University of Alberta

Examination of the Regulation and Localization of the Cytoskeletal Adaptor Protein Paxillin in Cytotoxic T Lymphocytes

by

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ABSTRACT

Paxillin is a multidomain adaptor protein that plays an integral role in the assembly of structural and signaling proteins at the focal adhesions of adherent cell types. The binding partners of paxillin include numerous proteins that are important for the tethering of integrins to actin, for actin dynamics, and for the disassembly of focal complexes to allow migration. This protein is ubiquitously expressed but until now has predominantly been studied in adhesive cell types such as fibroblasts. As a result, little is known of its function in immune cells. As part of the adaptive immune system, cytotoxic T lymphocytes (CTL) eliminate self cells that have become infected or transformed; as such they possess complex cytolytic machinery that is tightly regulated in its delivery. CTL become activated after contact between their clonotypic T cell receptor (TCR) and antigen-loaded major histocompatibility complexes (MHC) of a given target cell.

Activation of the TCR leads to a concatenation of signaling events, mainly propagated by phosphorylation, which ultimately lead to the release of lytic granules (degranulation) toward the target, which then dies by apoptosis. Active microtubule and actin cytoskeletons are essential to this process, as are the integrins that fasten the CTL to its target. The exact mechanisms by which the TCR controls the complex steps of degranulation remain incompletely characterized. Paxillin has been identified as one of the proteins phosphorylated downstream of the TCR, and as paxillin is involved in the regulation of integrins and the cytoskeleton, it seemed plausible that the protein might be relevant to CTL function. Firstly, I examined the regulation of TCR-induced paxillin phosphorylation, and found that paxillin was both serine and tyrosine phosphorylated, and that these events were regulated by distinct and independent pathways. Microscopy revealed that in CTL-target conjugates paxillin was located at the microtubule cytoskeleton and the immune synapse, particularly the periphery of the immune synapse, an area known to be rich in integrins. Paxillin association with the microtubule cytoskeleton was interesting in that it is a characteristic not yet observed in adherent cells, implying that the protein may be performing a novel function possibly related to CTL killing. The findings presented in this work contribute not only to our knowledge of paxillin regulation, but also to our understanding of the dynamic and specialized CTL and how they become activated to clear infection and malignancy.

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aa	amino acid
Ab	antibody
ADAP	adhesion and degranulation adaptor protein
AP-3	adaptor protein-3
APC	antigen presenting cell
ARF	ADP-ribosylation factor
BSA	bovine serum albumin
ConA	concanavalin-A
CTL	cytotoxic T lymphocyte
DAG	diacylglycerol
DC	dendritic cell
dCS	defined calf serum
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
D-PBS	Dulbecco's phosphate-buffered saline
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ERK	extracellular-regulated kinase
F-actin	filamentous actin
FA	focal adhesion
FAK	focal adhesion kinase
FasL	Fas ligand
FCS	fetal calf serum
Fyn	fibroblast Src/Yes novel gene
GAP	GTPase-activating protein
GEF	GTP exchange factor
GFP	green fluorescent protein
Grb2	growth factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase
GTP	guanine 5'-triphosphate
HDAC6	histone deacetylase 6
HGF	hepatocyte growth factor
Hic-5	hydrogen peroxide-inducible clone-5
HRP	horseradish peroxidase

ICAM-1	intercellular adhesion molecule-1
IL-2	interleukin-2
IP	immunoprecipitaton
IP_3	inositol 1, 4, 5-triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LAT	linker for activation of T cells
Lck	lymphocyte-specific cytoplasmic protein tyrosine kinase
LD	leucine-aspartic acid domain
LFA-1	leukocyte function-associated antigen-1
LIM	Lin-11 Isl-1 Mec-3 domain
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MEK	MAP/ERK kinase
MHC	major histocompatibility complex
MLCK	myosin light-chain kinase
MTOC	microtubule organizing centre
NK cell	natural killer cell
NP-40	nonidet P-40
OA	okadaic acid
PAK	p21 GTPase-activated kinase
PBS	paxillin-binding subdomain
PCR	polymerase chain reaction
PH	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4, 5 bisphosphate
PIP_3	phosphatidylinositol 3, 4, 5 triphosphate
PIX	PAK-interacting exchange factor
PKA	protein kinase A
PKC	protein kinase C
PLC-γ	phospholipase C-γ
PMA	phorbol myristyl acetate
PS	phosphoserine
PT	phosphothreonine
PTP-PEST	protein tyrosine phosphatase - Pro/Glu/Ser/Thr
PVDF	polyvinylidene difluoride
PY	phosphotyrosine
Pyk2	proline-rich tyrosine kinase 2
RAPL	regulation of adhesion and cell polarization enriched in lymphoid tissues
RasGRP	Ras guanyl nucleotide-releasing protein
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription-polymerase chain reaction

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
siRNA	small interfering ribonucleic acid
SKAP-55	Src kinase-associated phosphoprotein
SLP-76	SH2 domain-containing leukocyte protein-76
SMAC	supramolecular activation cluster
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SOS	son of sevenless homologue
TCR	T cell receptor
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
WB	Western blot
ZAP-70	zeta-associated protein-70

CHAPTER 1: Introduction

PART I. INNATE AND ADAPTIVE IMMUNITY

The immune system evolved as a means of protecting the organism against attack by pathogens in the form of viruses, bacteria, fungi, and parasites, and as they have progressed over time, so has the immune system adapted and diversified its means of defense. The immune system of mammals can be divided into two principal branches: the innate system and the adaptive system. Innate immunity is governed by a group of cell types that have developed to detect invading pathogens and to kill them indiscriminately. Compromise of the body's outer anatomical defences triggers a cascade of chemical messengers and the rapid influx of innate immune cells from the bloodstream. The principal cellular agents of the innate system include macrophages, monocytes, natural killer (NK) cells and the granulocytes eosinophils, basophils, and neutrophils. Invaders are destroyed by phagocytosis and by exposure to toxic chemical mediators. These cells require no previous contact with the pathogen to become active and are thus immediately available for defense; however they form no memory of specific pathogens and are often insufficient to fully clear infections. If the first function of the innate system is to contain invading pathogens, then its second function, no less important, is to alert and mobilize the adaptive immune system.

Dendritic cells (DCs) form an important bridge between the innate and adaptive immune systems; they are able take fragments of phagocytosed microorganisms and present them as antigen to the cells of the adaptive immune system. DCs constantly take up antigen in the periphery and migrate to the spleen and lymph nodes to initiate the adaptive immune response. Other antigen presenting cells (APC), such as macrophages, can further enhance activation of the response through similar mechanisms. T lymphocytes, which are important for cell-mediated immunity and cytokine secretion,

and B lymphocytes, which produce antibody as part of the humoral response, are the main effectors of adaptive immunity, and they differ from their innate counterparts in important ways. The lymphocytes are not indiscriminate killers of foreign invaders; they are each tailored for one antigen only and are effectively blind to all other antigens or pathogens. The establishment of an adaptive response must therefore begin anew with each pathogen encountered. The antigen specificity of T and B cells is conferred by their clonotypic T and B cell receptors. At any given time the body carries a complement of millions of randomly configured receptors; as lymphocytes have no inherent foreknowledge of specific pathogens it is the sheer numbers of the receptors and their random assembly that favours the detection of a given target. It is the function of the antigen-presenting cells to find those very rare T cells that express a TCR (T cell receptor) capable of recognizing the pathogen-derived antigens they have taken up. Antigen recognition by a lymphocyte is followed by clonal expansion; that is, the cell multiplies to produce a proliferation of cells all with the same antigenic specificity as the original. It is the rarity of specific T and B cells, as well as the time required for activation and expansion, which delays the onset of adaptive immunity to 5-7 days after initial antigen contact. This delay is compensated for by the ability of the adaptive immune system to develop memory, which allows for much more rapid and aggressive action should the pathogen be encountered again in a secondary response. The innate and adaptive immune systems each have their strengths and limitations, but cooperate to effectively prevent and control infections.

PART II. BIOLOGY OF T LYMPHOCYTES

T lymphocytes can be divided into two main categories, based on their expression of surface markers and their functions in pathogen clearance. The $CD4^+$, or helper, T cells (T_H) express CD4 in addition to the TCR and are restricted to recognizing antigen presented on Class II MHC (major histocompatibility complex) molecules by professional APCs such as dendritic cells, macrophages, and B cells. By contrast, the

CD8-expressing cytotoxic T lymphocytes (CTL) are restricted to antigen presented by Class I MHC molecules on the surface of nearly every cell in the body. The primary role of $CD4^+$ helper T cells is to facilitate the activation of other cell types such as naïve $CD8^+$ T cells and B cells and to manipulate the immune response through the production of cytokine messengers. In contrast to T_H the role of CTL is not regulatory; rather, they are adapted to the detection and elimination of self cells that are either virally-infected or tumour cells.

All T cells develop from the same progenitors and are committed to one lineage or the other in the thymus over the course of their development. The TCR is a heterodimer which in the majority of T cells is composed of an α-chain and a β-chain, the genes for which exist in multiple segments that must be recombined before they are expressed. The randomly assembled TCRs are just as likely to recognize self as foreign antigens and therefore TCR screening mechanisms are necessary to protect the organism from autoimmunity. The thymus provides an environment not only for T cell proliferation and development but also for T cell sorting, the end result being a mature T cell repertoire that is both competent to recognize MHC-antigen and tolerant to self-antigens. The small percentage of T cells that satisfy the rigourous requirements of thymic selection leave the thymus as $CD4^+$ or $CD8^+$ naïve T cells and take their place in the T cell repertoire.

PART II.a. ***T cell activation***

Naïve T cells that depart the thymus then circulate between the bloodstream, the spleen, and the peripheral lymphatic system, and are said to be "resting" T cells. Recirculation through the lymph nodes and spleen assures that as many T cells as possible will have the chance to screen antigens presented on APCs, mainly DCs. T cells new to a particular lymph node migrate rapidly and engage in many brief contacts with DCs (Mempel, Henrickson et al. 2004). When a circulating T cell makes contact with cognate MHC-antigen on the surface of a DC, it ceases to migrate and over the following

hours receives signals through the TCR and other receptors. An activation program is put into motion in which diverse signaling cascades trigger proliferation, differentiation, and effector mechanisms such as cytokine production and the production of cytolytic granules in the case of $CD8^+$ T cells. Eventually, the proliferated T cells exit the lymph node and search for sites of infection.

The signals triggered by contact between the TCR and antigen-MHC are essential for the differentiation of naïve T cells into effector cells with the ability to combat infection. These signals are propagated for the most part by a cascade of kinase-catalyzed phosphorylation events. The addition of phosphate groups to serine, threonine, or tyrosine residues constitutes a form of biochemical communication that disseminates the activation message from one protein to another and thus amplifies the cellular response. The two chains of the TCR possess short cytoplasmic domains and do not transmit activation signals themselves, but rather through an assemblage of other proteins that make up the TCR/CD3 complex. Six accessory chains make up the CD3 complex: a CD3ζ homodimer and CD3γε and δε heterodimers (Kuhns, Davis et al. 2006). All units of CD3 have at least one ITAM (immunoreceptor tyrosine-based activation motif), a module which contains tyrosine residues that are phosphorylated and transmit the activation signal to the next round of kinases. Aggregated with the TCR/CD3 complex are the co-receptors CD4 or CD8, depending on the lineage of the T cell. The extracellular domains of CD4 and CD8 can interact with MHC Class II and Class I, respectively, and aid in signal transmission.

When the TCR encounters an MHC presenting an appropriate antigen peptide the activation signal is transmitted across the plasma membrane by a mechanism that remains controversial but which may involve clustering or a conformational change of the TCR/CD3 (Choudhuri and van der Merwe 2007). Phosphorylation of the CD3 ITAMs is then catalyzed by the Src-family kinases Lck (lymphocyte-specific cytoplasmic protein-tyrosine kinase) and Fyn (fibroblast src/yes novel gene) (**Figure 1-1**). Lck interacts with

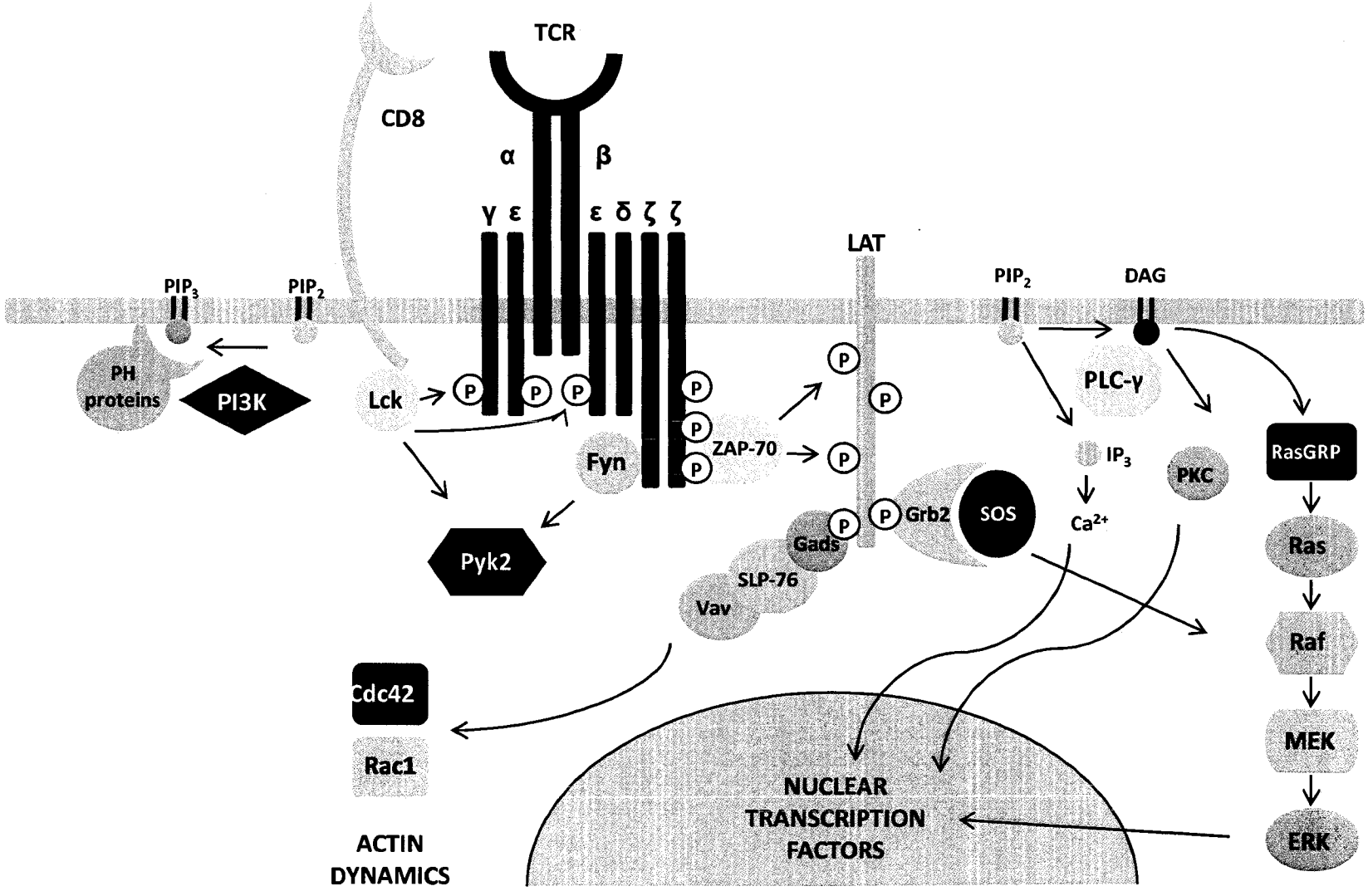


FIGURE 1-1. The principal signaling events downstream of the T cell receptor.

the cytoplasmic domains of CD4 and CD8 and thus is brought into close proximity to the TCR (Turner, Brodsky et al. 1990). Fyn appears to be associated with the TCR/CD3 complex directly (Samelson, Phillips et al. 1990). Phosphorylation of the ITAMs on CD3ζ creates docking sites for the Src homolgy 2 (SH2) domains of ZAP-70 (zeta-associated protein-70), which is then in a position to be phosphorylated and catalytically activated by Lck and Fyn (Chan, Iwashima et al. 1992; Iwashima, Irving et al. 1994; Chan, Dalton et al. 1995). ZAP-70 expands and diversifies the signal to other pathways, including the PLCγ1 (phospholipase C-γ_1) and Ras pathways. ZAP-70 accomplishes this via the phosphorylation of an intermediary: the transmembrane adaptor protein LAT (linker for activation of T cells) (Zhang, Sloan-Lancaster et al. 1998). The many phosphotyrosine residues on LAT create a scaffold for multiple protein interactions. LAT brings in PLC-γ1 and the Tec family kinases necessary to activate it (Horejsi, Zhang et al. 2004). Once in proximity to the plasma membrane, PLC-γ1 performs the critical function of hydrolyzing membrane PIP_2 (phosphatidylinositol 4, 5 bisphosphate) into two second messengers: DAG (diacylglycerol) and IP_3 (inositol 1, 4, 5-triphosphate). The former, DAG, activates PKC (protein kinase C) and RasGRP (Ras guanyl nucleotide-releasing protein), while the latter, IP_3, initiates the release of intracellular calcium stores and the activation of calcium-dependent transcription mechanisms (Cantrell 1996).

The Ras pathway is a ubiquitous pathway for the regulation of growth control and differentiation and is also a key element in TCR activation. Ras is a small membrane-associated GTPase which is made active through at least two known pathways downstream of the TCR. Phosphorylation of LAT creates a docking site for the adaptor Grb2 (growth factor receptor-bound protein 2), which in turn recruits SOS (son of sevenless homologue), a GTP exchange factor (GEF) which favours the binding of GTP by Ras and thus Ras activation (McCormick 1993; Buday, Egan et al. 1994). The second known means of Ras activation involves the DAG-dependent protein RasGRP, also an exchange factor for Ras (Dower, Stang et al. 2000; Ebinu, Stang et al. 2000). When Ras is active and bound to GTP, it facilitates the membrane translocation and activation of the

kinase Raf (also known as MAP kinase kinase kinase) which in its turn activates the dual-specficity kinase MEK (MAP/ERK kinase, also known as MAP kinase kinase) which finally phosphorylates ERK1 and ERK2 (extracellular signal-regulated kinase 1 and 2, also known as mitogen-activated protein kinase, MAPK) (Cantrell 1996). The phorbol ester PMA (phorbol myristyl acetate), a DAG analogue which bypasses the TCR-proximal signaling to directly activate PKC, also induces the phosphorylation of ERK (Downward, Graves et al. 1990). PMA recruits RasGRP to the membrane and also directly activates PKC. PKC can then phosphorylate and activate RasGRP, thus turning on the Ras-ERK pathway (Lorenzo, Kung et al. 2001; Teixeira, Stang et al. 2003; Zheng, Liu et al. 2005). PKC has been shown to be important for maintaining ERK activation at later time points after TCR stimulation with anti-CD3 immobilized on plastic (Puente, Stone et al. 2000). Active ERK can phosphorylate targets in the cytoplasm but exerts its control over proliferation and differentiation by migrating into the nucleus and activating transcriptional targets.

PI3K (phosphatidylinositol 3-kinase) is a lipid kinase that is activated soon after the initiation TCR signaling, though the mechanism of its activation remains controversial. PI3K is a heterodimer composed of regulatory and catalytic subunits, the former containing two SH2 domains (Deane and Fruman 2004). PI3K has been reported to associate with the CD3ζ chain (Exley, Varticovski et al. 1994) and may also be recruited by the LAT adaptor (Zhang, Sloan-Lancaster et al. 1998). The Class I PI3Ks catalyze the addition of a phosphate group to PIP_2 to produce PIP_3 (phosphatidylinositol 3, 4, 5 triphosphate) in the plasma membrane. PIP_3 acts as a lipid binding motif for pleckstrin homology (PH) domains and thus recruits PH domain-containing proteins to the membrane in the area of T cell signaling (Deane and Fruman 2004). Potential PH-containing targets of PI3K activation include AKT (also known as protein kinase B), which is involved in cell proliferation and survival, PLC-γ1, and Vav, which plays a role in cytoskeletal regulation (Kane and Weiss 2003). PI3K activity has been shown to be

necessary for the optimal TCR-induced activation of ERK, though how these two enzymes are linked to each other is unclear (Von Willebrand, Jascur et al. 1996).

As described here, engagement of the TCR by antigen-MHC triggers a web of signaling cascades that radiate from the central point and ramify the original signal into many outcomes. The terminus of many TCR-initiated pathways is the nucleus; genes controlling proliferation, differentiation, and cytokine production (particularly interleukin-2 in the case of $CD4^+$ T cells) are switched on and regulated. The cross-talk and redundancies built into the T cell activation pathways allow for a flexible and finely-tuned response to antigen types and levels that are not necessarily optimal. The plasticity inherent in the system also permits the integration of signals from receptors other than the TCR which contribute to T cell activation.

PART II.b. ***TCR Co-stimulation***

Interaction of the TCR with a corresponding antigen-MHC complex is necessary for the activation of naïve T cells but it is not sufficient. Naïve T cells which have not previously encountered antigen require additional signals, termed "co-stimulation," to clonally expand and become active effector T cells. Professional APCs bear not only Class II MHC molecules but co-stimulatory surface receptors that engage their ligands on T cells and create signals that amplify or modulate those derived from the TCR. The most well-known of these receptor pairs is the CD28-B7 pair. CD28 on T cells interacts with B7 on the surface of DCs or other APCs and synergizes with the TCR to amplify cell proliferation and the expression of cytokines, cytokine receptors, and other co-stimulatory molecules (Acuto and Michel 2003). While the signal from the TCR is termed "signal 1", the signal delivered through CD28 is referred to as "signal 2", and both are necessary. If both signals are not received, the T cell enters a state of anergy, or inactivity, which is permanent and persists even if the T cell later receives signals 1 and 2. Effector T cells and memory T cells differ from their naïve precursors in that they no

longer require co-stimulation. Co-stimulatory molecules other than CD28 have been identified, some of which prolong the activation signal received by the TCR while others, such as the integrin LFA-1 (lymphocyte function-associated antigen-1), contribute to adhesion. During the many hours of contact between a T cell and an APC these molecules interact with their ligands to generate a flow of different signals that must be integrated by the cell into a productive outcome.

PART II.c. ***Formation of the Immunological Synapse***

The point of contact between a T cell and an APC or between a CTL and a target is termed the immunological synapse, in reference to the exchange of signals and chemical messengers that is reminiscent of a neurological synapse. It was known that elements such as the TCR, LFA-1, and F-actin accumulated and clustered at the APC contact point and it was assumed that they were uniformly distributed across the immune synapse. The Kupfer group used microscopy to demonstrate that the components of the synapse are in fact segregated into two distinct phases to form a "bull's-eye" pattern termed the SMAC (supramolecular activation cluster) (Monks, Freiberg et al. 1998). The central portion of the bull's-eye was termed the central SMAC (cSMAC), while the outer ring was named the peripheral SMAC (pSMAC). The cSMAC contains surface receptors such as the TCR, the co-receptors CD4 or CD8, and the co-stimulatory receptor CD28, and is also a focal point for cytoplasmic signaling molecules such as PKC-θ, Lck, and Fyn. In contrast, adhesion molecules such as LFA-1 and other integrins, as well as the cytoskeletal adaptor talin are concentrated in the outer ring of the pSMAC. Highly glycosylated and bulky surface proteins such as CD45 and CD43 are excluded from the synapse and held in the distal SMAC (dSMAC).

How this arrangement is achieved is not completely understood yet it appears that the cytoskeleton may orchestrate the segregation of the receptors through the tethering of surface proteins such as integrins to actin. The reasons for SMAC formation are equally

uncertain. It has been proposed that the SMAC facilitates the perpetuation of signaling events, though an argument against this idea is that mature SMAC formation occurs only after the initial burst of activity of Lck and ZAP-70 (Lee, Holdorf et al. 2002). Another hypothesis proposes that molecular size dictates in part whether a given receptor is held in the cSMAC, pSMAC, or relegated to the dSMAC. This model posits that small receptors such as the TCR are better able to contact their ligands when large, bulky proteins that might otherwise block or shield them are removed (Huppa and Davis 2003). In addition to reasons of size, CD45, a protein tyrosine phosphatase, may be removed from the vicinity of the TCR to prevent its phosphatase activity from abrogating the phosphotyrosine cascades of TCR signaling. The SMAC may also aid in the directional delivery of effector molecules such as cytokines and, in the case of CTL, cytolytic mediators (topics specific to the role of the SMAC in CTL killing will be discussed later). It is clear that the formation and maintenance of the SMAC is a complex process that involves contributions from the cytoskeleton, adaptor proteins, and adhesion molecules, as well as from the TCR itself.

PART II.d. *Cytotoxic T lymphocytes*

When cells within the body become infected or develop into tumour cells, it is in the best interest of the organism to sacrifice its own cells in order to contain the invasion; within the adaptive immune system this task falls to the cytotoxic T lymphocytes (CTL). CTL are uniquely adapted for their function of killing self cells, but before they become functionally competent they must first encounter antigen and differentiate from naïve $CD8^+$ T cells (Olsen, Bou-Gharios et al. 1990). Naïve $CD8^+$ T cells circulate in the periphery as do their $CD4^+$ counterparts and express no cytolytic effector molecules. To become mature, $CD8^+$ T cells must receive TCR signals from antigen-MHC contact as well as co-stimulation, and must also receive growth and differentiation signals from IL-2 produced by $CD4^+$ helper T cells. These "licensing" conditions developed to prevent

autoimmunity and only when they are met does the $CD8^+$ T cell differentiate into a fully cytotoxic CTL.

The hallmark of an effector CTL is the formation of cytolytic granules, which requires the expression of effector molecules such as granzymes and perforin. An effector CTL may kill a detected target in one of two ways: by the exocytosis of cytolytic granules (degranulation) or by the expression of Fas ligand (FasL) which binds to Fas on the target and induces apoptosis. For the purposes of this work I will focus on degranulation, the mechanics of which will be discussed in the following paragraph. Effector CTL must contact antigen on the surface of a target cell in order to undergo degranulation. This time, however, no co-stimulation or cytokines are needed and any cell which expresses Class I MHC is potentially a CTL target. Granzymes and perforin are pre-stored and no protein synthesis is required for degranulation, making it a rapid process. Perforin is a critical ingredient of granules that oligomerizes to form a membrane pore which may compromise the target plasma membrane (Podack and Konigsberg 1984). When perforin is absent, as in the case of perforin-deficient mice, CTL killing ability is severely impaired (Kagi, Ledermann et al. 1994). Membrane damage is not the primary cause of target cell death, however. Granzymes, particularly the well-studied granzyme B, are serine proteases which cleave and activate caspases within the target cell to initiate cell death by apoptosis (Lord, Rajotte et al. 2003). When the granule contents are released toward the target cell, granzyme B is taken up by receptor-mediated endocytosis. Perforin is required for this process and it appears that perforin expedites the liberation of granzyme B from endocytic vesicles (Pinkoski, Hobman et al. 1998). Once unleashed into the cytoplasm, granzyme B cleaves caspase-3 and thus begins a chain reaction leading to cell death (Darmon, Nicholson et al. 1995). CTL must be in close contact with their target when granules are released and are therefore in danger of being killed by granzymes or perforin themselves. It was demonstrated that CTL can protect themselves via the inclusion of the protease cathepsin B in granules (Balaji, Schaschke et al. 2002). After degranulation, cathepsin B on the cell surface degrades cytolytic proteins.

Cathepsin B is one protective factor, but there are evidently others that can fill the same role (Baran, Ciccone et al. 2006), shielding the CTL from inadvertent cell suicide and allowing it to go in search of another target cell.

