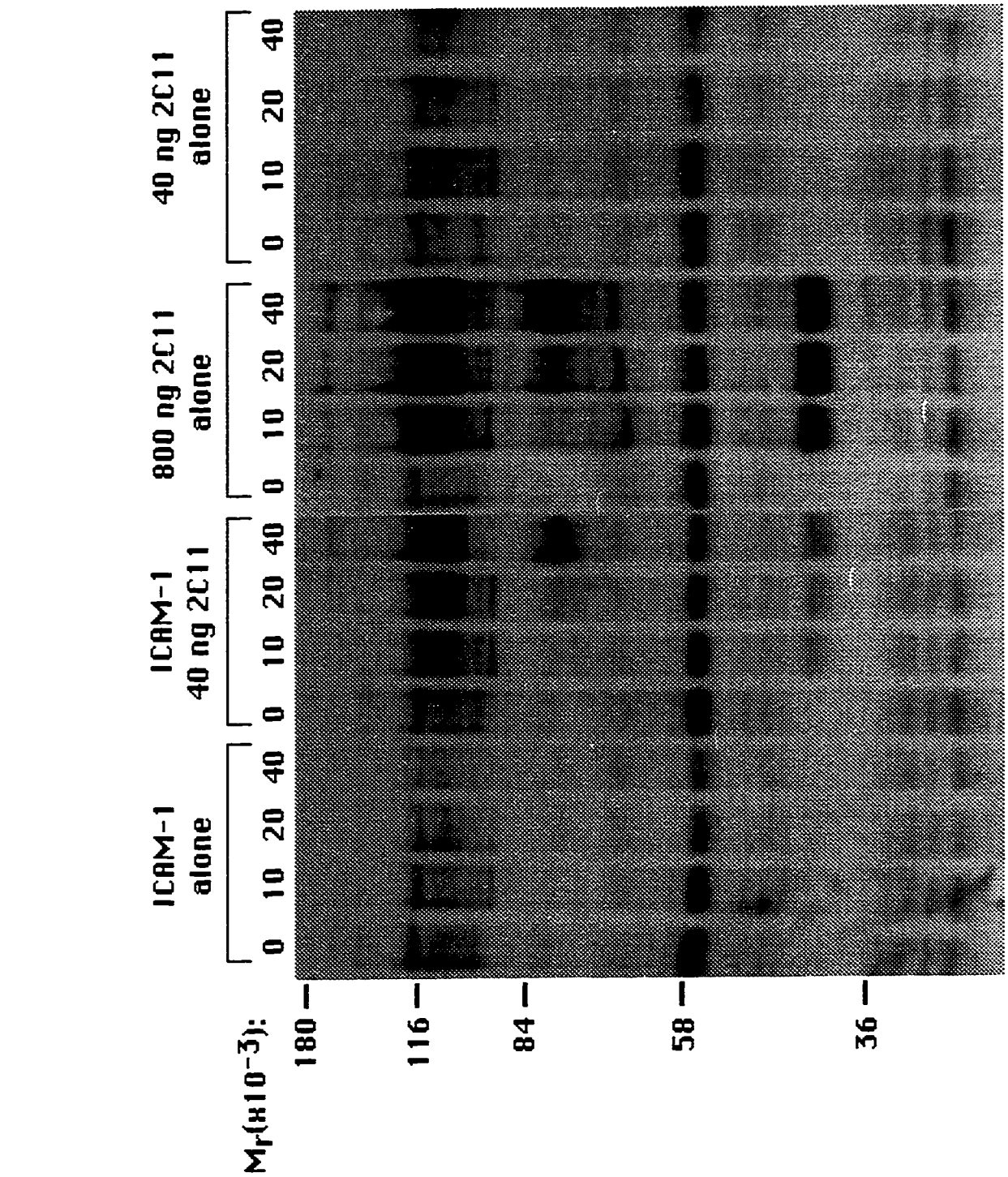


Figure 3-8. ICAM-1 must be on the same surface as the stimulating antibody to enhance degranulation. Latex beads were coated with 2C11 at 1 μg/ml (**A**) or 5 μg/ml (**B**). Beads were also coated with purified ICAM-1 at either 0.5 mg/ml (closed symbols) or 0.9 mg/ml (open symbols). 2C11-coated beads were either used alone (cross-box) or were mixed with ICAM-1-coated beads (circles). Alternatively, beads were co-immobilized with 2C11 and ICAM-1 (squares). ICAM-1-coated beads (triangles) were used alone as a negative control. Protein-coated beads were added to wells of a 96-well plate along with 1.5 X 10^5 clone 11 cells and incubated at 37°C for 4 hours after which the cell supernatants were assayed for serine esterase activity.

Figure 3-9. ICAM-1 does not induce tyrosine phosphorylation of cellular proteins in CTL clones, but does facilitate anti-CD3 stimulated phosphorylation. Clone 11 (1.5×10^5) cells were stimulated for various times with wells coated with ICAM-1 alone (20 ng/well), substimulatory 145-2C11 (40 ng/well) or both. As a positive control, the cells were maximally stimulated with 800 ng/well of 145-2C11. Cell lysates were subjected to SDS-PAGE and anti-phosphotyrosine immunoblotting. Cells added to BSA-coated wells give phosphorylation patterns identical to those shown in lanes 1-4 and 13-16.


ICAM-1 alone
0 10 20 40
ICAM-1 40 ng 2C11
0 10 20 40
800 ng 2C11 alone
0 10 20 40
40 ng 2C11 alone
0 10 20 40
Mr(x10-3):
180
116
84
58
36

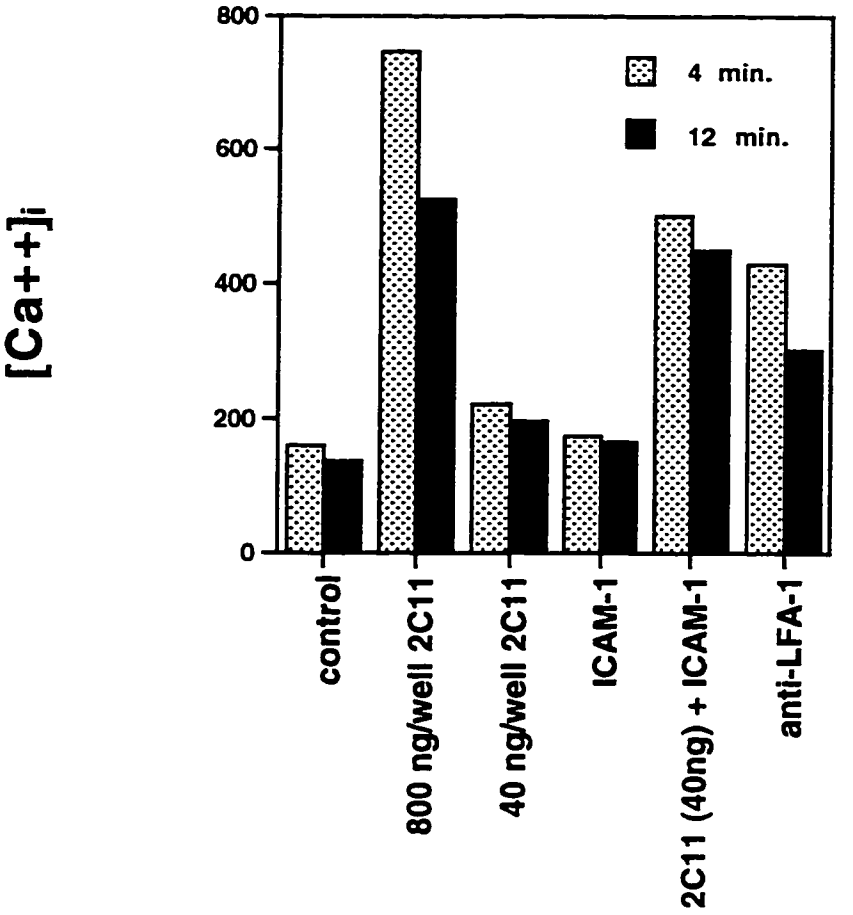


Figure 3-10. ICAM-1 does not stimulate a Ca++ flux in CTL. FLUO-3 loaded cells were incubated in wells of a microtitre plate coated with stimulatory amounts of 145-2C11 (800 ng/well), substimulatory amounts of 145-2C11 (40 ng/well), ICAM-1 alone (20 ng/well), substimulatory 145-2C11 with ICAM-1, or anti-LFA-1 (M17/5.2; 800 ng/well). After 4 and 12 minutes the intracellular Ca++ concentration was determined.

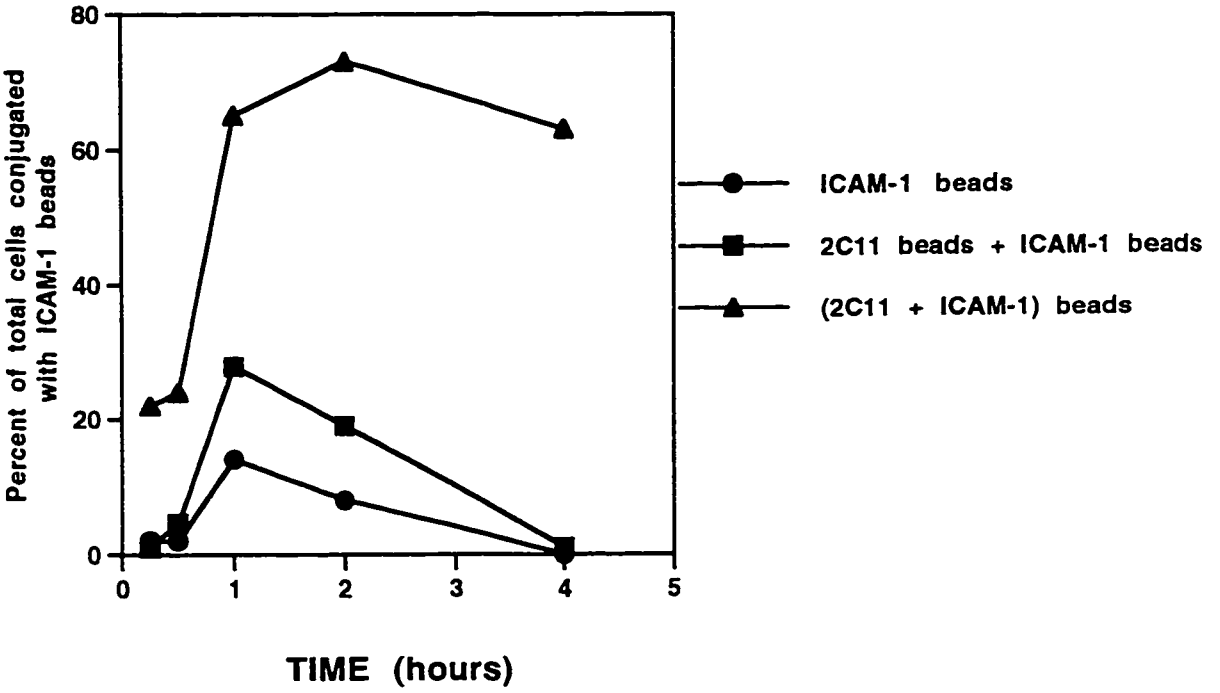


Figure 3-11. CTL adhere to ICAM-1 immobilized latex beads only when anti-CD3 is co-immobilized with the ICAM-1. Fluorescent beads were coated with ICAM-1 or ICAM-1 and 2C11 (anti-CD3). 2C11 alone was immobilized on non-fluorescent beads. The various beads were mixed with CTL clones (5:1 bead:cell ratio) and the percentage of CTL conjugated with fluorescent beads was determined at various times.

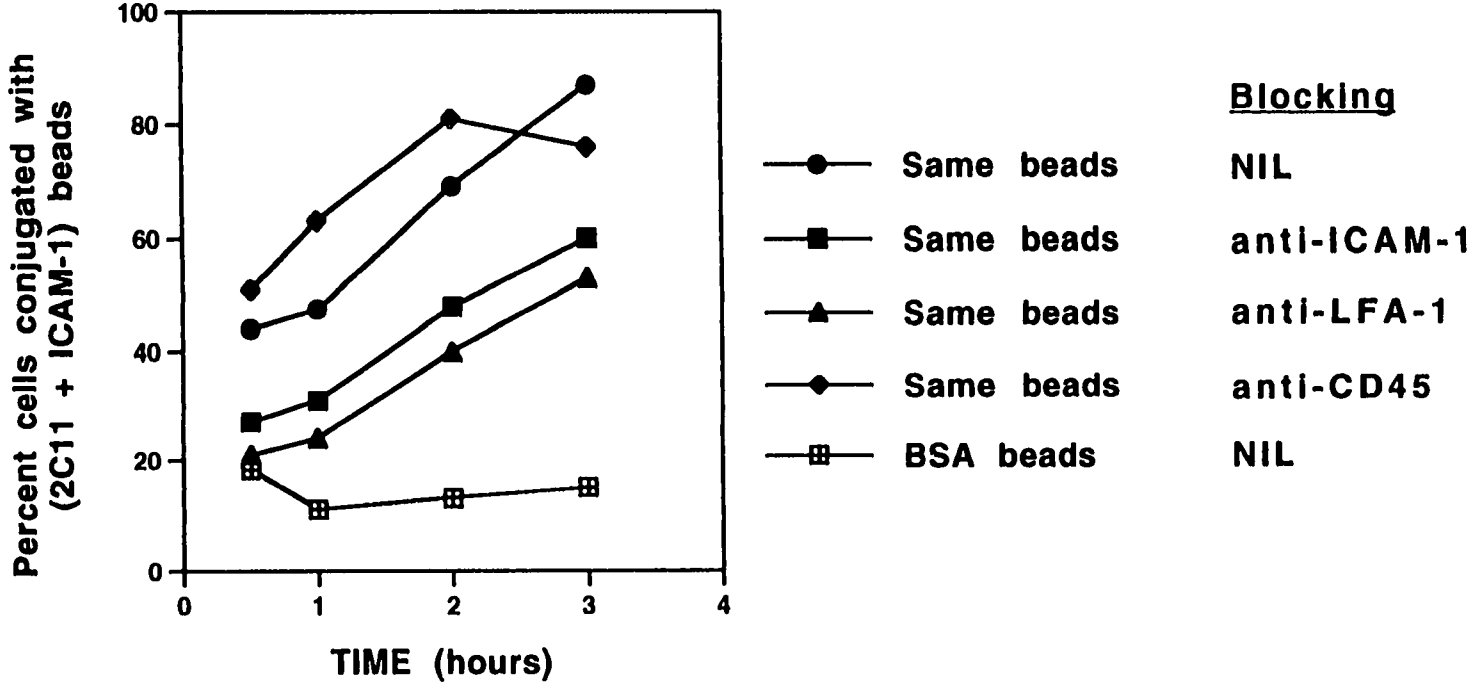


Figure 3-12. Antibodies against ICAM-1 and LFA-1 inhibit conjugate formation between clone 11 and (2C11 and ICAM-1)-coated beads. Latex beads were coated with BSA (control) or 2C11 and ICAM-1 (same). Cells and beads were mixed (5:1 bead:cell ratio) with or without antibodies to LFA-1 (M17/5.2), ICAM-1 (YN1/1.7) or CD45 (I3/2). The percentage of total CTL conjugated with beads was determined at various time intervals after initial mixing.

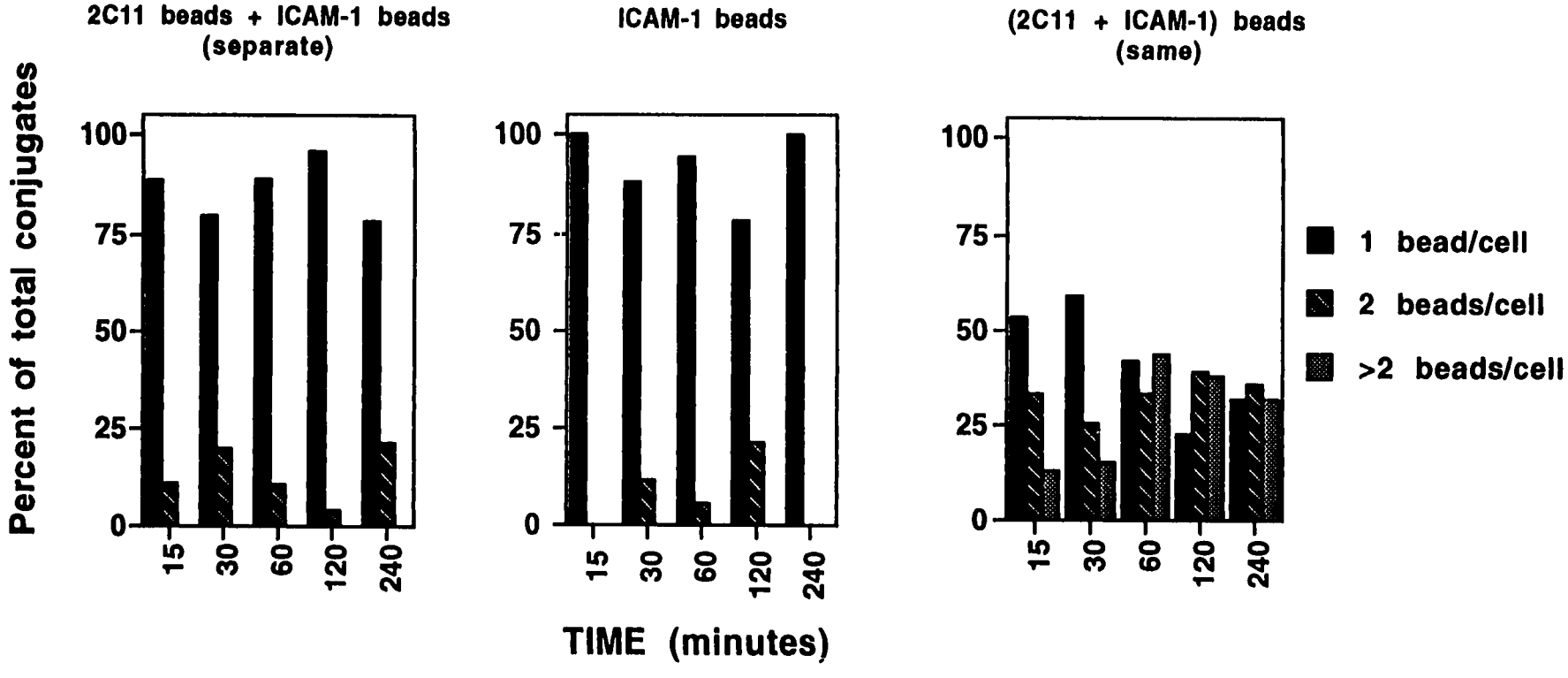


Figure 3-13. Analysis of CTL clones conjugated to ICAM-1 beads. Fluorescent beads were coated with ICAM-1 or ICAM-1 and 145-2C11 (same). 2C11 alone was immobilized on non-fluorescent beads. 2C11-coated and ICAM-1-coated beads were mixed (separate). The various beads were mixed with CTL clones and the number of CTL conjugated with fluorescent beads was determined at various times after initial mixing. Data is presented as the number of beads engaging each CTL in the CTL/bead conjugates.