PART II.e. ***Mechanism of CTL degranulation***

The highly refined program of CTL degranulation includes mechanisms not only for the release of cytotoxic mediators but also for the precise aiming of those mediators toward self cells that are virally-infected or in danger of becoming malignant. The targeting function is crucial; it prevents bystander killing of healthy cells and limits the kind of collateral tissue damage that is often caused by cells of the innate immune system. NK cells use the same mechanism of targeted granule release as CTL, though they express a distinct set of inhibitory and activating receptors to detect stressed and infected self cells.

The first contact between a CTL and a potential target cell involves a loose attachment based on the adhesion of integrins such as LFA-1 on the CTL to ICAM-1 (intercellular adhesion molecule-1) on the target. If the TCR encounters cognate antigen-MHC, integrin adhesion is strengthened (by mechanisms to be described below) and a tight conjugate is formed (**Figure 1-2A and B**). The MTOC (microtubule organizing centre) polarizes toward the immune synapse, as does the Golgi apparatus (Geiger, Rosen et al. 1982; Kupfer and Dennert 1984). The MTOC, a protein complex that is the anchoring point for the minus ends of microtubules, translocates toward the target until it is in close apposition to the membrane, where it oscillates back and forth (Kuhn and Poenie 2002). The interface between a $CD8^+$ CTL and its target forms a SMAC similar to that formed between a $CD4^+$ T cell and an APC, though as killing is a rapid process the CTL SMAC is more transient (Stinchcombe, Bossi et al. 2001). The MTOC is positioned in the centre of the adhesive ring that forms the pSMAC, and the granules then use the microtubules as guide wires to direct their traffic, sliding toward the minus ends at the MTOC by virtue of

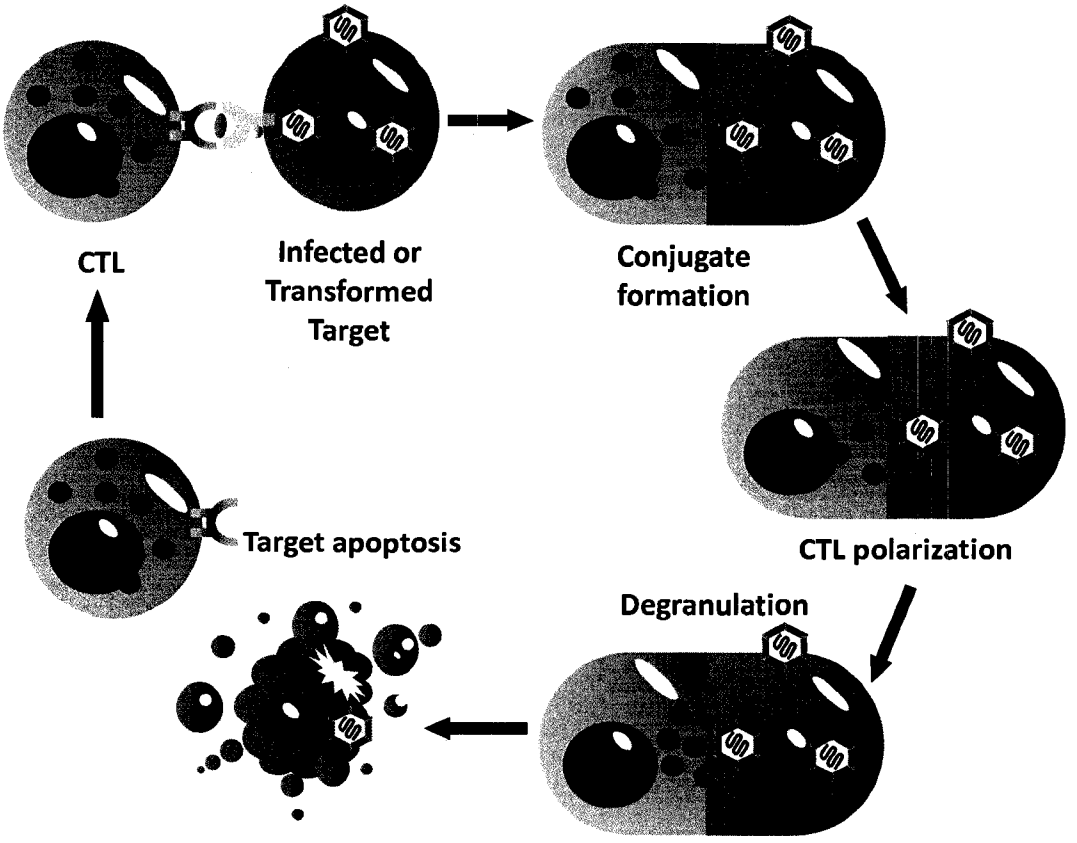


FIGURE 1-2A. The steps involved in the killing of an infected or transformed target by a cytotoxic T lymphocyte.

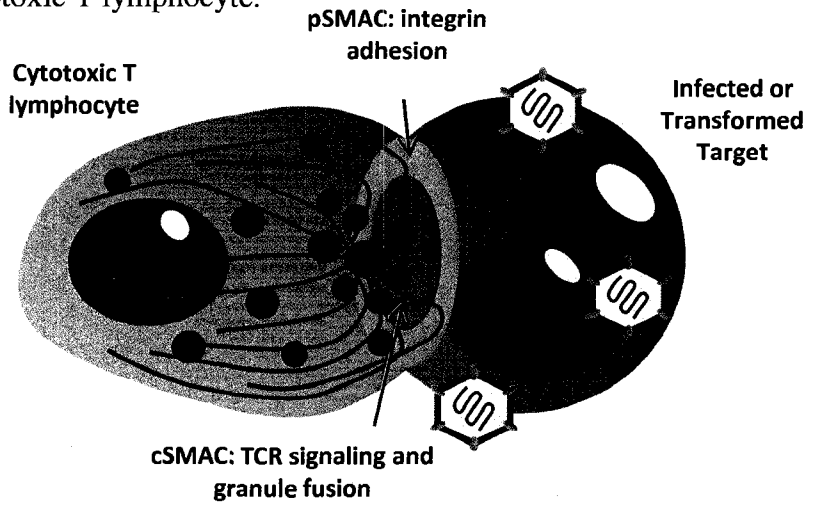


FIGURE 1-2B. A CTL-target conjugate. The immune synapse between the two cells consists of the central and peripheral SMACs, where TCR signaling and integrin adhesion occur, respectively. The MTOC (blue) polarizes toward the target until it is close to CTL membrane within the cSMAC. Cytolytic granules (red) traffic along the microtubules until they reach the MTOC and are then delivered to the membrane. Membrane fusion releases granule contents onto the target cell.

dynein motors (Burkhardt, McIlvain et al. 1993). The MTOC itself appears to complete the delivery of the granules to the plasma membrane (Stinchcombe, Majorovits et al. 2006).

Before degranulation can occur, granules must dock to the plasma membrane and be primed for exocytosis. Docking of the granules to the membrane is facilitated by the Ras-like GTPase Rab27a. The Rab family proteins are expressed on the surfaces of vesicles, "tag" them for delivery to specific target membranes, and facilitate vesicle docking. In Rab27a-deficient CTL, granules polarize to the SMAC but remain clustered around the MTOC and fail to dock to the plasma membrane, making the CTL incapable of killing targets (Haddad, Wu et al. 2001; Stinchcombe, Barral et al. 2001). Once the granules are moored in place within the cSMAC, in a zone called the secretory domain, the tethered vesicles must be primed for fusion in a process which in humans is known be dependent upon Munc 13-4 (Stinchcombe, Bossi et al. 2001; Feldmann, Callebaut et al. 2003; Menager, Menasche et al. 2007). The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) family of proteins, which facilitate fusion of many vesicle types, likely execute the final release of cytolytic granule contents into the intercellular space (Hong 2005). After delivering the "lethal hit" to the target, the CTL detaches itself by a mechanism which is as yet little understood. Exposure to the granzymes and perforin stored in the granules causes the infected target cell to die via apoptosis. Because the CTL does not deliver all of its granules at once, it is able to continue hunting for targets in the vicinity of the first.

Recent years have seen the publication of many studies advancing our knowledge of the mechanics of CTL granule exocytosis, and though the process is similar to that of vesicle exocytosis in many other cells, the TCR-specific signaling pathways that orchestrate these events are in many ways still unclear. It has been demonstrated that PKC, PI3K, and ERK are necessary for the onset of degranulation, but the relevant downstream substrates of these kinases remain unidentified (Nishimura, Burakoff et al.

1987; Berg, Puente et al. 1998; Wei, Gamero et al. 1998; Fuller, Ravichandran et al. 1999; Robertson, Mireau et al. 2005). Further studies will be required to identify the signaling mediators which orchestrate the dynamic processes of adhesion and degranulation that lead to the termination of compromised self cells by CTL.

PART III. CELL ADHESION AND MIGRATION

PART III.a. *Introduction to integrin attachment and focal adhesions*

The cells of the body exist within the framework of the extracellular matrix (ECM), and their ability to adhere to this substrate and to migrate within it is necessary for the normal development of the organism, for wound healing, and for the general maintenance of healthy tissues. Integrin receptors bind to proteins of the extracellular matrix such as fibronectin and collagen as well as proteins on other cells, thus anchoring the cell and providing stability to cell-cell interactions. Integrins consist of two heterodimerized transmembrane subunits: an alpha subunit and a beta subunit. Regulation of integrin adhesion can determine whether a cell adheres tightly and spreads on a surface or migrates in response to chemotactic factors. Many stimuli can affect the balance of adhesion and migration, among them a variety of growth factors, differentiation factors, cytokines, chemokines, and mechanical forces, each initiating signaling cascades that manipulate integrin behaviour. Though integrins are transmembrane proteins, it is cytoplasmic factors such as actin and signaling cascades that ultimately control their affinity and their ability to aggregate into membrane clusters.

Adherent cell types such as epithelial and smooth muscle cells form sturdy attachments to the ECM that are reinforced by actin stress fibres, yet these cells must also be capable of motility. Focal complexes and focal adhesions (FAs) coalesce at the points of integrin clustering and appear in microscopy images as punctate complexes situated at the membrane, often at the tips of stress fibres. The function of FAs is twofold,

composed as they are of a structural core with mechanical strength and an accumulation of signaling proteins which regulate adhesion and the cytoskeleton. Structurally, the attachment of integrin clusters to actin is a key feature of FAs as it allows the cell to generate the tension needed for motility. The signaling molecules recruited to FAs can promote either the solidification or dissolution of the attachments, the former being favoured at the leading edge of the migrating cell, the latter at the trailing edge (Wozniak, Modzelewska et al. 2004).

PART III.b. *Migration of adherent cells and lymphocytes*

Cell migration consists of a repeating cycle of discrete steps, all of which occur simultaneously in different parts of an actively moving cell: (1) polarized protrusion of the membrane, (2) attachment, (3) traction and translocation, and (4) retraction and detachment (Brown and Turner 2004). The first step, the extension of the cell membrane in the direction of migration, is mediated by the Rho family of p21 GTPases, particularly Rac1 and Cdc42. When bound to GTP these proteins promote local actin polymerization. The activation of Rac1 by growth factors, chemoattractants, or other stimuli induces the formation of sheet-like lamellipodia, while Cdc42 activation leads to the protrusion of finger-like filopodia. Rho itself promotes stress fibre formation, and all three family members are able to stimulate the aggregation of FAs within their respective membrane structures (Ridley and Hall 1992; Ridley, Paterson et al. 1992; Nobes and Hall 1995; Hall 1998). Adhesion, the second step, is mediated by integrin attachment and the formation of relatively transient focal complexes. Focal complexes are continually formed under the protruding edge of the cell and are subsequently disassembled within minutes; it is the turnover of these focal complexes which allows the cell to repeatedly put forward new membrane attachments after dissolving the old ones (Zaidel-Bar, Ballestrem et al. 2003). A more detailed description of focal complex turnover will be presented in Part IV. Under the influence of active Rho, transitory focal complexes may also mature into the more robust focal adhesions, characterized by an attachment to stress fibres. Both types

of adhesions are rich in tyrosine-phosphorylated proteins as well as α-actinin, an actin cross-linking protein which stabilizes the attached filaments (Laukaitis, Webb et al. 2001). Myosin contraction of actin fibres as promoted by MLCK (myosin light-chain kinase) generates the tension needed to pull the cell forward. Finally, the disintegration of ECM contacts by phosphatases and proteinases at the rear of the cell results in progressive detachment as the cell moves (Wozniak, Modzelewska et al. 2004; Mitra, Hanson et al. 2005).

Lymphocytes, in contrast to adherent cells, are rapidly motile and migrate extensively between the lymphatic system, the blood vessels, and the tissues. Interactions between the selectins and their ligands on endothelial cells allow T cells to roll along the blood vessel walls. Inflammatory markers on the vessel wall induce the cells to stop, an event which requires binding of the integrins LFA-1 and VLA-4 (very late antigen-4, α4β1) to ICAM-1 and VCAM-1 (vascular cell adhesion molecule-1), respectively. The cell can then extravasate through the vessel wall and into the inflamed tissue, where it migrates through the ECM in a direction dictated by the chemoattractant gradient.

Lymph node T cells are capable of migrating as quickly as 40μm/min but average 10μm/min (Mempel, Henrickson et al. 2004), as compared to fibroblasts which average 0.5μm/min (Samstag, Eibert et al. 2003). In order to achieve these high speeds, T cells do not form FAs as such but migrate in an amoeboid fashion characterized by an unstructured and diffuse distribution of adhesion (Friedl, Entschladen et al. 1998). Leukocytes differ from adherent cells not just in speed but in their ability to use integrin-dependent and independent means of locomotion on 2-dimensional substrates and 3-dimensional matrices, respectively (Friedl and Brocker 2000). The migrating cell first extends an F-actin-rich lamellipodium and filopodia, known as the leading edge, in which are concentrated chemokine receptors and LFA-1. The cell body, including the nucleus, follows, and is proceeded by the MTOC and the trailing uropod. Detachment of adhesive contacts then occurs at the rear of the cell. The TCR is uniformly distributed across the

adhesion area. Cross-linking of VLA-4 and LFA-1 can trigger motility (Hauzenberger, Klominek et al. 1997), whereas TCR engagement can deliver a signal which favours more stable binding to APCs and target cells (Dustin, Bromley et al. 1997). T cells in the 3-dimensional milieu of lymph nodes or tumours crawl along stromal cells or ECM fibres and make serial contacts with cells such as DCs or tumour cells (Bousso and Robey 2003; Bajenoff, Egen et al. 2006; Mrass, Takano et al. 2006). Firm cell-cell contact, as stabilized by integrins, is necessary for the type of receptor-ligand communication used by T cells; however, imaging has demonstrated that even when firmly conjugated with another cell the T cell continues to crawl, engaging in both stable adhesion and motility simultaneously. Improved techniques in the intravital microscopy of living lymph nodes and tissues has revealed how dynamic and flexible T cells are in their adhesive and migratory behaviour.

PART III.c. ***Integrin regulation in CTL-target conjugates***

Adhesion of a CTL to a target cell is mediated in large part by the integrin LFA-1 and its ligand ICAM-1 and the properties of this interaction are subject to modulation by signals from the TCR. CTL do not form punctate contacts but the pSMAC can be considered analogous to a focal adhesion in that it is an area in which integrins are clustered together with the proteins that regulate their adhesive properties. Adhesion of LFA-1 ($\alpha_L\beta_2$) to its ligand is governed both by the affinity of the individual receptors and by the avidity of the interaction, and both are enhanced after TCR engagement. Cytoplasmic signals resulting from TCR activation are transmitted back to the cell surface to modify the dynamics of integrin adhesion, and are thus termed "inside-out" signaling. Conversely, integrins such as LFA-1 may generate signals of their own upon binding of their ligand, known as "outside-in" signaling.

LFA-1 exists in low, intermediate, and high-affinity conformations, and the transitions between these states are regulated by binding of the ICAM-1 ligand itself and by inside-

out signals. The adaptor protein talin, which binds directly to the cytoplasmic tails of β-integrins, increases the affinity of integrins by inducing conformational changes. The avidity of LFA-1 is governed by the lateral mobility of the integrin in the membrane. LFA-1 must be mobile in order to collect into clusters, and the tethering of LFA-1 to actin reduces its mobility. Talin, in addition to its role in affinity regulation, acts to regulate integrin avidity by linking the receptors to actin fibres. In the current model, LFA-1 in resting T cells is tethered to actin by talin and has low mobility and affinity. Inside-out signals originating from the TCR result in the release of LFA-1 from actin, allowing the integrins to coalesce into clusters and create tight adhesion. High affinity, clustered LFA-1 is then re-attached to actin by talin in order to stabilize adhesion (Dustin, Bivona et al. 2004). The clustering of integrins and the concomitant strengthening of adhesion is critical to stabilizing interactions between T cells and APCs and between CTL and targets, and as a result inside-out signaling has become a significant area of study.

Inside-out integrin regulation is still incompletely understood, though some proteins have been identified which act as intermediaries between the TCR and integrins. The LAT adaptor protein recruits a complex of the adaptor protein ADAP (adhesion and degranulation adaptor protein, also known as Fyb/Slap) and SKAP-55 (Src kinase-associated phosphoprotein of 55 kDa), which is important for relaying the TCR signal to integrins. In the absence of ADAP, LFA-1 adhesion and clustering are impaired, as are conjugate formation and T cell activation (Griffiths, Krawczyk et al. 2001; Peterson, Woods et al. 2001; Mueller, Thomas et al. 2007). The ADAP-SKAP-55 complex recruits the GTPase Rap1 to the membrane (Kliche, Breitling et al. 2006) and the binding partner of Rap1, RAPL (regulation of adhesion and cell polarization enriched in lymphoid tissues), interacts directly with the α_L subunit of LFA-1. Expression of dominant negative mutants of Rap1 and RAPL in T cells resulted in decreased binding of LFA-1 to ICAM-1 and reduced conjugate formation between T cells and APC (Katagiri, Hattori et al. 2002; Katagiri, Maeda et al. 2003). Together, ADAP-SKAP-55 and Rap1-RAPL form a

conduit between the TCR and membrane integrins and act in concert to nucleate an integrin-anchored signaling complex that regulates both LFA-1 affinity and avidity (Burbach, Medeiros et al. 2007). There are undoubtedly additional components of inside-out signaling that have yet to be identified. Most studies of integrin regulation and inside-out signaling have concentrated on T cell interaction with APCs, although CTL also rely on tight adhesion to form conjugates with target cells and it is likely that the same mechanisms apply to both forms of cell contact.

PART IV. PAXILLIN, A CYTOSKELETAL ADAPTOR PROTEIN

Paxillin is multi-domain adaptor molecule that is an integral component of focal adhesions and that has been shown to marshall together proteins important to both signaling and structure. Originally identified as a tyrosine-phosphorylated protein from chicken embryo fibroblasts, paxillin was named for the latin word "paxillus", meaning peg, or stake, to reflect its structural function (Glenney and Zokas 1989). Paxillin localizes to punctate membrane complexes corresponding to focal adhesions (Turner, Glenney et al. 1990). The modular structure of paxillin allows for the accretion of a diverse group of binding partners including kinases, phosphatases, small GTPases, actin-binding proteins, and other adaptor proteins, and provides the proximity needed for these proteins to modify and regulate one another.

PART IV.a. *Paxillin Structure*

As a hub for numerous signaling and structural proteins, paxillin contains the different types of protein interaction domains necessary to attract its complement of binding partners (**Figure 1-3**). The N-terminal half of paxillin contains five leucine-aspartic acid (LD) domains - short motifs with the consensus sequence LDXLLXXL. LD domains form amphipathic α-helices in which the leucine residues are aligned on one face of the helix to form a hydrophobic patch flanked by negatively charged aspartic and glutamic

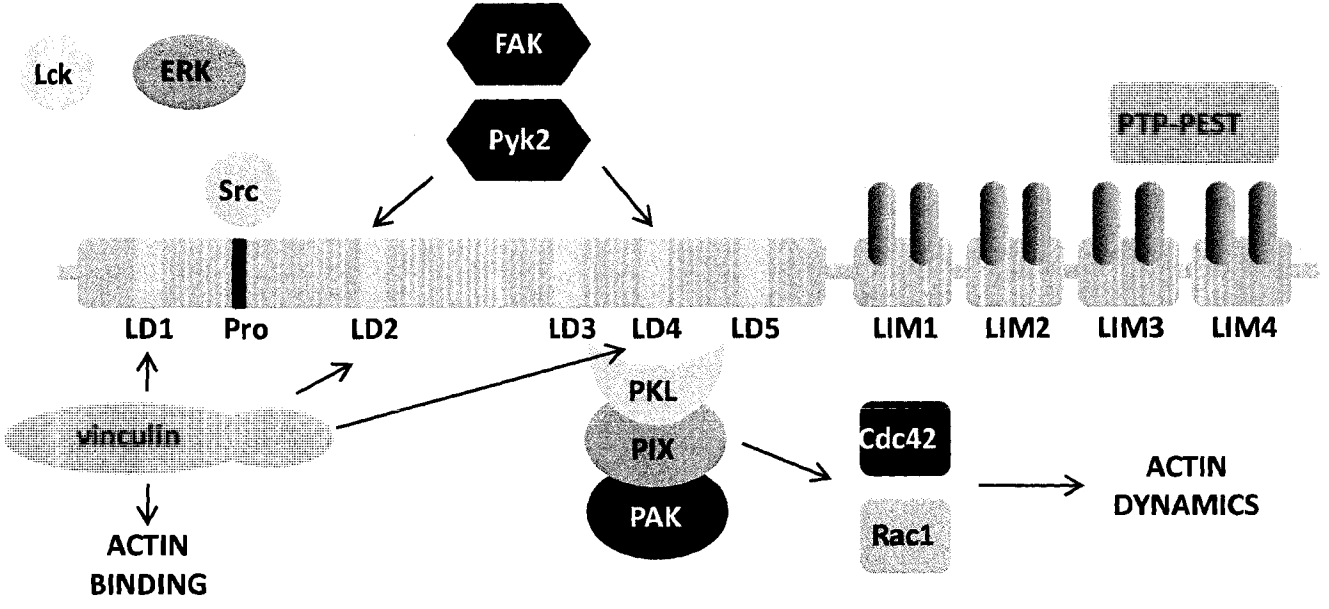


FIGURE 1-3. The domain structure of paxillin and a selection of its binding partners. The N-terminal half contains five leucine-aspartic acid (LD) domains and a proline-rich region (Pro). The C-terminal half of paxillin forms four double zinc finger LIM domains. Tyrosine, serine, and threonine phosphorylation sites are dispersed throughout. Paxillin is linked to actin via actopaxin (not shown) and vinculin, which can bind LDs 1, 2, and 4. The tyrosine kinases Src and FAK/Pyk2 interact with the Pro and LD2/LD4 domains, as shown. LD4 attracts a complex of PKL, the Cdc42/Rac1 GEF PIX, and PAK kinase. The tyrosine phosphatase PTP-PEST binds via the LIM3 and LIM4 domains. Lck has been shown to interact via an SH2-PY interaction, but the tyrosine residue responsible for this is unidentified. ERK phosphorylates serine residues of paxillin.

acid residues (Tumbarello, Brown et al. 2002). One binding module for the LD has been identified: the paxillin-binding subdomain (PBS). To date the PBS has been identified in several proteins though it appears that LD binding may not be confined to this type of module. Between LD1 and LD2 is a proline-rich region potentially able to interact with SH3 (Src homology 3) domains (Weng, Taylor et al. 1993). The C-terminal half of paxillin is composed of four Lin-11 Isl-1 Mec-3 (LIM) domains, each of which takes the form of a double zinc finger. Zinc atoms coordinated by cysteine and histidine residues maintain the finger-like structures. The LIM modules are found in many proteins and mediate protein-protein interactions by mechanisms as yet uncharacterized (Kadrmas and Beckerle 2004). Interspersed amid the LD and LIM domains are numerous serine, threonine, and tyrosine phosphorylation sites. Mass spectrometric mapping of chicken paxillin flagged 31 phosphoserine residues, 3 phosphothreonine residues, and 5 phosphotyrosine residues that translated to corresponding residues in mouse paxillin (Webb, Schroeder et al. 2005). The LD region contains 27 of these serine residues and all 5 of the tyrosines. Phosphotyrosine creates a docking site for proteins containing SH2 (Src homology 2) domains and phosphoserine may also modify protein-binding properties. LIM3 is required for the recruitment of paxillin into focal adhesions by an as-yet unknown mechanism that appears to be regulated by serine and threonine phosphorylation of the LIM domains (Brown, Perrotta et al. 1996; Brown, Perrotta et al. 1998).

Two family members of paxillin have been identified based on structure: leupaxin and hic-5. The former is mainly expressed by cells of hematopoietic derivation (and is named for leukocytes) and is only 45kDa compared to 68kDa for paxillin. Leupaxin shares 37% amino acid identity with paxillin and contains only four LD domains: LDs corresponding to LDs 1, 4 and 5 of paxillin as well as a unique LD2. The C-terminal four LIM domains are also present in leupaxin with an average of 72% identity, but the proline-rich sequence is absent (Lipsky, Beals et al. 1998). Hic-5 is 57% identical to paxillin, is widely expressed, and shares domains similar to the paxillin LD 1, 2, 4, and 5 domains.

It also contains a proline motif and the four LIM domains with an average of 63% identity (Thomas, Hagel et al. 1999). As a result of their conserved domains leupaxin and hic-5 can interact with some of the same binding partners as paxillin and are thought to fill similar roles.

PART IV.b. *Paxillin binding partners and function*

Paxillin has no catalytic activity of its own but performs its function by bringing signaling molecules into close proximity to each other and to the membrane. Paxillin is one of the first proteins to be recruited into nascent focal complexes (Laukaitis, Webb et al. 2001) but is not essential for their formation. Rather, the incorporation of paxillin and its binding partners into focal complexes is necessary for their disassembly during cell migration. Genetic deficiency of paxillin causes embryonic lethality in null mice at or before day 9.5, most likely as a result of faulty cell migration and adhesion leading to defective development. Paxillin-deficient embryonic stem cells and mouse-derived fibroblasts display delayed spreading on fibronectin and laminin and impaired migration (Hagel, George et al. 2002; Wade, Bohl et al. 2002). Over the course of numerous studies, it has been demonstrated that paxillin affects migration by accumulating structural actin proteins such as vinculin and actopaxin, actin regulators, tyrosine kinases such as FAK (focal adhesion kinase) and Src, and phosphatases such as PTP-PEST (protein tyrosine phosphatase - Pro/Glu/Ser/Thr).

PART IV.b.i. *Actin binding and regulation*

Transmembrane integrins bound to the ECM are anchored to actin fibres via a complex of paxillin and the actin linker proteins vinculin and actopaxin (Turner, Glenney et al. 1990; Nikolopoulos and Turner 2000). Vinculin binds to LDs 1, 2, and 4, and actopaxin to LDs 1 and 4 through PBS-LD interactions. In addition to physical actin linkage, paxillin affects actin dynamics through the recruitment of regulatory proteins.