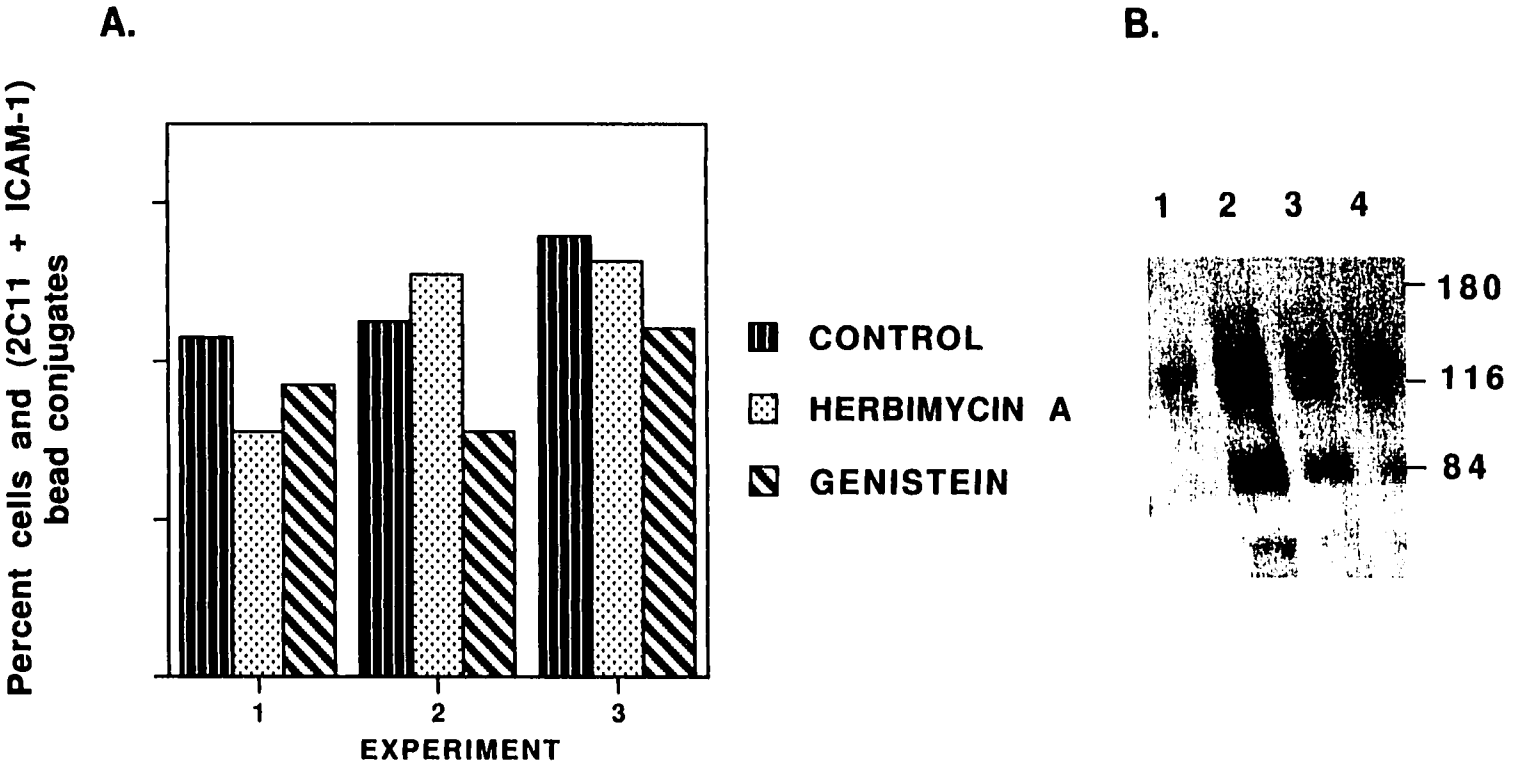


Figure 3-14. Stable adhesion between CTL and (ICAM-1 and anti-CD3) immobilized latex beads is not inhibited by tyrosine kinase inhibitors. **A.** ICAM-1 and anti-CD3 (145-2C11) co-immobilized on the same beads were mixed with CTL clones preincubated for 30 minutes in the absence (control) or presence of tyrosine kinase inhibitors herbimycin A (6 μg/ml) or genistein (50 μg/ml) and the percentage of CTL conjugated with latex beads was determined 2 hours after mixing. **B.** Clone 11 cells were stimulated for 30 minutes in a microtitre plate with a stimulatory amount of immobilized anti-CD3 (145-2C11) alone (lane 2) or first preincubated for 30 minutes in the presence of herbimycin A (lane 3) or genistein (lane 4) as in A. Cells were incubated on BSA-blocked wells as a control (lane 1). Proteins were run on 7.5% SDS-PAGE and phosphotyrosine immunoblotting was performed.

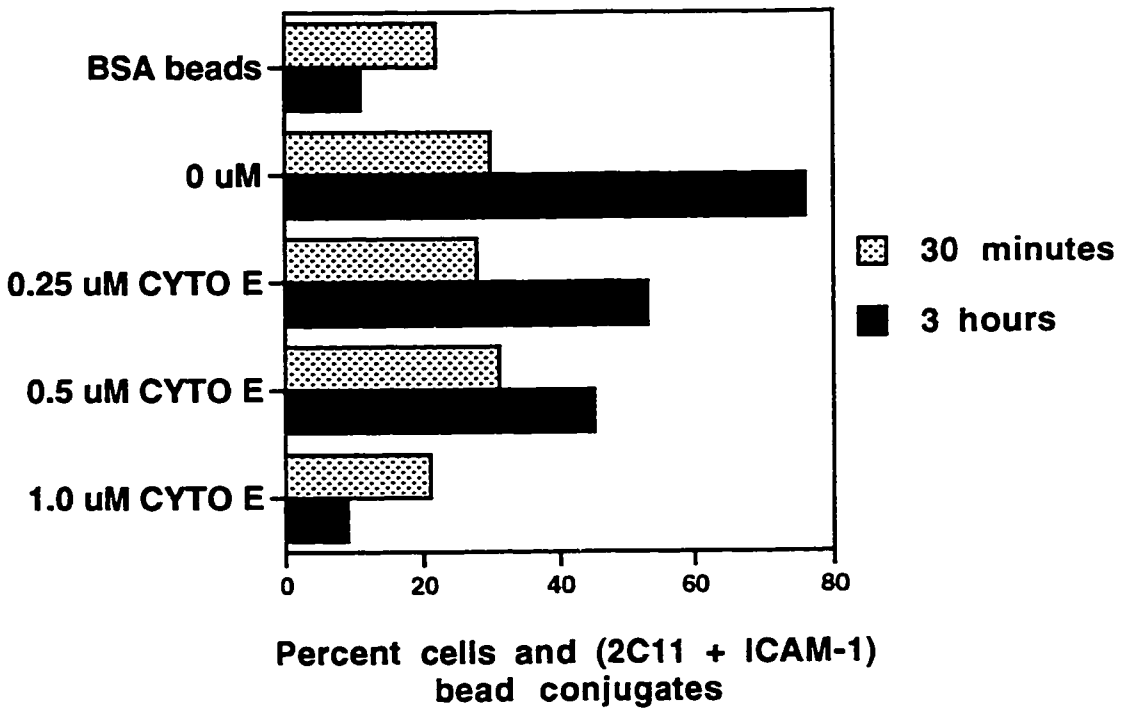


Figure 3-15. Conjugate formation between clone 11 and (2C11 and ICAM-1)-coated beads is inhibited by cytochalasin E. Latex beads were coated with BSA (control) or 2C11 and ICAM-1. Cells and beads were mixed (5:1 bead:cell ratio) with or without cytochalasin E and the percentage of CTL conjugated with beads was determined at 30 minutes and 3 hours after mixing.

CHAPTER IV

T Cell Receptor Engagement Induces Tyrosine Phosphorylation of FAK and Pyk2 and Their Association with Lck

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A. Introduction

Stimulation through the TCR results in tyrosine phosphorylation of a number of proteins which leads to functional activation of T cells. Identification of the substrates that become phosphorylated and defining their interactions with other signalling molecules will provide insight into the mechanisms of signalling cascades controlling T cell activation. We examined the contribution of a new family of non-receptor PTKs to the activation of CTLs and the regulation of these PTKs.

FAK is a non-receptor PTK that localizes to focal adhesions (Schaller et al., 1994; Schaller, 1996). Tyrosine phosphorylation and activation of FAK has been shown to occur upon integrin engagement and is thought to be linked to integrin signalling (Schaller et al., 1994; Schaller, 1996). More recently FAK has been shown to become tyrosine phosphorylated following stimulation of G-protein linked receptors, PTK receptors (Schaller, 1996), and the TCR (Maguire et al., 1995), and following oncogenic transformation (Guan et al., 1992), suggesting that FAK can play a role in signalling cascades initiated through a number of different receptors.

Proline rich tyrosine kinase 2 (Pyk2) was cloned and has 45% amino acid identity to FAK (Lev et al., 1995). Cell adhesion kinase β (CAKβ) (Sasaki et al., 1995) and the related adhesion focal tyrosine kinase (RAFTK) (Avraham et al., 1995) were also cloned as

FAK-related kinases and are identical to Pyk2. FAK and Pyk2 have been grouped as a new non-receptor PTK family. FAK and Pyk2 do not contain SH2 or SH3 domains common to several other signalling molecules. However, they contain several sites for binding of SH2/SH3 containing proteins including two proline rich regions (Lev et al., 1995; Hanks and Polte, 1997).

Initial characterization of Pyk2 in neuronal cell lines indicated that Pyk2 might be involved in regulating extracellular signals that lead to a Ca^{++} flux and MAP kinase activation (Lev et al., 1995; Dikic et al., 1996). Overexpression studies implicated Pyk2 in the JNK signalling pathway in response to stress signals (Tokiwa et al., 1996) and possibly leading to Src-dependent MAP kinase activation (Dikic et al., 1996).

Previous evidence prompted us to examine if Pyk2 might be involved in signalling during T cell activation. First, Pyk2 RNA transcripts are expressed in both rat spleen, in Jurkat T cells (Sasaki et al., 1995) and in human thymus (Avraham et al., 1995). Second, Pyk2 appears to be important for mediating signals in neuronal cells through an undefined mechanism (Lev et al., 1995). Finally, we have observed that proteins at around 116 kDa become tyrosine phosphorylated upon T cell activation (Berg and Ostergaard, 1995). Since little was known about the involvement of FAK during T lymphocyte activation, and Pyk2 had not been characterized in T cells, we sought to understand the contribution of FAK and Pyk2 to signalling events stimulated through the TCR complex in CTLs.

B. Results

Generation of specific antisera to FAK and Pyk2

In order to study the possible function of FAK and Pyk2 in CTLs, we generated specific polyclonal rabbit antisera to the two kinases. This was necessary in order to isolate and distinguish the kinases. FAK and Pyk2 migrate on SDS-PAGE gels at the same molecular weight. At the initiation of this study, no Pyk2-specific antisera was available and the available antisera against FAK cross-reacted with Pyk2.

A sequence of Pyk2 (AA 720-862) and of FAK (AA 699-845) were used to make GST fusion proteins as described in the Materials and Methods. By sequence comparison, these regions were most divergent. Briefly, the sequences were amplified by RT-PCR from Jurkat cell poly A RNA (Pyk2) or from CTL clone AB.1 RNA (FAK). The sequences were inserted into the pGEX vector in frame with the GST coding sequence. GST fusion proteins were expressed and purified to homogeneity. For generation of antisera, New Zealand White rabbits were injected subcutaneously with the purified GST fusion proteins and terminal bleed antisera obtained.

Stimulation of CTL clones

Throughout the studies in this chapter, we stimulated the non-transformed, antigen driven CTL clones, clone 11 or clone AB.1, with immobilized antibody to the TCR/CD3 complex using an antibody directed against CD3ε, 145-2C11 (2C11), or with BSA as a control. The conditions used have been established to induce a functional degranulation response from the CTL clones (Figure 3-2; Berg and Ostergaard, 1995).

The tyrosine kinases FAK and Pyk2 become tyrosine phosphorylated after TCR stimulation

CTL clone 11 (10^7) were stimulated for various times with immobilized anti-CD3. FAK or Pyk2 were then immunoprecipitated and analyzed for tyrosine phosphorylation. The results in Figure 4-1A show that both FAK (upper panel) and Pyk2 (lower panel) become tyrosine phosphorylated within 10 minutes after stimulation, with maximum phosphorylation occurring at 30 minutes and being sustained to at least 60 minutes. Although the kinetics of phosphorylation appear slow compared to those observed with soluble antibody, these kinetics are normal for increases in tyrosine phosphorylation when clones are stimulated with immobilized antibody (Berg and Ostergaard, 1995). No increased phosphorylation is seen in response to the BSA control.

When this blot is stripped and reprobed using the anti-Pyk2 antiserum, Pyk2 is observed only in the Pyk2 immunoprecipitate lanes (Figure 4-1B). Similar amounts of Pyk2 are immunoprecipitated from the BSA- and 2C11-stimulated cells (Figure 4-1B). These results demonstrate that antiserum specific for FAK does not immunoprecipitate a protein detected with anti-Pyk2 antiserum. Unfortunately, the reciprocal experiment cannot be done because the anti-FAK antiserum cannot be used for immunoblotting.

We also examined the labeling of FAK and Pyk2 with ^{32}P in an *in vitro* kinase assay. Immunoprecipitated FAK and Pyk2 undergo increased phosphate incorporation after TCR/CD3 complex stimulation in this kinase assay (Figure 4-2). These results demonstrate that increased tyrosine phosphorylation of FAK and Pyk2 after T cell stimulation correlates with increased phosphate incorporation in the kinase assay. This suggests that the catalytic activity of FAK and Pyk2 is regulated, either directly or indirectly, by tyrosine phosphorylation.

Tyrosine phosphorylated FAK and Pyk2 associate with the SH2 domain of Lck

p56Lck (Lck) and p59Fyn (Fyn) are members of the Src-related PTK family involved in T cell activation (Weiss and Littman, 1994; Qian and Weiss, 1997). FAK has been shown to be associated with Src and Fyn (Cobb et al., 1994). Also, an uncharacterized 120 kDa tyrosine phosphorylated protein has been shown to become associated with Lck via its SH2 domain upon activation of T cell hybridomas (Peri et al., 1993). We also see a tyrosine phosphorylated protein(s) of approximately 116 kDa that co-immunoprecipitates with Lck following anti-CD3-stimulation of CTL clone 11 (Figure 4-3).

We wanted to determine if the Lck-associated protein(s) was FAK and/or Pyk2 (Figure 4-4A). Following stimulation and immunoprecipitation of Lck as described, the immunoprecipitated proteins were incubated with phenyl phosphate. This treatment will

release any proteins that associate with Lck via a phosphotyrosine/SH2 interaction. From the phenyl phosphate eluates, we re-immunoprecipitated with antiserum specific for either FAK or Pyk2 and immunoblotted for phosphotyrosine. Tyrosine phosphorylated FAK (Figure 4-4B) and Pyk2 (Figure 4-4C) can be eluted from Lck immunoprecipitates and re-immunoprecipitated. These results demonstrate that FAK and Pyk2 can be found in Lck immunoprecipitates.

One possibility is that FAK and Pyk2 do not directly associate with Lck and that they are found in Lck complexes on account of their association with other Lck-associated proteins. To establish that FAK and/or Pyk2 can directly associate with Lck, immunoprecipitates of FAK and Pyk2 from anti-CD3-stimulated or control cells were blotted with a Lck-SH2-GST fusion protein. The Far Western blot shown in Figure 4-5 confirms that the SH2 domain of Lck can directly bind phosphorylated FAK and Pyk2 from anti-CD3-stimulated cells. No association occurs with FAK or Pyk2 from control cells (Figure 4-5).

Lck is not required for the induction of FAK or Pyk2 tyrosine phosphorylation

Given that Lck associates with both FAK and Pyk2, we were interested in knowing whether Lck was required for the induction of tyrosine phosphorylation of FAK and/or Pyk2. To examine this question we employed the human T leukemia cells, Jurkat, and the Lck-deficient variant cell line, JCaM1.6 (Straus and Weiss, 1992). Jurkat cells can be activated with soluble antibody rather than the immobilized or cross-linked antibody required for activation of the murine CTL clones used in Figure 4-1. We therefore used a shorter time point of 5 minutes which is when peak tyrosine phosphorylation is observed in kinetic studies with Jurkat cells.

As shown previously (Straus and Weiss, 1992), when Jurkat cells are stimulated with OKT3 (anti-CD3), there is an increase in the phosphorylation of a number of cellular

proteins from whole cell lysates (Figure 4-6A). Stimulated JCaM1.6 cells have a drastically reduced amount of total protein tyrosine phosphorylation (Figure 4-6A). Surprisingly, there is an increase in the tyrosine phosphorylation of proteins in the 116 kDa range in the JCaM1.6 cells (Figure 4-6A).

FAK and Pyk2 were immunoprecipitated from OKT3-stimulated Jurkat and JCaM1.6 cells to ascertain whether the 116 kDa protein(s) that increases in phosphorylation is FAK or Pyk2. There is an increase in the tyrosine phosphorylation of both FAK and Pyk2 upon stimulation of Jurkat cells (Figure 4-6B). This result parallels that seen for the CTL clones. Interestingly, an increase in tyrosine phosphorylation of FAK and Pyk2 in the Lck-deficient JCaM1.6 cells is also observed (Figure 4-6B). Therefore the increase in FAK and Pyk2 tyrosine phosphorylation is not solely dependent on Lck.

Fyn is not required for the induction of FAK and Pyk2 tyrosine phosphorylation

Lck and Fyn may have some redundant roles in the signalling pathways leading to T cell activation (Weiss, 1993). For this reason, we examined the possibility that the Fyn PTK could be required for the induction of FAK and/or Pyk2 tyrosine phosphorylation. To investigate, we used a CTL clone, IF10, derived from alloantigen-stimulated spleen cells from a Fyn knockout mouse (Atkinson et. al., 1996). No Fyn protein could be detected in the IF10 cells as compared to the Fyn competent clone 10/1 (Figure 4-7B).

IF10 clones were stimulated with anti-CD3 for the indicated times (Figure 4-7A). FAK or Pyk2 were then immunoprecipitated and their phosphotyrosine content analyzed. Both FAK and Pyk2 undergo tyrosine phosphorylation upon stimulation through the TCR/CD3 complex with maximum phosphorylation occurring within 20 minutes and being sustained to at least 60 minutes (Figure 4-7A). These results parallel those of the Fyn competent clones (Figure 4-1) and therefore the increase in FAK and Pyk2 tyrosine phosphorylation is not solely dependent on Fyn.

Ca^{++} is neither necessary nor sufficient for FAK or Pyk2 tyrosine phosphorylation

The initial characterization of Pyk2 was done in the PC12 neuronal cell line where it was found that Ca^{++} is both necessary and sufficient for Pyk2 tyrosine phosphorylation (Lev et al., 1995). This had not been determined for FAK. We investigated the possible role for Ca^{++} in the tyrosine phosphorylation of both FAK and Pyk2 in our CTL clones (Figure 4-8A). As shown earlier, clone 11 cells stimulated with immobilized anti-CD3 for 30 minutes have a strong induction of tyrosine phosphorylation of FAK and Pyk2 above that seen with the BSA control. Identical stimulation conditions, but in the presence of EGTA to chelate extracellular Ca^{++}, results in similar levels of FAK and Pyk2 phosphorylation. This suggests that extracellular Ca^{++} is not required for the tyrosine phosphorylation of these two kinases in CTL clones. Degranulation induced upon stimulation of the CTL clones is inhibited with EGTA treatment indicating that EGTA is functionally inhibiting CTL activation (Figure 4-8B).

We tested whether a Ca^{++} flux would induce phosphorylation of FAK or Pyk2. Clones were left in suspension as a control or the Ca^{++} ionophore A23187 was added (Figure 4-8A). Neither FAK nor Pyk2 became phosphorylated following such treatment. As an additional control, CTL clones were left in suspension and treated with both EGTA and A23187 (E and A) and this also had no effect. The lower panel of Figure 4-8A shows the same blot reprobed for Pyk2.

There remained the possibility that the tyrosine phosphorylation of FAK or Pyk2 had a requirement for intracellular Ca^{++}. To address this question, we stimulated the clone AB.1 cells with immobilized 2C11 or BSA in the presence or absence of BAPTA, a compound that inhibits the increase in intracellular Ca^{++} (Figure 4-9A). Immunoprecipitated FAK and Pyk2 from the CTL clones have a strong induction of tyrosine phosphorylation upon anti-CD3 stimulation in the presence of BAPTA that is indistinguishable from that in the absence of BAPTA. The inhibition of degranulation upon

stimulation of the CTL clones that is seen with BAPTA treatment indicates that BAPTA is functionally inhibiting CTL activation (Figure 4-9B). This data, together with that of EGTA treatment, indicates that Ca^{++} is not necessary nor sufficient for the tyrosine phosphorylation of FAK or Pyk2.

Pyk2 phosphate incorporation is abolished in CTL clones with phenyl phosphate treatment

We initially observed that FAK and Pyk2 have increased phosphate incorporation following stimulation in an *in vitro* kinase assay (Figure 4-2). Importantly, the assay was performed on FAK or Pyk2 immunoprecipitates and therefore, the readout was trans- or auto-phosphorylation. Subsequently we found an association between FAK and Lck and between Pyk2 and Lck (Figures 4-4 and 4-5). One possibility is that associated Lck or another kinase is responsible for the increase in phosphate incorporation of FAK or Pyk2. Further study of the regulation of tyrosine phosphorylation was only completed for Pyk2.

To address the question of Pyk2 tyrosine phosphorylation, we modified the *in vitro* kinase assay such that we could separate the contribution of Pyk2 auto- or trans-phosphorylation from that of the associated protein(s). As before, anti-CD3 stimulation of clone AB.1 cells results in increased phosphate incorporation (Figure 4-10). Alternatively, phenyl phosphate was included during the immunoprecipitation step of anti-Pyk2 incubation (IP) or of protein A bead capture (BEADS). This treatment inhibited the Pyk2 phosphate incorporation to near baseline level. The fact that phenyl phosphate treatment of Pyk2 immunoprecipitates abolishes the phosphate incorporation would suggest that additional PTK(s) may be responsible for Pyk2 phosphorylation by virtue of an association with Pyk2 via their SH2 domains.

Pyk2 tyrosine phosphorylation is inhibited in CTL clones with PP1 treatment

We tested the effect of PP1, a specific inhibitor of the Src PTK family (Hanke et al., 1996). We initially looked at the effect of PP1 on total protein tyrosine phosphorylation in the clone AB.1 cells (Figure 4-11). Cells stimulated with anti-CD3 (2C11) undergo robust tyrosine phosphorylation of whole cell lysate proteins as previously observed. However, when cells are preincubated with PP1, the level of phosphorylation is drastically reduced over a wide time course. This is not unexpected as the Src family of PTKs play a pivotal role in the tyrosine phosphorylation cascades early in T cell activation (Weiss and Littman, 1994; Qian and Weiss, 1997).

We also specifically examined the effect of PP1 on the tyrosine phosphorylation of Pyk2 in clone AB.1 cells. As established, Pyk2 is tyrosine phosphorylated in response to anti-CD3 stimulation compared to the BSA control (Figure 4-12). Pretreatment of cells with PP1 results in an extreme reduction in the tyrosine phosphorylation of Pyk2 over a broad time course. Similar results are found for FAK. There is some level of Src family-independent phosphorylation of Pyk2, however it appears to be small.

Pyk2 phosphate incorporation is abolished in CTL clones with PP1 treatment

We examined the effect of PP1 on Pyk2 phosphate incorporation using the *in vitro* kinase assay. Clone AB.1 cells were left untreated or were pretreated with PP1. Following anti-CD3 stimulation, Pyk2 was immunoprecipitated and subjected to an *in vitro* kinase assay. The increased phosphate incorporation seen in the absence of PP1 following stimulation is completely abolished in the presence of PP1 (Figure 4-13). Together, the results of the *in vitro* kinase assay analysis indicates that one or more members of the Src family of PTKs is responsible for Pyk2 phosphate incorporation.

C. Discussion

TCR-stimulated FAK and Pyk2 tyrosine phosphorylation

In our studies, we have found that FAK and Pyk2 become tyrosine phosphorylated in CTL clones upon stimulation of the TCR/CD3 complex. FAK has been shown to become tyrosine phosphorylated through engagement of a number of different receptors on various cell types (Schaller, 1996) and Pyk2 becomes phosphorylated in neuronal cells in response to stimuli that result in a Ca^{++} flux (Lev et al., 1995). We have also demonstrated that integrin engagement stimulates the tyrosine phosphorylation of both FAK and Pyk2 in T cells (Ma et al., 1997). Therefore phosphorylation of these kinases is not unique to TCR stimulation. However, this does not diminish the potential importance of FAK or Pyk2 in T cell activation. In these studies we are maximally stimulating cells with antibodies to the TCR complex resulting in high levels of FAK and Pyk2 phosphorylation. It is possible that when T cells are stimulated with antigen presented in the context of an antigen presenting cell, suboptimal levels of FAK and Pyk2 phosphorylation are induced through the TCR. The contribution of FAK and Pyk2 phosphorylation through integrins and other accessory molecules is then required for a threshold level of phosphorylation leading to T cell activation.

We demonstrate that FAK and Pyk2 associate with the SH2 domain of Lck after T cell stimulation. Interestingly, it has been shown that Lck containing a mutation in the SH2 domain cannot restore the earliest signalling events, such as tyrosine phosphorylation or Ca^{++} flux in JCaM1 cells. This suggests that the SH2 domain of Lck is required very early in the T cell activation cascade (Straus et al., 1996). One role for Lck in T cell activation is to phosphorylate the TCR ζ chains. This allows the recruitment of the tyrosine kinase ZAP70 to the TCR complex, leading to its phosphorylation and activation (Chan et al., 1992; Iwashima et al., 1994). Therefore, since Lck plays such a pivotal role in initiating signals following TCR triggering, proteins associated with Lck, such as FAK and Pyk2, are candidate signalling molecules to participate in the signalling cascades. Also, Lck has

been shown to be associated with both ZAP70 and TCR ζ (Duplay et al., 1994; Straus et al., 1996). Therefore, there are interactions between Lck and four signalling cascade proteins: FAK, Pyk2, TCR ζ, and ZAP70. Whether these proteins are part of one multimeric complex or are in independent associations is unknown. Certainly the phosphorylation of FAK and Pyk2 may lead to the initiation of independent signalling pathways, but it is intriguing to consider that the association of FAK and Pyk2 with Lck may be involved in the TCR ζ/ZAP70 activation pathway leading to T cell activation.

Pyk2 can associate with the SH2 domain of Src in neuronal cells (Dikic et al., 1996) and FAK can associate with both Fyn and Src in Src-transformed chicken embryo cells (Cobb et al., 1994). Our data does not eliminate the possibility that FAK and/or Pyk2 may associate with other Src-related kinases in T cells. Subsequent to the completion of this work, Fyn and Lck were found to associate with Pyk2 in thymocytes (Qian et al., 1997) and Jurkat T cells (Ganju et al., 1997).

Regulation of FAK and Pyk2 tyrosine phosphorylation

We have begun to investigate how the tyrosine phosphorylation of FAK and Pyk2 is regulated. We found that the tyrosine phosphorylation of both FAK and Pyk2 are not reduced by chelating extracellular or intracellular Ca^{++} nor does an ionophore induce FAK or Pyk2 phosphorylation. Thus, unlike neuronal cells (Lev et al., 1995), the phosphorylation of FAK and Pyk2 in CTL clones is not regulated by Ca^{++}.

By using Lck deficient JCaM1.6 cells and Fyn deficient clones, we have demonstrated that neither Lck nor Fyn is solely responsible for the initial tyrosine phosphorylation of FAK or Pyk2. This does not eliminate the possibility that the Src-related kinases could be involved in phosphorylating sites subsequent to the initial phosphorylation. These latter phosphorylation events could be important in either regulating the enzymatic activity of the kinases or in mediating interactions with other proteins. Following completion of this work, it was shown that Pyk2 is not

phosphorylated in thymocytes from Fyn deficient mice (Qian et al., 1997). Possible reasons for our disparate results are potential redundant roles of Lck and Fyn in our clones or that thymocytes have a different requirement for Pyk2 phosphorylation than mature T cell clones.

We modified the *in vitro* kinase assay to address the question of the regulation of tyrosine phosphorylation of Pyk2. We found that PP1 treatment lead to a near complete inhibition of phosphate incorporation. Similar inhibition was seen by disassociating proteins from Pyk2 using phenyl phosphate. Together these results suggest that the phosphorylation of Pyk2, and possibly FAK, may require the cooperation of members of the Src family of PTKs and/or other associated kinases for full phosphorylation. Lck and Fyn may serve some redundant roles during T cell activation (Weiss, 1993). It is possible that in the absence of one of the kinases, as is the case of the singly deficient cells, the other kinase could compensate, resulting in a similar level of total tyrosine phosphorylation. Therefore, in the absence of both Lck and Fyn, Pyk2 does not become phosphorylated to a significant extent. Another possibility is that Lck and Fyn phosphorylate different sites within FAK or Pyk2 which cannot be detected by the tyrosine phosphorylation immunoblotting. We conclude that members of the Src family of PTKs, likely in cooperation, are involved in the tyrosine phosphorylation of FAK and Pyk2.

Importance of FAK and Pyk2 phosphorylation in T cell activation

A recent study demonstrated that Pyk2 may link G-protein-coupled receptor signalling to MAP kinase activation by a functional association between Pyk2 and Src (Dikic et al., 1996). FAK associates with Fyn and Src (Cobb et al., 1994) and independent evidence has suggested that FAK is involved in the signalling pathways leading from integrin receptors to MAP kinase activation (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1997). Hence, there is a precedent that FAK and/or Pyk2 may be involved in signalling pathways that are dependent on their association with members of the

Src family PTKs. Our studies demonstrate that TCR-stimulated phosphorylation of FAK and Pyk2 results in association with the Src-related kinase Lck. This may prove to be important to T cell activation through the MAP kinase activation pathway.

FAK has been shown to participate in numerous protein-protein interactions with cytoskeletal and signalling proteins (Figure 1-4). We suggest a possible role for FAK and/or Pyk2 as a scaffolding protein in signalling cascades. As previously discussed, the tyrosine phosphorylation of FAK and Pyk2 may regulate their association with Lck and perhaps other signalling molecules. This may be very important in the formation of multimeric protein complexes needed to position signalling molecules in specific areas for T cell activation.

Our studies have allowed us to begin to delineate the role of FAK and Pyk2 in TCR-stimulated CTL activation. Their significance may be by virtue of their TCR-induced tyrosine phosphorylation and subsequent association with other signalling molecules leading to potential changes in their subcellular localization and/or their kinase activity.

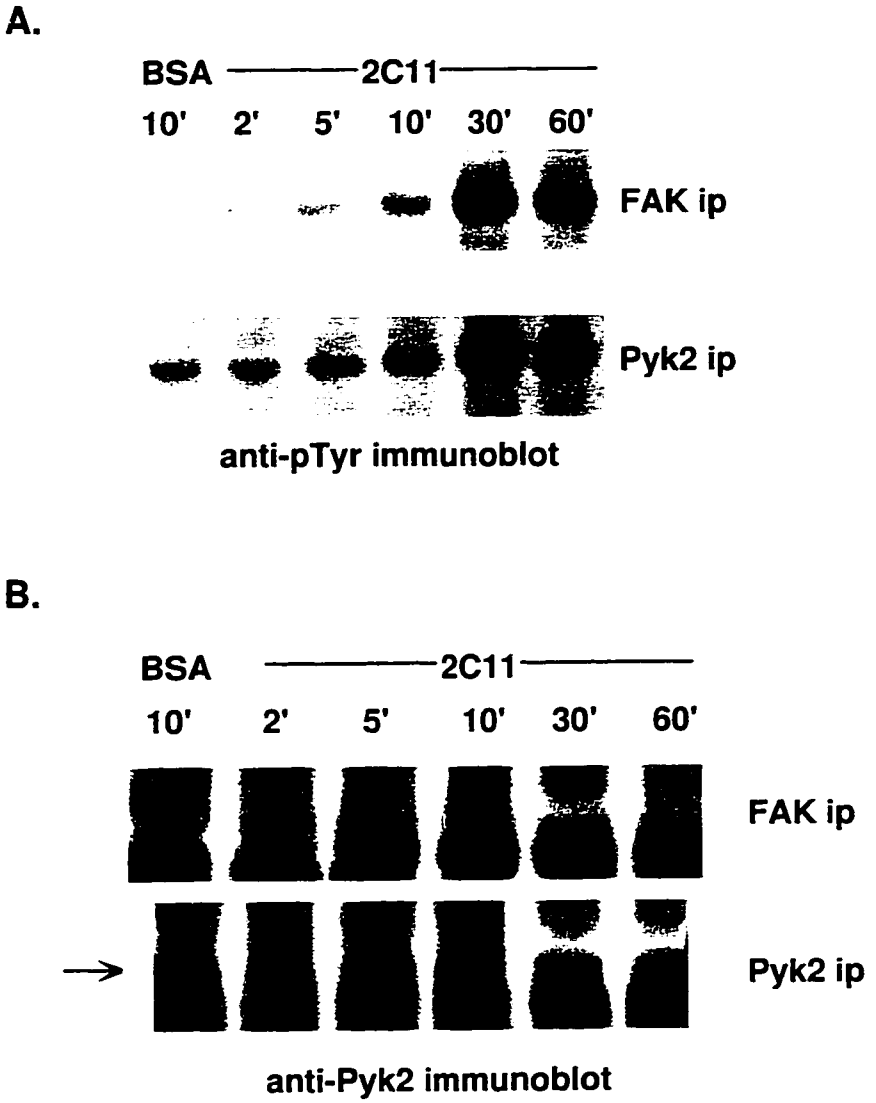


Figure 4-1. Stimulation through the TCR/CD3 complex leads to the phosphorylation of FAK and Pyk2. **A.** Clone 11 cells (10^7) were plated on dishes coated with 145-2C11 (2C11) or BSA. After the indicated time, cells were lysed and immunoprecipitates were prepared with either anti-FAK or anti-Pyk2 antisera. Proteins were subjected to 7.5% SDS-PAGE and immunoblotted with anti-phosphotyrosine. **B.** The anti-phosphotyrosine blot from A. was stripped and reprobed using anti-Pyk2 antisera. Arrow indicates Pyk2.

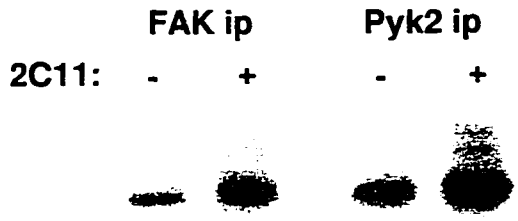


Figure 4-2. Phosphorylation of FAK and Pyk2 in an *in vitro* kinase assay is increased after T cell stimulation. Clone AB.1 cells were stimulated with immobilized 2C11 (+) or plated on BSA (-) as a control for 30 minutes and lysed. FAK and Pyk2 immunoprecipitates were prepared and subjected to an *in vitro* kinase assay. The exposure times were 2 hours for FAK and 30 minutes for Pyk2.

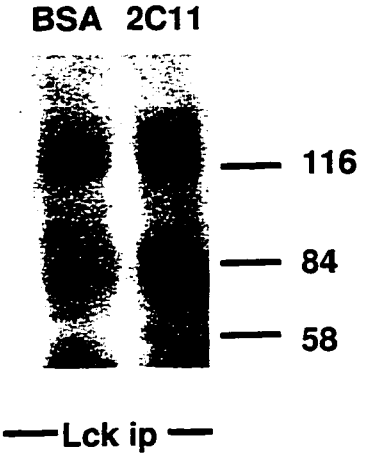


Figure 4-3. Tyrosine phosphorylated proteins co-immunoprecipitate with p56Lck. Clone 11 cells (10^7) were plated on dishes coated with 145-2C11 (2C11) or coated with BSA alone and lysed after 30 minutes. Immunoprecipitates were prepared with anti-Lck antisera, subjected to 7.5% SDS-PAGE, and immunoblotted with anti-phosphotyrosine.

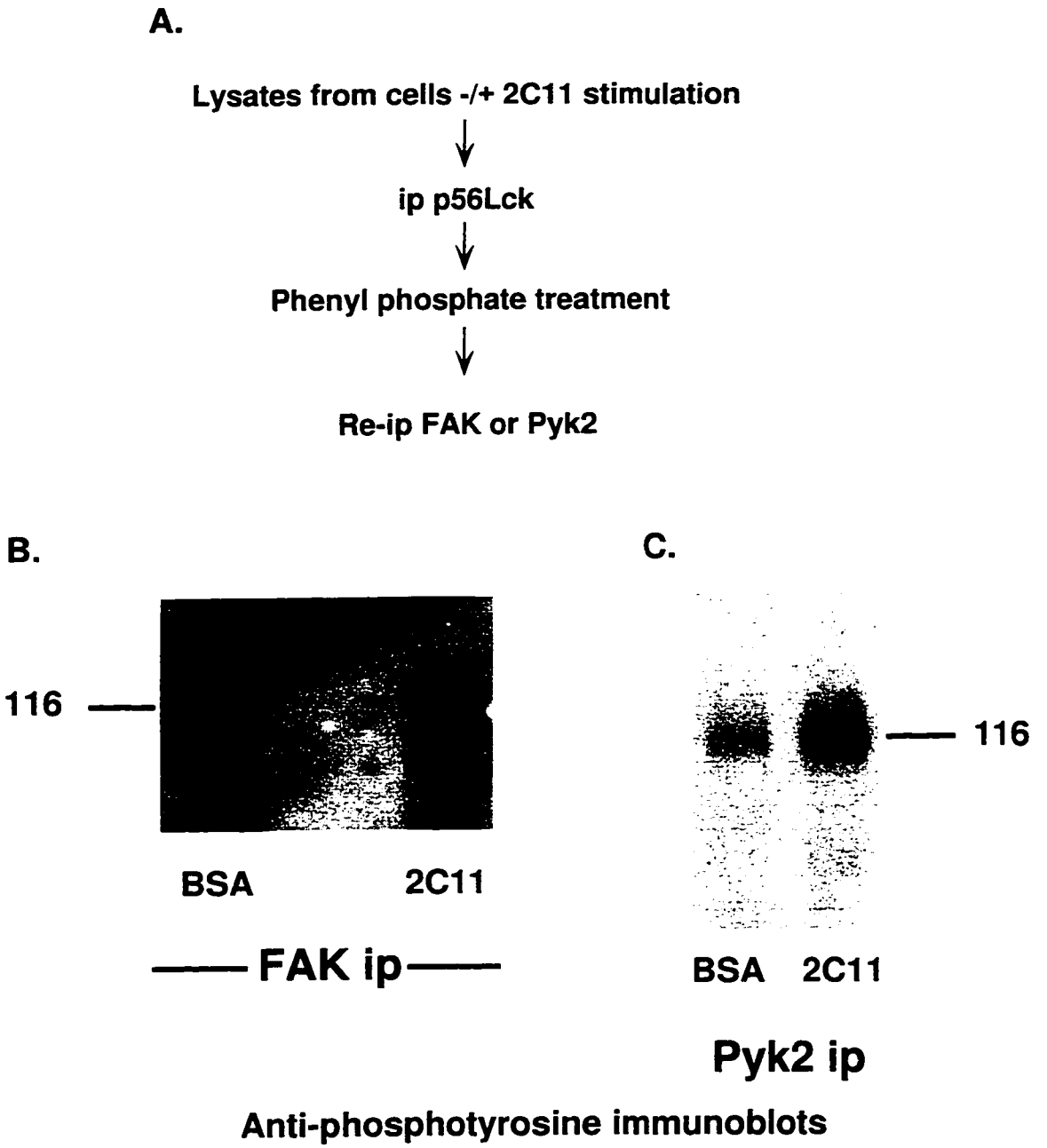


Figure 4-4. FAK and Pyk2 associate with Lck. **A.** CTL clones (10^7) were plated on immobilized BSA or 2C11 for 30 minutes, lysed, and Lck immunoprecipitated. Immunoprecipitates were treated with phenyl phosphate for 2 hours, washed, and eluates were subjected to anti-FAK or anti-Pyk2 immunoprecipitation. Proteins were run on 7.5% SDS-PAGE and immunoblotted with anti-phosphotyrosine. **B.** FAK re-immunoprecipitated from clone AB.1 cells. **C.** Pyk2 re-immunoprecipitated from clone 11 cells.

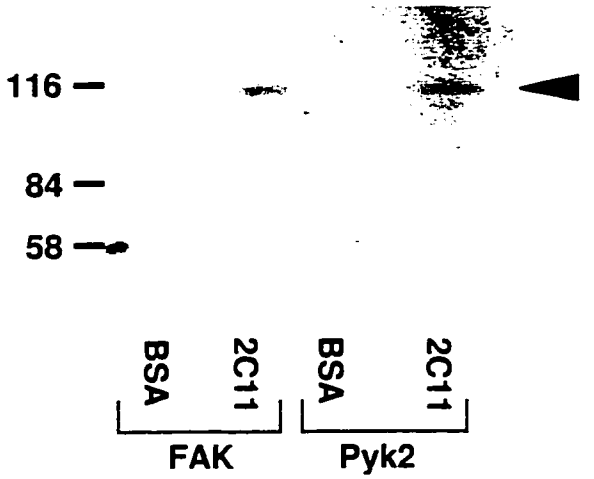


Figure 4-5. FAK and Pyk2 directly associate with the SH2 domain of Lck. FAK and Pyk2 were immunoprecipitated from 6 X 10^7 clone AB.1 cells which were plated on BSA- or 2C11-coated dishes. Proteins were blotted with GST-Lck-SH2 for one hour at 1 µg/ml, followed by extensive washing. The bound GST fusion proteins were detected using anti-GST antisera and protein A coupled to horseradish peroxidase. This figure was generated by Dr. Hanne Ostergaard.