The LD4 domain interacts with the PBS-containing protein PKL (paxillin kinase linker, a GTPase-activating protein for ADP-ribosylation factor proteins, or ARF-GAP) which in turn recruits PIX (PAK-interacting exchange factor), a guanine nucleotide exchange factor (GEF) for the Rho family of GTPases, and PAK (p21 GTPase-activated kinase) (Turner, Brown et al. 1999; Brown, West et al. 2002). The PIX GEF activates the small p21 Rho GTPases Cdc42 and Rac1, encouraging membrane protrusion (**Figure 1-4**). In turn, the serine/threonine kinase activity of PAK is triggered by its association with GTP-bound Cdc42 and Rac1 (Manser, Leung et al. 1994). PAK itself is known to modulate other actin-regulating proteins to decrease the formation of stress fibres and promote focal adhesion turnover (Manser, Huang et al. 1997; Kiosses, Daniels et al. 1999; Bokoch 2003). PAK can also phosphorylate serine 273 in the LD4 domain of paxillin to further promote the formation of the paxillin-PKL-PIX-PAK complex and an increase in cell motility (Nayal, Webb et al. 2006). Reconstitution of paxillin-null fibroblasts with a paxillin ΔLD4 deletion mutant failed to restore FA turnover as compared to wild type murine embryonic fibroblasts (Webb, Donais et al. 2004). Likewise, overexpression of a paxillin ΔLD4 deletion mutant in neuroblastoma cells resulted in the formation of fewer and smaller lamellipodia, and expression of a small LD4 fragment in NIH 3T3 cells reduced cell migration in a wound assay (Turner, Brown et al. 1999). It is interesting to note that paxillin has also been associated with a decrease in Rac1 activity (Nishiya, Kiosses et al. 2005). This was attributed to Git1, an ARF-GAP related to PKL which binds to LD4 of paxillin, but the mechanism through which Rac1 is inhibited not yet clear. In summary, the paxillin LD4 domain recruits to the membrane a selection of proteins associated with aspects of actin regulation, particularly membrane protrusion (PIX, Cdc42 and Rac) and focal adhesion turnover (PAK).

PART IV.b.ii. ***Kinases: FAK, Src, and ERK***

Within the hierarchy of focal contact signaling, the tyrosine kinases Src and FAK occupy a high level of control and are capable of manipulating many cellular elements. The LD2 and LD4 domains of paxillin cooperate to bind the PBS of FAK (Brown, Perrotta et al. 1996; Thomas, Cooley et al. 1999; Gao, Prutzman et al. 2004), a well-studied tyrosine kinase that is recruited to FAs and phosphorylated by Src family kinases in response to integrin binding and growth factor receptors (Schaller, Borgman et al. 1992). Src kinases also bind to paxillin either directly, through interaction of the Src SH3 domain with the paxillin proline-rich region, or indirectly, through association with FAK (Weng, Taylor et al. 1993). When fibroblasts from mice deficient in the Src kinases Src, Yes, and Fyn (SYF-/-) were analyzed, they displayed a lack of tyrosine phosphorylation of FA proteins and a defect in migration. Embryonic lethality of these mice occurred at the same point as that of paxillin-deficient mice: day 9.5 (Klinghoffer, Sachsenmaier et al. 1999). Chicken embryo fibroblasts expressing dominant-negative Src were observed to have abnormally large, extended FAs, suggesting that Src kinases are not required for FA formation per se, but for their disassembly (Fincham and Frame 1998).

FAK, like Src, is dispensable for FA formation, but rather the activation of FAK promotes the turnover of FAs and thus migration (Ilic, Furuta et al. 1995; Webb, Donais et al. 2004). The importance of Src in FA turnover is related to its activation of FAK, as demonstrated by the inability of a FAK mutant lacking the Src interaction site (Y397F) to restore normal FA disassembly in FAK-null fibroblasts (Schaller, Hildebrand et al. 1994; Webb, Donais et al. 2004). The means by which FAK can regulate FA turnover are manifold and involve many downstream effector proteins (**Figure 1-4**). The FAK-associated mechanisms include but are not limited to the principal effector proteins that I have outlined here, and there may exist as-yet unidentified substrates and levels of FAK function. One mechanism of FA turnover involves the FAK-mediated phosphorylation of the actin cross-linking protein α-actinin, its subsequent detachment from actin, and the

concomitant dispersal of stress fibre-integrin attachments (Izaguirre, Aguirre et al. 2001; Mitra, Hanson et al. 2005). FAK-dependent activation of proteinases such as calpain is also likely to play a role in the breakdown of focal adhesions. Calpain proteolysis releases integrin clusters from actin, thus breaking down focal contacts and permitting migration to continue (Dourdin, Bhatt et al. 2001; Bhatt, Kaverina et al. 2002; Carragher, Westhoff et al. 2003). FAK may also influence actomyosin contraction both positively and negatively through its recruitment of Rho-regulating proteins that ultimately modulate MLCK activity and stress fibre tension. Increased activation of Rho leads to the consolidation of stress fibres and focal adhesions, whereas Rho inhibition favours the dissolution of contacts (Mitra, Hanson et al. 2005). Lastly, FAK is required for phosphorylation of PKL and consequently for its recruitment to FAs via the LD4 of paxillin, along with the PKL-PIX-PAK complex (Brown, Cary et al. 2005).

FAK and Src are also necessary for the tyrosine phosphorylation of paxillin itself. When paxillin-FAK association is disrupted or when dominant-negative FAK is introduced, paxillin tyrosine phosphorylation is reduced (Richardson and Parsons 1996; Thomas, Cooley et al. 1999). Deficiencies in Src or FAK do not however impair the translocation of paxillin into focal adhesions (Webb, Donais et al. 2004). Tyrosines 31 and 118 of paxillin have been identified as targets of Src or FAK which, when phosphorylated, dock with SH2-containing proteins(Schaller and Sasaki 1997; Schaller and Schaefer 2001). A phosphorylation-defective Y31F/Y118F mutant of paxillin failed to restore FA turnover in paxillin-null cells, demonstrating the importance of these residues to paxillin function (Webb, Donais et al. 2004).

ERK is well-characterized as a nuclear regulator of gene expression, but the active kinase is also recruited into new focal contacts and acts on cytoskeletal targets in a mechanism critical for focal adhesion turnover (Fincham, James et al. 2000). The Ras pathway, which culminates in the activation of ERK, can be activated by a number of stimuli including adhesion, growth factors, and chemoattractants. Src and FAK were

shown to be at the root of the pathway when it was demonstrated that upon ECM adhesion Src-activated FAK could directly recruit Grb2 and SOS, the upstream activator of Ras (Schlaepfer, Hanks et al. 1994). Inhibition of ERK in adherent cells resulted in impaired migration; a defect which could be attributed to attenuated activation of MLCK and the ensuing weakening of myosin contractile force. Further, it was shown that MLCK can be directly phosphorylated by ERK *in vitro* (Klemke, Cai et al. 1997). A published examination of focal contact turnover in murine embryonic fibroblasts established by several experimental avenues that ERK and MLCK activities are critical for FA turnover. For example, reconstitution of FAK-null cells with constitutively active MEK successfully reinstated FA turnover, and treatment of wild type cells with an MLCK inhibitor prevented FA turnover (Webb, Donais et al. 2004). A likely explanation for the MLCK finding is that actin tension itself may encourage turnover of focal contacts (Crowley and Horwitz 1995). Combined, these studies have demonstrated that activation of ERK and subsequently MLCK is an important outcome of Src-FAK signaling with respect to FA turnover (**Figure 1-4**).

Paxillin is also a part of the FAK-ERK axis; not only does paxillin provide a scaffold for the enhancement of FAK activation, but the adaptor is a substrate and binding partner of ERK (Ku and Meier 2000; Ishibe, Joly et al. 2003). Initial phosphorylation by ERK is permissive of many subsequent serine phosphorylation events (Cai, Li et al. 2006) and the paxillin-ERK interaction is required for optimal motility in epithelial cells (Ishibe, Joly et al. 2004). The involvement of ERK in paxillin- and FAK-associated signaling represents a departure from the canonical view of this kinase as a transcriptional activator and establishes a role for ERK in adhesion and motility.

PART IV.b.iii. *The PTP-PEST Phosphatase*

A major feature of focal adhesion formation is the tyrosine phosphorylation of proteins included in the complexes and consequently it is not surprising that phosphatase

activity accompanies the disassembly of focal contacts. The LIM3 and LIM4 domains of paxillin recruit the non-receptor phosphatase PTP-PEST to membrane complexes (Shen, Schneider et al. 1998; Angers-Loustau, Cote et al. 1999; Cote, Turner et al. 1999). PTP-PEST dephosphorylates various targets and mediates paxillin dephosphorylation, either directly or indirectly (Shen, Lyons et al. 2000). PTP-PEST-null cells are characterized by impaired migration, an overabundance of focal adhesions, and hyperphosphorylation of paxillin and FAK, all of which are consistent with a role for PTP-PEST in the turnover of focal contacts (Angers-Loustau, Cote et al. 1999). Moreover, the association between PTP-PEST and paxillin is needed for these functions (Jamieson, Tumbarello et al. 2005). Mice deficient in PTP-PEST die *in utero* at day 9.5 – 10.5 (Sirois, Cote et al. 2006).

Paxillin resides at the centre of a complex web of interconnected proteins, the common feature among them being their ability to regulate focal adhesions and stress fibres. Since its discovery, a great proliferation of work has characterized the binding interactions of paxillin and their ultimate effects on the cell. The confines of the present work have compelled me to convey a somewhat abbreviated representation of paxillin function and I have restricted myself to those aspects which are the best-characterized and the most relevant to the experiments presented in the following chapters. In general, paxillin appears to be important not so much for adhesion as for the detachment of adhesion, particularly the disassembly of focal contacts that occurs near the leading edge and allows the cell to continue its cycle of protrusion and motility. As exemplified by the lethality of paxillin deficiency, the ability of cells such as epithelial cells, fibroblasts, neurons, and smooth muscle cells to migrate within the extracellular medium is essential to the normal development and maintenance of the organism. The dangers of disregulated migration include the spread of malignant cells, and indeed both FAK and paxillin have garnered attention of late as regulators of tumour motility and metastasis. The picture of paxillin function is far from complete as this adaptor protein continues to be studied in different cell types and under different conditions.

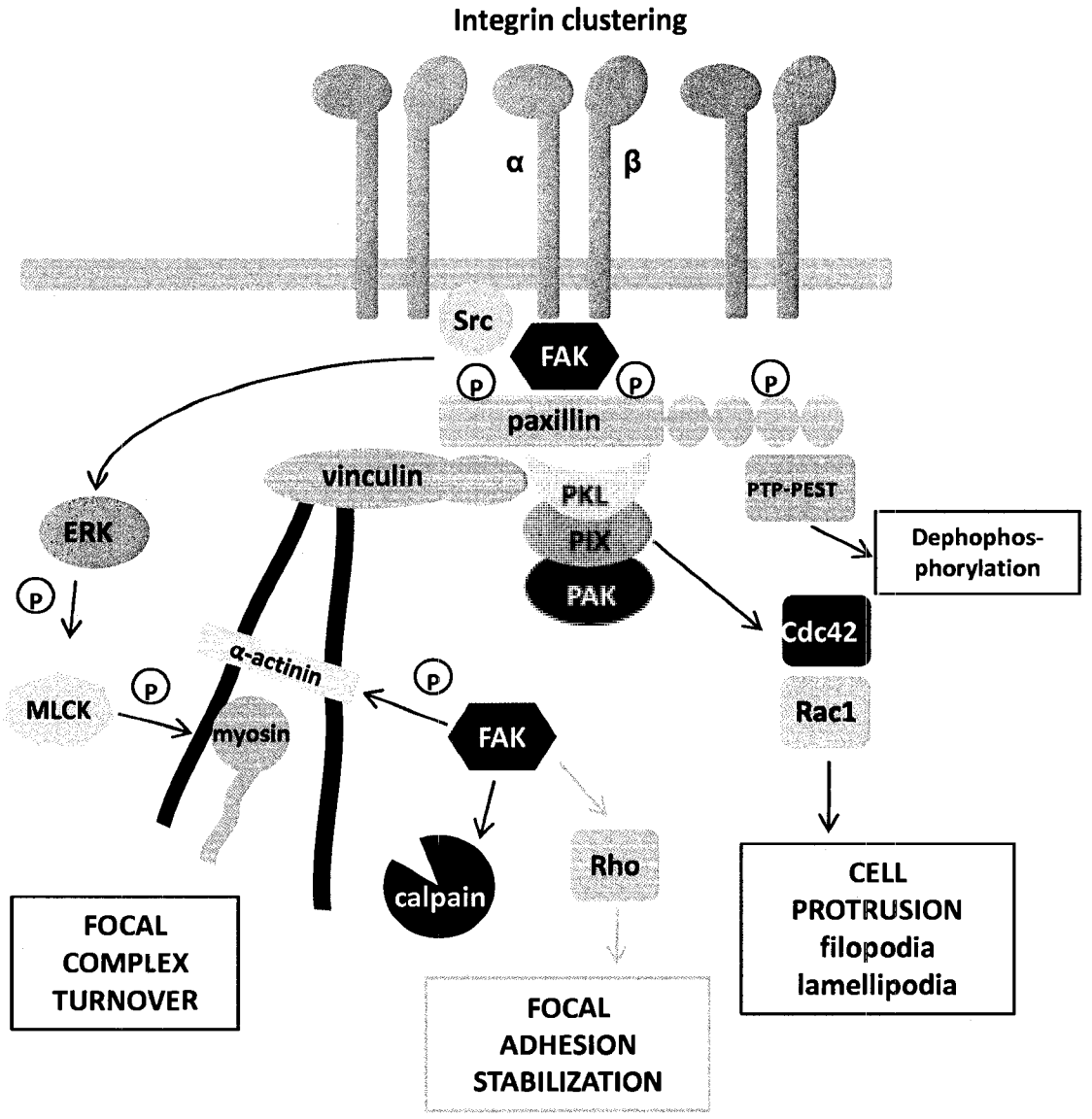


FIGURE 1-4. Focal complex structure. For the sake of space, some elements have been omitted or simplified. Src, FAK, and paxillin are recruited early into new focal complexes, initiating a cascade of tyrosine phosphorylation. Actin dynamics and cell protrusion are promoted by Cdc42 and Rac1 (black arrows and box). Turnover of focal complexes (red arrows and boxes) is essential for cell motility. FAK activity leads to the disengagent of α-actinin from actin and increased proteolytic activity of calpain. ERK activation of MLCK leads to increased myosin contractility, which encourages the breakdown of focal complexes. PTP-PEST recruitment by paxillin also favours turnover by dephosphorylating focal complex proteins. Conversely, activation of Rho leads to a slowing of migration due to the maturation of focal complexes into more stable focal adhesions (green arrow and box).

PART IV.c. *Paxillin in T cells*

PART IV.c.i. *Localization*

To date paxillin has been characterized primarily in adherent cell types, but those studies which have examined paxillin in cells of hematopoietic lineage have uncovered significant differences in paxillin regulation and function, these possibly being a reflection of the disparate ways in which adherent cells and leukocytes adhere and migrate. As alluded to above, one of the most salient differences between lymphocytes and adherent cells is the lack of focal adhesions in the former. A microscopic examination of paxillin localization in T cells revealed paxillin to be localized at the MTOC; a subcellular distribution not previously seen for the protein (Herreros, Rodriguez-Fernandez et al. 2000). The regulation and function of this association have not yet been addressed though it introduces the possibility of a novel role for the MTOC-associated paxillin. Indeed, during the writing of this thesis a publication investigating the role of JNK (c-Jun N-terminal kinase), a MAPK family member related to ERK, in NK cell killing also included a figure demonstrating that paxillin knockdown by siRNA (small interfering RNA) inhibited NK target lysis (Li, Ge et al. 2008). There were no supporting experiments presented and therefore we do not know at what stage NK killing was blocked. Needless to say, this result was very interesting to us as it opens the possibility of paxillin playing a role in the process of CTL target cell cytolysis.

PART IV.c.ii. *Binding partners: Pyk2 and Lck*

Lymphocytes express a somewhat different complement of proteins than adherent cells, the result being a shift in the paxillin binding partners. For instance, T cells preferentially express the FAK family member Pyk2 (proline-rich tyrosine kinase 2), which contains a PBS domain and interacts with paxillin (Li and Earp 1997; Ostergaard, Lou et al. 1998). Pyk2 is distinct from FAK in that its expression is mainly restricted to

the hematopoietic and neuronal lineages and it is not localized to focal adhesions but the cytoplasm (Schaller and Sasaki 1997; Zheng, Xing et al. 1998; Ostergaard and Lysechko 2005). In T and NK cells Pyk2 is recruited to the MTOC (Rodriguez-Fernandez, Gomez et al. 1999; Sancho, Nieto et al. 2000; Rodriguez-Fernandez, Sanchez-Martin et al. 2002). Like FAK, Pyk2 is activated by Src kinases, Fyn being the preferred enzyme downstream of TCR activation (Qian, Lev et al. 1997). The function of Pyk2 in T cell activation is not fully understood, yet Pyk2 has been shown to contribute to NK cell MTOC reorientation and cytotoxicity (Gismondi, Jacobelli et al. 2000; Sancho, Nieto et al. 2000). In Pyk2-deficient macrophages cell polarity, membrane protrusion, and migration are impaired, suggesting that Pyk2 may share some functions with FAK (Okigaki, Davis et al. 2003). That paxillin and Pyk2 associate in T cells and are both localized to the MTOC leads to the speculation that the two proteins may cooperate to perform a novel function in this cell type.

T cells express a specific pair of Src kinases: Lck and Fyn. Paxillin was shown to bind to Lck in T cells; however the interaction required not the SH3 domain of Lck but its SH2 domain, which presumably docks to a phosphotyrosine residue of paxillin (Ostergaard, Lou et al. 1998). Also unique to T cells are the diverse signaling mechanisms initiated upon contact with antigen-MHC. Paxillin is heavily phosphorylated in response to TCR activation and can therefore be considered a component of TCR signaling cascades (Ostergaard, Lou et al. 1998).

PTP-PEST

The interaction between paxillin and PTP-PEST may have an extra dimension in T cells as it has been published that PTP-PEST regulates antigen receptor signaling. In A20 B cells, overexpression of PTP-PEST caused a reduction in IL-2 production as well as reductions in phosphorylated Pyk2 and FAK after B cell receptor activation. This effect was dependent upon PTP-PEST-paxillin interaction (Davidson and Veillette 2001). The

evidence suggests that paxillin recruitment of PTP-PEST may provide a means for the down- modulation of lymphocyte signaling as the cell transitions between activation and resting effector states. PTP-PEST may also play a role in actin and immune synapse regulation. PTP-PEST phosphatase activity acts in opposition to Fyn kinase activity to negatively regulate actin polymerization and SMAC formation (Badour, Zhang et al. 2004).

PKL/PIX/PAK complex

The PKL/PIX/PAK complex is intact in T cells, activated in response to the TCR, and recruited to the immunological synapse between Jurkat T cells and APC (Ku, Yablonski et al. 2001; Phee, Abraham et al. 2005). The complex does not appear to interact with paxillin in Jurkat T cells or CTL, however ((Ku, Yablonski et al. 2001) and Laura Mireau, unpublished observation); a striking difference from focal adhesions. The complex appears to be involved in T cell activation, but how this occurs is yet to be described.

The α_4 integrin cytoplasmic tail

Direct binding of paxillin to the cytoplasmic tails of integrins does not appear to be a major means of recruitment of paxillin to focal adhesions, but there does exist a unique and highly specific interaction between α_4 integrin and paxillin (Liu, Thomas et al. 1999). The α_4 subunit coupled with β_1 make up VLA-4, an integrin principally expressed on leukocytes which mediates rolling, extravasation, and trafficking by binding to VCAM-1 and fibronectin (Alon, Kassner et al. 1995; Berlin, Bargatze et al. 1995; Weber and Springer 1998). Analysis of α_4-null chimeric mice (total α_4 deficiency is embryonic lethal) revealed faulty development of B and T lymphocytes (Rose, Han et al. 2002). The α_4 subunit behaves differently from other integrins in that it promotes motility rather than spreading and adhesion (Rose, Han et al. 2002). Upon further characterization it was

discovered that the LD domains of paxillin mediate the interaction with α_4 despite the lack of a PBS domain in the integrin subunit. A segment encompassing LDs 3 and 4 was sufficient for binding of α_4 though no one LD domain was essential (Liu, Kiosses et al. 2002). On the other side, a small sequence of 9 amino acids in α_4 is sufficient for paxillin binding and the phosphorylation by PKA (protein kinase A) of serine 988 within this region disrupts the association (Liu and Ginsberg 2000; Han, Liu et al. 2001). A series of publications have profited from this knowledge of α_4–paxillin binding properties to address the function of the interaction.

Functionally, the dynamics of the α_4-paxillin interaction contribute to the enhancement of motility that is characteristic of α_4 integrin engagement. Disruption of the association by the introduction of a dominant-negative α_4-binding paxillin fragment (Ala^{176}- Asp^{275}) or various α_4 mutations (S988D phosphomimetic, Y991A, E983A) resulted in the prevention of Pyk2/FAK activation in addition to generally increased spreading and impaired migration on the substrate (Liu, Thomas et al. 1999; Liu and Ginsberg 2000; Liu, Kiosses et al. 2002; Han, Rose et al. 2003). The reconstitution of α_4–deficient Jurkat T cells with an S988A mutant (constitutively associated with paxillin) was expected to enhance migration but instead inhibited it (Han, Rose et al. 2003). In particular, S988A-expressing cells failed to form stable, polarized lamellipodia (Goldfinger, Han et al. 2003). The model which emerged from the ensuing studies proposed that α_4 at the cell periphery was phosphorylated by PKA at the leading edge, thus displacing paxillin. Displacement of paxillin removed the Git1 ARF-GAP from the vicinity, allowing the activation of Rac1, the extension of a polarized lamellipodium, and migration of the cell. Association of α_4 (non-phosphorylated) with paxillin at the lateral and trailing edges of the cell limited Rac1 activation in those areas and effectively polarized the cell. The exact mechanism by which Git1 inhibits Rac1 has not been described (Goldfinger, Han et al. 2003; Nishiya, Kiosses et al. 2005). This knowledge was applied to engineer an *in vivo* model in which an α_4 Y991A mutation abrogating paxillin binding was knocked into mice. The animals had no major developmental defects but did show defective

mononuclear leukocyte trafficking to the area of the peritonitis induced by the authors (Feral, Rose et al. 2006).

The interaction between paxillin and α_4 is an interesting example of motility regulation by paxillin and suggests that the adaptor protein may function in novel ways in T cells. In T cells specifically, VLA-4 ($\alpha_4\beta_1$) engagement by VCAM-1 is able to enhance LFA-1 activity and migration through *trans*-regulation. This phenomenon is dependent upon paxillin-α_4 binding and is attributable to the paxillin-dependent activation of FAK and Pyk2, which creates signals that regulate LFA-1 (Rose, Liu et al. 2003). Considering this information, it is tempting to speculate that paxillin may be involved in other LFA-1 mediated adhesion events such as APC and target cell binding by helper and cytotoxic T cells. In any event, based on the distinctive localization and binding partners of paxillin in T cells, together with preliminary functional data (Li, Ge et al. 2008), it appears probable that the adaptor protein is impacting on a set of cellular functions that is particular to T cells and distinct from those previously described in other, adherent cell types.

Study Objectives

The principal aim of this study is to examine aspects of paxillin regulation and function in $CD8^+$ cytotoxic T cells. The specific questions addressed were as follows:

1) How is the TCR-triggered phosphorylation of paxillin regulated, and are tyrosine and serine phosphorylations modulated separately?

2) What is the subcellular localization of paxillin in CTL, and what domains of the adaptor protein are required for its normal recruitment?

3) How is the localization of paxillin regulated by TCR and integrin engagement in CTL?

By answering the questions above I will contribute to the general knowledge of paxillin function and, more specifically, begin to describe its role in the overall program of CTL activity. Many aspects of CTL regulation and cytotoxicity remain obscure, and the larger aim of this study is to expose more details of the mechanisms governing their ability to be activated by and to eliminate cells altered by viruses and malignancy.

CHAPTER 2: Methods and Materials

Cells

Non-transformed murine H-2^b–specific CTL clone lines AB.1 (H-2^d) and Clone 11 (H-$2^{d/k}$) were derived from mice and have been described previously (Blakely, Gorman et al. 1987; Kane, Sherman et al. 1989). The CTL clones were cultured in RPMI supplemented with 10% fetal calf serum (FCS), 100μg/mL penicillin/streptomycin, 2mM L-glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 53nM β-mercaptoethanol, and 10 units/mL of recombinant IL-2. The clones were stimulated weekly with irradiated allogeneic splenocytes from C57BL/6 mice. Experiments were performed with the clones 4-6 days after splenocyte stimulation. In general, AB.1 clones were used for target signaling experiments, while Clone 11 were used for all transfections and therefore most imaging experiments. Most signaling experiments were repeated in both clone types with similar results.

The 1F10 clones were produced in our laboratory from Fyn-deficient mice. The Fyn -/- clones were generated as H-2^d (BALB/c)-specific by continuous mixed lymphocyte culture. The 1F10 CTL were stimulated weekly with irradiated allogeneic BALB/c splenocytes; otherwise culture conditions were identical to those described above for AB.1 and Clone11.

The L1210 (H-2^d) and L1210 K^bD^d target cell lines were a gift from Dr. K. P. Kane (University of Alberta, Edmonton, AB). The latter were stably transfected with a chimeric Class I MHC K^bD^d construct and therefore were maintained in 1mg/mL neomycin/G418. L1210 cells were cultured in DMEM medium supplemented with 8% defined calf serum (dCS).

$CD8^+$ splenocytes were purified by negative selection from C57BL/6 splenocytes using the MACS magnetic bead separation system ($CD8^+$ T cell isolation kit, Miltenyi Biotec, Auburn, CA). Concanavalin A (ConA) blasts were generated by treating C57BL/6 splenocytes at 5 x 10^6/mL with 2μg/mL ConA for 48 hours. ConA blasts were

cultured in RPMI containing 10% FCS, L-glutamine, sodium pyruvate, and β-mercaptoethanol.

Antibodies

Anti-paxillin, anti-Lck, and the anti-Pyk2 mouse monoclonal used for Western blotting were purchased from BD-Transduction Labs (Mississauga, ON). Anti-phospho-ERK (Thr202/Tyr204) was obtained from Cell Signaling Technologies (Danvers, MA), and anti-ERK1/ERK2 from Zymed (San Francisco, CA). Anti-Fyn was obtained from Abcam (Cambridge, MA). Anti-phospho-tyrosine (PY72), anti-LFA-1 (M17/5.2), and anti-CD3ε (145-2C11) were purified from hybridomas as described previously (Berg and Ostergaard, 1998, Berg and Ostergaard 1995). The rabbit polyclonal antibody to Pyk2 , F245, was generated by our laboratory against a peptide corresponding to amino acids 720-826 fused to GST, and has been described previously (Berg and Ostergaard, 1997). The antibody against α-tubulin used for microscopy was purchased from Abcam (Cambridge, MA). Anti-GFP mouse monoclonal and rabbit polyclonal antibodies were purchased from Molecular Probes/Invitrogen (Carlsbad, CA), as were the secondary antibodies used for confocal: anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 488, anti-rabbit Alexa Fluor 594, and anti-rat Alexa Fluor 488. Rhodamine-coupled anti-mouse, rabbit anti-mouse Ig (for immunopreciptation), and horseradish peroxidase (HRP)-coupled anti-mouse Ig, were obtained from Jackson Immunoresearch (West Grove, PA).

Reagents

Protein A-coupled Sepharose was purchased from GE Healthcare (Piscataway, NJ). Latex beads were obtained from Interfacial Dynamics Corporation (Eugene OR). Protein A-HRP was purchased from Pierce (Rockford, IL). Wortmannin, okadaic acid, PP2 and PP3 were purchased from Calbiochem (San Diego, CA). The MEK inhibitor U1026 and

phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (Oakville, ON). Cell Tracker Blue CMAC was obtained from Molecular Probes (Invitrogen, Carlsbad, CA). The Fyn plasmid was a gift from Dr. Chris Bleackley (University of Alberta, Edmonton, AB). For paxillin knockdown experiments, scrambled control oligos and siGenome ON-TARGET plus paxillin siRNA oligos were designed and synthesized by Dharmacon Research Inc. (Lafayette, CO). The sequence for the paxillin oligos was as follows: 5' GGCAAAGCGUACUGUCGUAUU 3' (sense strand).