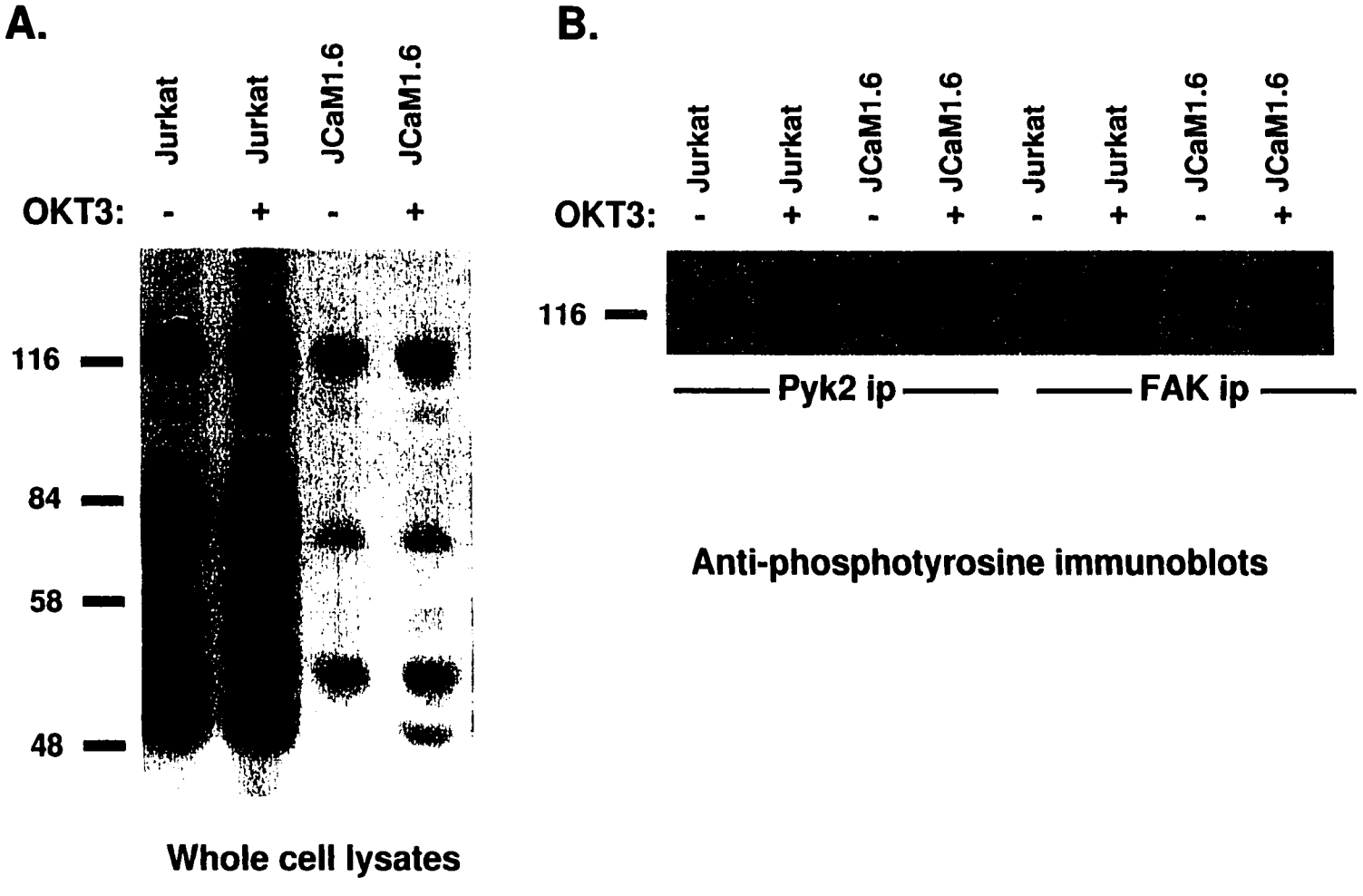


Figure 4-6. Lck is not required for tyrosine phosphorylation of FAK and Pyk2. Jurkat or JCaM1.6 cells (3.25 X 10^6) were treated with soluble OKT3 (anti-CD3) for 5 minutes at 37°C. Anti-phosphotyrosine immunoblots of postnuclear lysates **(A)** and anti-FAK and anti-Pyk2 **(B)** immunoprecipitates.

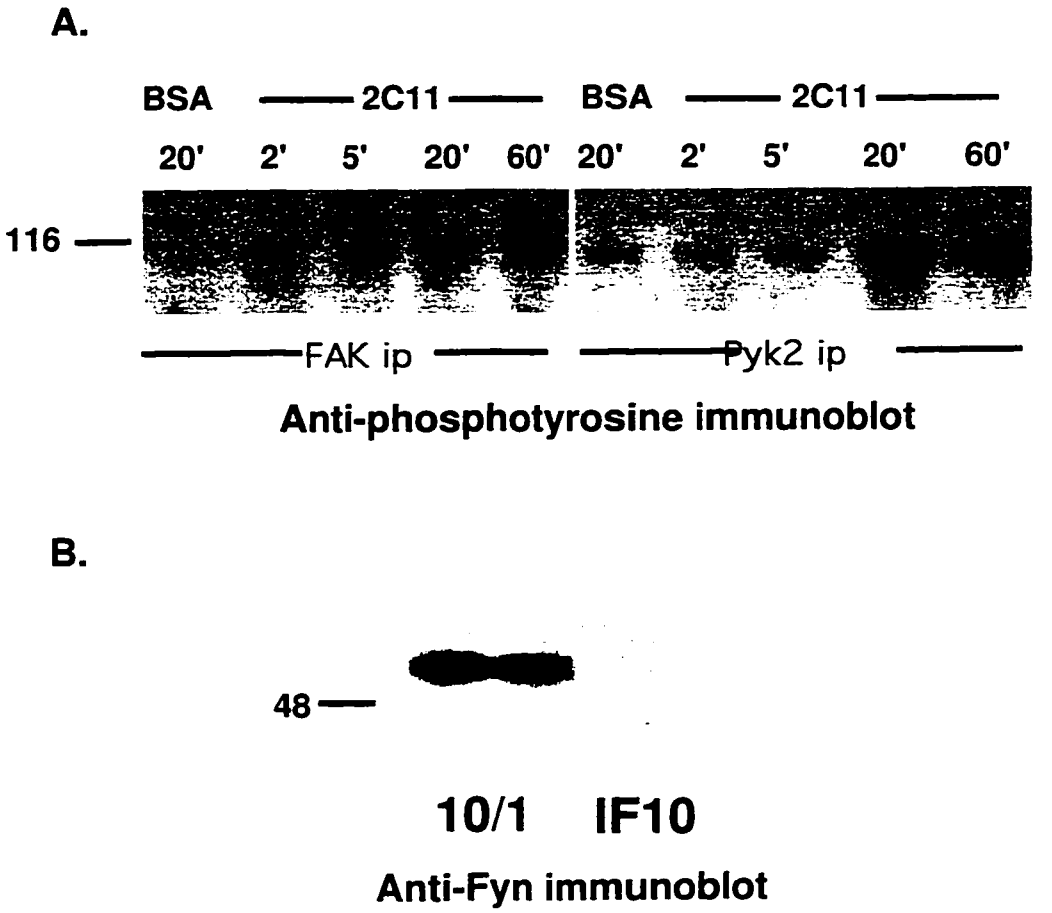


Figure 4-7. Fyn is not required for tyrosine phosphorylation of FAK and Pyk2. **A.** Clone IF10 cells (5 X 10^6) were plated on dishes coated with 145-2C11 (2C11) or coated with BSA alone. After the indicated time, the cells were lysed, and immunoprecipitates were prepared with either anti-FAK or anti-Pyk2 antisera. Proteins were subjected to 7.5% SDS-PAGE and immunoblotted with anti-phosphotyrosine. **B.** Postnuclear lysates from clone 10/1 (Fyn +/+) and clone IF10 (Fyn -/-) were run on 7.5% SDS-PAGE and immunoblotted for Fyn.

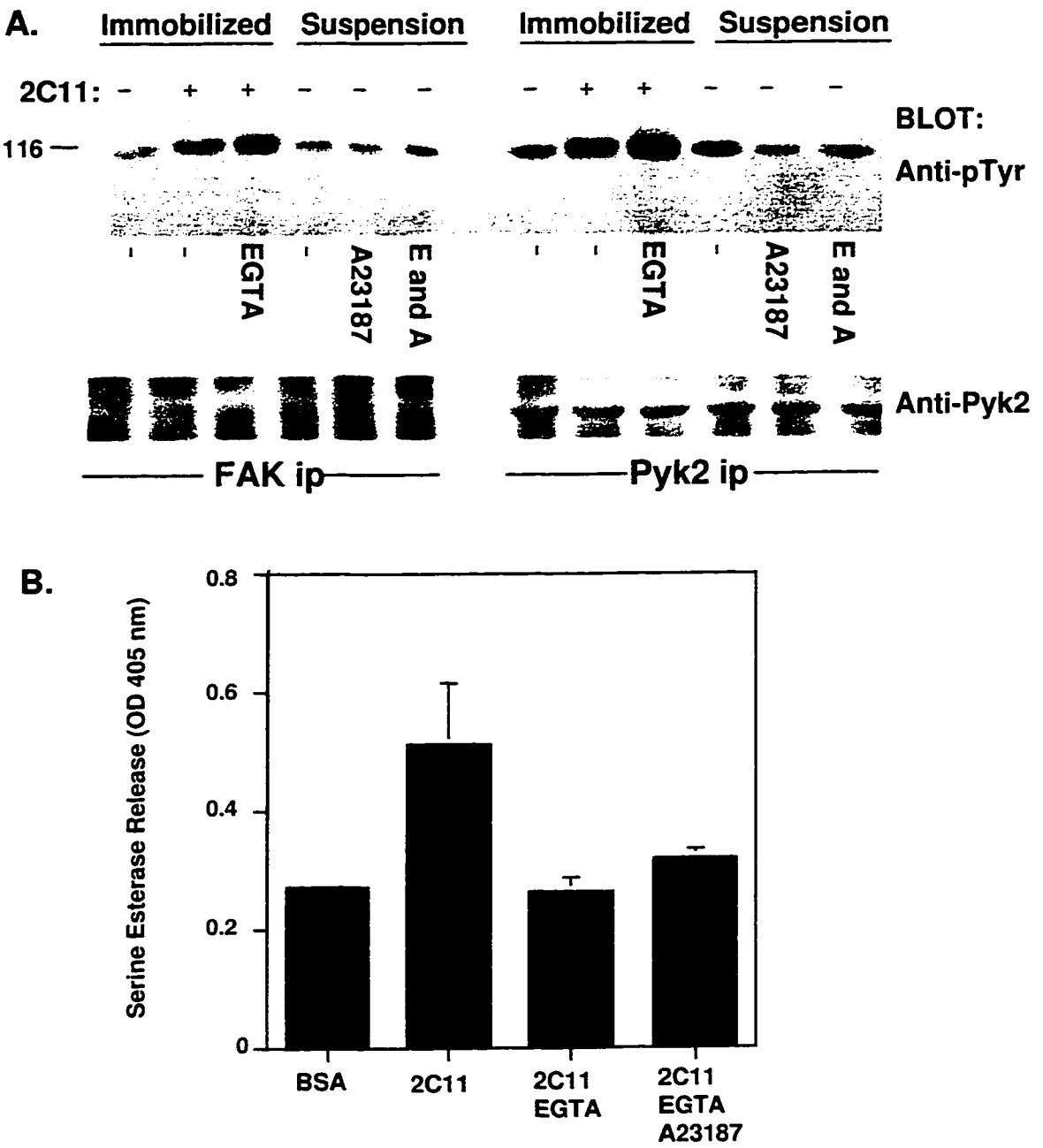


Figure 4-8. Ca^{++} is neither necessary nor sufficient for tyrosine phosphorylation of FAK and Pyk2. **A.** Clone 11 cells were plated on immobilized BSA (-) or 2C11 (+) for 30 minutes at 37°C with or without EGTA pretreatment for 30 minutes at 4°C. Unstimulated cells in suspension (-) or with added A23187 were incubated for 2 minutes at 37°C. Cells were similarly pretreated with EGTA and stimulated with A23187 (control; E and A). Cells were lysed and FAK or Pyk2 immunoprecipitated. Proteins were run on 7.5% SDS-PAGE and immunoblotted for phosphotyrosine. The lower panel is the same blot stripped and reprobed using the anti-Pyk2 antisera. **B.** Cells treated identical to A. were tested for the release of serine esterase.

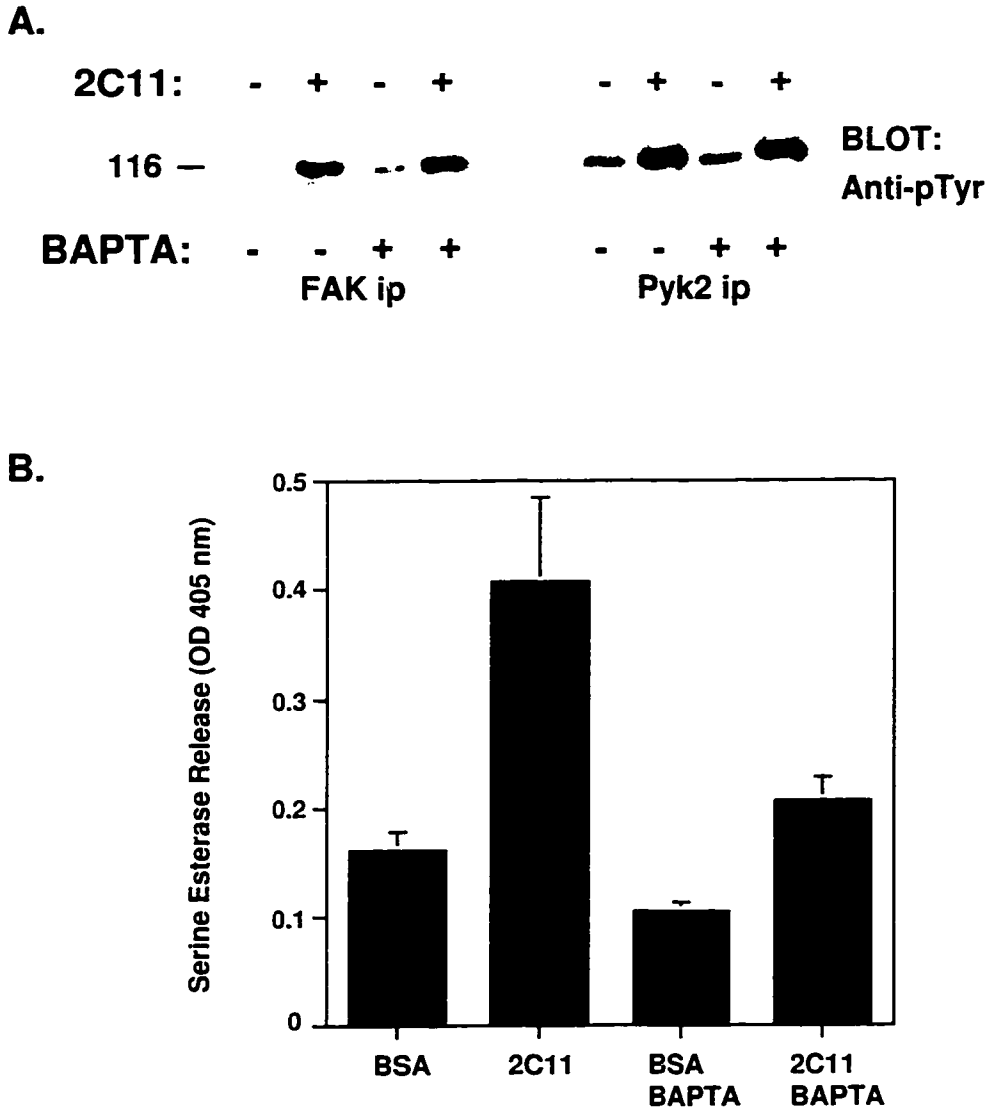


Figure 4-9. Tyrosine phosphorylation of FAK and Pyk2 does not require intracellular Ca^{++}. **A.** Clone AB.1 cells were plated on immobilized BSA (-) or 2C11 (+) for 30 minutes at 37°C with or without BAPTA pretreatment for 30 minutes at 4°C. Following stimulation, cells were lysed and FAK or Pyk2 immunoprecipitated. Proteins were run on 7.5% SDS-PAGE and immunoblotted for phosphotyrosine. **B.** Cells treated identical to A. were tested for the release of serine esterase.

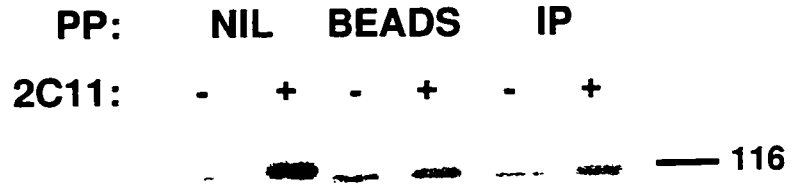


Figure 4-10. Increased phosphorylation of anti-CD3-stimulated Pyk2 in an *in vitro* kinase assay is abolished with phenyl phosphate treatment. Clone AB.1 cells (10^7) were plated on immobilized 2C11 (+) or BSA (-) for 30 minutes and lysed. Phenyl phosphate (PP) was included in either the antibody (IP) or bead (BEADS) incubation stage of Pyk2 immunoprecipitation. Immunoprecipitates were subjected to an *in vitro* kinase assay and run on 7.5% SDS-PAGE.

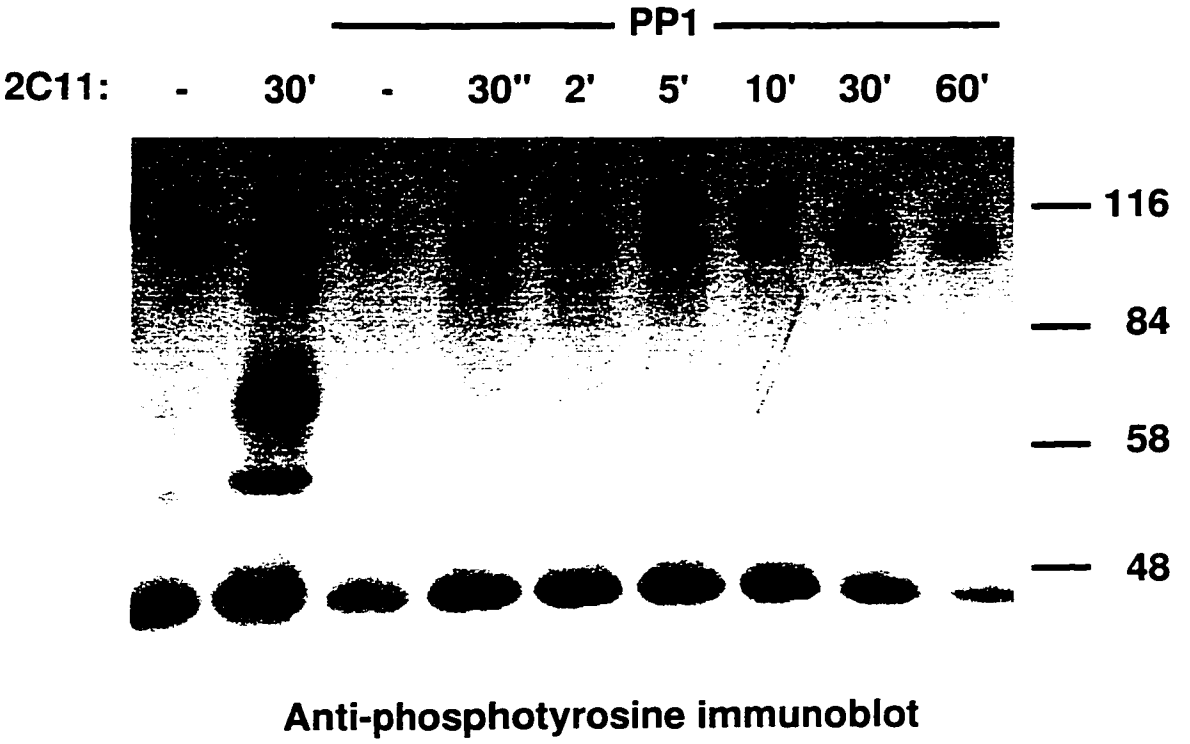


Figure 4-11. PP1 inhibition of anti-CD3-stimulated protein tyrosine phosphorylation in CTL clones. Clone AB.1 cells were untreated or pretreated with 10 μM PP1 and incubated for 30 minutes at 4°C. Cells were plated on immobilized BSA (-) or 2C11 for the indicated times. Whole cell lysates were run on 7.5% SDS-PAGE and anti-phosphotyrosine immunoblotting performed.