Cloning of murine paxillin

Paxillin was cloned from RNA extracted from murine splenocytes using Trizol (Invitrogen, Carlsbad, CA). First strand synthesis was catalyzed with the Superscript II reverse transcriptase and oligo(dT) primers (Invitrogen, Carlsbad, CA). The resulting cDNA was used as a template for the specific amplification of paxillin using the PAX1 and PAX2 primers (**see Table 2-1**). Full-length paxillin (aa 1-557) was first ligated into the pDrive cloning plasmid (PCR cloning kit, Qiagen, Mississauga, ON) by TA cloning. The sequence of the full-length paxillin insert was verified by DNA sequencing (performed by University of Alberta Molecular Biology Facility) using PAX3, PAX4, PAX5, and PAX6 primers. For transfer into pEGFP-C1 (Clontech, Mountain View, CA), an N-terminal forward primer containing an EcoRI site (EcoPax) and a C-terminal reverse primer containing a SalI site (SalPax) were used for amplification, using the pDrive-paxillin vector as a template. Pfu Turbo DNA polymerase was the high-fidelity polymerase used (Stratagene, La Jolla, CA). The resulting PCR product was purified using the Qiaquick PCR purification kit (Qiagen, Mississauga, ON) and digested with EcoRI and SalI (Invitrogen, Carlsbad, CA) at 37°C for 4h or overnight. The empty pEGFP-C1 plasmid was digested in parallel. The digests were analyzed by agarose gel electrophoresis on 0.75% agarose gels in TAE supplemented with 1mM guanosine. Excision of the bands was followed by extraction of the DNA with glass wool columns, followed by phenol-chloroform extraction. The paxillin insert was ligated into pEGFP-

C1 with T4 DNA ligase (Invitrogen, Carlsbad, CA) overnight at 15°C, creating a fusion of EGFP to the N-terminus of paxillin.

Further constructs of paxillin were made containing the N-terminal half only (pEGFP-NT, aa 1-323), the C-terminal half only (pEGFP-CT, aa 319-557), LD domain truncations of the N-terminal portion (LD1-4 aa 1-296, LD1-3 aa 1-264, LD1-2 aa1-219), and LD truncations from the full-length molecule (LD1trunc aa 136-557, LD2trunc aa 214-557). In addition, a construct was made containing only the LIM2/3 portion of paxillin (aa 376-503). All were prepared in the pEGFP-C1 plasmid using the same method of PCR amplification, digestion, and ligation described above. EcoRI was always used as the N-terminal cloning site, and SalI as the C-terminal cloning site. The primers used to amplify these segments are listed in **Table 2-1.**

Transformation of each construct into Library Efficiency DH5α competent cells (Invitrogen, Carlsbad, CA) was done according to manufacturer's directions, using kanamycin selection. Endotoxin-free DNA preparations for the purpose of transfection into eukaryotic cells were made using the Endo-free Plasmid Maxi Kit (Qiagen, Mississauga, ON).

Construct	Primers used	Sequence (5'-3')
full-length paxillin	PAX1	GGACCAGCCATGGACGACCTC
	PAX2	GTGCGTGGCGGCACTGGCTGG
Full-length paxillin (pEGFP cloning)	EcoPax	CTCTTG**GAATTC**AGCCATG-GACGACCTCGA
	SalPax	CTCTCTCT**GTCGAC**GTGCGT-GGCGGCACT
pEGFP-NT	EcoPax/**SalNterm**	TCTCTCTC**GTCGAC**GACCCCTTTGGCAAC
pEGFP-CT	**EcoC-term**/SalPax	CTCTTG**GAATTC**CGTTGCCAAAGGGGTCTGT
pEGFP-LIM2/3	EcoLIM2	TCTCTC**GAATTC**CCACAGCCTCTTCTCCCC
	SalLIM3	CTCTCTTA**GTCGAC**GCCGGAGCACAGCGA
pEGFP-LD1-4	EcoPax/**LD5trunc**	TCTGTCTG**GTCGAC**GGGCTTGGAGAGTCCCCC
pEGFP-LD1-3	EcoPax/**LD4trunc**	TGTGTCTG**GTCGAC**CCTGGTGGCAGAGGAGGC
pEGFP-LD1-2	EcoPax/**LD3trunc**	TGTGTCTG**GTCGAC**GCTCTCCACGCTGGGCCG
pEGFP-LD1trunc	SalPax/**LD1trunc**	TCTCTG**GAATTC**CTCCTCCCTGGGCAGCAAC
pEGFP-LD1-2trunc	SalPax/**LD1/LD2trunc**	TCTGTG**GAATTC**GCGGCCCAGCGTGGAGAGC
pEGFP-LD2-4	LD1trunc/LD5trunc	See above
Sequencing primers	PAX3	AGCAGAGCCCTCACCTA
	PAX4	CGAGTCACCTCCAGCCAG
	PAX5	CCCACGCTGCTACTACTG
	PAX6	TGCCAGAAGCCCATCACG

Table 2-1. Primers used in the cloning and sequencing of paxillin, and in the production of truncation mutants. Restriction sites are shown in bold text.

Site-directed mutagenesis of paxillin

Site-directed mutagenesis of specific amino acids within paxillin was performed by PCR using mutagenic primers and full-length pEGFP-paxillin as a template. The primers were designed such that they did not overlap completely (overhang of 9 bp), according to the

protocol laid out by Zheng, *et al.* (2004) (Zheng, Baumann et al. 2004). The PCR mix consisted of 50ng template DNA, 0.4μM of each primer, 200μM dNTPs, and 2.5 units Pfu Turbo polymerase. Serine residues 83 and 130 were mutated to alanines for the constructs pEGFP-S83A and pEGFP-S83A/S130A. Tyrosine to phenylalanine substitutions were made for residues Tyr^{31}, Tyr^{118}, and Tyr^{181}. Mutations in LIM domains 2 and 3 consisted of cysteine to alanine substitutions (pEGFP-C411A/C470A). After mutagenic PCR, the products were purified using the Qiaquick PCR purification kit (Qiagen, Mississauga, ON) and digested with DpnI (Invitrogen, Carlsbad, CA) for 1-2 hours to remove template DNA. The DpnI was then heat inactivated for 15min at 75°C, and a sample of the mix was transformed into DH5α. Resulting bacterial colonies were screened for the desired mutation by DNA sequencing. In the cases where more than one mutation was desired, the plasmid containing the first mutation was used as a PCR template for producing the second mutation. Primers used for the engineering of each mutation and their sequences are listed in **Table 2-2.**

Construct	Primer name	Sequence (5'-3')
C411A	C411Amutf	TTCTTCTGTGCCCAGGCTGGAGCCTTCTTTGGTCCAGAAGG
	C411Amutr	ACCAAAGAAGGCTCCAGCCTGGGCACAGAAGAAGTGTTCCGG
C470A	C470Amutf	GAATGCTTTGTGTGCAGGGAGGCCTTCACACCCTTCGTCAACGGC
	C470Amutr	GCCGTTGACGAAGGGTGTGAAGGCCTCCCTGCACACAAAGCATTC
S83A	fS83Amut	CACCAGCAGCCTCCGGCCCCACTGCCCGTGTAC
	rS83Amut	GTACACGGGCAGTGGGGCCGGAGGCTGCTGGTG
S130A	fS130Amut	AAGTCGGCAGAGCCCGCACCTACCGTCATGAGCTCCTCCCTG
	rS130Amut	GCTCATGACGGTAGGTGCGGGCTCTGCCGACTTCTGCTTGTT
Y31F	fY31Fmut	TCAGAGGAGCCCCCCTTCTCCTACCCAACTGGAAACCACACA
	rY31Fmut	TCCAGTTGGGTAGGAGAAGGGGGGCTCCTCTGACAAGAACAC
Y118F	fY118Fmut	GCAGGCGAGGAAGAGCACGTCTTCAGCTTCCCCAACAAG
	rY118Fmut	GCTTGTTGGGGAAGCTGAAGACGTGCTCTTCCTCGCCTGC

Table 2-2. Primers used in the site-directed mutagenesis of paxillin.

Deletion mutagenesis of paxillin

Targeted deletions of LD domains and the proline-rich domain of paxillin were engineered via a step-wise PCR method. A forward primer on the C-terminal side of the deletion was designed to be used with the reverse SalPax primer to amplify the portion of paxillin C-terminal to the deletion. A reverse primer on the N-terminal side of the deletion, to be used with EcoPax to amplify the segment of paxillin N-terminal to the deletion, was engineered to contain a short 11 bp bridge sequence complementary to the C-terminal side of the deletion. PCR amplification using full-length pEGFP-paxillin as a template was performed with each of the two primer pairs separately. The resulting fragments were separated by gel electrophoresis and bands of the correct size were

excised. After purification, samples of each fragment were mixed for a second PCR, this with the full-length EcoPax and SalPax primers to create a "joined" product in which both flanking segments were contiguous, with the desired deletion made. Successful deletion was confirmed by DNA sequencing. This technique was used to delete LD4 (Δ aa 265-274), LD3 (Δ aa 220-226), LD2 (Δ aa 143-154), a larger LD2-4 segment (Δ aa 55-274), and the proline region (Δ aa 45-54). Double deletions were made by subjecting the first completed mutant plasmid to a second round of deletion mutagenesis. Information on the primers used for deletion mutagenesis can be found in **Table 2-3**.

Construct	Primer name	Sequence (5'-3')
ΔPro	Pro del f	AGCGAGGCCCTCAATGGCACGGTC
	Pro del r	AGGGCCTCGCTΔCGCAATCTCCTGGTATGT
ΔLD2	LD2 del f	GTGCAGCACAGCCCGCCTGGCTTC
	LD2 del r	CTGTGCTGCACΔGAGGTTGCTGCCCAGGGA
ΔLD3	LD3 del f	TCCGTGCCCAGCCCTGTCCCGGCC
	LD3 del r	CTGGGCACGGAΔGCTCTCCACGCTGGGCCG
ΔLD4	LD4 del f	GATTTCAAGTTCATGGCCCAGGGG
	LD4 del r	AACTTGAAATCΔCCTGGTGGCAGAGGAGGC
ΔLD2-4	LD2-LD4 del f	GATTTCAAGTTCATGGCCCAGGGG
	LD2-LD4 del r	AACTTGAAATCΔGGACGGTGGTGGTGGGAC

Table 2-3. Primers used to produce deletion mutations in paxillin. The site of the deletion is marked by "Δ".

Antibody Immobilization

Immobilization of anti-CD3 was achieved by incubating plastic, 60mm, non-tissue culture treated plates with 10μg/mL 145-2C11 antibody overnight at 4°C. In cases where small numbers of cells were to be used (1-2x10^5 cells), 96-well plastic plates were coated instead. Prior to use, the plates were washed three times with Dulbecco's phosphate buffered saline (D-PBS) containing calcium and magnesium and blocked for 30-60min at 37°C with 2% bovine serum albumin (BSA) in PBS. The blocking agent was washed out three times with PBS before the addition of cells.

Latex beads were diluted to 1 x 10^7 beads/mL in PBS and coated either with 5 μg/mL anti-CD3 or 5 μg/mL anti-LFA-1. The beads were allowed to rotate at 4°C overnight. The next day, an equal volume of 2% BSA in PBS was added to block the beads, followed by rotation at room temperature for 30 min. The beads were pelleted at 3300xg for 2 min and then washed 3 times with 0.1% BSA in PBS. Finally, the beads were resuspended to a concentration of 1 x 10^7 beads/mL in PBS.

Cell stimulation with immobilized anti-CD3

CTL clones were harvested and resuspended at a concentration of 2x10^7 cells/mL in PBS containing calcium and magnesium. If drug treatment was desired, CTL were incubated with the appropriate concentration of drug or carrier control for the required time (see following paragraph for details). 1x10^7 cells were added to 145-2C11-coated plates (prepared as described above) and incubated at 37°C for 25 minutes, if not stated otherwise. Cells were lysed in the dishes for 20min at 4°C by the addition of 1mL 1.5% NP-40 lysis buffer, for a final concentration of 1% NP-40. The lysis buffer consisted of the following: 1.5% NP-40, 5mM EDTA, 150mM NaCl, 10mM Tris pH 7.6, and 1mM vanadate (VO_4) as a phosphatase inhibitor. Lysates were removed from the dishes and the nuclei were pelleted by centrifugation at 16000xg for 5 min. A sample of post-nuclear lysate corresponding to 2.7x10^5 cell equivalents (40μL) was removed from the lysates for SDS-PAGE gel analysis. In cases where the pellet material itself was to be analyzed, 150μL of 1x Laemmli reducing sample buffer was added to the pellet, which was broken up and resuspended with repeated boiling and pipetting. 50μL of this was then loaded on an SDS-PAGE gel. In cases where only a lysate was required, 1-2 x 10^5 CTL were added in 40μL PBS to a 145-2C11-coated well of a 96-well plate and incubated at 37°C, followed by the direct addition of 40μL 2x Laemmli reducing sample buffer. The samples were then removed from the wells and boiled for 4 min prior to SDS-PAGE.

Drug treatment with wortmannin (30nM or 100nM, as indicated) or U1026 (10μM or 30μM, as indicated) was carried out at 37°C for 30min, with DMSO as a carrier control. The Src-kinase inhibitor PP2 and its inactive analogue, PP3, were used at a final concentration of 10μM and incubated with the cells on ice for 15 min. The serine phosphatase inhibitor okadaic acid was used at 1μM and incubated at 37° for 30min.

Target cell stimulation of CTL

CTL clones were mixed with targets cells with (L1210 K^bD^d) or without (L1210) the appropriate allogeneic Class I MHC on their surface, at an effector:target (E:T) ratio of 1:2. Before mixing, the cells were resuspended in 2% FCS in PBS. 2×10^5 CTL in 200μL were mixed with 4×10^5 L1210 K^bD^d or L1210 targets in 200μL and centrifuged at 100xg for 1 min to facilitate conjugation. After incubation at 37°C for the desired time, the supernatant was removed and the cells resuspended in 50μL 1x Laemmli reducing sample buffer. The samples were then boiled for 4 minutes for SDS-PAGE analysis.

Cell stimulation with PMA

Stimulation of CTL with the phorbol ester PMA was done by adding PMA to the required number of CTL in PBS to a final concentration of 100ng/mL. The CTL were then incubated for 10 minutes at 37°C. The cells were pelleted at 2300xg for 2 minutes and then lysed in NP-40 lysis buffer for 20 minutes as usual.

Immunoprecipitation

Paxillin immunoprecipitation (IP) was performed by incubating the post-nuclear lysates prepared as described above (containing 1×10^7 cell equivalents) with 1.5μg anti-paxillin antibody for 15 minutes followed by 3.45μg (1.5μL) rabbit anti-mouse Ig for 15

minutes, and finally 20μL of a 50% slurry of protein A Sepharose beads. IPs were incubated overnight at 4°C with rotation. For Pyk2 IPs, 7μL of F245 rabbit antiserum was added first, followed by protein A beads. For GFP IPs, 7μg mouse anti-GFP was used. Beads were pelleted and washed three times with NP-40 lysis buffer, then prepared for SDS-PAGE by addition of 60μL 1x Laemmli reducing sample buffer and boiling for 4 minutes.

SDS-PAGE and Western blotting

Cell lysates and IPs were analyzed by electrophoresis on 7.5% or 8.5% acrylamide SDS-PAGE gels run overnight at 6mA. The proteins were then transferred to PVDF membranes for Western blot analysis. In lieu of blocking, blot membranes were dried completely prior to the addition of antibody. The desired primary and HRP-coupled secondary antibodies were incubated on the membrane and detected with the enhanced chemiluminescence (ECL) system (Perkin Elmer, Life Science Products, Boston, MA). Antibodies were stripped off the membrane with buffer containing β-mercaptoethanol, SDS and Tris-HCl pH 6.7 at 56°C for 30 min to allow for sequential immunoblots of the same membrane. To protect phoshorylated amino acid residues, immunoblotting for phosphotyrosine or phospho-ERK was always performed in 4% BSA rather than 5% skim milk, which was used for all other antibodies.

Removal of dead cells

Prior to siRNA treatment, dead cells were removed by density centrifugation. OptiPrep density barrier medium from Sigma-Aldrich (Oakville, ON) was diluted 1:2 in RPMI, for a total volume of 3mL. The cell suspension was layered over top and centrifuged at 800xg for 15 min. The cell band was harvested, washed, and resuspended in the desired medium.

Transfection CTL with plasmid DNA or siRNA

Transfection of CTL was accomplished by nucleofection using the Amaxa mouse T cell kit according to manufacturer's directions (Amaxa Biosciences, Gaithersburg, MD) CTL clones were harvested 4 days after splenocyte stimulation and $1x10^6$ cells per sample were dispensed into separate tubes and pelleted at 2300xg for 2 min. The supernatant was removed and the pellet resuspended in 100μL nucleofection solution. 4μg of plasmid DNA was added, and the sample was nucleofected using the X-01 program on the Amaxa nucleofector. Cells were immediately transferred into 1mL of pre-warmed medium (Mouse T Cell Nucleofector Medium) supplemented with medium components A and B, 10% clone FCS, 2mM L-glutamine, 53nM β-mercaptoethanol, and 10units/mL recombinant IL-2. 24 hours after nucleofection, transfection efficiency and GFP expression was assessed by flow cytometry, and cells were used for experiments.

For nucleofection of siRNA oligos, 7μg of paxillin siRNA or non-targeting scrambled oligos (Dharmacon Research Inc., Lafayette, CO) was added to the cells in nucleofection solution, under RNase-free conditions. The cells were first subjected to density centrifugation to remove dead cells and improve viability (see above). CTL transfected with siRNA were split 1:2 24 hours after nucleofection and cultured for a total 48 hours prior to use. Knockdown of paxillin was assessed by analysis of cell lysates ($1x10^5$ cells) and comparison between paxillin and actin loading control immunoblots. Densitometry was performed using the Li-Cor Odyssey Application Software (Li-Cor Biosciences, Lincoln, NE). The integrated intensities of the paxillin and actin bands were determined by the software relative to the background intensity. Intensity ratios of paxillin to actin were then calculated for both the mock (or scrambled) and paxillin siRNA samples, and used to determine the percentage knockdown according to the formula:

$$\% \text{ knockdown} = 100 - \left[\frac{(\text{pax:actin})_{\text{siRNA}}}{(\text{pax:actin})_{\text{mock}}} \times 100 \right]$$

Preparation of CTL for confocal microscopy

Target cells used for the conjugates in preparation for microscopy were labeled prior to use with 7μM CMAC Cell Tracker Blue dye (Molecular Probes, Invitrogen, Carlsbad, CA) for 20 min at 37°C in DMEM media. Following a 30 minute incubation in fresh medium, the cells were washed twice in PBS and resuspended at the desired concentration.

In the case of transfected cells, 1 well of CTL (4-5x 10^5cells after 24h), (resuspended in 100μL PBS) was combined with 4 x 10^5 L1210 K^bD^d target cells (in 100μL PBS). In the case of non-transfected CTL, 6x10^5 CTL were mixed with 3x10^5 L1210 K^bD^d for an E:T ratio of 2:1. For bead imaging, 4x10^5 CTL were mixed with 5x10^5 L1210 targets and 6x10^5 beads. The cells were pelleted at 100xg for 1 min to facilitate conjugate formation and incubated at 37°C for 2 minutes. Conjugates were gently vortexed and transferred using a wide-bore pipet tip onto poly-L-lysine-coated coverslips (each placed in a well of a 24-well plate) and allowed to settle and adhere for 7 minutes at room temperature. Fixation and permeabilization were then performed by treatment with 4% paraformaldehyde in PBS for 10 min and 0.2% NP-40 in PBS for 5 min, respectively. Cells were then washed twice with 2% FCS in PBS. Cells were stained with the appropriate primary (45-60min) and fluorochrome-coupled secondary (30-45min) antibodies in the same 2% FCS buffer at room temperature in a dark chamber. Between antibodies, coverslips were washed quickly once and then 4 times for 3 minutes each in 2% FCS buffer. Stained coverslips were finally mounted on microscope slides in a droplet of glycerol and sealed with tape. Images were acquired using a Zeiss LSM 510 confocal microscope with a x40 oil-immersion objective (N. A. 1.3). Alexa 488 and rhodamine/Alexa 594 dyes were excited using 488nM argon and 543nm HeNe lasers, respectively. CTL and targets were distinguished by Cell Tracker Blue staining of the targets, visualized with the 351nm UV laser.

Though dozens of conjugates were viewed during any given imaging experiment, by necessity I was required to choose a sample of representative cells of which to take permanent images. I only took images of those cells which were clearly conjugated to a target cell, unobstructed, and in which the microtubule cytoskeleton, MTOC, and target cell interface were in the same optical plane, clearly visible, and easily distinguished from each other. In general, between 10 and 25 total images of each construct were taken, over at least 3 experiments. From each sampling of images, I then selected a representative image to be presented here in the thesis. Given that the goal of many of the imaging experiments was to assess paxillin localization to the microtubule cytoskeleton and target interface, preference was given to those images which displayed these structures distinctly, for ease of analysis. With respect to the imaging of cells expressing EGFP fusion proteins, I attempted to image those cells expressing a medium level of EGFP, as those with low levels were often too dim and those expressing high levels often had EGFP proteins throughout the cytoplasm and were too bright for cell architecture to be plainly seen. An additional control was performed in the cases of EGFP-paxillin, EGFP-NT, EGFP-CT, EGFP-ΔLD2-4, and EGFP-LD2-4: to eliminate the possibility of cross-reactivity between the tubulin and EGFP staining, which would lead to a false result, each of the fusion proteins was imaged with staining for EGFP alone, with no second colour.

Statement on animal use

All animal studies were approved by the University Animal Policy and Welfare Committee at the University of Alberta and adhered to the guidelines put forward by the Canadian Council on Animal Care.

Reproducibility of Results

Unless otherwise stated, experiments were repeated a minimum of three times. Data representative of all experiments were chosen for presentation.

CHAPTER 3: Regulation of paxillin phosphorylation downstream of the T cell receptor

A. Introduction

Cytotoxic T lymphocytes (CTL) constitute a primary immune defense against tumourous and virally-infected cells and are highly specialized for their task of detecting and eliminating altered self cells. Recognition of cells bearing viral or tumour antigens occurs via interaction of the clonotypic T cell receptor (TCR) with the antigen-loaded major histocompatibility complex (MHC) of the target cell. To effectively kill a detected target, the CTL must form a tightly adhered conjugate with the target, polarize its cytoskeleton and cytolytic granules, and finally release the granule contents in the direction of the abnormal cell. In $CD4^+$ T effector cells, TCR signaling leads to transcriptional activation and secretion of interleukin-2 and other cytokines which allow the helper T cells to manipulate the immune response. In contrast, the primary outcome of TCR stimulation in $CD8^+$ CTL effectors is the succession of events leading to degranulation. Activation of the TCR initiates many signaling cascades that eventually drive physical events such as actin polymerization, polarization of the microtubule cytoskeleton and microtubule organizing centre (MTOC), and granule movement toward the target. Despite the large amount of data concerning TCR signaling in T cells, there is much that remains unknown about the exact nature and mechanisms of the signaling cascades that lead to degranulation in CTL.

The signals that are triggered upon contact of the TCR-CD3 complex with antigen are propagated mainly by a series of phosphorylation events targeted to tyrosine, serine, and threonine residues. Phosphorylation of tyrosine can mediate both enzyme catalytic activity, as in the case of Src-family kinases (Zamoyska, Basson et al. 2003), and protein-protein interactions, as in the case of Src homology 2 (SH2) domains that bind to phosphotyrosine motifs. The interplay of kinases and phosphatases is therefore of key

importance to the initiation and control of T cell activation. The earliest stages of the TCR cascade include the phosphorylation of the TCR protein complex itself by Src-family kinases such as Lck and Fyn, followed by a concatenation of events leading to the activation of such proteins as phospholipase C-γ (PLC-γ), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), Ras, and transcriptional activators such as extracellular signal-regulated kinase (ERK) (Cantrell 1996). The kinase proline-rich tyrosine kinase 2 (Pyk2) is also activated by Src-family kinases Lck and Fyn (Berg and Ostergaard 1997; Qian, Lev et al. 1997). These TCR-proximal elements must transmit signals to coordinate the entire array of regulatory and effector proteins necessary for activation and degranulation and therefore all outcomes can ultimately be traced back to early events.

Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase that is activated soon after TCR stimulation and which produces lipid second messengers that influence downstream signaling events. Class I PI3Ks catalyze the addition of a phosphate group to phosphatidylinositol 3, 4 bisphosphate (PIP_2) to produce phosphatidylinositol 3, 4, 5 triphosphate (PIP_3) in the plasma membrane (Deane and Fruman 2004). PI3K catalytic activity can be inhibited irreversibly and reversibly by the drugs wortmannin and LY-294002, respectively. Inhibition of PI3K activity with wortmannin inhibits CTL degranulation as induced by antigen-bearing target cells (Fuller, Ravichandran et al. 1999) but treatment with the phorbol ester PMA and ionomycin, which bypasses TCR signaling, restores degranulation in the presence of wortmannin. PI3K kinase is therefore required for the signaling that leads to degranulation but not for the physical process of degranulation itself (Robertson, Mireau et al. 2005). The chain of events leading from PI3K to degranulation has yet to be deciphered. PI3K activation is also necessary for optimal activation of ERK downstream of the TCR, but again the mechanism is unknown (Von Willebrand, Jascur et al. 1996).

A ubiquitous regulator of growth and differentiation in response to diverse cell stimuli, ERK, or mitogen-activated protein kinase (MAPK), is a serine/threonine kinase that constitutes the terminal step of the Ras pathway. Inhibition of MAP/ERK kinase (MEK) activity, and thus ERK activity, is possible with drug inhibitors such as U0126, and this method is frequently used to test the cellular consequences of ERK deficiency. ERK is well-known as a transcriptional activator of nuclear targets in T cells but it has also been shown that ERK activity is necessary for degranulation by CTL and natural killer (NK) cells (Berg, Puente et al. 1998; Wei, Gamero et al. 1998; Jiang, Zhong et al. 2000), suggesting that there are non-transcriptional ERK targets involved in this process. What these target proteins are and how they regulate degranulation downstream of ERK, PI3K, and the TCR are questions yet to be answered.

Paxillin is a multidomain cytoskeletal adaptor protein which in adherent cell types is a vital component of focal adhesions and plays roles both in structure, by linking integrins to actin filaments, and in signaling, by gathering signaling molecules to control the balance of adhesion and motility (Brown and Turner 2004). Paxillin is widely expressed and is regulated in response to a broad spectrum of cell stimuli. In CTL, paxillin is phosphorylated in response to TCR stimulation and interacts with Pyk2 and Lck, both proteins implicated in T cell signaling (Ostergaard, Lou et al. 1998). The protein interaction domains of paxillin include five N-terminal leucine-aspartic acid (LD) motifs, a proline-rich domain, and four C-terminal lin-11 isl-1 mec-3 (LIM) domains, in addition to numerous phosphorylation sites. The study of paxillin regulation by phosphorylation is complicated by the number of sites present in the protein and the combination of both tyrosine and serine/threonine phosphorylation. Mass spectrometric analysis indicated extensive phosphorylation of chicken paxillin and identified 31 phosphoserine residues, 3 phosphothreonine residues, and 5 phosphotyrosine residues that corresponded to mouse paxillin (Webb, Schroeder et al. 2005).

Tyrosine phosphorylation of paxillin is induced by stimuli such as integrin adhesion and growth factors (Brown and Turner 2004) and has been shown to be catalyzed *in vitro* by the Pyk2 family member focal adhesion kinase (FAK), an important component of focal adhesions, and also by Src kinase (Schaller and Parsons 1995; Schaller and Schaefer 2001). Serine phosphorylation is likely performed by several enzymes in series, but ERK has been identified as one of the key catalysts in the process. In EL4 thymoma cells, ERK was shown to phosphorylate paxillin in response to PMA treatment (Ku and Meier 2000). Later, specific sites for ERK were identified: serine 83 and serine 130 (Ishibe, Joly et al. 2004; Cai, Li et al. 2006). The effect of this extensive post-translational modification on paxillin function is unclear, though one possibility is that varying degrees and forms of phosphorylation fine-tune the ability of paxillin to interact with other proteins. This could be accomplished by creating SH2 binding sites, by changing the conformation and thus the availability of binding motifs, or by altering electrostatic and stearic characteristics. Given the potential ramifications of paxillin phospho-regulation, these modifications are likely to be tightly controlled by signaling systems within the cell.

Though the function of paxillin has been extensively studied in adherent cell types such as fibroblasts, it is unclear what its function might be in CTL, which do not form classical focal adhesions. A recent publication demonstrated that paxillin expression is necessary for effective NK cell target lysis (Li, Ge et al. 2008), but it is unknown how paxillin might be important. In this study I have conducted an examination of the regulation of paxillin downstream of the TCR, with particular focus on its phosphorylation, in an effort to place paxillin into the larger framework of CTL signaling and activation. My results demonstrated that though tyrosine and serine phosphorylation are both induced by TCR activation they are regulated by distinct mechanisms. The former is regulated by the Fyn pathway, the latter by PI3K and ERK; thus two branches of TCR signaling converge at a common target protein. Furthermore, the two forms of phosphorylation are independent of one another, as mutation of key serine residues failed

to affect tyrosine phosphorylation and vice versa. The existence of a complex system of paxillin regulation downstream of the TCR suggests that the adaptor protein may be involved in events important to CTL function.

B. Results

TCR signaling results in both tyrosine and serine phosphorylation of paxillin

My experiments made use of two types of CTL clones: Clone 11 ($H\text{-}2^{d/k}$) and AB.1 ($H\text{-}2^{d}$). Both were selected as anti-$H\text{-}2K^{b}$ (allogeneic MHC) clones. In the experiments presented in this chapter, AB.1 clones were used for the target cell experiments while Clone 11 clones were used for the other experiments shown. It is worth noting, however, that most TCR signaling experiments have been performed with both cell types, with similar results.

In order to determine the effect of TCR signaling upon paxillin in CTL, a biochemical approach was taken wherein non-transformed CTL clones were stimulated with immobilized anti-CD3, lysed, and analyzed by SDS-PAGE and immunoblot. Anti-CD3 immobilized on plastic induces the tyrosine phosphorylation of many proteins and this response can be used as an indication of TCR stimulation, as can the phosphorylation of ERK. As **Figure 3-1A** demonstrates, the widespread increase in phosphotyrosine can be detected as early as 2 minutes after TCR stimulation. Phospho-ERK is detectable after 5 minutes, and increases over time. Paxillin was observed to undergo a molecular weight shift over the course of stimulation, with the higher molecular weight forms becoming more prevalent over time.

In addition to antibody stimulation, I examined the effect of allogeneic target cell contact on paxillin. CTL were mixed with L1210 ($H\text{-}2^{d}$) target cells either untransfected or transfected with a chimeric, allogeneic MHC Class I molecule (L1210 $K^{b}D^{d}$) appropriate for activation of the $H\text{-}2K^{b}$-specific AB.1 clones. Conjugates were allowed

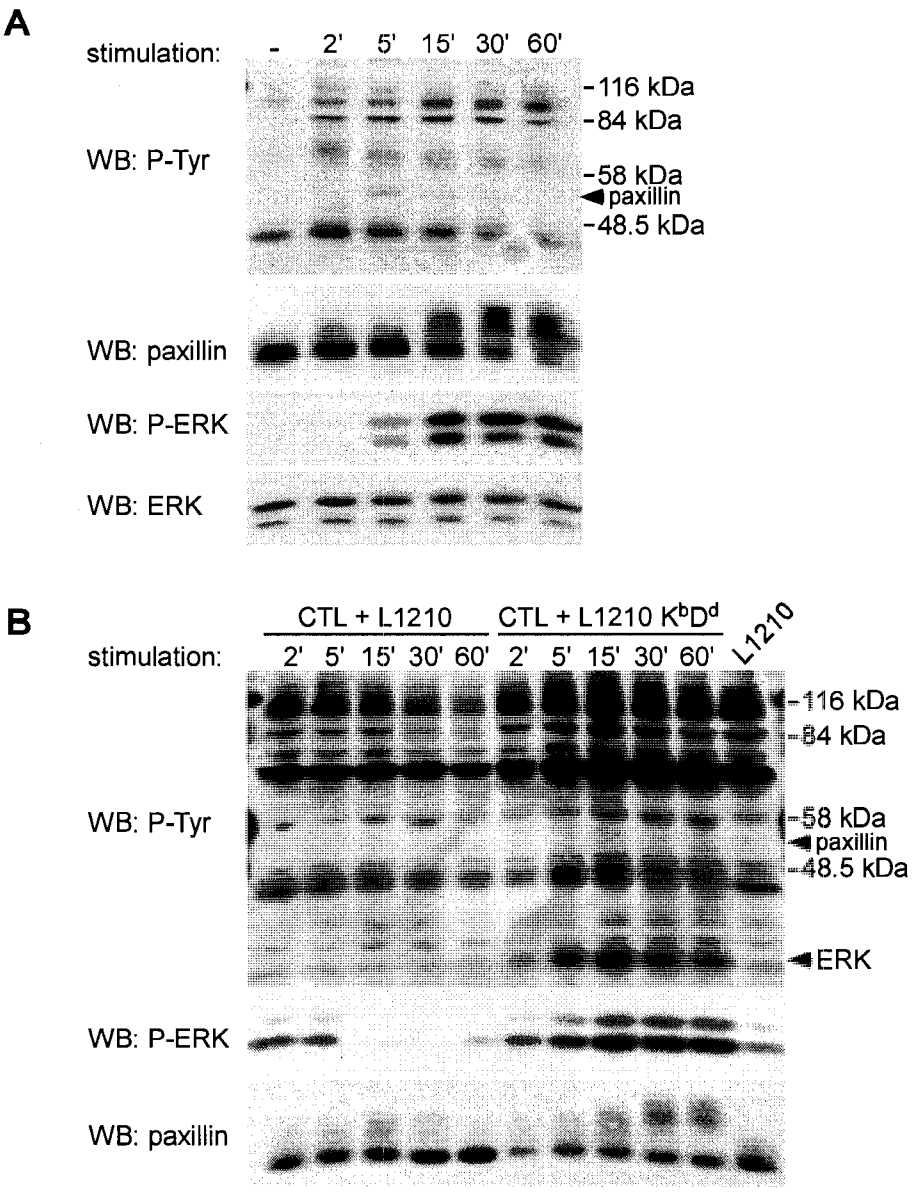


FIGURE 3-1. Paxillin is phosphorylated in response to anti-CD3 and target cell stimulation. (A) Clone 11 CTL clones were stimulated for the time period shown at 37°C with anti-CD3 immobilized onto plastic. The cells were then lysed with Laemmli reducing sample buffer and analyzed by SDS-PAGE and sequential Western blots (WB) with the antibodies listed above. (B) AB.1 CTL clones were mixed with L1210 target cells either untransfected or transfected with an allogeneic Class I MHC molecule ($L1210K^bD^d$) for the times given. The conjugates were lysed and analyzed as above. The paxillin arrows indicate the approximate migration of the protein in the lysate, but do not necessarily indicate the paxillin band itself.

to form and after a given amount of time were lysed and analyzed as before. CTL contact with L1210 cells triggered a modest increase in overall phosphotyrosine (PY), but as expected L1210 K^bD^d cells induced a much more robust increase (**Figure 3-1B**). CTL conjugated with L1210 cells displayed low levels of phospho-ERK at early time points but this disappeared after approximately 5 minutes. In contrast, conjugation with L1210 K^bD^d induced a high level of phospho-ERK in the CTL which was sustained over the 60 minute assay. The molecular weight shift of paxillin followed a similar trend: a minimal and transient gel shift was seen in response to L1210 cells, but L1210 K^bD^d cells engendered more pronounced and sustained gel retardation. The weak signaling induced by the L1210 cells can be explained by the complex nature of the stimulus, which does not involve the TCR but which does involve integrins such as LFA-1 and other receptors. As observed, this engagement results in some signals such as ERK activation, whereas the negative control for the antibody stimulation experiments provides no receptor engagement and no signal whatsoever.

It is known that the gel retardation of paxillin is due to phosphorylation as the shift is eliminated by treatment with alkaline phosphatase (Laura Mireau, unpublished observation). To further characterize the nature of the paxillin molecular weight shift, I analyzed immunoprecipitates of paxillin for phosphotyrosine (PY). Immunoprecipitation (IP) was found to be necessary for the accurate illustration of PY on paxillin due to interference from the high number of tyrosine-phosphorylated proteins present in CTL lysates. Though some basal PY was detected, TCR activation with anti-CD3 triggered an increase in phosphorylation of tyrosine residues on paxillin over time (**Fig 3-2A**) as has been demonstrated before (Ostergaard, Lou et al. 1998). To demonstrate the contribution of phosphoserine (PS) to the observed gel retardation, CTL were treated with 1μM okadaic acid, an inhibitor of serine phosphatases, before anti-CD3 stimulation. Given that ERK is also a target of a serine kinase, it was expected that okadaic acid would enhance ERK activation by shifting the equilibrium in favour of phospho-ERK. Indeed, after treatment with okadaic acid, phospho-ERK was detected even in lysates of

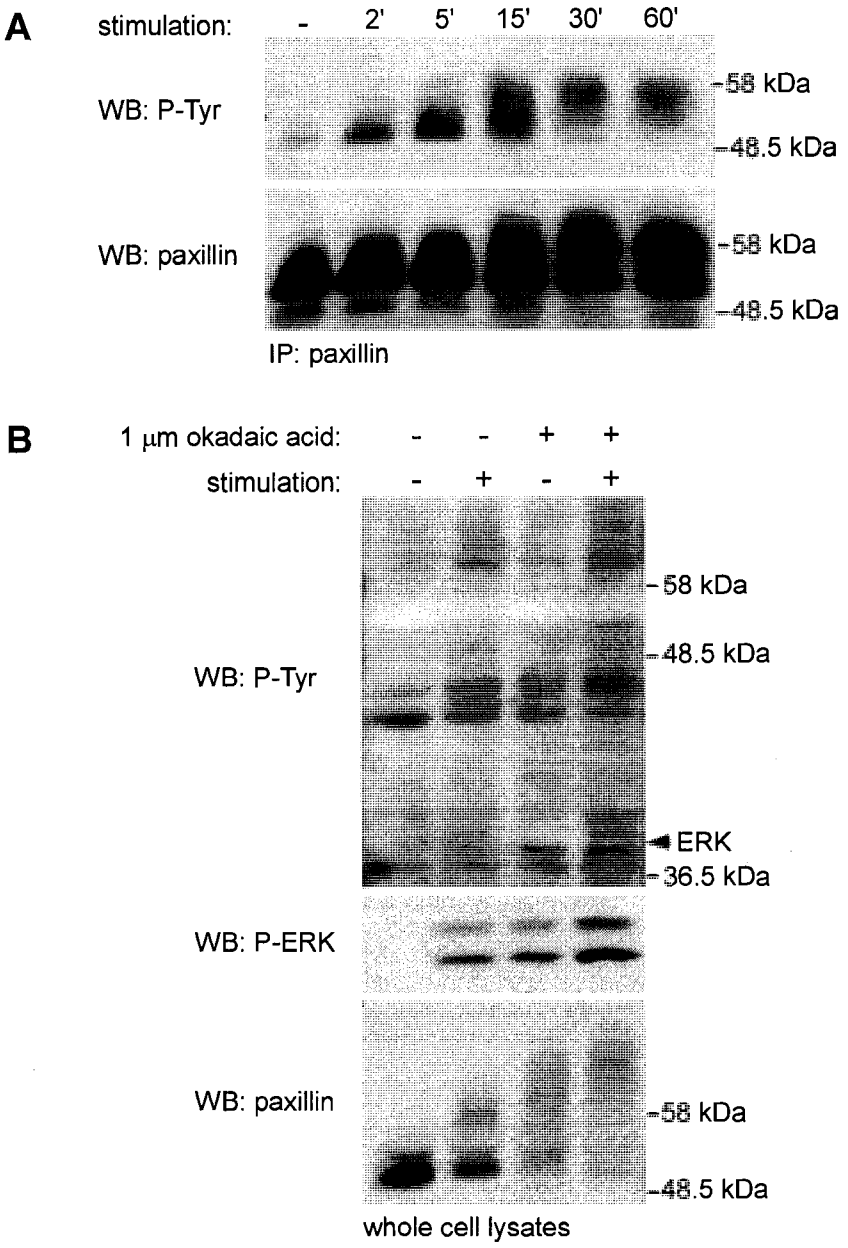


FIGURE 3-2. Paxillin is both serine and tyrosine phosphorylated in response to anti-CD3. (A) Clone 11 CTL clones were stimulated for the time period indicated at 37°C with anti-CD3 immobilized onto plastic. The cells were lysed with 1% NP-40 and post-nuclear lysates were subjected to immunoprecipitation (IP) with anti-paxillin. The IPs were analyzed by SDS-PAGE and sequential Western blots with the antibodies listed above. (B) Clone 11 were treated with 1μM okadaic acid or carrier control for 30 minutes at 37°C. CTL were then either left unstimulated or stimulated at 37°C with immobilized anti-CD3 for 35 minutes. The cells were then lysed and analyzed by SDS-PAGE and Western blot.

unstimulated T cells, and the paxillin molecular weight shift was greatly accentuated both before and after anti-CD3 treatment (**Fig 3-2B**). The outcome of the drug treatment suggests that PS contributes to the molecular weight shift of paxillin. Activation of TCR signaling in CTL resulted in both the serine and tyrosine phosphorylation of paxillin, and the pathways leading to these events were the subject of the next series of experiments.

Paxillin serine phosphorylation is dependent upon PI3K

PI3K is a lipid kinase upstream of many signaling pathways in CTL and is necessary for degranulation of CTL and NK cells (Fuller, Ravichandran et al. 1999; Jiang, Zhong et al. 2000; Robertson, Mireau et al. 2005). Numerous kinases are regulated directly or indirectly by PI3K, including, but not limited to, AKT (also known as protein kinase B), the Tec kinases, isoforms of PKC, and ERK (Deane and Fruman 2004). The enzyme can be inhibited irreversibly with wortmannin, and this treatment was applied to CTL clones prior to anti-CD3 stimulation to determine whether PI3K was required for paxillin phosphorylation. Controls to ensure efficacy of the drug in our system had been performed previously (Robertson, Mireau et al. 2005). At concentrations of 30nM and 100nM, wortmannin did not appreciably decrease overall levels of PY, though some attenuation of ERK activation was observed, in agreement with published reports (Von Willebrand, Jascur et al. 1996). The molecular weight shift of paxillin was also both delayed and reduced by wortmannin treatment (**Fig 3-3A**). Because this result was not sufficient to conclude whether it was serine or tyrosine phosphorylation being affected by wortmannin, I analyzed immunoprecipitates of paxillin. As shown in **Figure 3-3B**, inhibition of PI3K did not affect tyrosine phosphorylation. This result not only illustrated that paxillin serine phosphorylation was regulated by PI3K, but also implied that the two forms of phosphorylation were regulated by separate TCR-derived signaling pathways.

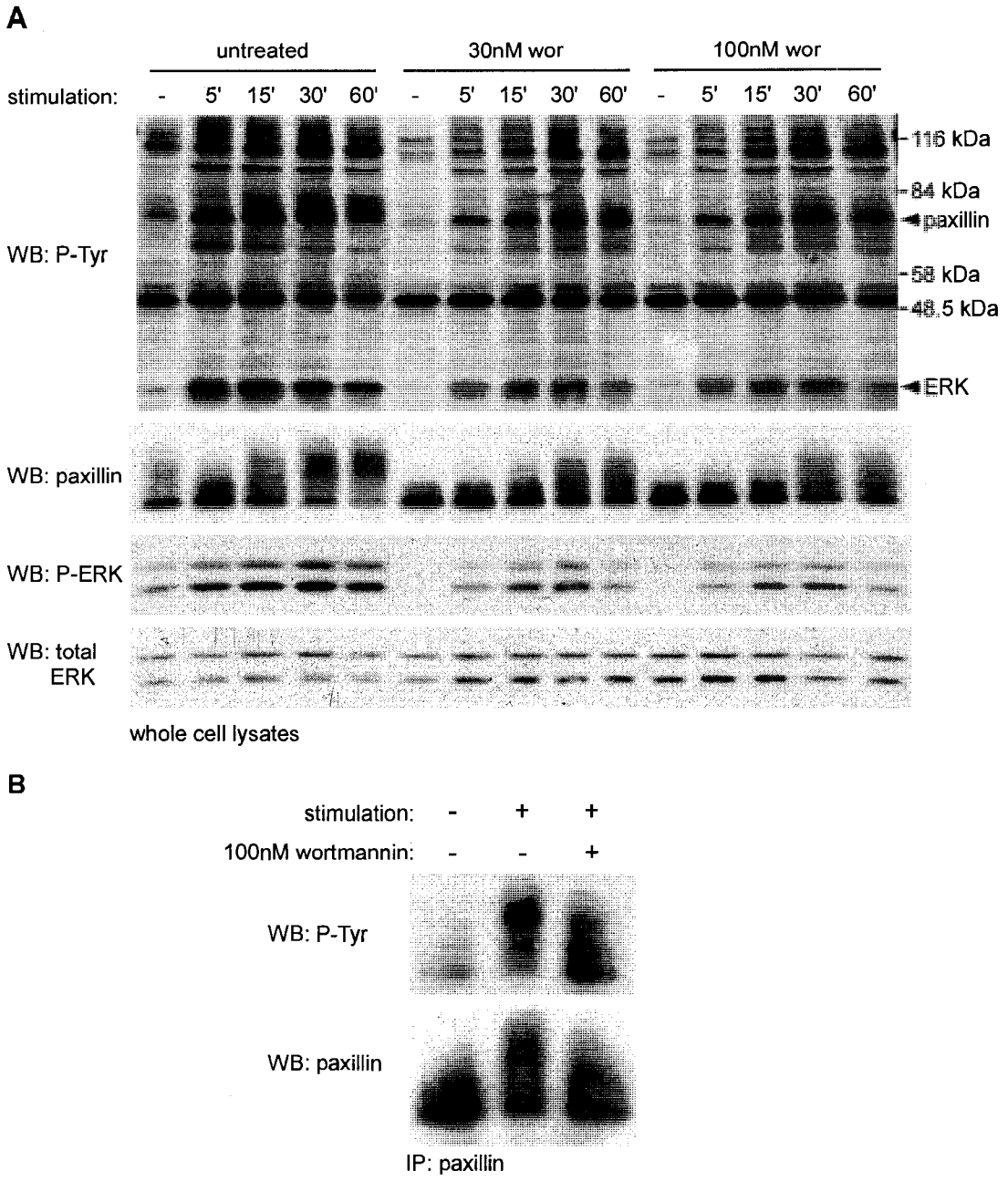


FIGURE 3-3. Paxillin serine, but not tyrosine, phosphorylation is dependent upon PI3K activity. (A) CTL clones were pre-treated with either 30nM or 100nM of the PI3K inhibitor wortmannin or carrier control for 30 minutes at 37°C. Cells were then stimulated for the indicated time on immobilized anti-CD3 and lysed with Laemmli reducing sample buffer. Lysates were analyzed by SDS-PAGE and sequential Western blotting with the antibodies indicated. (B) Clone 11 CTL were pre-treated with 100nM wortmannin or carrier control for 30 minutes and either left unstimulated or stimulated on immobilized anti-CD3 for 25 minutes. The cells were lysed with 1% NP-40 and post-nuclear lysates were subjected to immunoprecipitation with anti-paxillin. The IPs were analyzed by SDS-PAGE and sequential Western blots with the antibodies listed above.

Paxillin serine phosphorylation is dependent on ERK

Given that wortmannin treatment reduced ERK activation and that ERK was reported to phosphorylate paxillin (Ku and Meier 2000), I next tested whether inhibition of ERK activation with U0126 had an effect upon paxillin. CTL were treated with 10μM or 30μM of the MEK inhibitor U0126 prior to TCR stimulation with immobilized anti-CD3. Treatment with U0126 did not reduce overall TCR-triggered PY, which is expected given that ERK activation occurs in the later stages of TCR signaling. Phosphorylation of ERK by MEK was completely eliminated at the higher concentration of U0126, an indication that the drug was having the desired effect. The U0126 inhibitor is specific for the kinases MEK1 and MEK2, which activate ERK1 and ERK2 only (Roux and Blenis 2004). The possibility that U0126 is acting on paxillin through MEK targets other than ERK1 and 2 is therefore low. The gel-retardation shift of paxillin was reduced to an even greater extent than was observed after wortmannin treatment (**Fig 3-4A**). As before, immunoprecipitates of paxillin were analyzed for tyrosine phosphorylation. Inhibition of ERK activation did not reduce paxillin tyrosine phosphorylation, but did reduce the gel mobility shift, which is mainly a result of serine phosphorylation (**Fig 3-4B**). Once again, the data implicated that phosphoserine and phosphotyrosine were induced by separate signaling pathways. My results also suggested the existence of a pathway leading from the TCR to PI3K, then to ERK, and finally to paxillin.

ERK triggers paxillin phosphorylation downstream of PI3K

PI3K activation is a TCR-proximal event; that is, it occurs early in the sequence of TCR-triggered signaling and can be prevented only by the early obstruction of TCR signaling. ERK activation, in contrast, occurs later and can be induced by the phorbol ester PMA (a diacylglycerol analogue), thus bypassing the TCR (Downward, Graves et al. 1990). PMA circumvents the need for membrane-proximal TCR signaling by recruiting the Ras guanyl nucleotide releasing protein (RasGRP) to the membrane and

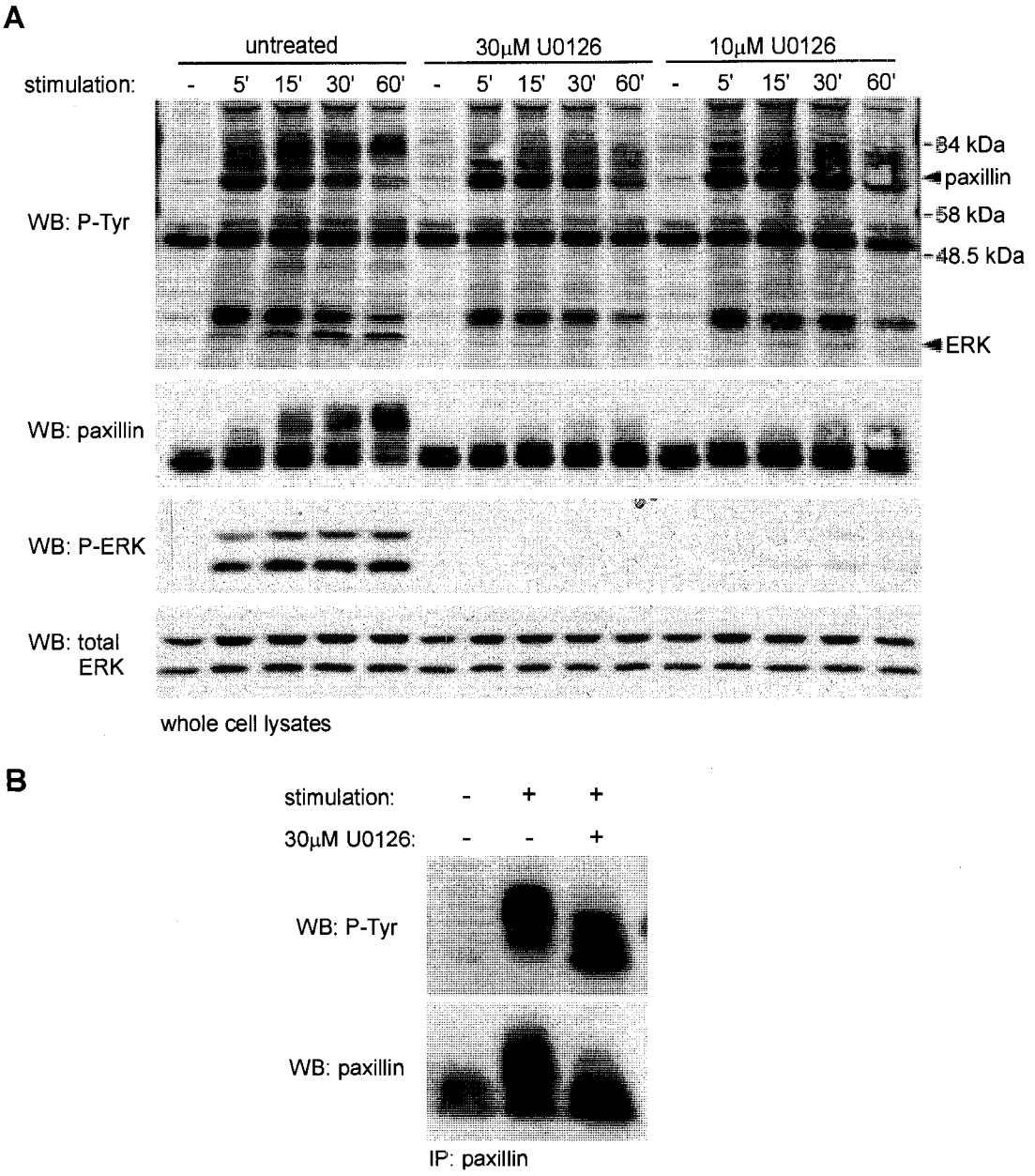


FIGURE 3-4. Paxillin serine, but not tyrosine, phosphorylation is dependent upon ERK activity. (A) CTL clones were pre-treated with either 10μM or 30μM of the MEK inhibitor U0126 or carrier control for 30 minutes at 37°C. Cells were then stimulated for the indicated time on immobilized anti-CD3 and lysed with Laemmli reducing sample buffer. Lysates were analyzed by SDS-PAGE and sequential Western blotting with the antibodies indicated. (B) Clone 11 CTL were pre-treated with 30μM U0126 or carrier control for 30 minutes and either left unstimulated or stimulated on immobilized anti-CD3 for 25 minutes. The cells were lysed with 1% NP-40 and post-nuclear lysates were subjected to immunoprecipitation with anti-paxillin. The IPs were analyzed by SDS-PAGE and sequential Western blots with the antibodies listed above.

directly activating PKC, which then activates RasGRP and thus the Ras-ERK pathway (Lorenzo, Kung et al. 2001; Teixeira, Stang et al. 2003; Zheng, Liu et al. 2005). CTL were treated with wortmannin or U0126 and then stimulated with either anti-CD3 or PMA (**Fig 3-5**). After TCR stimulation of untreated CTL, ERK and paxillin were phosphorylated (lane 5). Both of these events were partially inhibited by wortmannin (lane 6), but completely prevented by U0126 treatment (lane 7). When PMA was used as a stimulus, wortmannin was no longer capable of reducing ERK activation (lane 3), confirming that PMA was bypassing the TCR and PI3K signaling. The paxillin molecular weight shift was also mostly restored in the presence of PMA. In contrast, treatment with the MEK inhibitor U0126 prevented ERK activation and paxillin phosphorylation regardless of the stimulus applied (lanes 4 and 7). These results implied that the dependence of paxillin phosphorylation upon PI3K is confined to the activation of ERK, and that PI3K itself does not modify paxillin.