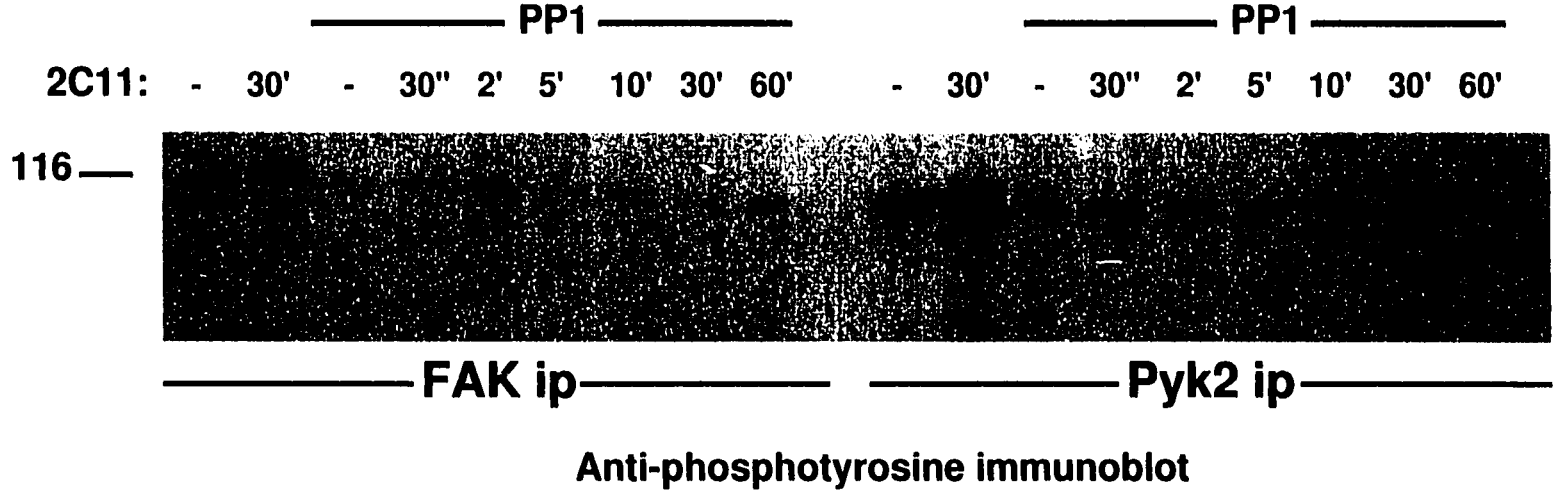
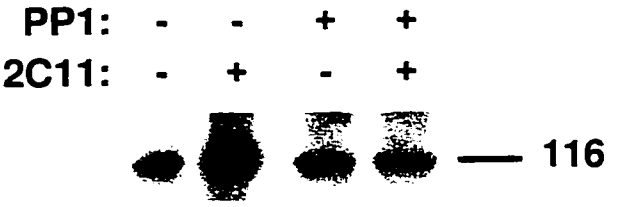


Figure 4-12. PP1 inhibition of anti-CD3-stimulated protein tyrosine phosphorylation of FAK and Pyk2. Clone AB.1 cells were untreated or pretreated with 10 μM PP1 and incubated for 30 minutes at 4°C. Cells were plated on immobilized BSA (-) or 2C11 for the indicated times. Cells were lysed and FAK or Pyk2 immunoprecipitated. Proteins were run on 7.5% SDS-PAGE and immunoblotted for anti-phosphotyrosine.



In vitro kinase assay

Figure 4-13. Increased phosphorylation of anti-CD3-stimulated Pyk2 in an *in vitro* kinase assay is abolished with PP1 treatment. Clone AB.1 cells were incubated for 30 minutes at 4°C with or without PP1. Cells (10^7) were plated on immobilized 2C11 (+) or BSA (-) for 30 minutes and lysed. Pyk2 was immunoprecipitated and subjected to an *in vitro* kinase assay.

Chapter V

Differential Signalling in Response to TCR/CD3 Complex Ligation: Sustained Signalling is Required for CTL Degranulation

A. Introduction

TCR/CD3 engagement triggers a myriad of intracellular signalling events ultimately leading to a functional outcome such as cytokine secretion, proliferation or cell-mediated killing. In recent years, many of the key signalling molecules, how they interact with one another, and their place in the signalling cascades beyond TCR engagement have been elucidated (Weiss and Littman, 1994; Cantrell, 1996). Following TCR engagement the tyrosine kinase p56Lck (Lck) is thought to phosphorylate the immunoreceptor tyrosine based activation motif (ITAM) sequences in the TCR zeta (ζ) chains. The tyrosine kinase ZAP70 subsequently associates with the phosphorylated ζ chains and activation of ZAP70 leads to downstream signalling events (Qian and Weiss, 1997). Further downstream, through a mechanism that is not fully defined in T cells, activation of the Ras pathway leads to MAP kinase activation (Cantrell, 1996).

The study of altered peptide ligands (APL) has contributed to our understanding of T cell activation (Evavold et al., 1993; Sloan-Lancaster and Allen, 1996). Th cells engaging APL have altered TCR ζ chain tyrosine phosphorylation and altered association with, and activation of, ZAP70 (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). Engagement of APL leads to altered signalling in $CD8^+$ cells in the absence of serine esterase release and cytolysis (Reis e Sousa et al., 1996). A hierarchy of cellular responses has been demonstrated in Th cells depending on whether the stimulus is an agonist, partial

agonist or antagonist (Rabinowitz et al., 1996). The different cellular responses following stimulation with APL indicate that the events of T cell activation can be uncoupled from one another and that TCR/CD3 complex engagement does not always lead to complete activation.

It has been suggested that a threshold of TCR occupancy is necessary for sustained signalling and subsequent T cell activation (Valitutti et al., 1995a; Valitutti et al., 1996). The formation of intracellular signalling complexes could be an obligatory consequence of TCR occupancy that is necessary for full cellular activation (Wange and Samelson, 1996). Given this information, it seemed probable that the method of T cell stimulation may influence the activation outcome. We tested the functional differences in stimulating CTL clones with antibody to the TCR/CD3 complex in immobilized and soluble cross-linked form. Similar to the study of APL, we utilized this experimental approach to examine the requirements for T cell activation. In this chapter, we present evidence supporting the need for sustained signalling through the TCR/CD3 complex to generate downstream signalling responses and T cell activation as measured by CTL degranulation.

B. Results

Cell stimulation

We stimulated the CTL clones, clone AB.1 or clone 11, through the TCR/CD3 complex using an antibody specific for CD3ε, 145-2C11 (2C11), or for the TCR, H57-597 (H57). For immobilized (IMM) stimulation, cells were plated on antibody-coated wells of 96-well microtiter plates or antibody-coated 60 mm petri dishes. Alternatively, cells were incubated with soluble anti-CD3 for 30 minutes on ice and used directly (Soluble 2C11) or were cross-linked (XL) with secondary goat (or rabbit) anti-hamster (GAH) antibody. Cells were incubated at 37°C.

CTL degranulation requires an immobilized stimulus

We demonstrated that immobilized antibody to CD3ε (IMM 2C11) stimulates a dose-dependent degranulation response in CTL clone AB.1 as measured by the release of serine esterase (Figure 5-1; Berg and Ostergaard, 1995). In contrast, when we performed the same experiment with soluble cross-linked antibody to CD3ε (XL 2C11) this stimulation condition was unable to evoke a degranulation response (Figure 5-1). Degranulation was not observed over a wide range of primary antibody (2C11) concentrations with two concentrations of secondary GAH antibody; no cross-linking conditions were found that could stimulate degranulation above the background control of BSA alone. Soluble 2C11 alone did not induce a degranulation response. Ice pretreatment had no effect on the degranulation in response to immobilized anti-CD3 stimulation.

Similarly, immobilized anti-TCR (H57) stimulation at 20 μg/ml (high) induced robust degranulation whereas soluble cross-linked anti-TCR did not. As controls, wells coated with BSA or immobilized substimulatory anti-TCR (1.0 μg/ml; low) did not induce degranulation (Figure 5-2). This experiment was performed with clone 11 indicating that both clone AB.1 and clone 11 are capable of degranulating in response to TCR/CD3 complex stimulation.

Immobilized, but not soluble cross-linked antibody to TCR/CD3 complex leads to sustained tyrosine phosphorylation

Since degranulation is a downstream event following TCR engagement and it is not triggered upon cross-linking soluble antibody against the TCR complex, we examined a more membrane proximal signalling event. We initially examined the tyrosine phosphorylation of total cellular proteins following both stimulation conditions as this is the earliest event detected thus far following TCR engagement (Mustelin, 1994).

When CTL clones are stimulated with soluble cross-linked antibody to CD3 there is an early and transient tyrosine phosphorylation of a number of proteins (Figure 5-3).

Phosphorylation of the majority of proteins occurs by 5 minutes and begins to diminish by 20 minutes. Immobilized anti-CD3-stimulated CTL clones have similar levels of protein tyrosine phosphorylation at 10 minutes compared to soluble cross-linked antibody stimulation, but phosphorylation is sustained until at least 60 minutes. Both stimulation conditions yield similar patterns of phosphorylation with apparently similar proteins becoming phosphorylated to the same extent. The major difference appears to be the duration. Even considering an initial lag time for cells to contact the immobilized antibody, the duration of tyrosine phosphorylation is about 15 minutes for soluble cross-linked stimulation as compared to 50 minutes for immobilized conditions.

We specifically examined several intracellular proteins to compare the duration of their tyrosine phosphorylation to that of the whole cell lysate proteins. Following anti-CD3 stimulation under the two different conditions, the non-receptor PTKs, FAK and Pyk2, were immunoprecipitated with polyclonal antisera and their phosphotyrosine content analyzed. Figure 5-4A shows that under immobilized stimulation conditions, FAK becomes tyrosine phosphorylated by 15 minutes and that this is sustained to at least 45 minutes. Similar kinetics of tyrosine phosphorylation are seen for Pyk2 (Figure 5-4B). Following soluble cross-linked anti-CD3 stimulation, FAK undergoes a very transient tyrosine phosphorylation that is only apparent at 5 minutes after stimulation (Figure 5-4A). The kinetics of Pyk2 tyrosine phosphorylation, examined in a narrower time interval, reached a maximum at 2 minutes and decreased after 15 minutes (Figure 5-4B).

In addition to signalling proteins, the cytoskeleton protein paxillin was also examined. We have previously shown that upon stimulation with immobilized anti-CD3, paxillin is tyrosine phosphorylated and undergoes a dramatic mobility shift from approximately 60 kDa to 80 kDa when subjected to SDS-PAGE (Ostergaard et al., 1998). We examined the mobility shift of paxillin in whole cell lysates of stimulated cells. With soluble cross-linked anti-CD3, paxillin is seen to shift in its mobility starting at 10 minutes and this begins to decrease after 40 minutes (Figure 5-4C). Immobilized anti-CD3

stimulation leads to a sustained mobility shift in paxillin beginning at 20 minutes and sustained for until at least 90 minutes. This mobility shift may be due to tyrosine and/or serine/threonine phosphorylation (Batt and Roberts, 1998). The phosphorylation of FAK and Pyk2 and the mobility shift of paxillin have similar kinetics to that of the whole cell lysate proteins with the events triggered by the immobilized stimulus being sustained and those by soluble cross-linked stimulus being more transient.

The CTLs are therefore capable of quantitatively and qualitatively similar tyrosine phosphorylation events under both stimulation conditions, with the duration of the signal being the only obvious difference. This indicates that the immobilized stimulus allows for sustained tyrosine phosphorylation that is not achieved when soluble cross-linked stimulation is used. These results demonstrate that induction of tyrosine phosphorylation by a TCR agonist does not necessarily lead to a functional response.

p56Lck undergoes a mobility shift only with immobilized antibody stimulation

TCR/CD3 complex stimulation leads to the activation of p56Lck. The phosphorylation and enzymatic activity of Lck plays a pivotal role in the downstream tyrosine phosphorylation events that result in T cell activation (Abraham et al., 1991; Straus and Weiss, 1992; Straus et al., 1996). It has previously been shown that p56Lck undergoes a mobility shift to p60Lck following anti-CD3 stimulation (Veillette et al., 1988a; Marth et al., 1989; Caplan and Baniyash, 1996). This shift may be due to increased serine phosphorylation of Lck (Veillette et al., 1988a; Marth et al., 1989), the function of which is not clearly understood.

We wanted to determine if our stimulation conditions lead to a migratory shift in Lck. CTL clones were stimulated with either soluble cross-linked or immobilized anti-CD3 and immunoprecipitated Lck was run on a 8.5% low bis SDS-PAGE gel which has high resolution in the 40-60 kDa range. The gel was probed with a monoclonal antibody to Lck.

The resulting blot (Figure 5-5) indicates that following soluble cross-linked anti-CD3 stimulation there is a dominant low MW form of Lck (arrow 1) and a minor higher MW form (arrow 2). Lck from CTLs stimulated with immobilized anti-CD3 undergo a more extensive mobility shift to the higher MW form (arrow 2). Also, two even higher MW forms of Lck (arrows 3 and 4) are present at 30 minutes following immobilized antibody stimulation and they are sustained to at least 60 minutes. This would indicate that only under conditions of immobilized stimulation is an extensive mobility shift in Lck observed. The higher molecular weight forms could be due to serine phosphorylation (Veillette et al., 1988a; Marth et al., 1989).

Failure to induce degranulation by soluble cross-linked antibody is not restored with increased intracellular Ca^{++} or PKC activation

Recently it has been reported that the Ca^{++} flux in T cells is more sustained following stimulation with surface-attached ligands than soluble ligands (Hashemi et al., 1996). One possibility is that the soluble cross-linking conditions do not stimulate a sustained Ca^{++} flux and it was shown that Ca^{++} is required for degranulation by CTL clone AB.1 (Ostergaard et al., 1987). If this is the case then we should be able to restore degranulation in the CTL clones with a Ca^{++} ionophore.

Together, the Ca^{++} ionophore A23187 and the phorbol ester PMA trigger CTL degranulation whereas either alone does not (Figure 5-6). Treatment of cells with A23187 along with soluble cross-linked anti-CD3 stimulation does not induce degranulation in the CTL clone AB.1. Addition of PMA following soluble cross-linking antibody stimulation also does not induce degranulation. Degranulation in response to treatment with both A23187 and PMA and soluble cross-linked 2C11 serves as an additional control to show that stimulation with soluble cross-linked anti-CD3 is not interfering with the ability of the CTL clone AB.1 to degranulate. This indicates that the defect in CTL degranulation is not

simply a lack of increased intracellular Ca^{++} or PKC activation, but suggests that both pathways are insufficiently triggered by soluble cross-linked antibodies.

Sustained MAP kinase activation requires an immobilized stimulus

Since both stimulation conditions induce tyrosine phosphorylation in the CTL clones whereas degranulation is not triggered with soluble cross-linked antibody, we examined another downstream effector outcome following TCR complex stimulation, the consensus MAP kinase pathway. Previous data in fibroblasts indicated that MAP kinase activation was dependent on cell anchorage and that greater MAP kinase activation occurred in cells adhering to fibronectin than in those in suspension (Lin et al., 1997b). It is possible that similar criteria are necessary for T cells even though T cells are not considered adherent. CTL clones were stimulated as described and whole cell lysates run on a low-bis 15% SDS-PAGE and immunoblotted for MAP kinase (Figure 5-7). MAP kinase exhibits decreased mobility due to threonine and tyrosine phosphorylation which can be used to assess the extent of activation of the enzyme when low-bis gels are employed (Seger and Krebs, 1995). Following soluble cross-linked anti-CD3 stimulation there is transient and partial activation of p42 MAP kinase for a 5 minute duration as indicated by the mobility shift. Following immobilized antibody stimulation the mobility shift is evident at 20 minutes and some shifted material is present even at 60 minutes. Interestingly, the extent of p42 MAP kinase activation is significantly greater after stimulation with immobilized antibodies. Thus, for sustained MAP kinase activation, an immobilized stimulus is required.

An intact cytoskeleton is required for sustained tyrosine phosphorylation

Upon incubation with soluble cross-linked antibody there will be a uniform distribution of antibody on the cell surface with most receptors engaged as compared to the polarized engagement with immobilized antibody. We established that preventing actin

polymerization by treating the CTL clones with cytochalasin E inhibits degranulation in response to immobilized anti-CD3 or anti-TCR stimulation (Figure 5-8). This demonstrates that CTLs require an intact cytoskeleton to degranulate. It is plausible that the tyrosine phosphorylation induced with immobilized anti-CD3 requires an intact cytoskeleton, possibly for surface receptor redistribution, in order to be sustained. We hypothesized that inhibiting cytoskeleton assembly with cytochalasin E treatment would alter the tyrosine phosphorylation differently in the cells stimulated in the two different ways.

CTL clones were treated with cytochalasin E prior to stimulation at 37°C for various times and tyrosine phosphorylation of total cellular proteins was examined. Figure 5-9A shows that cells stimulated with immobilized 2C11 had a near complete inhibition of protein tyrosine phosphorylation in the presence of cytochalasin E (right) as compared to the normal pattern of tyrosine phosphorylation (left). Cells stimulated with soluble cross-linked 2C11 (Figure 5-9B) undergo the same tyrosine phosphorylation events in the absence (left) and presence (right) of cytochalasin E. Consequently, in contrast to the immobilized stimulus, the soluble cross-linked antibody-induced phosphorylation is not inhibited by cytoskeleton disruption.

C. Discussion

We have shown that the manner in which CTLs are stimulated, by an immobilized or a soluble cross-linked antibody to the TCR/CD3 complex, results in dramatically different outcomes. Both stimulation conditions trigger qualitatively and quantitatively similar patterns of cellular protein tyrosine phosphorylation, but of different duration. The phosphorylation induced by soluble cross-linked conditions is transient whereas it is sustained when induced by immobilized stimulation. Although soluble cross-linked antibodies can trigger tyrosine phosphorylation, degranulation is not observed. One may speculate that sustained phosphorylation is a necessary prerequisite for further downstream

effector functions such as degranulation. The immobilized antibody condition is also necessary to achieve sustained MAP kinase activation and to induce an extensive mobility shift in Lck. It is therefore evident that tyrosine phosphorylation is not necessarily an indicator of, and can be uncoupled from, a functional response. Clearly not all stimulation conditions are equal; when cells are stimulated with soluble antibody the signalling results must be interpreted carefully as a functional response may not be stimulated.

Valitutti et al. (1995a; 1996) have shown that the effector responses elicited from CTLs can be uncoupled from one another and this is attributed to different levels of TCR occupancy. It was also demonstrated that anti-CD3 stimulated apoptosis and cytokine secretion by a Th hybridoma can be uncoupled from each other by using immobilized versus soluble antibody (Glickstein et al., 1996). Furthermore, it has been suggested that T cells have the ability to count the number of TCRs triggered with MHC/peptide (Viola and Lanzavecchia, 1996) and in the absence of threshold TCR engagement, there is no cell activation. Although our data supports that a minimum TCR engagement at the cell surface is necessary for T cell activation, our results suggest that there is more to activating T cells than simple receptor occupancy, as stimulation with soluble cross-linked antibody will result in essentially all the TCR complexes being engaged. Consistent with our data, it has been shown that prolonged TCR/CD3 engagement is needed for a sustained Ca^{++} flux which is required for T cell activation (Donnadieu et al., 1994). We propose that under immobilized antibody conditions there is a prolonged and sufficient engagement of the TCR complex to allow sustained tyrosine phosphorylation and MAP kinase activation, Lck mobility shift, and degranulation, none of which is achieved with cross-linked antibody.

TCR engagement leads to down-regulation of TCR surface expression by internalization (Valitutti et al., 1995b; Viola and Lanzavecchia, 1996). Engagement of the TCR complex on T cells with soluble cross-linked 2C11, using the same conditions that were used for this study, leads to TCR down-regulation (DeBell et al., 1992), but this is apparently insufficient for activation, as indicated by the lack of degranulation (Figure 5-1).

Moreover, recent studies have demonstrated that TCR down-regulation can occur in the absence of T cell activation and TCR down-regulation does not necessarily lead to subsequent T cell activation (Cai et al., 1997; Salio et al., 1997). Taken together, these data indicate that TCR down-regulation and T cell activation can be uncoupled.

Intracellular signalling events that lead to T cell activation require multimeric complexes to form between signalling molecules and the cytoskeleton (Wange and Samelson, 1996). Wulfing et al. (1997) suggest that the nature of the stimulus will dictate the assembly of intracellular molecules and hence the outcome of stimulation. The duration of TCR engagement is important to the formation of such complexes and the subsequent signalling events. Shaw and Dustin (1997) suggest that T cell activation is attained only after the correct signalling complexes have formed and that this requires a given amount of time. The time is needed in order to provide a stimulus of threshold strength and duration. We propose that the threshold is not achieved with soluble cross-linked antibody and the absence of these events may result in the lack of effector functions such as degranulation. The necessary strength and/or duration of stimulus is achieved only with the immobilized stimulus. We speculate that the Lck mobility shift that only occurs following immobilized stimulation may contribute to the formation of such protein complexes possibly through protein-protein interactions as a result of serine phosphorylation (Marth et al., 1989).

Previous evidence has shown that the cytoskeleton is necessary for T cell effector functions (O'Rourke et al., 1991) and that this may be required to sustain the intracellular signals (Valitutti et al., 1995a). We found that an intact cytoskeleton is required for the sustained tyrosine phosphorylation induced by immobilized antibody, but is not required for the transient phosphorylation induced by soluble cross-linked antibody. We predict that the cytoskeleton is required for cell spreading on the antibody-coated surface and engagement of sufficient receptors for threshold stimulation. As already discussed, however, TCR occupancy alone is not sufficient for T cell activation and we conclude that the cytoskeleton is required for the subsequent sustained phosphorylation. The

cytoskeleton, either directly or indirectly, is likely needed for the assembly of signalling molecules into multimeric complexes necessary for activation and subsequent effector functions such as degranulation.

Our data shows that MAP kinase is activated only transiently with cross-linked antibody, but with immobilized antibody MAP kinase is activated to a greater extent and is sustained. Based on a number of studies in neuronal cells, Marshall (1995) proposes that the duration of MAP kinase activation leads to functionally different responses. Although our systems differ, our data is in agreement with this model in that a longer duration of MAP kinase activation correlates with a degranulation response in CTL.

Taken together, our results suggest that T cell occupancy and induction of tyrosine phosphorylation alone are not sufficient for T cell activation. The duration of TCR stimulated signals, which likely involves cytoskeletal rearrangements, is critical to attain a functional response.

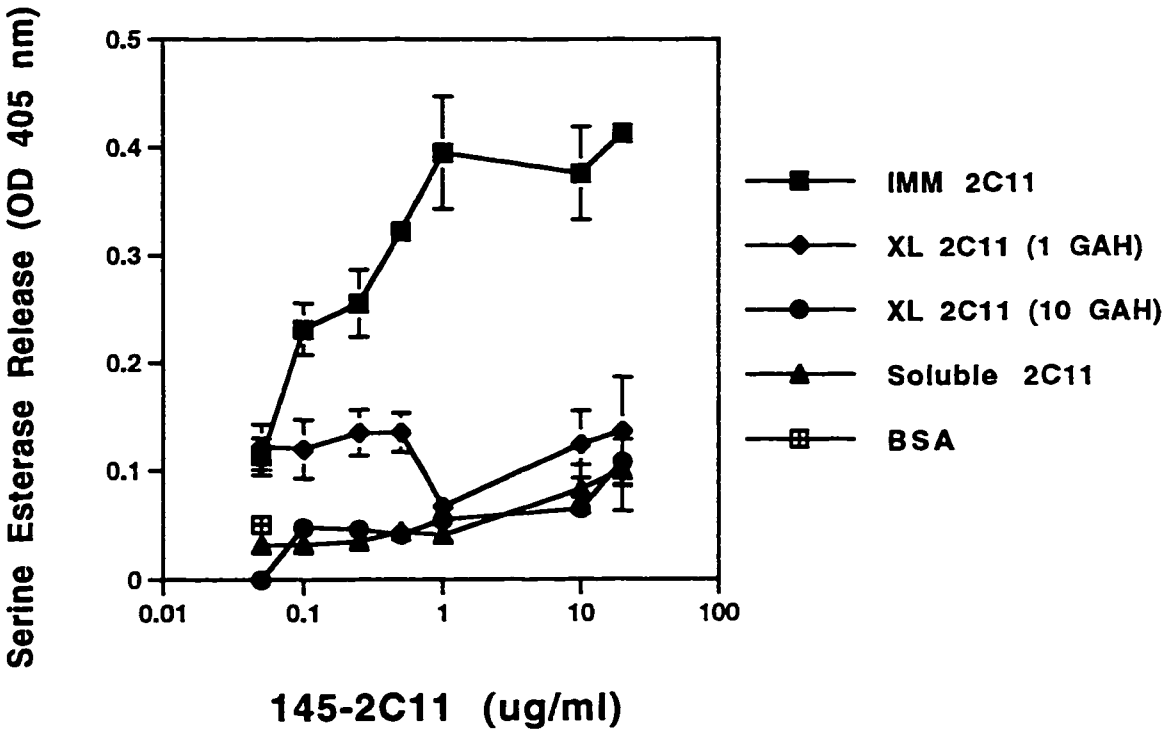


Figure 5-1. Immobilized, but not soluble or cross-linked anti-CD3 triggers CTL degranulation. Clone AB.1 cells (1.5 X 10^5) were stimulated with immobilized anti-CD3 (IMM 2C11) at 0.05 to 20.0 μg/ml (squares). Alternatively, cells were stimulated with 0.05 to 20.0 μg/ml soluble 2C11 alone (triangles) or followed by cross-linking (XL) with goat anti-hamster IgG at 1.0 μg/ml (diamonds) or 10.0 μg/ml (circles). The blocking protein BSA was used as a background control. Cells were incubated at 37°C for 4 hours and supernatants were assayed for serine esterase release.

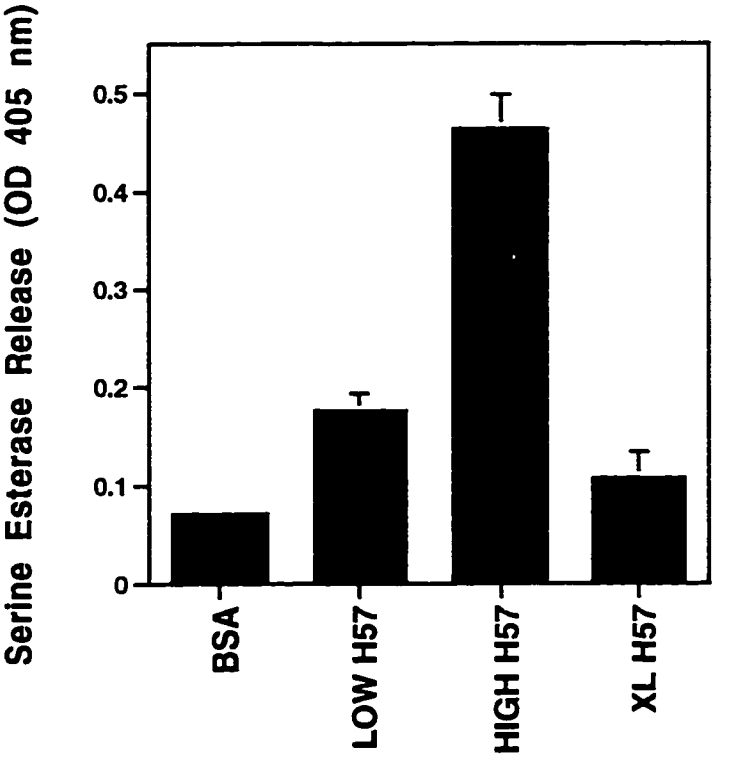


Figure 5-2. Immobilized, but not soluble cross-linked anti-TCR triggers CTL degranulation. Clone 11 cells (1.5 X 10^5) were stimulated with immobilized anti-TCR (H57) at 1.0 µg/ml (low) and 10.0 µg/ml (high). Alternatively, cells were stimulated with 10.0 µg/ml H57 and cross-linked with goat anti-hamster IgG at 10.0 µg/ml (XL). The blocking protein BSA is used as the control. Cells were incubated at 37°C for 4 hours and supernatants were assayed for serine esterase release.

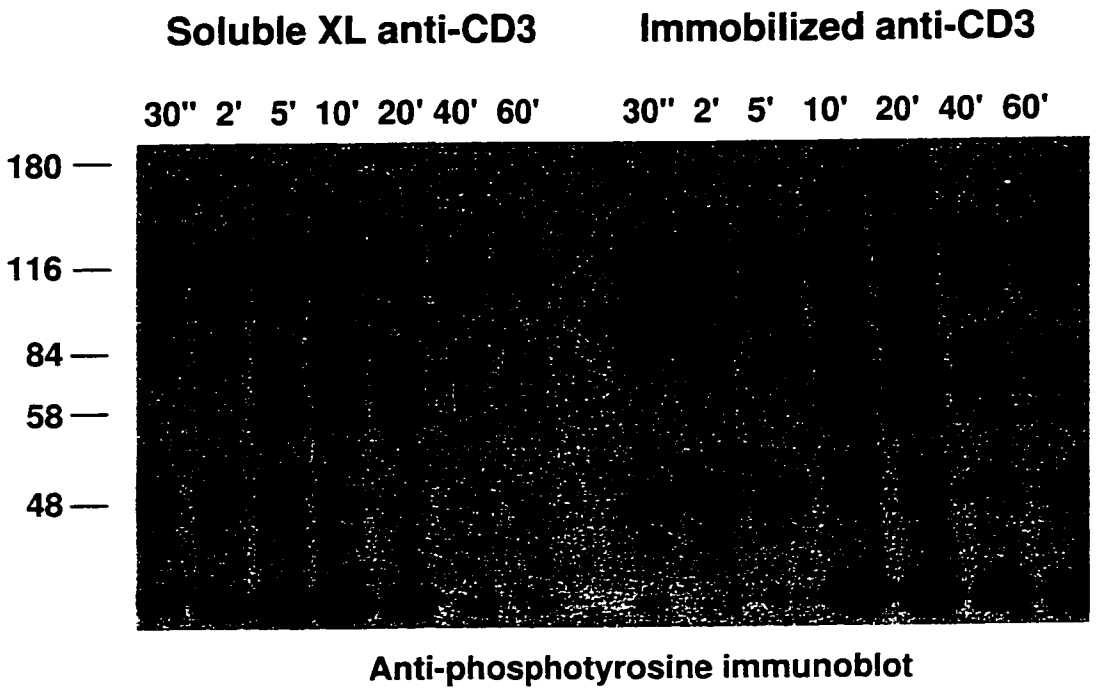
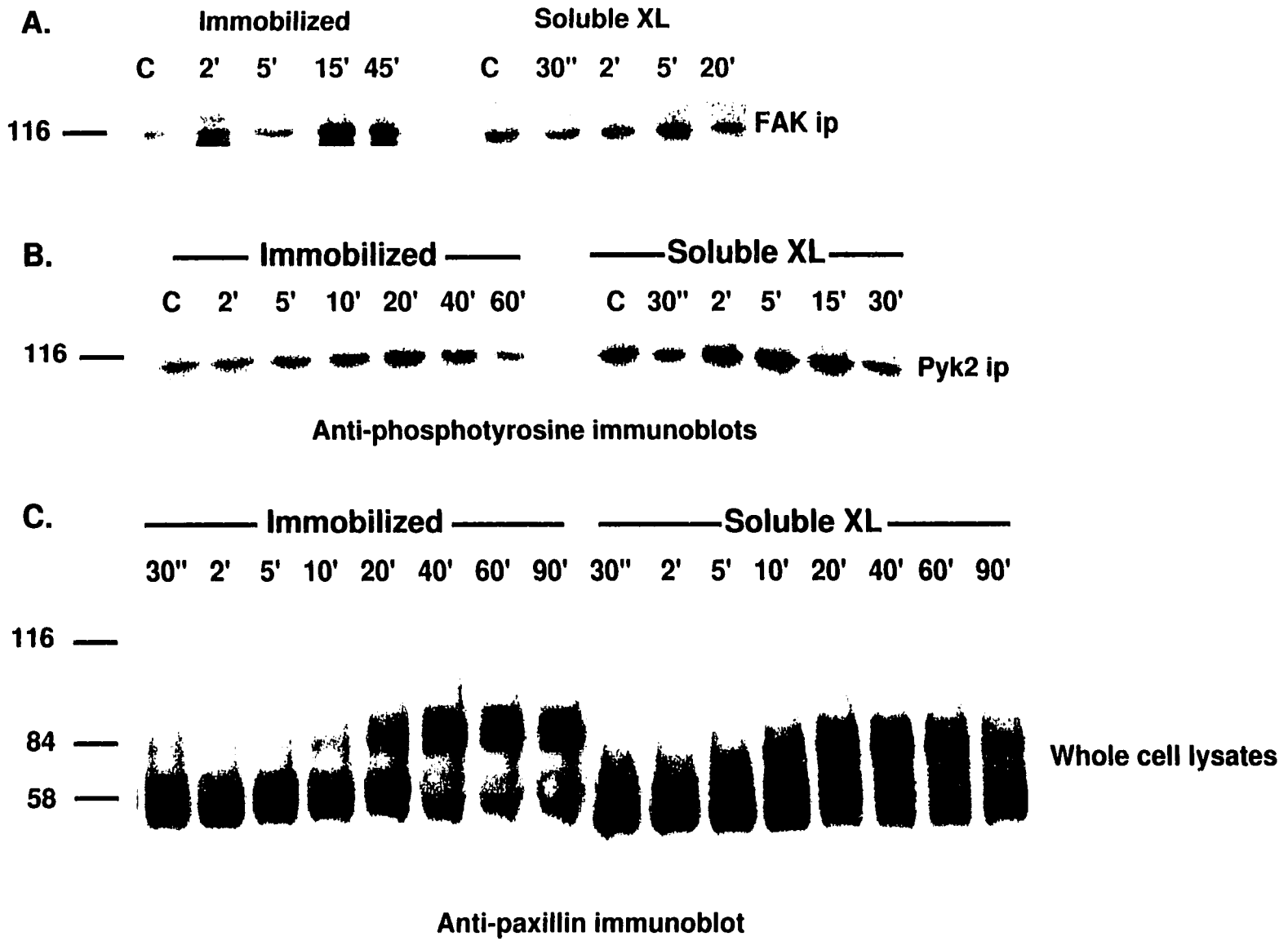


Figure 5-3. Kinetics of protein tyrosine phosphorylation of total cell lysates following anti-CD3 stimulation. Clone AB.1 cells (1.5×10^5) were stimulated with soluble anti-CD3 (2C11) and cross-linked (XL) with goat anti-hamster IgG each at 10 µg/ml or with immobilized anti-CD3 at 20 µg/ml and incubated at 37°C for the indicated times. Cell lysates were subjected to 7.5% SDS-PAGE and anti-phosphotyrosine immunoblotting.

Figure 5-4. Kinetics of FAK and Pyk2 tyrosine phosphorylation and paxillin mobility shift following anti-CD3 stimulation. 4.4 X 10^6 (**A**), 10 X 10^6 (**B**) and 1.5 X 10^5 (**C**) clone 11 cells were stimulated with soluble 2C11 and cross-linked (XL) with goat anti-hamster IgG each at 10 µg/ml or with immobilized 2C11 at 12.5 µg/ml (**A and B**) or 20 µg/ml (**C**). Cells were incubated at 37°C for the indicated times and lysed. Immunoprecipitated FAK (**A**) and Pyk2 (**B**) or whole cell lysates (**C**) were subjected to 7.5% SDS-PAGE and anti-phosphotyrosine (**A and B**) or anti-paxillin (**C**) immunoblotting. Soluble control (C) is goat anti-hamster secondary antibody only and immobilized control is BSA.


A.
Immobilized
Soluble XL
C 2' 5' 15' 45'
C 30" 2' 5' 20'
116
FAK ip
B.
Immobilized
Soluble XL
C 2' 5' 10' 20' 40' 60'
C 30" 2' 5' 15' 30'
116
Pyk2 ip
Anti-phosphotyrosine immunoblots
C.
Immobilized
Soluble XL
30" 2' 5' 10' 20' 40' 60' 90'
30" 2' 5' 10' 20' 40' 60' 90'
116
84
58
Whole cell lysates
Anti-paxillin immunoblot

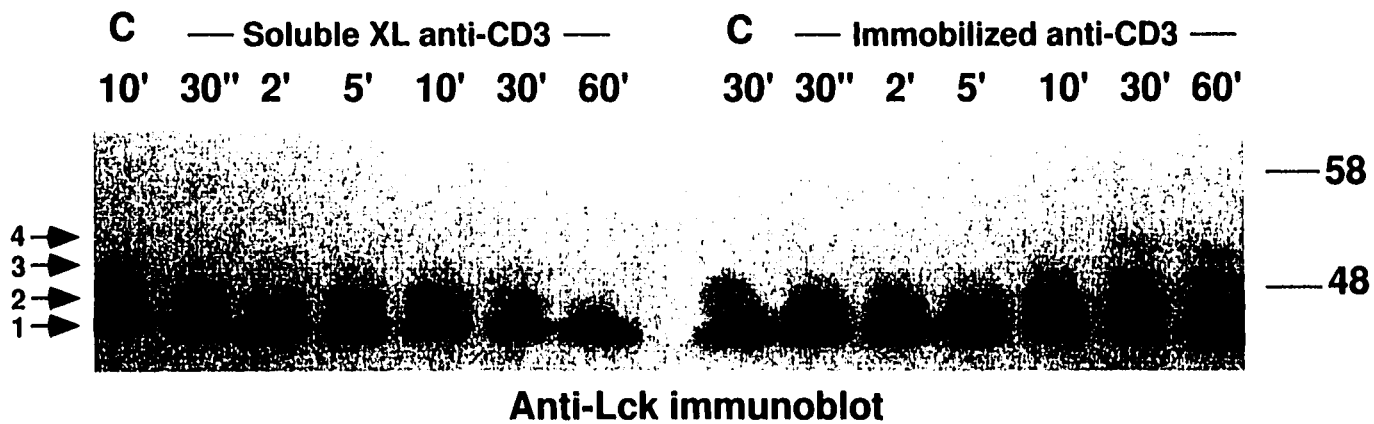


Figure 5-5. Mobility shift of p56Lck following immobilized and cross-linked anti-CD3 stimulation. Clone AB.1 cells (1.5 X 10^5) were stimulated with soluble cross-linked anti-CD3 at 10 µg/ml and goat anti-hamster IgG at 10 µg/ml (XL 2C11) or immobilized 2C11 at 20 µg/ml and incubated at 37°C for the indicated times. Soluble control (C) is goat anti-hamster IgG only and immobilized control is BSA. Cell lysates were subjected to 8.5% low-bis SDS-PAGE and anti-p56Lck immunoblotting.

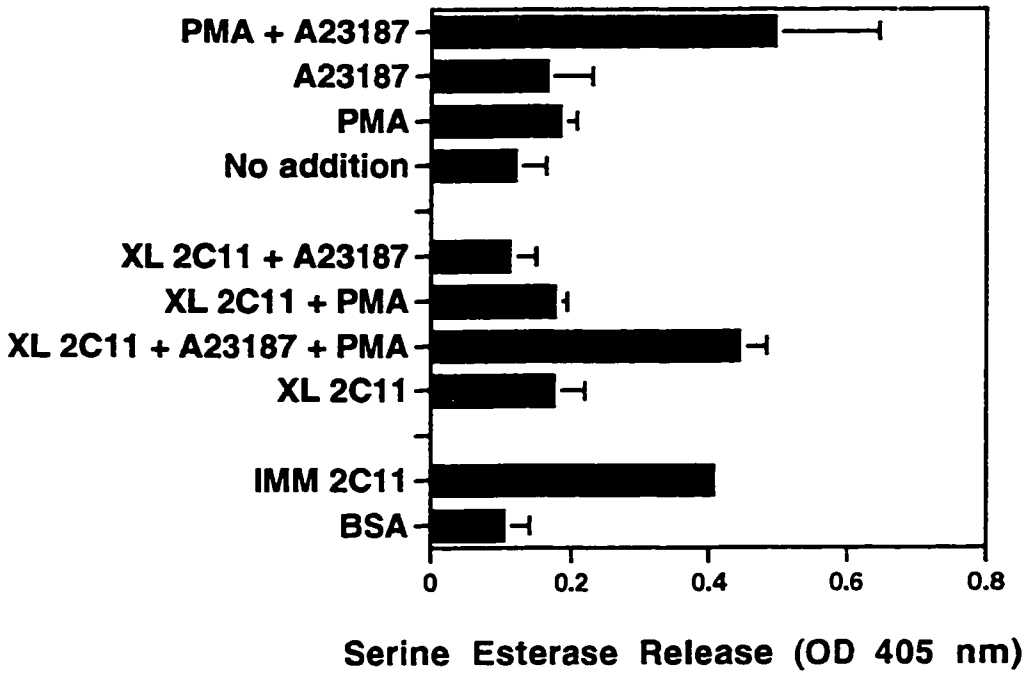


Figure 5-6. Failure to induce degranulation by soluble cross-linked antibody is not restored with PMA or A23187. Clone AB.1 cells (1.5 X 10^5) were stimulated with PMA (125 ng/ml) and/or A23187 (50 μM) alone or with 10 μg/ml soluble cross-linked (XL) 2C11. BSA and immobilized (IMM) 2C11 (20 μg/ml) were used as controls. Cells were incubated at 37°C for 4 hours and supernatants were assayed for serine esterase release.

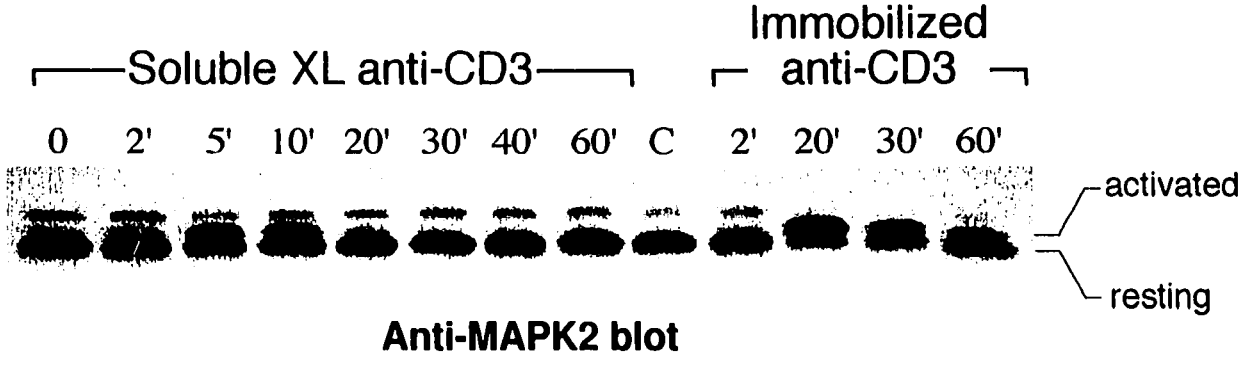


Figure 5-7. Kinetics of MAP kinase activation following immobilized and soluble cross-linked anti-CD3 stimulation. Clone AB.1 cells (1.5 X 10^5) were stimulated with soluble cross-linked anti-CD3 (XL 2C11) at 10 µg/ml and rabbit anti-hamster IgG at 5 µg/ml or immobilized 2C11 at 20 µg/ml and incubated at 37°C for the indicated times. Whole cell lysates were run on 15% low-bis SDS-PAGE and anti-MAPK2 immunoblotting was performed. The data for this figure was generated by Lawrence Puente.