The drug PP2 arrests TCR signaling in the first stage by inhibiting Src-family kinases such as Lck and Fyn. By shutting off the TCR, and thus PI3K, with PP2 and then stimulating the CTL with PMA, I was able to isolate the contribution of ERK to paxillin phosphorylation. In CTL left untreated or treated with the inactive PP2 analogue PP3, both anti-CD3 and PMA resulted in a global increase in PY, as well ERK activation (**Fig 3-6A, lanes 2-5**). In the presence of PP2, however, the widespread induction of tyrosine phosphorylation in response to anti-CD3 was greatly reduced, as were the activation of ERK and the molecular weight shift of paxillin (lane 6). When PMA was used in lieu of anti-CD3, however, ERK activity was unaffected and paxillin was phosphorylated (lane 7). This result corresponded to that shown in Figure 3-5, and supported the conclusion that it is ERK, and not another protein downstream of PI3K, that is mediating paxillin serine phosphorylation. Immunoprecipitation of paxillin further revealed that PP2 prevented the induction of paxillin tyrosine phosphorylation, a loss not compensated for by PMA stimulation as was the loss of serine phosphorylation (**Fig 3-6B, lanes 6 and 7**). Despite the lack of paxillin tyrosine phosphorylation in the presence of PP2, the

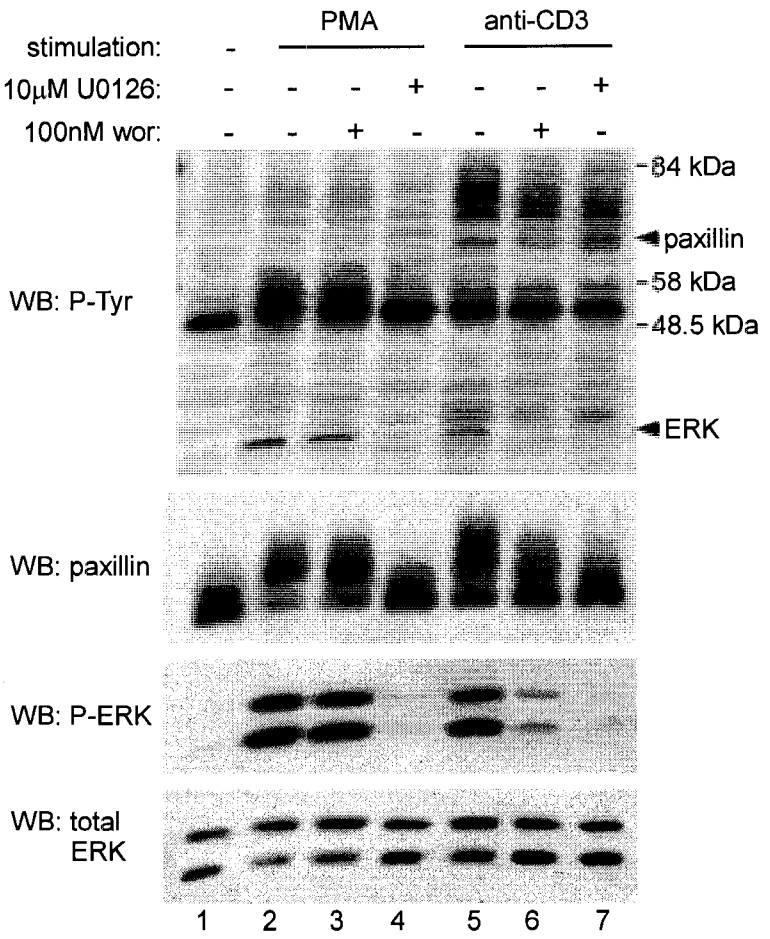


FIGURE 3-5. PMA bypasses TCR and PI3K signaling to directly activate ERK, resulting in paxillin phosphorylation. CTL clones were left untreated (lanes 1, 2, and 5) or pre-treated with either 10μM of the MEK inhibitor U0126 (lanes 4 and 7) or 100nM of the PI3K inhibitor wortmannin (lanes 3 and 6). Stimulation was triggered either by 100ng/mL of the phorbol ester PMA for 10 minutes (lanes 2, 3, and 4) or by immobilized anti-CD3 for 25 minutes (lanes 5, 6, and 7). The cells were lysed with Laemmli reducing sample buffer. Lysates were analyzed by SDS-PAGE and sequential Western blotting with the antibodies indicated. The paxillin arrow indicates the approximate migration of the protein in the lysate, but does not necessarily indicate the paxillin band itself.

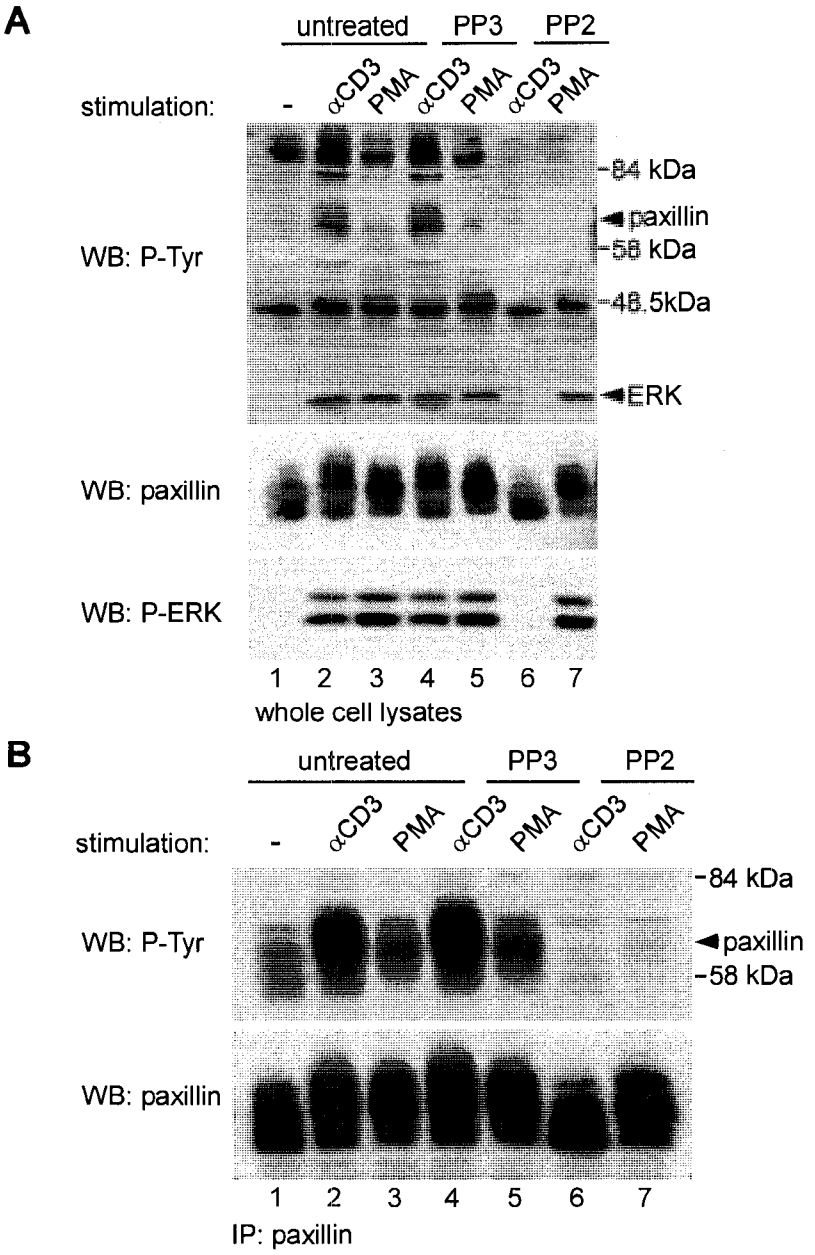


FIGURE 3-6. Serine phosphorylation of paxillin can be triggered by PMA even in the absence of tyrosine phosphorylation. (A) CTL clones were left untreated (lanes 1, 2, and 3) or pre-treated with 10μM of the Src-family kinase inhibitor PP2 (lanes 6 and 7) or its inactive analogue PP3 (lanes 4 and 5). Stimulation was triggered either by immobilized anti-CD3 for 25 minutes (lanes 2, 4, and 6) or by 100ng/mL of the phorbol ester PMA for 10 minutes (lanes 3, 5, and 7). The cells were lysed with 1% NP-40. Lysates were analyzed by SDS-PAGE and sequential Western blotting with the antibodies indicated. (B) The lysates from (A) were subjected to immunoprecipitation with anti-paxillin. The IPs were analyzed by SDS-PAGE and sequential Western blots with the antibodies listed above.

molecular weight shift remained, showing that the mobility shift is due to serine phosphorylation and that it is possible to obtain serine phosphorylation without tyrosine phosphorylation. Inhibition of Src kinases eliminated even the basal level of paxillin PY seen in unstimulated cells. Further, PMA did not appear to induce an increase in paxillin PY above the basal level, even in the absence of PP2 (lanes 3 and 5). It can then be inferred that the signaling proteins responsible for controlling paxillin tyrosine phosphorylation lie upstream of the PMA target PKC but downstream of the Src kinases Lck and Fyn.

Paxillin interaction with Pyk2 does not require ERK

Tyrosine phosphorylation of paxillin has been associated with both Src family kinase and Pyk2 kinase activities (Schaller and Parsons 1995). An association between paxillin and the non-receptor tyrosine kinase Pyk2 has been reported previously (Li and Earp 1997; Ostergaard, Lou et al. 1998), and I aimed to replicate this experiment in the presence of U0126 to determine the effect of ERK inhibition on this association. Binding of paxillin to Pyk2 was demonstrated in both paxillin and Pyk2 co-immunoprecipitates and the interaction was constitutive and independent of TCR signaling (**Fig 3-7A**). Treatment with U0126 did not reduce the amount of association between the two proteins, demonstrating that serine phosphorylation is not required for binding. This is consistent with reports that it is the LD2 and LD4 domains which mediate the interaction between Pyk2 or FAK and paxillin (Brown, Perrotta et al. 1996; Thomas, Cooley et al. 1999). Another publication (Liu, Yu et al. 2002) reported that the paxillin interaction with FAK was enhanced after treatment of mIMCD-3 epithelial cells with hepatocyte growth factor (HGF) and that this interaction was reduced by U0126 treatment. The disparity in our results implies that the characteristics of Pyk2-paxillin association in CTL differ from those of FAK-paxillin binding in adherent cells.

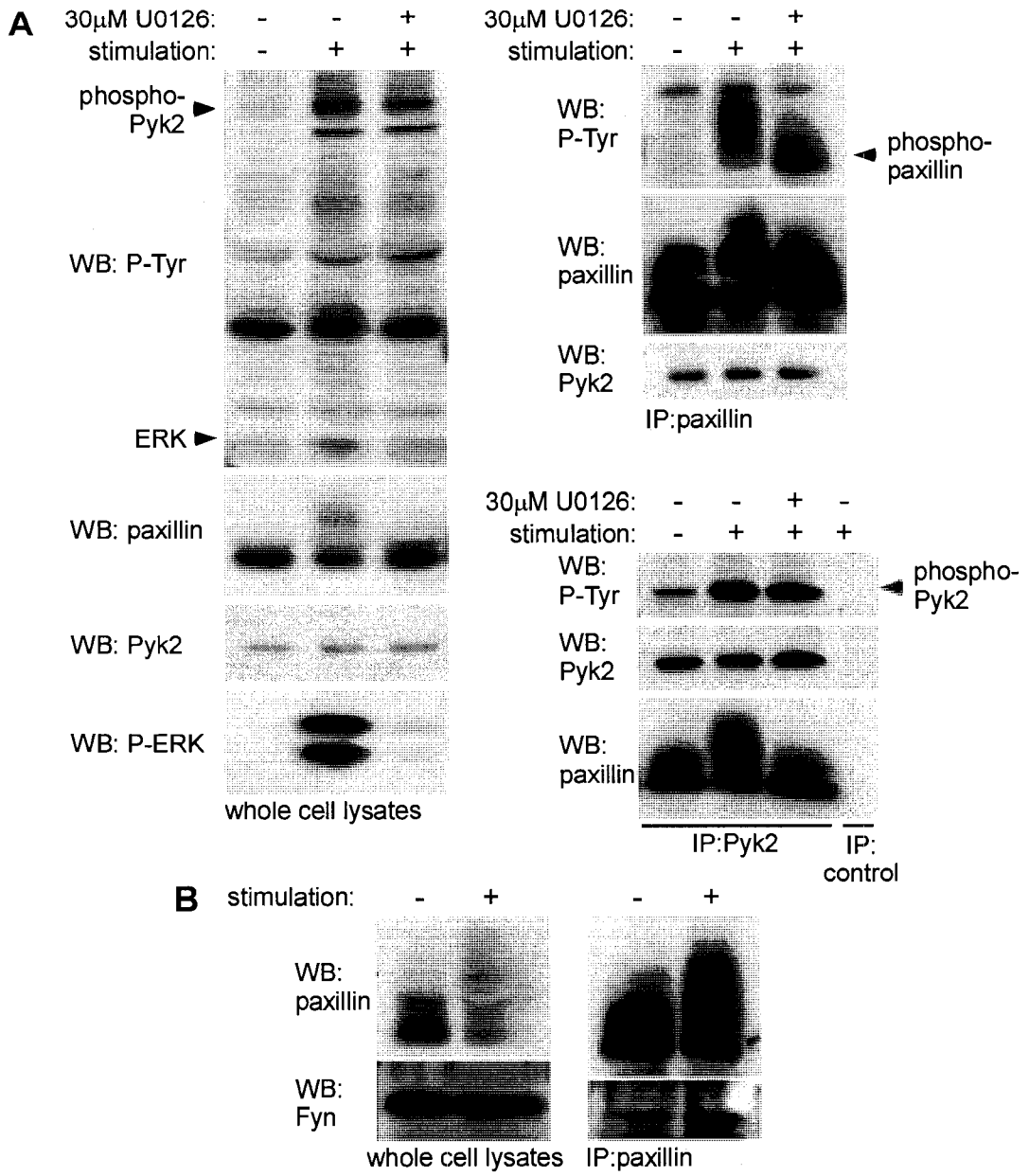


FIGURE 3-7. Paxillin associates with Pyk2 and the Src-family kinase Fyn. (A) CTL clones left untreated or treated with 30μM U0126 were stimulated on immobilized anti-CD3 for 25minutes and lysed with 1% NP-40. The lysates were either used directly (upper left panel) or subjected to IP with anti-paxillin (upper right panel), anti-Pyk2 (lower right panel) or a control IP (lower right panel), as indicated. The resulting Western blots were sequentially probed with the antibodies indicated. (B) Untreated CTL clones were left unstimulated or were activated by immobilized anti-CD3. The lysates (left panel) were subjected to IP with anti-paxillin (right panel). The resulting Western blot was probed for paxillin and Fyn, as shown. Note: though broken into separate panels, both (A) and (B) each consist of the results from one experiment.

Paxillin interacts with the Src family kinase Fyn

The interaction between paxillin and Lck is mediated by binding of the Lck SH2 domain to an as yet unidentified PY residue on paxillin (Ostergaard, Lou et al. 1998). Given the structural similarity between Lck and Fyn (Zamoyska, Basson et al. 2003) I tested for an interaction between paxillin and Fyn. Fyn was detected in paxillin immunoprecipitates regardless of whether the CTL were left unstimulated or stimulated with anti-CD3 (**Fig 3-7B**). This differs from the interaction between paxillin and Lck, which is enhanced after TCR stimulation presumably as a result of increased paxillin tyrosine phosphorylation. It is possible that Fyn does not bind directly to paxillin, but rather through a complex with Pyk2. Our CTL clones do not express high amounts of Fyn, especially when compared to Jurkat T cells (Lysechko 2007), and the antibodies available for Fyn were not particularly good for immunoprecipitation or Western blot. As a result, I was not able to confirm the association by Fyn IP. In future, a glutathione S-transferase (GST) pull-down approach similar to the one taken by our laboratory to detect the Lck-paxillin interaction (Ostergaard, Lou et al. 1998) may be useful in characterizing paxillin-Fyn binding.

Tyrosine phosphorylation of paxillin is regulated by the Src-kinase Fyn

The two principal Src-family kinases of T cells, Lck and Fyn, are subtly different in subcellular localization and function though otherwise very similar in structural organization: each contains a Src homology 3 (SH3) domain capable of interacting with proline-rich domains, an SH2 domain, and a kinase domain (Zamoyska, Basson et al. 2003). The divergent functions of the two enzymes were revealed when mice were engineered to be deficient in each of the genes. In Fyn-null mice, T cell development appeared to proceed normally though a defect was recorded in their proliferation and the calcium flux that follows TCR activation. In mature T cells however the deficiency in calcium flux was lessened and proliferation was normal (Appleby, Gross et al. 1992;

Stein, Lee et al. 1992). A far more extreme phenotype was observed in Lck-deficient animals, whose profound defect in thymic development resulted in a near-complete absence of mature T cells in the periphery (Molina, Kishihara et al. 1992). Clearly, the two Src kinases fulfill different roles in T cells and are not functionally redundant. Further evidence to this effect was obtained upon examination of the subcellular localization of Lck and Fyn in Jurkat T cells and human T lymphoblasts. Lck was detected at the membrane and in pericentrosomal vesicles, while Fyn localized mainly to the microtubule cytoskeleton and MTOC (Ley, Marsh et al. 1994).

Making use of non-transformed CTL clones developed from Fyn -/- mice (1F10 CTL), I tested the effect of Fyn deficiency on paxillin phosphorylation in response to anti-CD3 stimulation. Lysates of Fyn-/- clones were analyzed and confirmed to have no expression of the enzyme (**Fig 3-8A**). Global levels of TCR-triggered tyrosine phosphorylation were somewhat reduced in Fyn -/- CTL compared to wild type (**Fig 3-8B**). Levels of ERK activation were similar between the two cell types however, which suggests that later TCR signaling events were proceeding normally in the absence of Fyn. Serine phosphorylation of paxillin, as measured by its molecular weight shift, was also comparable in both cell types. This result correlates with the similar degree of ERK activation observed and further supports the finding that the TCR-proximal Src-kinase Fyn is not directly involved in regulating serine phosphorylation of paxillin.

When immunoprecipitates were examined it was observed that in Fyn-deficient cells both basal and TCR-activated tyrosine phosphorylation of paxillin was greatly inhibited (**Fig 3-8C**), though not completely absent. The reduction in PY could be explained either by a lack of direct phosphorylation by Fyn or by a decrease in the activity of Pyk2, which associates with paxillin and is regulated by Src-kinases. In Fyn -/- clones, basal and anti-CD3-triggered Pyk2 phosphorylation was somewhat reduced, which was evident in both cell lysates and Pyk2 IPs (**Fig 3-8B and D**). This smaller difference could however be partly due to differences inherent in the two CTL clones used. It does seem that the

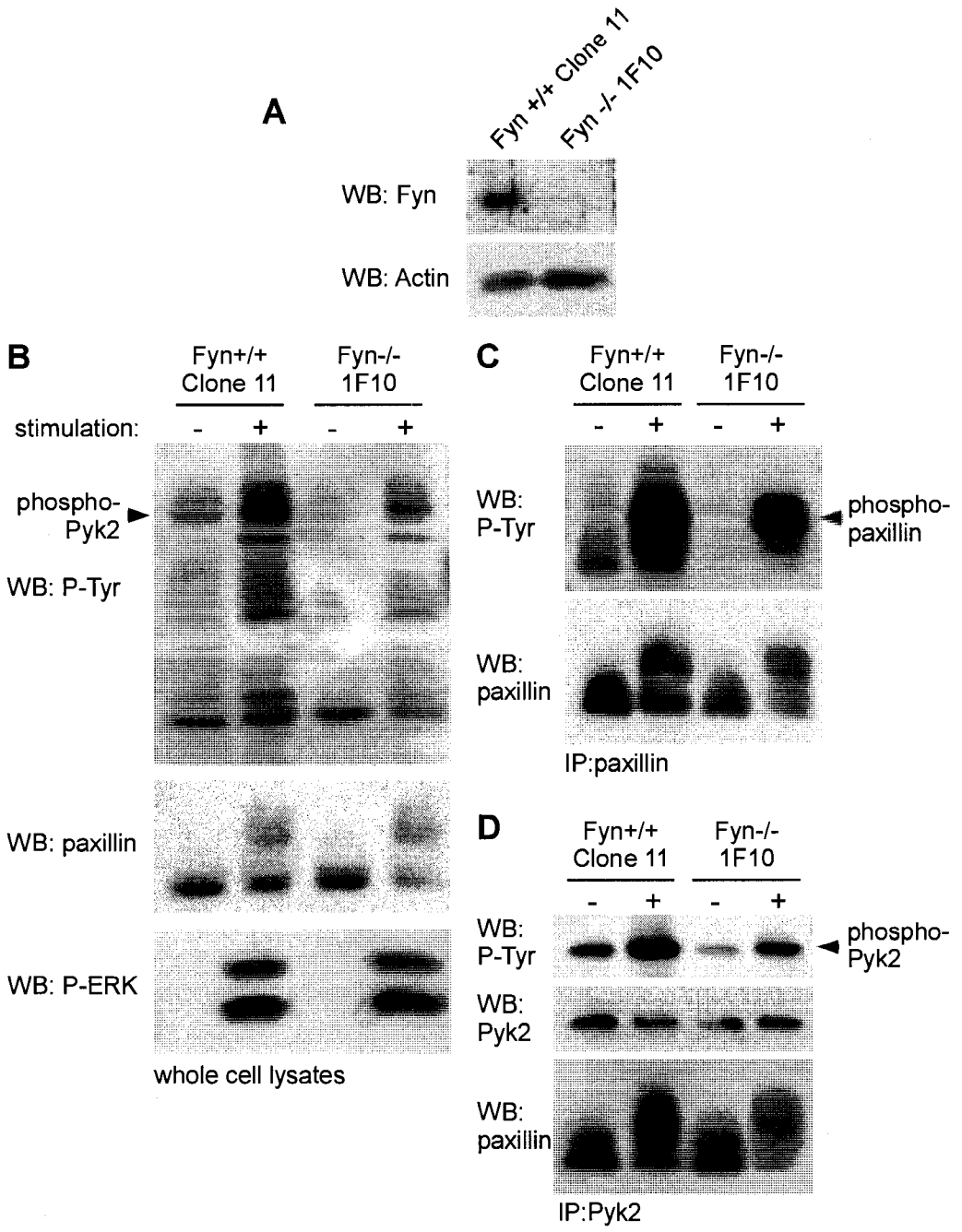


FIGURE 3-8. Paxillin tyrosine phosphorylation is at least partly dependent upon Fyn. (A) Clone 11 CTL and Fyn-deficient 1F10 CTL were lysed with Laemmli sample buffer. Lysates were applied to a gel and analyzed by Western blotting for the expression of Fyn. The membrane was reprobed for actin as a loading control. (B) Clone 11 and 1F10 CTL were left unstimulated or stimulated with immobilized anti-CD3 for 25 minutes and lysates were analyzed by SDS-PAGE and sequential Western blotting with the antibodies indicated. (C) The cells were treated as in (B) but the post-nuclear lysates were subjected to immunoprecipitation with anti-paxillin. (D) The cells were treated as in (B) but the post-nuclear lysates were subjected to immunoprecipitation with anti-Pyk2.

reduction in paxillin PY is greater than can be explained simply by the decrease in TCR signaling, which was minor. Fyn may therefore be the preferred kinase in paxillin phosphorylation, be it direct or indirect. It is likely that Lck is responsible for the residual paxillin tyrosine phosphorylation and that Lck can compensate for some Fyn function. As a means of supporting these results, I attempted to transfect the Fyn -/- 1F10 CTL with an EGFP-tagged Fyn construct. Unfortunately, unlike the other CTL clones used in this study, these cells could only be transfected at very low efficiency and with low survival and the experiment could not be completed. These results must therefore be considered preliminary until such time as a more detailed and direct examination of the relationship between Fyn and paxillin can be conducted.

Ser^{83} and Ser^{130} are key phosphorylation sites that permit further modifications of paxillin

The extensive serine phosphorylation of paxillin is unlikely to be entirely catalyzed by ERK even if ERK is required to instigate the process. A recent publication by Cai and colleagues (Cai, Li et al. 2006) clarified a mechanism for the initiation of paxillin serine phosphorylation that involves an ERK-dependent priming event at serine 130 followed by phosphorylation of serine 126 by glycogen synthase kinase 3 (GSK3). In addition to Ser^{130}, Ser^{83} was reported to be a target for ERK kinase (Ishibe, Joly et al. 2004). In view of these publications, I sought to examine the contribution of these two serine residues to paxillin signaling by engineering mutations in wild type paxillin. Full-length murine paxillin was fused to the C-terminus of enhanced green fluorescent protein (EGFP) and then altered by PCR-based mutagenesis in order to replace serines 83 and 130 with alanine residues. I created two mutants: one bearing a single S83A substitution and one a double S83A/S130A substitution. CTL transfected with the EGFP-paxillin fusion proteins were either left unstimulated or were activated through the TCR with immobilized anti-CD3. Wild type paxillin (EGFP-paxillin) was phosphorylated in response to anti-CD3, though the gel retardation was less than that of endogenous paxillin

due to the larger size of the fusion protein and the reduced separation of the bands (**Fig 3-9, lane 2**). The S83A mutation partly reduced the molecular weight shift (lane 4), but when Ser^{130} was mutated in tandem with Ser^{83}, paxillin phosphorylation was almost completely prevented (lane 6). The molecular weight shift of endogenous paxillin was not affected by any of the three transfected constructs. My result supported the previously published material and demonstrated that in CTL Ser^{83} and Ser^{130} of paxillin constitute key residues whose modification enables a sequence of downstream phosphorylation events.

Paxillin serine and tyrosine phosphorylation are independent of one another

I next tested whether a loss of serine phosphorylation affected the ability of paxillin to be tyrosine phosphorylated. Immunoprecipitates of EGFP were prepared from the lysates of CTL that were transfected with either pEGFP-paxillin or pEGFP-S83A/S130A and stimulated with anti-CD3. The corresponding cell lysates showed that once again the S83A/S130A double mutation prevented TCR-induced paxillin serine phosphorylation. Tyrosine phosphorylation of both the wild type and S83A/S130A paxillin was readily detected after TCR activation, despite the loss of serine phosphorylation in the latter (**Fig 3-10**). Serine phosphorylation of paxillin is therefore not required for tyrosine phosphorylation, further supporting the existence of separate regulatory mechanisms for the two forms of post-translational modification of paxillin.