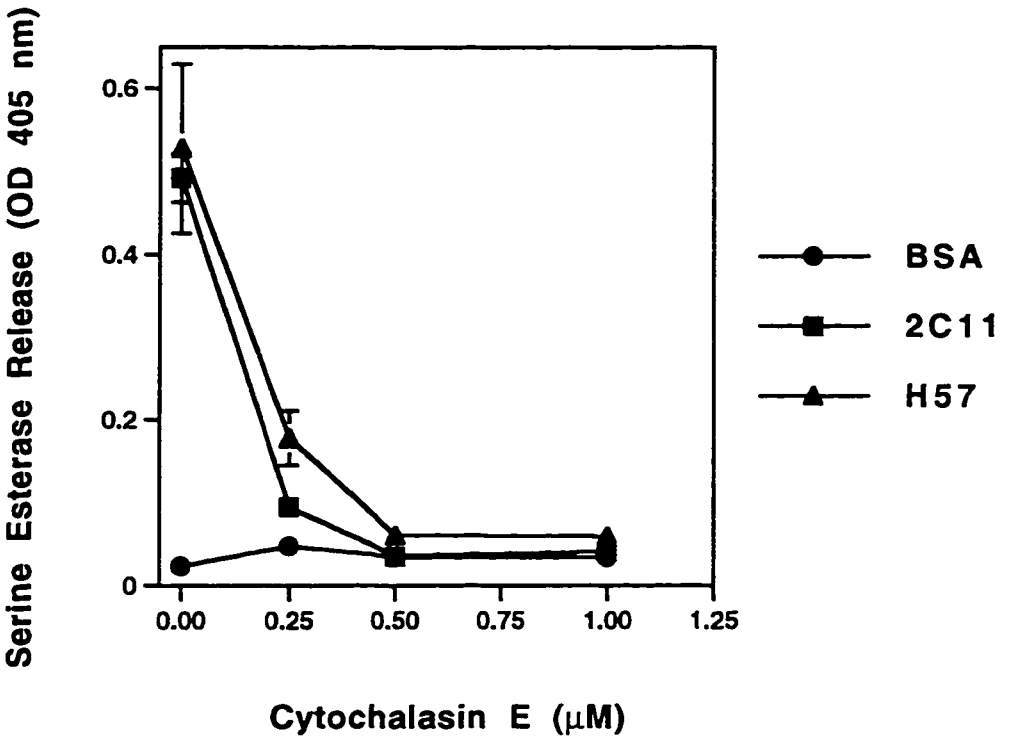


Figure 5-8. Cytochalasin E inhibits immobilized anti-CD3 or anti-TCR-stimulated degranulation. Clone 11 cells (1.5 X 10^5) were stimulated with immobilized anti-CD3 (2C11) or anti-TCR (H57) at 20 μg/ml in the absence or presence of 0.25 to 1.0 μM cytochalasin E. BSA was used as the control. Cells were incubated at 37°C for 4 hours and supernatants were assayed for serine esterase release.

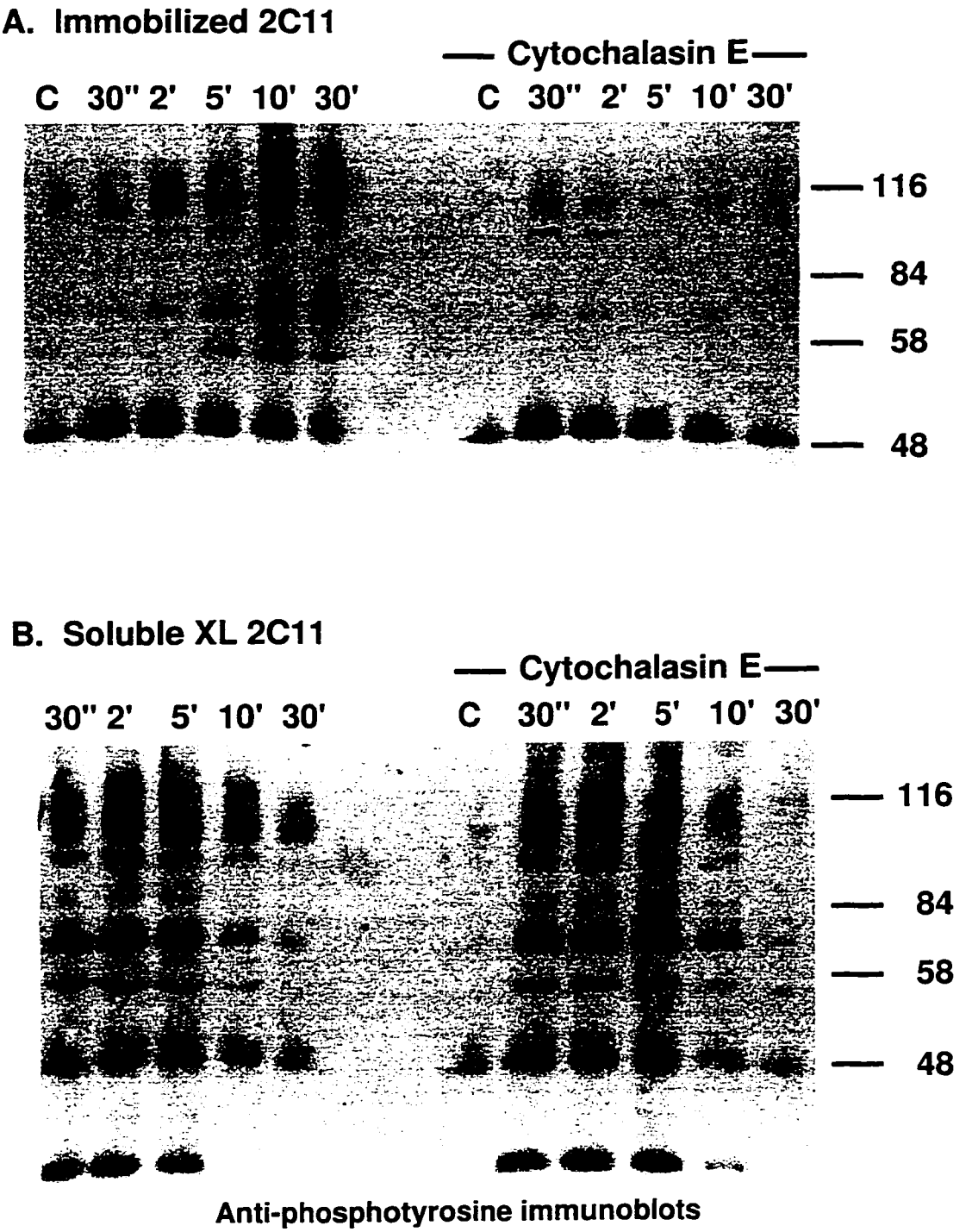


Figure 5-9. An intact cytoskeleton is required for tyrosine phosphorylation induced by immobilized but not by cross-linked anti-CD3. Clone AB.1 cells (1.5×10^5) were stimulated with immobilized 2C11 at 20 µg/ml or with soluble cross-linked (XL) 2C11 at 10 µg/ml and goat anti-hamster IgG at 10 µg/ml for the indicated times in the absence (left) or presence (right) of 10 µM cytochalasin E. Cell lysates were subjected to 7.5% SDS-PAGE and anti-phosphotyrosine immunoblotting.

CHAPTER VI

General Discussion

A. Summary of results

Firstly, we found that the interaction of CTL LFA-1 with ligand ICAM-1 facilitates degranulation, tyrosine phosphorylation, and an increase in Ca^{++}. LFA-1 engagement of ICAM-1 does not stimulate signalling events such as tyrosine phosphorylation or a Ca^{++} flux in CTLs. ICAM-1 must be on the same surface as the stimulating signal (anti-CD3) in order to facilitate degranulation and in order for the CTL to engage in stable cell/bead conjugates. We conclude that the LFA-1/ICAM-1 interaction is serving an adhesive role in CTL activation.

Secondly, we found that both FAK and Pyk2 are tyrosine phosphorylated in response to TCR/CD3 complex stimulation and associate with the SH2 domain of Lck in CTLs. The phosphorylation of FAK and Pyk2 is not regulated by Ca^{++}. It appears that neither Lck nor Fyn is solely responsible for the phosphorylation of FAK and Pyk2, however, we suggest a potential contribution of the Src family of PTKs in FAK and Pyk2 phosphorylation.

Lastly, by comparing stimulation of CTL clones with immobilized and soluble cross-linked antibody, we demonstrate that an immobilized stimulus is required to trigger sustained tyrosine phosphorylation and MAP kinase activation, a mobility shift in Lck, and degranulation. The sustained tyrosine phosphorylation and degranulation require a functioning cytoskeleton.

Therefore, we have examined several aspects of T cell activation: the contribution of the LFA-1/ICAM-1 interaction, the involvement of non-receptor PTKs FAK and Pyk2, and the requirements for TCR triggering. Together, these aspects can be woven into a discussion on the stimulation requirements for T cell activation and what role adhesion, cell

surface molecule clustering, and intracellular signalling complexes play in this intricate process.

B. The cytoskeleton and T cell activation

As discussed in the introduction, cellular processes such as adhesion, movement, and cell-cell conjugation require a functioning cytoskeleton (Pavalko and Otey, 1994). The cytoskeleton is also intimately involved in T cell activation. T cell stimulation with cross-linked anti-CD3 results in an increase in polymerized actin and in inositol phospholipid hydrolysis (DeBell et al., 1992; Phatak and Packman, 1994). As well, T cell stimulation triggers the cytoskeletal protein spectrin to undergo dramatic reorganization including moving from the cytoplasmic fraction to the plasma membrane (Lee et al., 1988). This indicates that there are changes in both cytoskeleton and signalling components following T cell stimulation. Additionally, anti-CD3-stimulated Jurkat cells display small, actin-rich pseudopods as well as numerous actin-rich branched pseudopods at sites of antibody attachment (Parsey and Lewis, 1993).

In cell-cell conjugates such as Th/APC or CTL/target cell, the T cell MTOC and Golgi apparatus rearrange to orient towards the conjugated cell (Kupfer and Singer, 1989). LFA-1 and talin co-localize to cell-cell contact areas (Kupfer and Singer, 1989), are associated following PMA stimulation (Kupfer et al., 1990), and LFA-1 moves to the detergent insoluble fraction after anti-CD3 treatment (Pardi et al., 1992), suggesting a link between the reorganization of cell surface and cytoskeletal proteins. Studies using CTLs with electroporated fluorescent dextrans to examine cytoplasmic structure show an increase in the dextran concentration at areas of CTL/target cell conjugation with both broad and fine protrusions towards the target (Waters et al., 1996). It is clear that cytoskeleton rearrangements and function may be a necessary prerequisite for T cell activation.

Treatment of cells with drugs that disrupt actin polymerization (cytochalasin D) prevents IFNγ production (Valitutti et al., 1995a), further supporting the proposal that the

actin cytoskeleton is involved in T cell activation. Our data also supports this hypothesis. We and other have shown that cells treated with cytochalasins do not degranulate, do not form conjugates with ICAM-1 and anti-CD3 coated latex beads, and do not undergo increases in tyrosine phosphorylation (O'Rourke et al., 1991). The cytoskeleton is interconnected to T cell triggering, signalling, and activation and this will be discussed throughout the chapter.

C. The cytoskeleton and multi-protein complexes

Cytoskeleton function is necessary for early signalling events and effector functions of T cell activation. The cytoskeleton may be participating by localizing proteins to the interface of interacting cell surfaces and rearranging intracellular proteins into functional signalling complexes (Shaw and Dustin, 1997). The models of Wange and Samelson (1996) and Shaw and Dustin (1997) propose that proteins must come together in specific and regulated multi-protein complexes in order for signal transduction to occur. These complexes are formed by protein-protein interactions involving both cytoskeleton and signalling proteins. Multi-protein complexes could contribute to signalling in several ways. Co-localization of two proteins in the same microdomain could facilitate events such as phosphorylation and regulate catalytic activity that would not occur otherwise. Protein-protein interactions can result in further changes such as focal adhesion formation, subsequent interactions leading to formation of a protein scaffold, and signalling events (Schaller and Parsons, 1994). Conserved protein modules such as the SH2 and SH3 domains are key to these events (Pawson, 1995).

Potential role for FAK as a scaffolding protein

FAK interacts with many signalling and cytoskeletal proteins (Figure 1-4 and references therein). We propose that the ability of FAK to act as a scaffolding protein may partially account for its involvement in T cell activation. FAK binding to cytoskeleton

proteins talin and paxillin, integrin cytoplasmic domains, and signalling proteins could prove fruitful for T cell activation. Firstly, by mediating all these interactions FAK may link the required elements for T cell activation upon integrin and TCR triggering, for example. Indeed, we have found that both TCR and integrin (anti-β3 cross-linking or β3 ligand vitronectin) stimulation leads to tyrosine phosphorylation of FAK in CTLs (Ma et al., 1997).

Secondly, FAK is suitably positioned to be a scaffold within the multimeric protein complexes. FAK associates with Src, Fyn, Cas, Graf, PI3K, Nck, PLCγ, Csk, and Syk (Figure 1-4 and references therein). Moreover, these proteins can in turn associate with other proteins. For example, FAK-associated Cas also binds to Crk and Src and Crk in turn also binds Sos and C3G, two guanine nucleotide exchange factors for Ras. Paxillin can also bind Crk, Src, and Csk. Therefore the interaction of FAK with proteins such as Cas and paxillin potentially positions FAK to regulate many signal transduction pathways necessary for T cell activation. Lastly, the FAK-associated proteins could influence the activity of FAK and in turn effect signal transduction. Like FAK, Pyk2 associates with Cas and paxillin and therefore has the potential to interact with other proteins in an analogous manner (Astier et al., 1997b).

A consequential problem of the data demonstrating this plethora of interactions, however, is that the interactions have been delineated in many cell types under many different conditions. Certainly not all the demonstrated protein-protein interactions will occur in all cell types. It is also feasible that microdomains exist within the cell where some interactions will occur and some will not. In fact, cell type specific interactions and microdomains may serve as a mechanism to insure specificity and regulation to signalling.

Multi-protein complexes: LAT, Vav, and SLP-76

Other molecules may be important to the TCR signalling pathways by mediating protein-protein interactions. LAT is a candidate molecule as it undergoes TCR-stimulated

tyrosine phosphorylation and associates with multiple signalling proteins including Grb2, PI3K, and PLCγ1, (Zhang et al., 1998). Cells overexpressing a mutant form of LAT, unable to associate with these proteins, lacks transcriptional activation of NF-AT and AP-1 (Zhang et al., 1998). Vav and SLP-76 may be scaffold proteins as they contain many motifs for mediating protein-protein interactions. For example, SLP-76 binds Grb2, PLCγ1, and Vav (Qian and Weiss, 1997), potentially linking multiple signalling pathways. Furthermore, Vav associates with ZAP70 and this is necessary for downstream phosphorylation events and signalling, at least in B cells. In the absence of the association between Vav and ZAP70, phosphorylation of Vav, SLP-76, Shc, and ZAP70 does not occur (Wu et al., 1996; Wu et al., 1997). Interestingly, in the absence of these phosphorylation events, ZAP70 is still catalytically active and binds TCR ζ chains (Wu et al., 1997). This suggests that there are multiple thresholds or levels of complexes needed for T cell activation.

Aspects of the TCR-triggered phosphorylation cascade

We demonstrated that both FAK and Pyk2 associate with Lck in CTLs following TCR stimulation (Berg and Ostergaard, 1997). These associations may be relevant to TCR-triggered signalling in two ways. FAK and Pyk2 may be linked with the early signal transduction events for which Lck is needed (Straus and Weiss, 1992; Straus et al., 1996). Secondly, in addition to the critical role Lck mediates by tyrosine phosphorylating TCR ζ (Chan et al., 1992; Iwashima et al., 1994), Lck may serve other roles for T cell activation.

T cells with CD4 molecules unable to associate with Lck were still antigen-responsive (Zerbib et al., 1994). This not only indicates that CD4 can act as a coreceptor independent of Lck, but that Lck may participate in T cell signal transduction by a mechanism independent of CD4 association. There is also evidence that Lck can participate in T cell signal transduction by a kinase-independent mechanism (Xu and Littman, 1993; Collins and Burakoff, 1993). These results imply that multiple domains of Lck are

important in T cell activation. This is supported by findings that Lck SH2 and SH3 domains are required for enhanced tyrosine phosphorylation induced with TCR and CD4 co-cross-linking (Lee-Fruman et al., 1996).

Lck may participate in signal transduction through protein-protein interactions and hence complex formation (Figure 6-1). Lck associates with both FAK and Pyk2 which in turn associate with a multitude of cytoskeletal and signalling proteins as discussed. This implies that the association with FAK or Pyk2 may be a means for Lck, via cytoskeleton-associated complexes, to localize to the TCR ζ chains and any other substrates. Furthermore, Lck may be key in mediating specific protein-protein interactions necessary for downstream signal transduction. Our finding that paxillin associates with Lck after TCR stimulation (Ostergaard et al., 1998) and that paxillin associates with Pyk2 constitutively (Ganju et al., 1997; Ostergaard et al., 1998) lends further support to the idea of Lck being a component of multi-protein complexes necessary for signal transduction downstream (Figure 6-1).

Phosphorylated TCR ζ chains are associated with the actin cytoskeleton in activated mature T cells (Caplan et al., 1995; Rozdzial et al., 1995). The association of the cytoskeleton with TCR ζ imparts an appealing prospect of how the cell surface signal transduction proteins are linked to the cell interior and how triggering the TCR/CD3 complex can lead to signalling events. Linking to the cytoskeleton in this manner, as will be discussed in more detail, may be a means of clustering and complex formation.

D. Sustained signalling

We correlate CTL effector functions such as degranulation with sustained tyrosine phosphorylation events. The sustained tyrosine phosphorylation requires an immobilized stimulus. As discussed extensively in chapter V, we propose that an immobilized stimulus is necessary to sustain signalling to achieve a threshold of TCR engagement and subsequent intracellular events.

Although there is tyrosine phosphorylation of proteins induced with soluble cross-linked anti-CD3 stimulation, such as that demonstrated for FAK, Pyk2, and paxillin, this is only transient. As well, these molecules are likely distributed over the entire cell. As discussed, we speculate that this does not result in the threshold of protein complexes necessary for activation. Certainly the extensive mobility shift in Lck is only achieved with the immobilized antibody. The increase in size of Lck may be important in mediating protein-protein interactions. These interactions may be particularly relevant in consideration of the previous discussion on Lck and its role in T cell activation.

We show that the sustained tyrosine phosphorylation requires an intact cytoskeleton. This supports the involvement of the cytoskeleton and adhesion in sustained signalling and T cell activation. Interestingly, we observe sustained paxillin phosphorylation with immobilized anti-CD3 stimulation only. Phosphorylated paxillin binds FAK (Schaller, 1996) and as discussed, paxillin associates with Lck after TCR stimulation (Ostergaard et al., 1998). This may be a mechanism whereby sustained phosphorylation, cytoskeleton elements, and intracellular complexes converge to achieve activation.

E. Integrins, the cytoskeleton, and clustering

Exactly how integrins mediate a signalling function is unknown. As discussed in the introduction, the integrin/cytoskeleton association may be a key mechanism (Collins et al., 1994). Much attention has focused on clustering of integrins as a mechanism to bring proteins into association with one another in specific, high affinity combinations thereby facilitating signalling (Schwartz, 1992). This is analogous to the discussion above on the formation of protein scaffolds following TCR stimulation. Differences in the redistribution of various cytoskeletal proteins toward antibody-coated beads are observed upon integrin occupancy and upon integrin aggregation. Maximum cytoskeleton protein accumulation was found with both integrin occupancy and aggregation (Miyamoto et al., 1995). This

suggests an intimate link between integrin engagement and clustering and the subsequent cytoskeleton rearrangements. This has implications in both integrin-mediated signal transduction and adhesion properties.