After demonstrating that PY is not dependent upon serine phosphorylation I next sought to test the reverse question: whether tyrosine modification of paxillin was necessary to obtain PS. Paxillin contains 5 tyrosine residues that are known to be phosphorylated: tyrosines 31, 40, 88, 118, and 181 (Webb, Schroeder et al. 2005); three of them were addressed here. A previous study examining the tyrosine phosphorylation sites of paxillin found that mutation of Tyr^{31} or Tyr^{118} to phenylalanine reduced the detection of PY. Even more dramatic was the effect when both tyrosines were mutated in

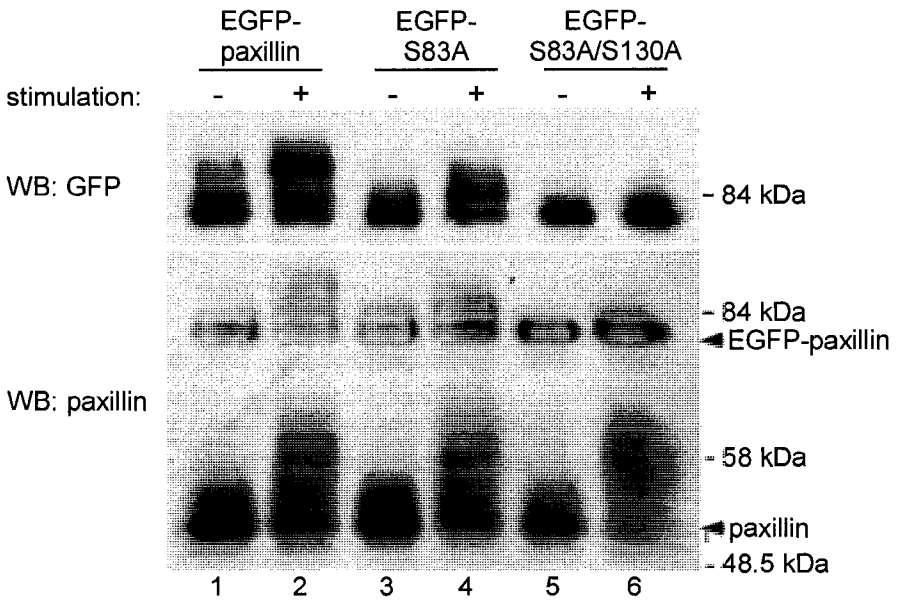


FIGURE 3-9. Phosphorylation of Ser^{83} and Ser^{130} of paxillin is critical for permitting downstream serine phosphorylation events. Clone 11 CTL were transfected with EGFP-tagged paxillin constructs: pEGFP-paxillin (lanes 1 and 2), or the serine mutants pEGFP-S83A (lanes 3 and 4), or pEGFP-S83A/S130A (lanes 5 and 6). The cells were left unstimulated (lanes 1, 3, and 5) or stimulated with immobilized anti-CD3 (lanes 2, 4, and 6) for 25 minutes. The cells were lysed with Laemmli reducing sample buffer. Lysates were analyzed by SDS-PAGE and sequential Western blotting with the antibodies indicated.

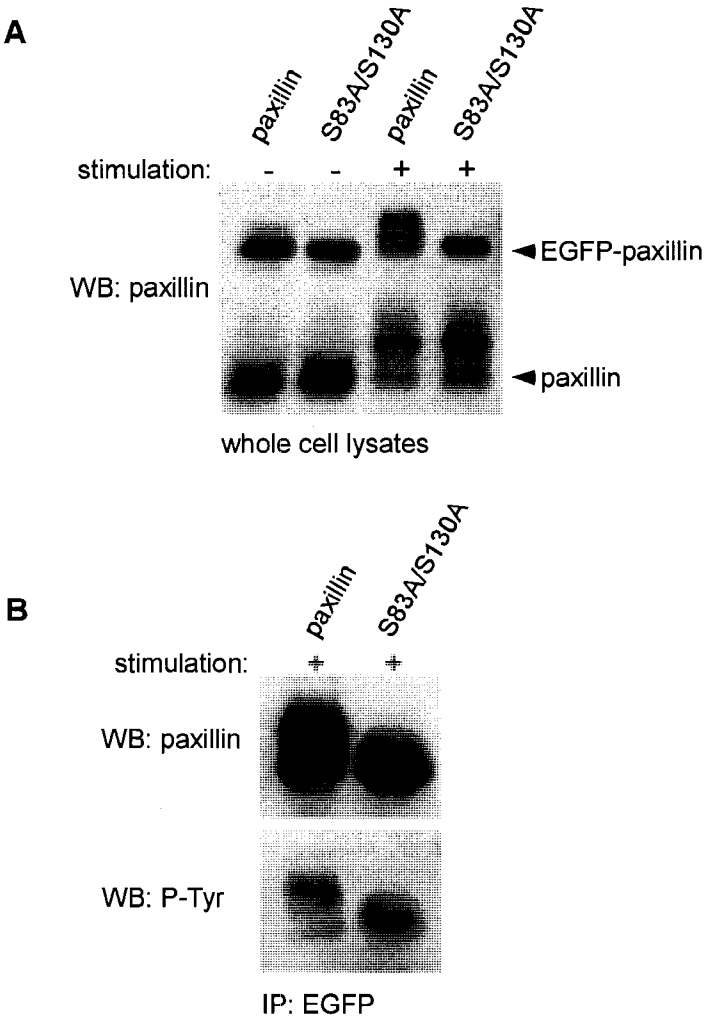


FIGURE 3-10. Tyrosine phosphorylation of paxillin occurs independently of serine phosphorylation. (A) Clone 11 CTL were transfected with EGFP-tagged paxillin constructs: pEGFP-paxillin, or the serine mutant pEGFP-S83A/S130A. The cells were left unstimulated or stimulated with immobilized anti-CD3. The cells were lysed with Laemmli reducing sample buffer. (B) The cells were treated as in (A) but were lysed in 1% NP-40 and the post-nuclear lysates were subjected to immunoprecipitation with anti-GFP. Lysates and IPs were analyzed by SDS-PAGE and sequential Western blotting with the antibodies indicated.

tandem: tyrosine phosphorylation of paxillin was almost completely eliminated (Schaller and Schaefer 2001). I produced a set of tyrosine to phenylalanine mutants within the pre-existing pEGFP-paxillin construct. The following mutants were transfected into CTL: pEGFP-Y31F (Figure 3-11 lanes 2 and 7), pEGFP-Y118F (lanes 3 and 8), pEGFP-Y181F (lanes 4 and 9), and the double mutant pEGFP-Y31F/Y118F (lanes 5 and 10). Unstimulated or anti-CD3-stimulated lysates of CTL transfected with each of the tyrosine mutants were analyzed by SDS-PAGE and immunoblot. In this case a greater amount of global PY was detected in the unstimulated transfected cells than is normally detected in non-transfected cells. Possibly this is a result of the transfection process itself and perhaps some cell activation occurs during the nucleofection technique used. However, activated ERK, a hallmark of TCR activation, and phosphorylation of endogenous paxillin were only detected in the anti-CD3 stimulated lysates. The transfected EGFP constructs were all expressed at similar levels and all tyrosine mutants were observed to undergo a molecular weight shift identical to that of wild type paxillin in response to TCR activation (**Fig 3-11**). Phosphorylation of Tyr^{31}, Tyr^{118}, and Tyr^{181} of paxillin in CTL is therefore dispensable for the serine phosphoryation that occurs as a result of ERK and PI3K.

C. Discussion

This study has established that paxillin is phosphorylated in response to TCR activation in CTL and that the serine and tyrosine phosphorylation of paxillin are separately and independently regulated. Serine phosphorylation is controlled through the PI3K and ERK pathway and appears to occur as a chain of many events that is initiated by the phosphorylation of Ser^{83} and Ser^{130} by ERK (**Fig 3-9**). I have not demonstrated direct phosphorylation of paxillin by ERK in this study, though there is much published data to suggest that this occurs. Paxillin was phosphorylated by ERK *in vitro*, and a paxillin-ERK association was detected by co-immunoprecipitation from murine mIMCD-3 epithelial cells (Ku and Meier 2000; Liu, Yu et al. 2002). Regulation of paxillin

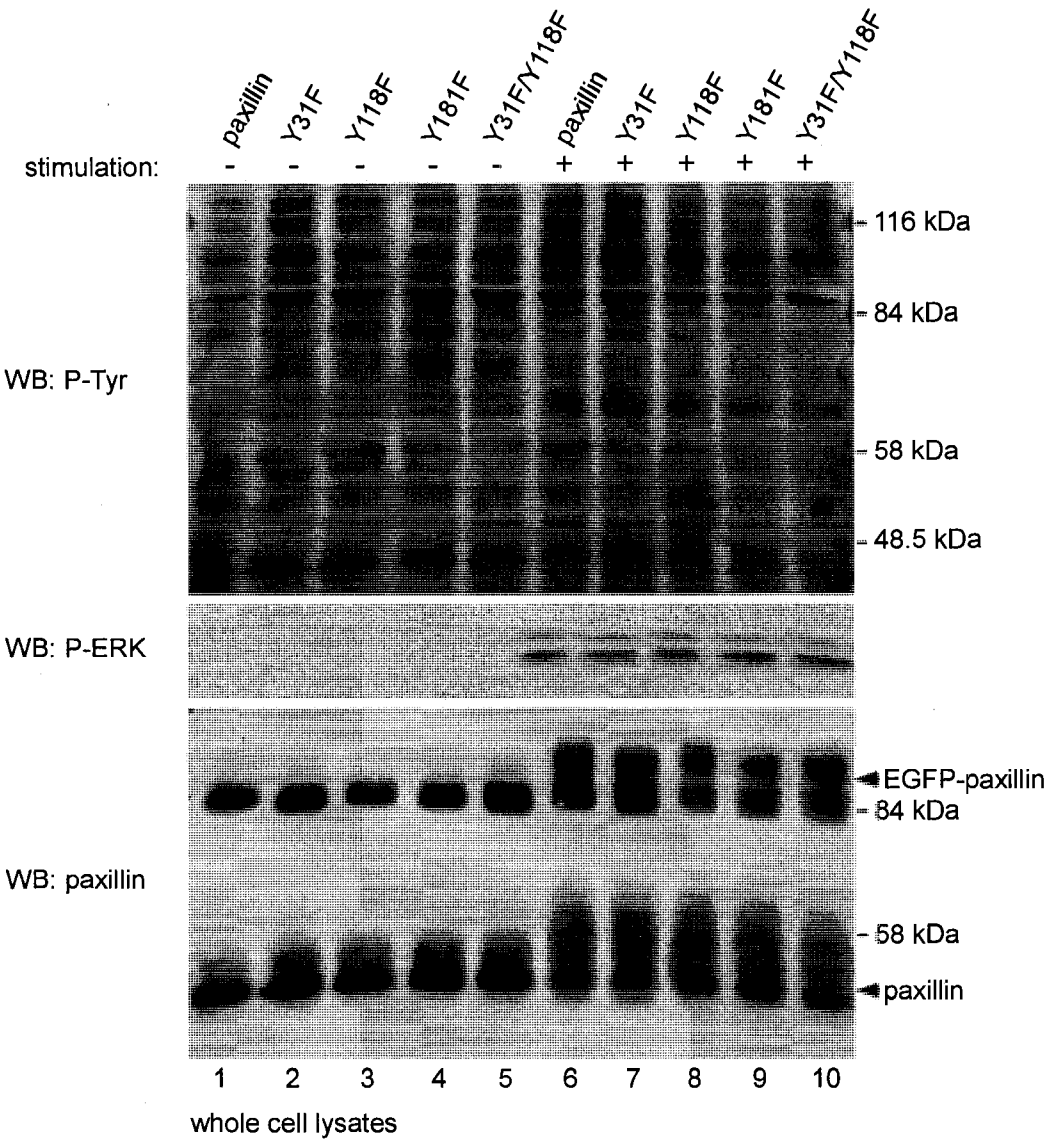


FIGURE 3-11. Serine phosphorylation of paxillin occurs independently of the phosphorylation of tyrosines 31, 118, and 181. Clone 11 CTL were transfected with EGFP-tagged paxillin constructs: pEGFP-paxillin (lane 1 and 6), or the tyrosine mutants pEGFP-Y31F (lanes 2 and 7), pEGFP-Y118F (lanes 3 and 8), pEGFP-Y181F (lanes 4 and 9), or pEGFP-Y31F/Y118F (lanes 5 and 10). The cells were either left unstimulated (lanes 1-5) or stimulated with immobilized anti-CD3 (lanes 6-10) and lysed with Laemmli reducing sample buffer. The lysates were subjected to SDS-PAGE and sequential Western blotting with the antibodies indicated.

downstream of ERK was demonstrated in EL4 thymoma cells in response to PMA and in mIMCD-3 epithelial cells in response to hepatocyte growth factor (HGF) (Ku and Meier 2000; Liu, Yu et al. 2002). Later, specific sites for ERK were identified: serine 83 and serine 130 (Ishibe, Joly et al. 2004; Cai, Li et al. 2006).

It is interesting to note that the Cantley group published a paper in which they reported an association between ERK and paxillin that was dependent upon the phosphorylation of Tyr^{118} of paxillin (Ishibe, Joly et al. 2003). Furthermore, they detected a complex consisting of paxillin and the Ras pathway members Raf, MEK, and ERK after stimulation with HGF. Such a complex was expected to facilitate the activation of ERK at the sites of focal adhesions. In a later paper, the same group demonstrated that mutation of the Tyr^{118} site ^{118}YSFP to ^{118}ASFP or ^{118}ASVA not only prevented ERK binding but also reduced phosphorylation at Ser^{83} (Ishibe, Joly et al. 2004). My own data contrasts with these results, as mutation of Tyr^{118} to phenylalanine (Y118F) had no effect on serine phosphorylation of paxillin (**Fig 3-11**). This outcome suggests that either ERK does not associate with paxillin through phospho-Tyr^{118} in our system or that the association is minor and unnecessary for phosphorylation. Also, it might be that phosphorylation of the tyrosine is dispensable and that my substitution of a phenylalanine as opposed to the alanine used by the Cantley group preserved the structure of a binding site (ERK has no SH2 domain). Another published report found that mutation of Tyr^{118} (Y118F in this case) did not prevent ERK-dependent phosphorylation of Ser^{126} or the molecular weight shift of paxillin in RAW264.7 macrophages treated with LPS (Cai, Li et al. 2006). The discrepancy in the results may be due to the different amino acid residues used as substitutes or to variations in ERK and paxillin regulation between the adherent mIMCD-3 epithelial cells and the hematopoietically-derived cells used in the current study and in the study by Cai and colleagues.

I attempted to show an association between ERK and paxillin by co-immunoprecipitation but was not able to detect ERK in paxillin IPs and the ERK antibodies available were not suitable for an IP of ERK. Given the many functions and interactions engaged in by ERK it is reasonable to speculate that a small portion of the total protein may associate with paxillin and that such an interaction may be relatively transient, making it difficult to detect an association by immunoblotting. The experiments described have nonetheless established the existence of a pathway from the TCR to paxillin via PI3K and ERK, and that the modification of paxillin downstream of ERK is confined to serine phosphorylation and independent of tyrosine phosphorylation. This pathway to paxillin serine phosphorylation is dependent on Src kinases and the TCR-proximal signals they initiate, though, as will be described below, Src kinases likely play a more direct role in paxillin tyrosine phosphorylation.

Tyrosine phosphorylation of paxillin in response to TCR activation was also detected and found to be regulated not by ERK but by the Src kinases, particularly Fyn. Fyn and Lck, though both Src-family kinases, perform different functions within the context of T cell activation. Lck has been shown to be essential for T cell development (Molina et al., 1992) and TCR signaling. Though Fyn also complexes with the TCR, activation-induced proliferation of T cells proceeds normally in its absence (Samelson, Phillips et al. 1990; Appleby, Gross et al. 1992; Stein, Lee et al. 1992). Indeed, I found that ERK activation downstream of the TCR was comparable in wild type and Fyn -/- CTL (**Fig 3-8B**). An association between the Lck SH2 domain and paxillin has been shown previously (Ostergaard, Lou et al. 1998), but because Lck is fundamental to TCR activation it is difficult to isolate its contribution to downstream events from its role in signaling initiation. Src-family kinases are able to phosphorylate paxillin, as are FAK and Pyk2 (Schaller and Parsons 1995; Schaller and Schaefer 2001). Treatment with the Src kinase inhibitor PP2 abolished not only TCR-induced but also basal tyrosine phosphorylation of paxillin **(Fig 3-6B)**, implying that even in resting cells a low level of Src kinase activity exists. In contrast, the inhibition of paxillin PY observed in Fyn -/- CTL inhibition was

incomplete, suggesting that Lck is capable of mediating some tyrosine phosphorylation, be it directly or indirectly. It is possible that paxillin is dependent upon Fyn not for direct phosphorylation, but for the activation of Pyk2; the experiments performed do not allow us to distinguish between the two possibilities. Though Pyk2 can interact with both Lck and Fyn, it is Fyn which appears to be the preferred kinase for the activation of Pyk2 (Berg and Ostergaard 1997; Qian, Lev et al. 1997). The interaction between Pyk2 and paxillin is constitutive and is not affected by TCR signaling (**Fig 3-7A and Berg, 1998**), but Pyk2 requires phosphorylation to become catalytically active and is thus Fyn-dependent. Pyk2 associates with the LD2 and LD4 domains of paxillin (Brown, Perrotta et al. 1996; Thomas, Cooley et al. 1999) in a manner that is independent of serine phosphorylation. It not surprising then that the creation of phosphoserine sites is not required for Pyk2 association or for tyrosine phosphorylation (**Fig 3-7A**). This study has examined the regulation of tyrosine and serine phosphorylation and found that paxillin exists as a convergence point for the ERK pathway and a distinct Fyn/Pyk2 pathway, which implies that each may manipulate paxillin function in a different way and thus exert control over the cellular outcome.

The addition of phosphate to either tyrosine or serine/threonine residues biochemically alters the target protein and influences its function in ways that can be either dramatic or subtle. Paxillin is a particularly complex example of this as it contains no fewer than 31 potential phosphoserine (PS) sites, 3 phosphothreonine (PT) sites, and 5 phosphotyrosine (PY) sites (Webb, Schroeder et al. 2005). The functional outcome of each of these post-translational modifications is unknown, though previous studies have succeeded in singling out PS and PY sites of particular importance. In the case of adaptor proteins such as paxillin, tyrosine phosphorylation is commonly associated with protein-protein interactions as PY can create a binding site for SH2 domains. The SH2 domain of Lck mediates its interaction with paxillin, though the exact tyrosine residue responsible for binding has not yet been pinpointed (Ostergaard, Lou et al. 1998). In CTL, the tyrosine phosphorylation observed downstream of the TCR may allow paxillin to interact with

SH2-containing proteins such as Lck and Fyn. It is interesting that Fyn association with paxillin is not increased upon TCR activation (**Fig 3-7B**). This suggests that the SH3 domain of Fyn may be mediating binding with the proline region of paxillin, as has been published for Src (Weng, Taylor et al. 1993), or that Fyn is binding via Pyk2. Further experiments will be required to compare the conditions of Lck and Fyn binding.

Serine phosphorylation can also influence protein interactions. For instance, phosphorylation by ERK of Ser^{130} creates a consensus priming site for glycogen synthase kinase-3 (GSK-3), which then adds a phosphate group to Ser^{126} and initiates a PS cascade (Cai, Li et al. 2006). Another example is the phosphorylation of Ser^{273} by p21-activated kinase (PAK), which promoted the recruitment of the complex consisting of paxillin kinase linker (PKL), PAK-interacting exchange factor (PIX), and PAK (the PKL/PIX/PAK complex) to regulate focal adhesion turnover (Nayal, Webb et al. 2006). Serine/threonine phosphorylation has also been shown to influence the recruitment of paxillin to focal adhesions. Experiments have suggested that LIM3 is responsible for focal adhesion targeting of paxillin and that sites within the LIM2 and LIM3 domains, when phosphorylated, encourage paxillin to translocate into the focal complexes (Brown, Perrotta et al. 1996; Brown, Perrotta et al. 1998). The mechanism is unknown but is thought to involve structural changes provoked by the phosphorylation events.

I have demonstrated that two sites of paxillin, Ser^{83} and Ser^{130}, are key to allowing all the ERK-dependent downstream phosphorylations that contribute to the molecular weight shift of paxillin. Mutation of Ser^{83} partly inhibited the molecular weight shift of paxillin, which suggests that Ser^{83} alone is important for allowing further modifications. Inhibition of the molecular weight shift was almost complete when Ser^{83} and Ser^{130} were mutated in tandem (**Fig 3-9**). Phospho-specific antibodies have been generated to both PS and PY sites and might have allowed a more detailed examination of the sites modified downstream of the TCR, but immunoblots with these antibodies were not successful. Given the published literature, the extensive TCR-induced serine

phosphorylation of paxillin could potentially affect protein interactions and the localization of paxillin in CTL. The fact that PS and PY are independently regulated insinuates not only that each type of post-translational modification is unnecessary for the association of kinases of the opposite type, but also that each may perform a different type of function. Further experiments will be required to examine the functional consequences of both serine and tyrosine modifications downstream of the TCR in CTL and the mechanisms responsible.

This study has not directly addressed the question of what role paxillin plays in the overall picture of CTL activation and degranulation though certain suggestions can be made based on the proteins that interact with and regulate paxillin. Pyk2 is a family member of FAK, but its expression is confined mainly to the hematopoietic and nervous systems. It is activated in response to integrin engagement and TCR signaling and though its function in T cells is unclear it is implicated in such diverse functions as regulation of the MTOC, TCR signaling, cell adhesion and spreading, and interleukin-2 (IL-2) signaling (Ostergaard and Lysechko 2005). The constitutive association between paxillin and Pyk2 implies that, in addition to being phosphorylated by Pyk2, paxillin may be needed to recruit the kinase to specific sites within the cell or may form protein complexes relevant to its function.

Both ERK and PI3K are needed for optimal degranulation by CTL (Berg, Puente et al. 1998; Fuller, Ravichandran et al. 1999), though PI3K is needed not for the mechanical process of degranulation per se, but for the signaling that triggers it (LM's data (Robertson, Mireau et al. 2005)). It is not yet clear which downstream targets of these enzymes are important for degranulation. It is clear however that ERK must regulate degranulation through a non-transcriptional target as this process does not require new synthesis of proteins. Based on the data presented here, it is impossible to determine whether paxillin is one of the targets of ERK necessary for degranulation; the connection between paxillin and ERK is correlative only and further experiments will be needed to

test whether paxillin itself is necessary for target cell killing. The requirement of paxillin for NK target lysis is a compelling piece of evidence to suggest that paxillin may play a role in CTL cytotoxicity (Li, Ge et al. 2008), especially given the similarities between the cytolytic mechanisms of the two cell types.

The elements critical to degranulation include the formation of a stable conjugate with the target cell, the reorientation of the MTOC and microtubules, the transport of granules along the tubules, and ultimately the fusion of granules with the plasma membrane at the site of target contact (Radoja, Frey et al. 2006). All are complicated processes involving many regulatory proteins. Paxillin has been well-characterized as a scaffolding protein integral to the regulation of integrin-based adhesion and cytoskeletal changes in adherent cell types (Brown and Turner 2004) and it is possible that paxillin can mediate the integrin-dependent process of target cell conjugation. Alternatively, there is a potential role for the adaptor protein in the dynamic reorganization of actin and microtubules associated with CTL activation, which seems plausible in light of its association with Pyk2 and Fyn. The next series of experiments will expand upon the current study to examine paxillin localization in CTL and address more directly its function in target cell killing.

CHAPTER 4: The subcellular localization of paxillin in cytotoxic T cells

A. Introduction

For adherent cell types such as fibroblasts and smooth muscle cells, adhesion to the extracellular matrix is necessary for cellular function and is accomplished through the formation of focal adhesions. At focal adhesions, the binding of a cluster of transmembrane integrins to the extracellular matrix creates an anchor point to which actin is attached through a complex of other proteins. The 68 kDa protein paxillin forms an integral component of focal adhesions and as an adaptor protein physically tethers actin, via binding to vinculin and actopaxin, to integrins adhered to the extracellular substrate (Turner, Glenney et al. 1990; Nikolopoulos and Turner 2000). Aside from its structural role, paxillin also acts as a hub for the gathering of signaling complexes that influence such functions as actin regulation, motility, adhesion, and focal adhesion turnover. Among these signaling molecules are focal adhesion kinase (FAK) and its family member proline-rich tyrosine kinase 2 (Pyk2) (Turner and Miller 1994; Brown, Perrotta et al. 1996; Li and Earp 1997; Ostergaard, Lou et al. 1998). Paxillin contains five N-terminal leucine-aspartic acid (LD) motifs, four C-terminal lin-11 isl-1 mec-3 (LIM) domains, and a proline-rich domain, all of which interact with target proteins (Turner and Miller 1994). Mutation of these domains often leads to a disruption of paxillin function and this method has been instructive in characterizing the nature and function of interactions between paxillin and other proteins.

Intracellular staining of paxillin in adherent cells shows it to be localized to punctate membrane-associated complexes at the ends of actin stress fibres and at the edge of membrane protrusions (Glenney and Zokas 1989; Turner, Glenney et al. 1990; Zaidel-Bar, Ballestrem et al. 2003). Focal adhesion targeting of paxillin has been linked to the LIM3 domain (Brown, Perrotta et al. 1996), though it is unknown how this recruitment is effected. Paxillin is widely expressed in many cell types and is present in the

hematopoietic system, including lymphocytes (Salgia, Li et al. 1995). In human T cells paxillin displays a different cellular distribution than in adherent cells and co-localizes with the microtubule organizing centre (MTOC) (Herreros, Rodriguez-Fernandez et al. 2000). The LIM2 and LIM3 domains were later found to mediate binding to α-tubulin in pull-down experiments using glutathione S-transferase (GST)-paxillin fusion proteins (Brown and Turner 2002). The molecular requirements for paxillin localization to the MTOC were not investigated, and neither was the function of paxillin at this site. Another significant difference between adherent cells and lymphocytes is the lack of classical focal adhesions in the latter (Friedl, Entschladen et al. 1998). Though their attachment is mediated by integrins, T cells adhere transiently to the extracellular surface and remain highly motile, allowing them to migrate quickly within the lymphatic system and to infiltrate tissues from blood vessels.

Integrin adhesion and microtubule dynamics are of particular importance in the function of cytotoxic T lymphocytes (CTL), which are required to attach to and kill self cells that bear antigens resulting from transformation or viral infection. CTL screen for foreign antigens through interactions of their T cell receptor (TCR) with Class I major histocompatibility complexes (MHC) on the surface of potential targets. In the event that a foreign antigen is detected, the CTL adheres tightly to the target cell in a process known as conjugate formation which is dependent upon integrins, particularly leukocyte functional antigen-1 (LFA-1) (Kinashi 2005). TCR engagement results in the recruitment of necessary signaling molecules to the point of contact between the CTL and target, where they form what is termed the supramolecular activation cluster (SMAC), or immune synapse (Monks, Freiberg et al. 1998). Signaling initiates many downstream events, among them the mobilization of the microtublule cytoskeleton. The MTOC, at which the minus ends of microtubules are anchored, polarizes toward the immune synapse (Geiger, Rosen et al. 1982; Kupfer and Dennert 1984) and the microtubules are then used as scaffolds to guide the movement of cytolytic granules containing granzymes and perforin toward the target (Burkhardt, McIlvain et al. 1993; Stinchcombe, Majorovits

et al. 2006). Once at the membrane, the granules fuse with the plasma membrane and their contents are released in the direction of the abnormal cell. The target cell then undergoes apoptosis while the CTL detaches and searches for another target. Natural killer (NK) cells kill targets in much the same manner as CTL and paxillin has recently been shown to be important for NK cytolysis, though it is not known which of the above stages is paxillin-dependent (Li, Ge et al. 2008).

Given the role of paxillin in integrin regulation and its association with the MTOC in T cells, I sought to examine the localization of paxillin in CTL under resting and activated conditions. In the current study, confocal imaging experiments were used to demonstrate that paxillin is associated with the MTOC and with the microtubules themselves, and is also recruited to the CTL-target contact point. Mutagenesis of paxillin further showed that localization to these sites is dependent upon the N-terminal, LD-containing half of the molecule rather than the C-terminal LIM domains and that a segment of paxillin containing the LD2-LD4 domains (aa 136-296) is sufficient for recruitment to the microtubules and immune synapse.