Data showing that the binding of LFA-1 to ICAM-1 differed depending on the stimulation conditions infers that there are two alternative mechanisms for LFA-1-mediated adhesion. Following stimulation with phorbol esters the cell is required to spread to get adhesion to ICAM-1 and this is dependent on Ca^{++} and PKC (Stewart et al., 1996). However, anti-TCR and Mg^{++} stimulation alone induces high affinity ICAM-1 binding and is inhibited with Ca^{++} (Stewart et al., 1996). Ca^{++} mobilizers such as ionomycin and thapsigargin increase the Ca^{++}-dependent adhesion of T cells to ICAM-1 (Stewart et al., 1998). This appears to be the result of LFA-1 clustering in the membrane following release from the restraint of the cytoskeleton (Stewart et al., 1998). The proposed mechanism is via the Ca^{++}-dependent protease calpain (Stewart et al., 1998). An entertaining caveat is that in platelets FAK has been shown to be cleaved by calpain resulting in its own dissociation from the cytoskeletal fraction (Cooray et al., 1996). Similar findings are found in transfected cells expressing an $\alpha 4\beta 1$ integrin with a mutated cytoplasmic domain that retains VCAM-binding capability, but does not adhere due to a lack of integrin clustering (Yauch et al., 1997). Additionally, treatment with cytochalasin D restores the ability of the cells to adhere suggesting that the cytoskeleton is partially restraining the integrin molecules (Yauch et al., 1997). This data emphasizes the potential requirement of integrin clustering for T cell activation.

Recent work has identified cytohesin-1 as a novel protein expressed in the spleen and thymus that interacts with the cytoplasmic domain of $\beta 2$ integrin. Overexpression of cytohesin-1 leads to LFA-1-dependent binding to ICAM-1 (Kolanus et al., 1996). Additionally, a serine/threonine kinase, ILK (integrin-linked kinase), associates with and phosphorylates $\beta 1$ cytoplasmic domain peptides (Hannigan et al., 1996). The association

between the integrin molecules and these newly defined signalling proteins may be another mechanism for regulating integrin-mediated adhesion and cell activation.

Integrins as mechanoreceptors

One aspect of cellular recognition of either a target cell or of ECM that remains undefined is how the physical interaction is transmitted to the cell and is translated to a response. In other words, how is the force transmitted. By a measurement called the *transmembrane force transfer*, it was shown that a cell has the ability to resist mechanical deformation in response to integrin ligands, but not to non-specific ligands (Wang et al., 1993). This resistance relied on cell microfilaments, microtubules, and intermediate filaments (Wang et al., 1993). This would indicate that the integrin receptors have the ability to act as mechanoreceptors by transferring mechanical signals to the cytoskeleton.

This curious phenomenon may be very important to the interpretation of our results. We found that an immobilized stimulus is required for sustained tyrosine phosphorylation, Lck mobility shift, sustained MAP kinase activation, and degranulation. Although difficult to directly compare T cell integrin engagement of ECM to TCR engagement of anti-CD3, due to differences in affinity, perhaps under the immobilized antibody stimulation conditions, the TCR can act analogous to a mechanoreceptor. Under the immobilized antibody stimulation conditions the cells have a physical barrier, one which a force can be exerted against. The immobilized antibody provides polarity to the interaction. The result is signal transduction across the membrane to the cytoskeleton and subsequent signalling pathways. With soluble cross-linked antibody, there is no polarity and no force being exerted. Hence the membrane remains fluid with the entire cell surface covered with antibody and the cytoskeleton is unable to respond.

F. Co-signalling

We concluded that the interaction between LFA-1 and ICAM-1 plays a primarily adhesive role to T cell activation. CTLs stimulated with soluble anti-TCR bound to non-antigenic class I MHC and degranulated (O'Rourke et al., 1990) and had inositol phosphate generation (O'Rourke and Mescher, 1992). Both LFA-1/ICAM-1 and CD8/class I MHC interactions are examples of triggered binding which can influence T cell activation. However, the outcomes are different given that the LFA-1/ICAM-1 interaction does not lead to unique signalling events (Berg and Ostergaard, 1995) and the CD8/MHC class I interaction leads to co-signalling. We have examined $CD8^+$ CTLs only; there may be a functional difference between $CD4^+$ and $CD8^+$ cells in that anti-TCR treatment of $CD4^+$ cells resulted in an increase in adhesion to FN and VN, but not to non-antigenic MHC class II (O'Rourke and Lasam, 1995).

G. Down-modulation of activation responses

All the data presented and discussed thus far examines signalling events leading to activation. Equally important to the function of the immune system and its capacity to be effective is the ability to down-modulate the immune response. Relevant to the data herein and above discussions, tyrosine phosphorylation events and subsequent protein-protein interactions could help turn off an immune response.

We discussed in chapter V that the shift in Lck mobility observed following immobilized anti-CD3 stimulation is potentially important for the functional response of CTL degranulation. Persistent TCR engagement with APC presenting MHC/peptide leads to a decrease in total protein tyrosine phosphorylation and a decrease in phosphorylation of TCR ζ and CD3ε concomitant with a decrease in CD4 surface expression and a shift of Lck to 59 kDa (Lee et al., 1997). This shift in mobility of Lck may be relevant to down-modulation of the T cell response as we observe high MW forms of Lck at 60 minutes after stimulation. Furthermore, a constitutively active form of Lck (Lck^{F505}) results in decreased

surface expression of the TCR complex due to degradation (D'Oro et al., 1997). Together these are potential mechanisms to down-modulate further TCR triggering and signal transduction.

Following TCR-stimulated autophosphorylation of Lck at Y397, Lck and Csk associate and this leads to an increase in phosphorylation of Lck Y505 by Csk (Bougeret et al., 1996). Since Lck Y505 is its negative regulatory site and phosphorylation inactivates its catalytic activity, this serves as a mechanism to down-modulate Lck activity. Moreover, Y192 in the Lck SH2 domain is phosphorylated after stimulation (Couture et al., 1996). This results in a reduced ability of Lck to bind phosphorylated proteins and to participate in downstream signalling events (Couture et al., 1996). Therefore this could also be a mechanism for regulating the formation of multi-protein complexes discussed above. It appears that Lck is balancing phosphorylation events and protein associations that both positively and negatively regulate its role in T cell activation. Although these examples demonstrate down-modulation of Lck, it is likely that many, if not all, proteins are controlled in analogous fashions.

LFA-1/ICAM-1-mediated adhesion is subject to regulatory mechanisms in addition to the 'inside-outside signalling' discussed. The coordinated involvement of PKC- and PKA-dependent pathways regulate LFA-1/ICAM-1 adhesion (Dustin and Springer, 1989). Evidence suggests that PKC-dependent pathways are responsible for early adhesive events (Haverstick and Gray, 1992); as discussed, the mechanism may be via a Ca^{++}-dependent release from, and re-association with, the cytoskeleton (Stewart et al., 1996; Stewart et al., 1998). PKA-dependent pathways may be responsible for later events (Haverstick and Gray, 1992). In agreement, more recent work indicates that elevations in cAMP and activation of PKA lead to de-adherence of T cells from ICAM-1 expressing cells (Rovere et al., 1996). Therefore additional signalling pathways serve as feedback mechanisms for regulating cell adhesion and immune responses.

H. Lessons from altered peptide ligands and partial signalling

The study of altered peptide ligands (APLs) has been extremely informative in delineating the requirements for T cell activation (Evavold et al., 1993; Sloan-Lancaster and Allen, 1996). The process of activation was originally thought to be an on/off mechanism. This was challenged by findings including those that proliferation and cytokine synthesis can be uncoupled from cytolysis (Evavold et al., 1993) and that APL engagement can lead to T cell anergy (Sloan-Lancaster et al., 1993). A pivotal finding to the field was that the TCR ζ chain was only partially phosphorylated following APL stimulation in comparison to the fully phosphorylated form following antigenic stimulation. Consequently, the association and activation of ZAP70 was perturbed (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). This demonstrates that the TCR-stimulated signalling pathways can produce a gradient of signalling functions depending on the nature of the peptide engaged.

Kersh and Allen (1996) discuss partial T cell activation as a result of T cells responding to different TCR ligands. In this case the ligands can be structurally different, such as APLs, or the kinetics of the ligand interaction with the TCR can be different. We postulate that stimulation with soluble cross-linked and immobilized antibody results in kinetically different effector outcomes such as transient versus sustained tyrosine phosphorylation. In this manner, the soluble cross-linked antibody delivers a signal analogous to that of an APL, resulting in partial activation. Like APLs, soluble cross-linked anti-CD3 did not achieve the threshold stimulation conditions.

Valitutti and Lanzavecchia (1997) suggest a relationship between the partial activation seen with APL stimulation and the partial activation that occurs in the absence of serial TCR triggering. The latter is proposed to occur when the TCR/MHC/peptide complex dissociate before full activatory signals are delivered. The end result of TCR complex engagement with soluble cross-linked antibody is also partial activation. Whatever the mechanism, full or partial activation of cells will depend on factors including the duration and type of TCR engagement, cell polarization, clustering, and multi-protein

complex formation. Both our system of differentially stimulating CTL clones and the study of APLs is a means to dissect the normal pathways of T cell signalling and activation.

I. Overall model of CTL activation

Based on data presented in this thesis and work by others, I am proposing the following model.

During immune surveillance, CTL/target cell contact occurs. This weak and transient adhesion begins with accessory molecules on the two opposing cell surfaces such as between LFA-1 and ICAM-1. At this point, few TCRs are engaged. In the presence of the correct peptide and CTL recognition, the weak adhesion will be maintained. At this point there may be some membrane proximal events occurring including some transient PTK activation and tyrosine phosphorylation. As a result of TCR triggering changes such as increased LFA-1 avidity will occur. We know that having ICAM-1 and the TCR stimulatory signal on the same surface results in increased adhesion between the CTL and target cell surfaces. As the threshold of sufficient adhesion is achieved, more surface area of the two opposing cells is in contact and therefore more TCRs are engaged with antigen-specific peptide/MHC complexes. The duration of adhesion and engagement is important. At the same time, CD8 is activated and binds to class I MHC. The result is sustained tyrosine phosphorylation. The tyrosine phosphorylation events and early signals, such as increases in Ca^{++}, are part of the second threshold. The duration of the phosphorylation is critical to the signal transduction events. The result is the formation of intracellular multi-protein signalling complexes analogous to the focal adhesion complex. The sustained signalling events and the formation of multimeric complexes of proteins require an active and functional cytoskeleton. These complexes and subsequent downstream signalling events will involve multiple non-receptor PTKs such as Lck, FAK, and Pyk2. The third threshold to achieve is that of maintaining a stable "cap" region for full T cell activation. This will require TCR/MHC/peptide complexes, accessory molecules, and the participation

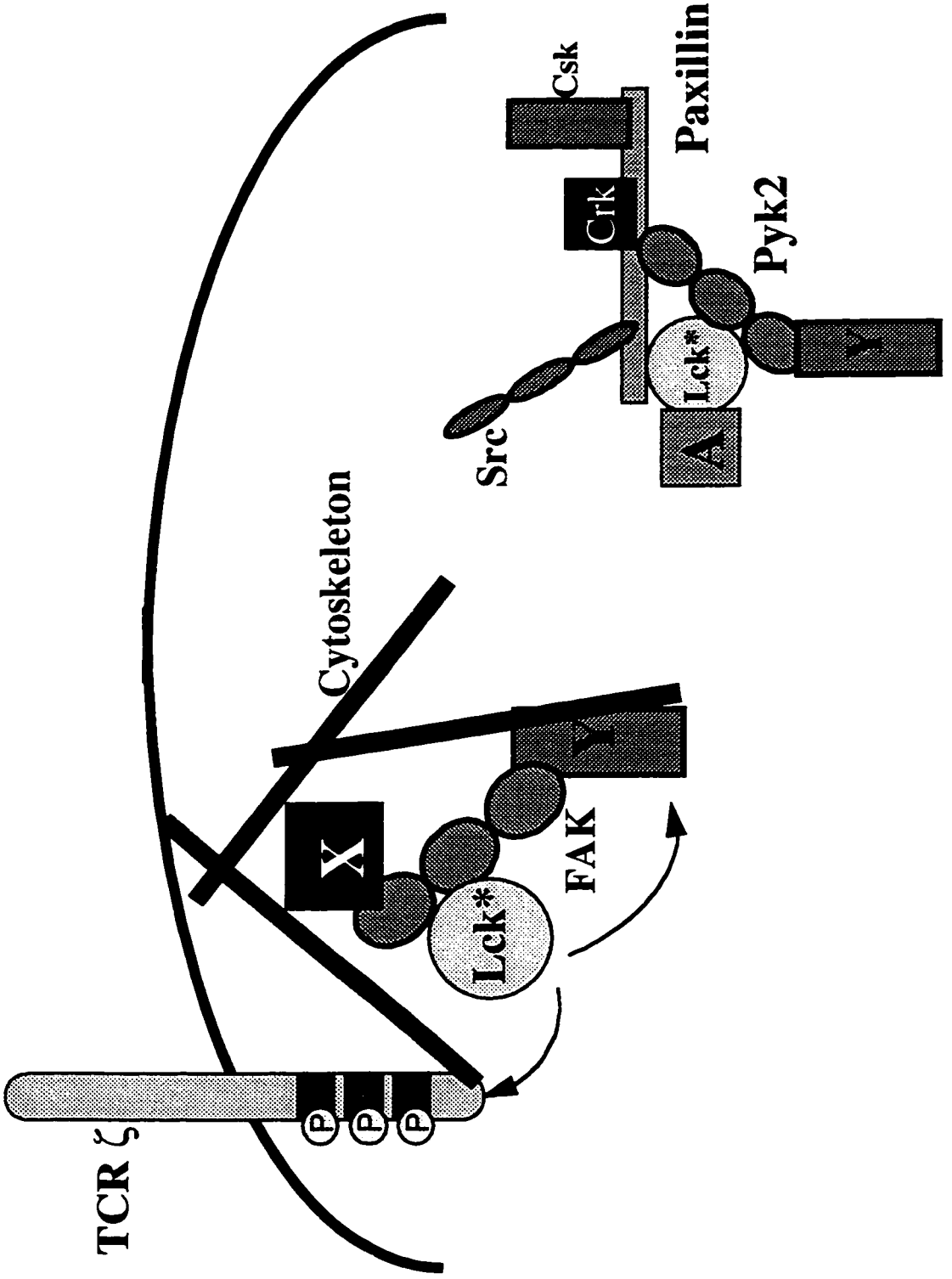
of intracellular cytoskeleton and signalling proteins. The result will be T cell activation. If the threshold checkpoints are not achieved, the result will be incomplete T cell activation.

J. Future directions

Continuation of these studies would examine the regulation of the tyrosine phosphorylation of FAK and Pyk2 at specific sites to determine what kinases are responsible for individual phosphorylation events. In addition, the relative contributions of integrin- and TCR-mediated stimulation of FAK and Pyk2 phosphorylation would be investigated in an attempt to determine if the signalling pathways stimulated are unique, the same or convergent.

The future direction in the investigation of the requirements for triggering the TCR/CD3 complex would involve determining the composition, nature, and regulation of multi-protein complexes. These studies would go beyond defining associations between individual proteins and would require determining the composition of proteins in multimeric complexes, how the assembly and activity of such complexes is regulated, and how this is linked to T cell activation.

Figure 6-1. Participation of Lck in signal transduction. Following TCR triggering, activated Lck (Lck*) phosphorylates the TCR ζ ITAM sequences which is a critical step in T cell activation. Lck may participate in signal transduction by means other than its kinase activity. FAK associates with Lck and a multitude of other cytoskeletal and signalling proteins (X). In this manner, Lck may be co-localized with its substrates, TCR ζ or an undefined substrate Y. Alternatively, Lck may mediate a specific protein-protein interaction (example is A), which is a key component of a multi-protein complex necessary for signal transduction. For example, Lck associates with paxillin after TCR stimulation and paxillin associates with both FAK and Pyk2 and other signalling proteins such as Src, Csk and Crk. Thus Lck could potentially be a necessary link between different signalling molecules and therefore pathways.


TCR ζ
P
P
P
Cytoskeleton
X
Lck*
FAK
Y
Src
Csk
Crk
Paxillin
A
Lck*
Pyk2
Y

CHAPTER VII

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IMAGE EVALUATION
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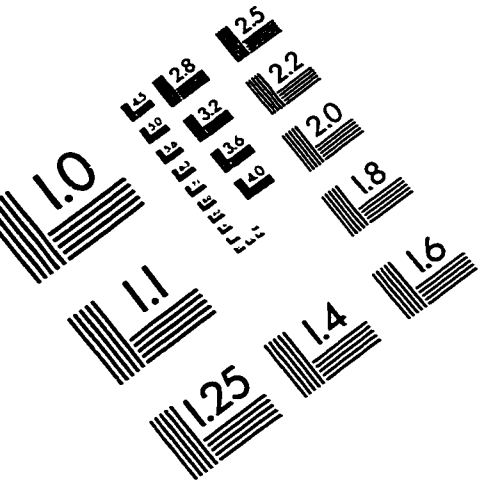

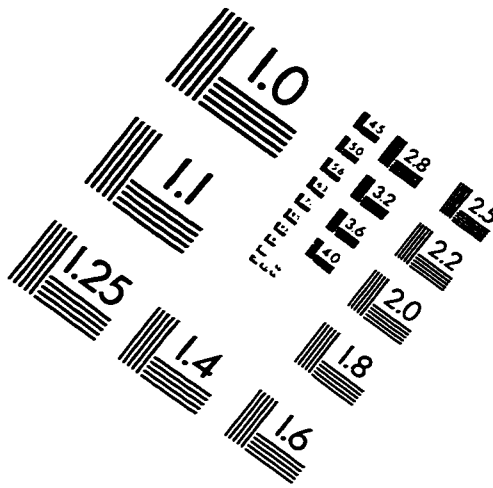

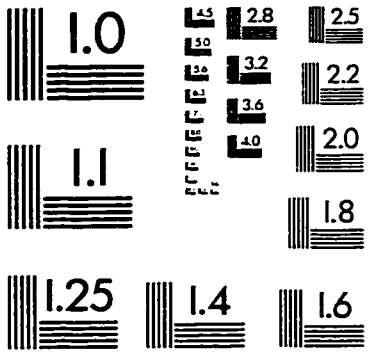

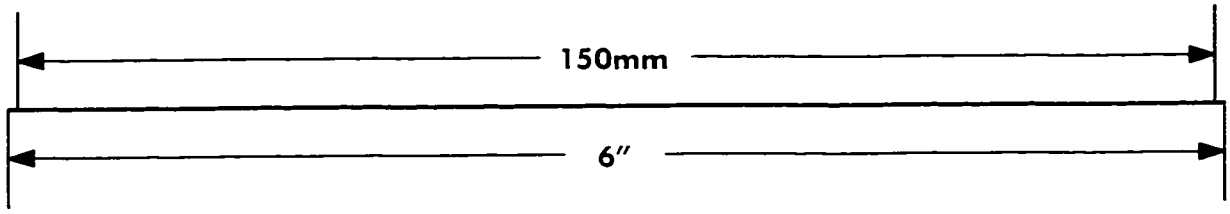

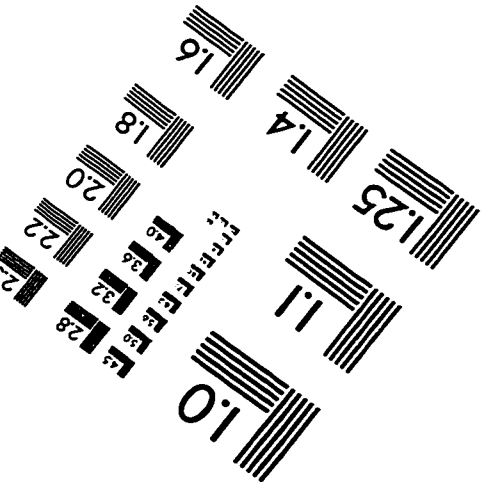

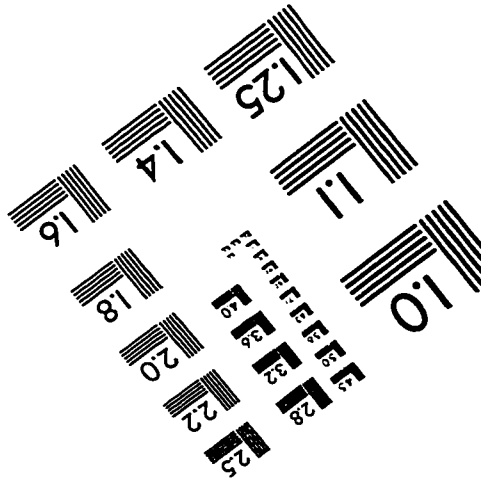

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