B. Results

Paxillin localizes to the microtubule cytoskeleton and sites of integrin engagement

My first aim was to examine the localization of endogenous paxillin in CTL both resting and engaged by a target cell. Non-transformed effector CTL clones were stained for paxillin and α-tubulin and examined by confocal microscopy. Paxillin was found to co-localize with tubulin at both the MTOC and the microtubules. In addition, paxillin was enriched at the leading edge of CTL migrating on the poly L-lysine used to coat the coverslip (**Fig 4-1A**). To determine the effect of target cell contact on paxillin localization, CTL clones were mixed with target cells bearing a chimeric allogeneic MHC Class I molecule (L1210 K^bD^d) and imaged by confocal microscopy. Association of

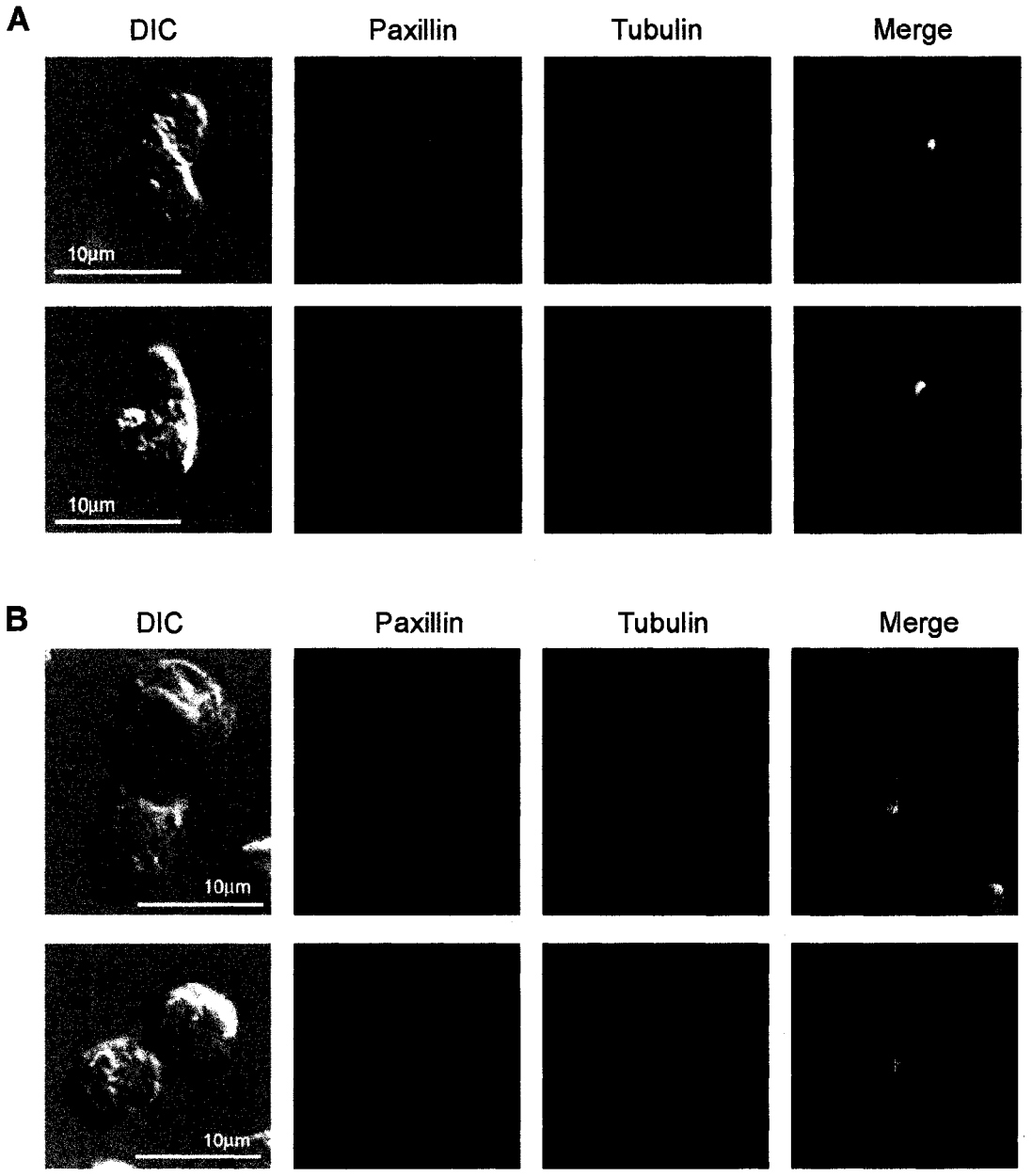


FIGURE 4-1. Endogenous paxillin localizes to the MTOC, microtubules, and target cell interface. CTL clones were adhered onto poly-L-lysine coverslips either (A) alone or (B) conjugated with allogeneic L1210 K^bD^d target cells. The target cells were pre-labeled with CellTracker Blue CMAC. The cells were then fixed, permeabilized, and stained for paxillin and α-tubulin. The red fluochrome used was rhodamine and the green fluorochrome Alexa Fluor 488. Shown is a single optical section, imaged using a Zeiss LSM confocal microscope. Note that the images have been pseudocoloured for the sake of consistency with upcoming images.

paxillin with the MTOC and microtubules was maintained under conditions of stimulation, and paxillin was also heavily enriched at the contact point between the CTL and the target (**Fig 4-1B**). The CTL-target interface, or SMAC, contains not only signaling molecules involved in T cell activation but also integrins and adhesive proteins, similar to the leading edge. Paxillin did not appear to be associated with the membrane outside of the SMAC or leading edge, though it is difficult to ascertain this for certain from imaging experiments. The microscopy results do show some cytoplasmic staining of the CTL, and it is likely that a certain portion of paxillin does exist free in the cytoplasm, in addition to being localized to the target interface and the microtubules.

Localization of paxillin in CTL is mediated by the N-terminal LD domains

In order to address the role of the paxillin LD and LIM protein interaction domains in recruitment to the MTOC and SMAC, the N-terminus of full-length mouse paxillin (aa 1-557) was fused to enhanced green fluorescent protein (EGFP) and this construct was subsequently altered by a series of deletion and truncation mutations (**Fig 4-2**). First, the localization of EGFP-paxillin was assessed to ensure that it corresponded with that of the endogenous protein. CTL were transfected with either empty pEGFP vector or pEGFP-paxillin, mixed with target cells, and stained for both EGFP and α-tubulin. Whereas uncoupled EGFP displayed diffuse cellular staining, the EGFP-paxillin fusion protein localized to the microtubule cytoskeleton and was recruited to the target cell interface **(Fig 4-3A and B)**. I therefore considered the EGFP-paxillin construct to be an appropriate platform for further mutagenesis and localization studies.

In a previous study, the LIM2 and LIM3 domains were identified as being capable of binding to α-tubulin when segments of paxillin were fused to glutathione S-transferase (GST) and used to pull down binding partners from Chinese hamster ovary (CHO) cell lysates (Brown and Turner 2002). Each LIM domain contains cysteine- or histidine-based coordination sites for two zinc atoms, and mutation of any of these residues causes

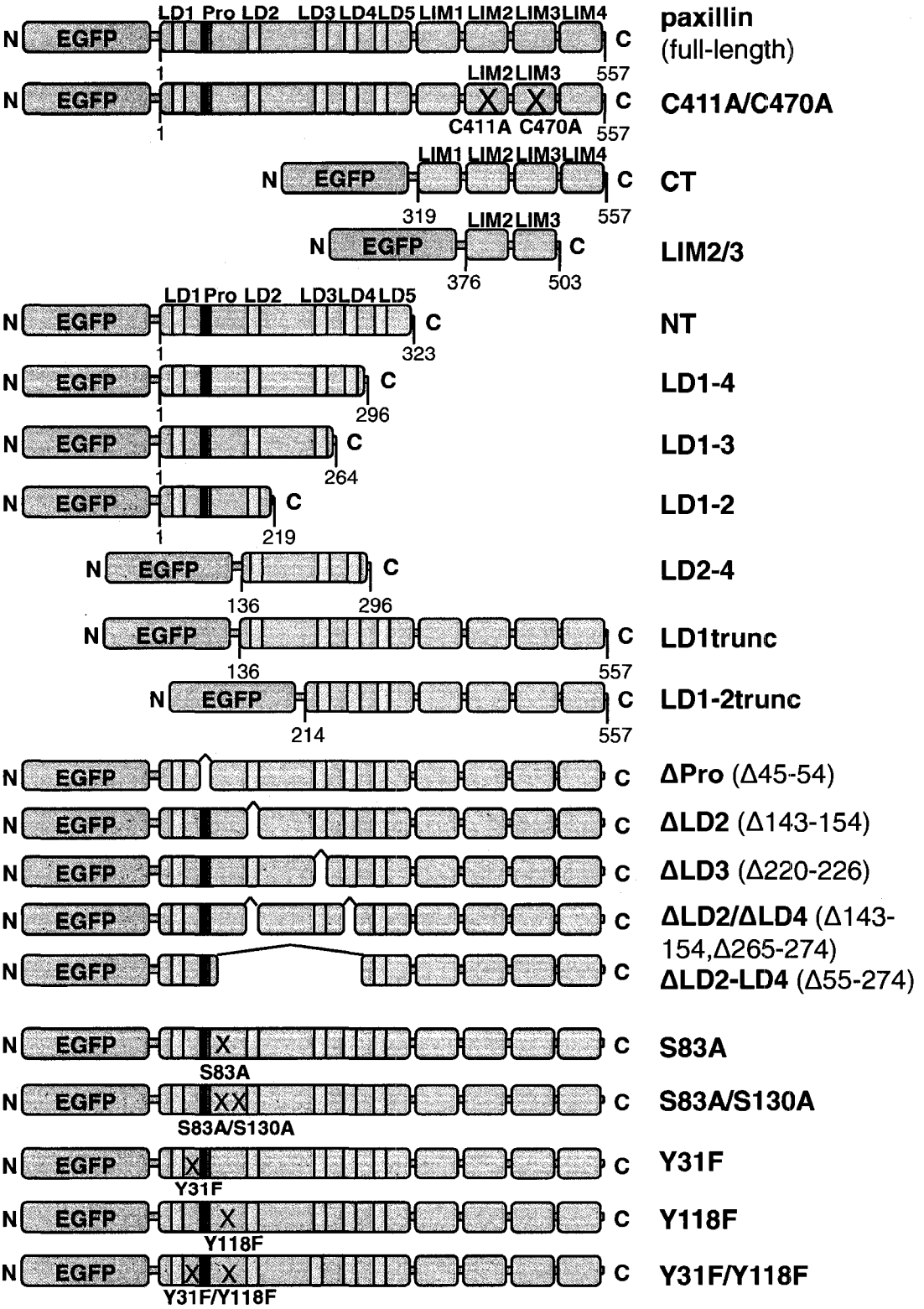


FIGURE 4-2. Mutant constructs of paxillin. The numbers listed correspond to the amino acids included in the construct.

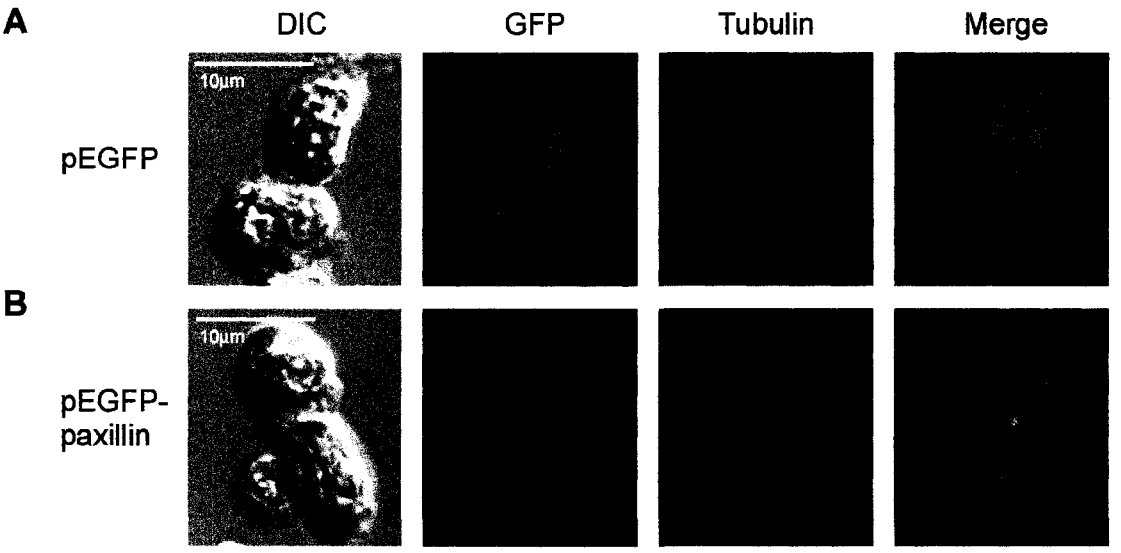


FIGURE 4-3. EGFP-paxillin localizes to the microtubule cytoskeleton and to the target cell interface. CTL were transfected with (A) empty pEGFP vector or (B) pEGFP-paxillin and conjugated to allogeneic L1210 K^bD^d target cells for 2 minutes. The conjugates were adhered to poly-L-lysine coverslips, fixed, permeabilized, and stained with anti-GFP and anti-α-tubulin. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

structural disruption of the domain (Kadrmas and Beckerle 2004). Mutation of the zinc-chelating cysteine residues in LIM2 and LIM3 (C411A/C470A) abolished binding to α-tubulin (Brown and Turner 2002). Given these results, I sought to determine whether similar mutations would disrupt paxillin recruitment to the MTOC and microtubules. Two constructs were created for this purpose: one containing a LIM2-LIM3 fragment fused to EGFP, and the other containing full-length paxillin bearing the C411A and C470A point mutations. Microscopic analysis revealed that the LIM2 and LIM3 domains alone were not sufficient to effect recruitment to the microtubule cytoskeleton, and in fact this protein was collected mainly in the nucleus (**Fig 4-4B**). Reciprocally, the removal of critical cysteine residues had no effect upon paxillin binding to the MTOC and microtubules (**Fig 4-4A**).

I then addressed whether microtubule recruitment was mediated by the N-terminal or C-terminal regions of the paxillin molecule. The N-terminal (NT, aa 1-323) and C-terminal (CT, aa 319-557) halves of paxillin, containing the five LD domains and the four LIM domains, respectively, were each fused to EGFP and transfected into CTL. The paxillin NT localized normally to the microtubule cytoskeleton and was recruited to the target interface (**Fig 4-5A**) whereas the paxillin CT was not, and, like the LIM2-LIM3 segment, was concentrated mainly in the nucleus of the cell (**Fig 4-5B**). I concluded from this result that the domain(s) responsible for paxillin localization was located in the LD domain region, and performed a more detailed analysis of this area.

A segment containing LD2-LD4 is sufficient for paxillin localization

Successive truncations were made of the N-terminal portion of paxillin, resulting in the following segments, which contained no LIM domains: LD1-4 (aa 1-296), LD1-3 (aa 1-264), and LD1-2 (aa 1-219). Truncation of LD5 and LD4 had no effect upon paxillin localization (**Fig 4-6B and C**), though truncation of LD3 partially disrupted MTOC and interface recruitment (**Fig 4-6D**). The segment containing LD1-2, though able to localize to the MTOC and SMAC, also displayed a diffuse cytoplasmic staining pattern not

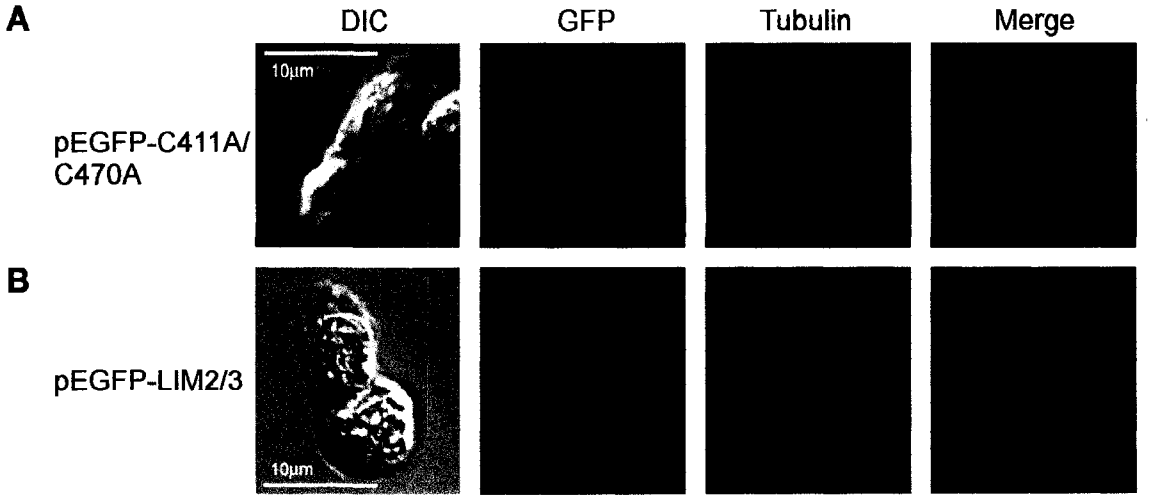


FIGURE 4-4. The LIM2 and LIM3 domains do not mediate localization of paxillin. CTL were transfected with (A) pEGFP-C411A/C470A, a construct of paxillin with mutations in LIM2 and LIM3, or (B) pEGFP-LIM2/3, containing a fragment encompassing LIM2 and LIM3 only. The transfected CTL were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

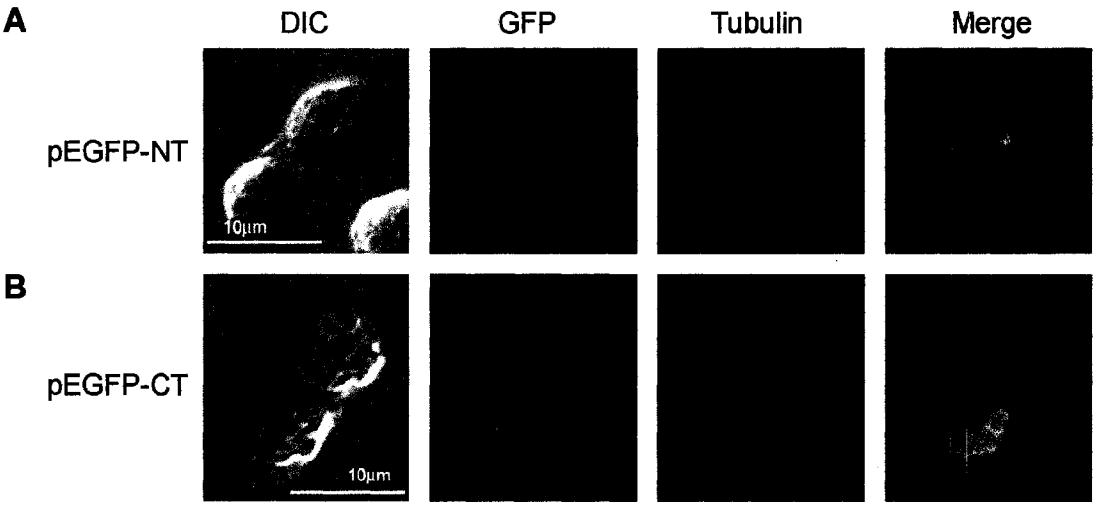


FIGURE 4-5. The N-terminal, LD-containing portion of paxillin is sufficient for localization to the MTOC and target interface. CTL were transfected with (A) pEGFP-NT, a construct expressing only the N-terminal half of paxillin, and (B) pEGFP-CT, a construct expressing the four C-terminal LIM domains of paxillin. The transfected CTL were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

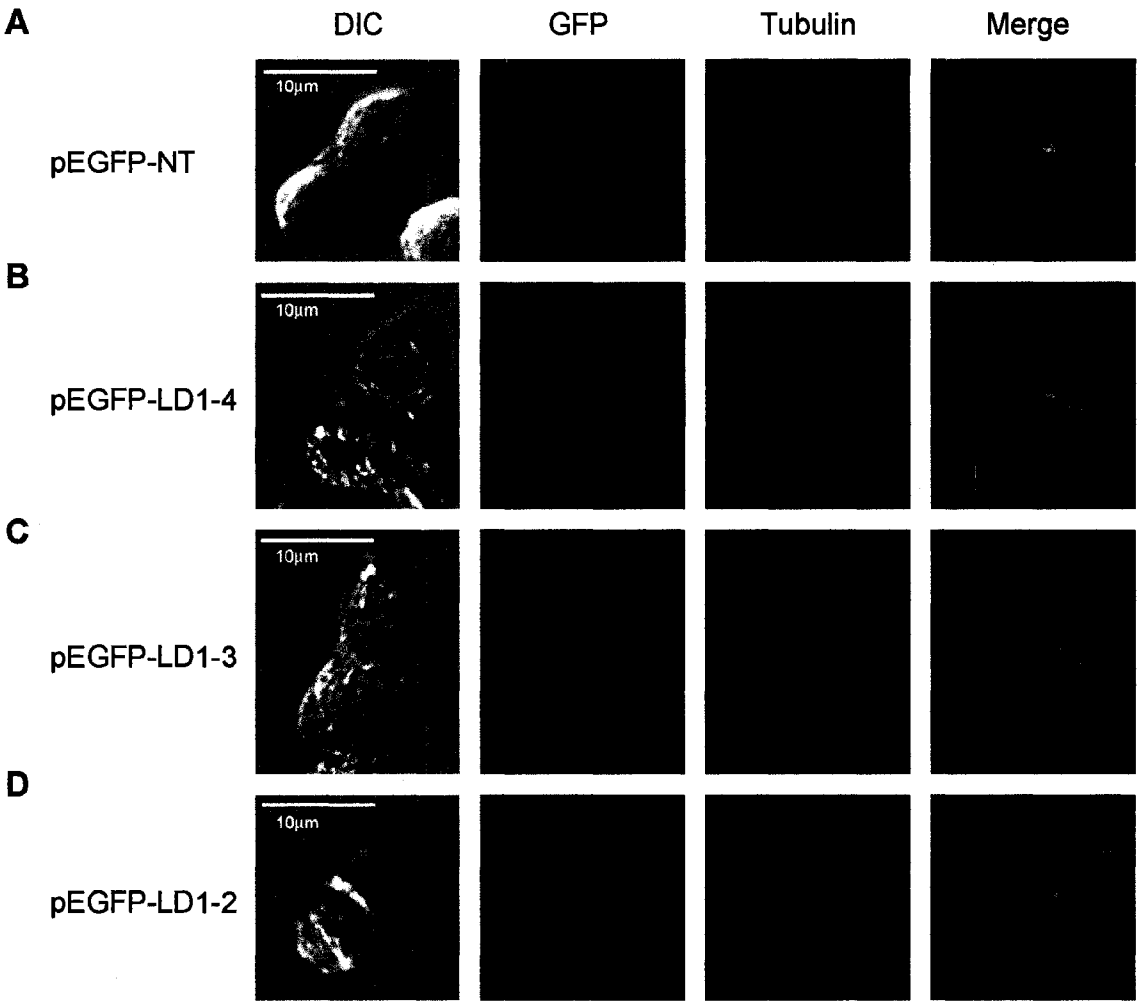


FIGURE 4-6. Truncation of LD3, 4, and 5 results in more diffuse localization of paxillin. CTL were transfected with (A) pEGFP-NT, EGFP coupled the N-terminal half of paxillin, (B) pEGFP-LD1-4, a construct with LD5 truncated, (C) pEGFP-LD1-3, a construct with LD5 and LD4 truncated, and (D) pEGFP-LD1-2, a construct with LDs 3, 4, and 5 truncated. The expressed proteins contain no LIM domains. The transfected CTL were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

observed for the other segments. This result suggested that although a component of LD1-2 was able to bind the MTOC and be recruited to the SMAC, either tertiary structural disruption in such a small segment reduced its ability to interact with its target, or other LD domains were required to enhance and support binding. A similar result was obtained in past studies of FAK and Pyk2 interactions with paxillin, which occur at both the LD2 and LD4 domains. Though each domain alone is capable of binding FAK or Pyk2, maximal binding is achieved with both sites present (Brown, Perrotta et al. 1996; Thomas, Cooley et al. 1999). I postulated that a similar phenomenon may be responsible for the partial disruption of MTOC binding in this case, and a closer dissection of the LD domains was therefore conducted.

Truncation of the N-terminal LD domains from full-length paxillin resulted in EGFP fusion proteins lacking LD1 (LD1trunc, aa 136-557) and LD1-2 (LD1-2trunc, aa 214-557). Neither mutation disrupted paxillin localization in CTL (**Fig 4-7A and B**). This result, together with the only partial disruption in localization observed for the LD1-2 segment in Figure 5-6, suggested that though either LD1 or LD2 are capable of MTOC and interface recruitment, neither is necessary. Based on this result I concluded that more than one LD domain is capable of mediating paxillin localization.

To further examine the contribution of each LD domain, a PCR-based method was employed to delete individual LD domains and the proline-rich region from full-length paxillin. Deletion of the proline domain (ΔPro Δ45-54) had no effect upon localization (**Fig 4-8A**). Likewise, removal of the individual LD2 (ΔLD2 Δaa 143-154) and LD3 (ΔLD3 Δaa 220-226) domains did not disrupt paxillin binding to the MTOC and immune synapse, further supporting the argument that more than one LD domain is involved (**Fig 4-8B and C**). I next deleted a larger segment encompassing the LD2, 3, and 4 regions (ΔLD2-4 Δaa 55-274). This construct, though capable of interacting with the MTOC and target interface, also displayed diffuse staining within the cell and some nuclear staining (**Fig 4-8D**). Colocalization between the MTOC and EGFP-ΔLD2-4 was confirmed by the staining intensity profiles for tubulin and EGFP, which were compared to those of the

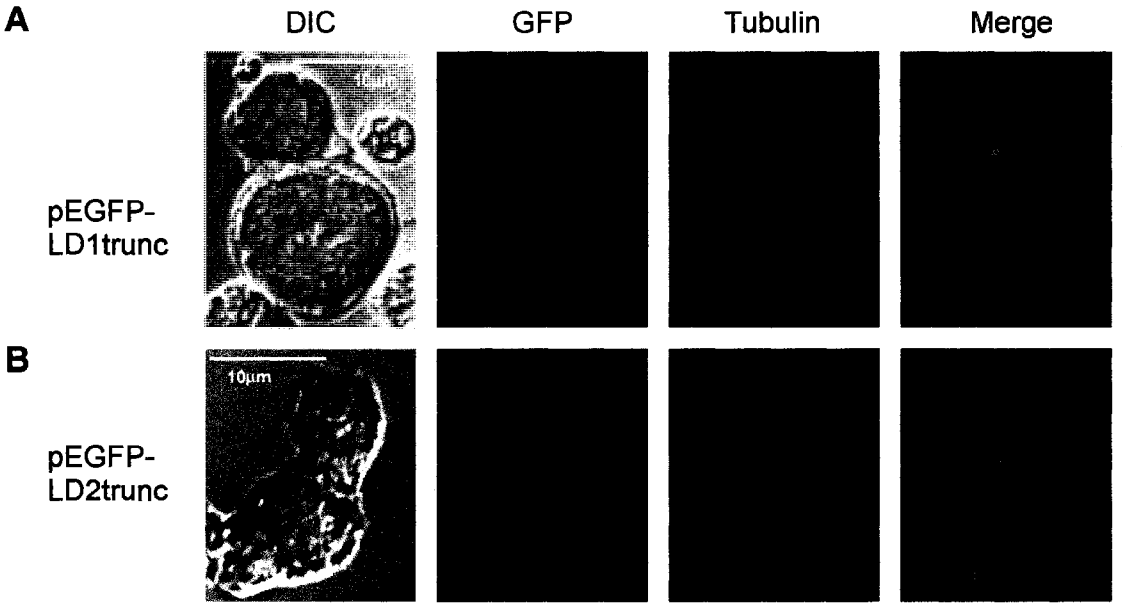
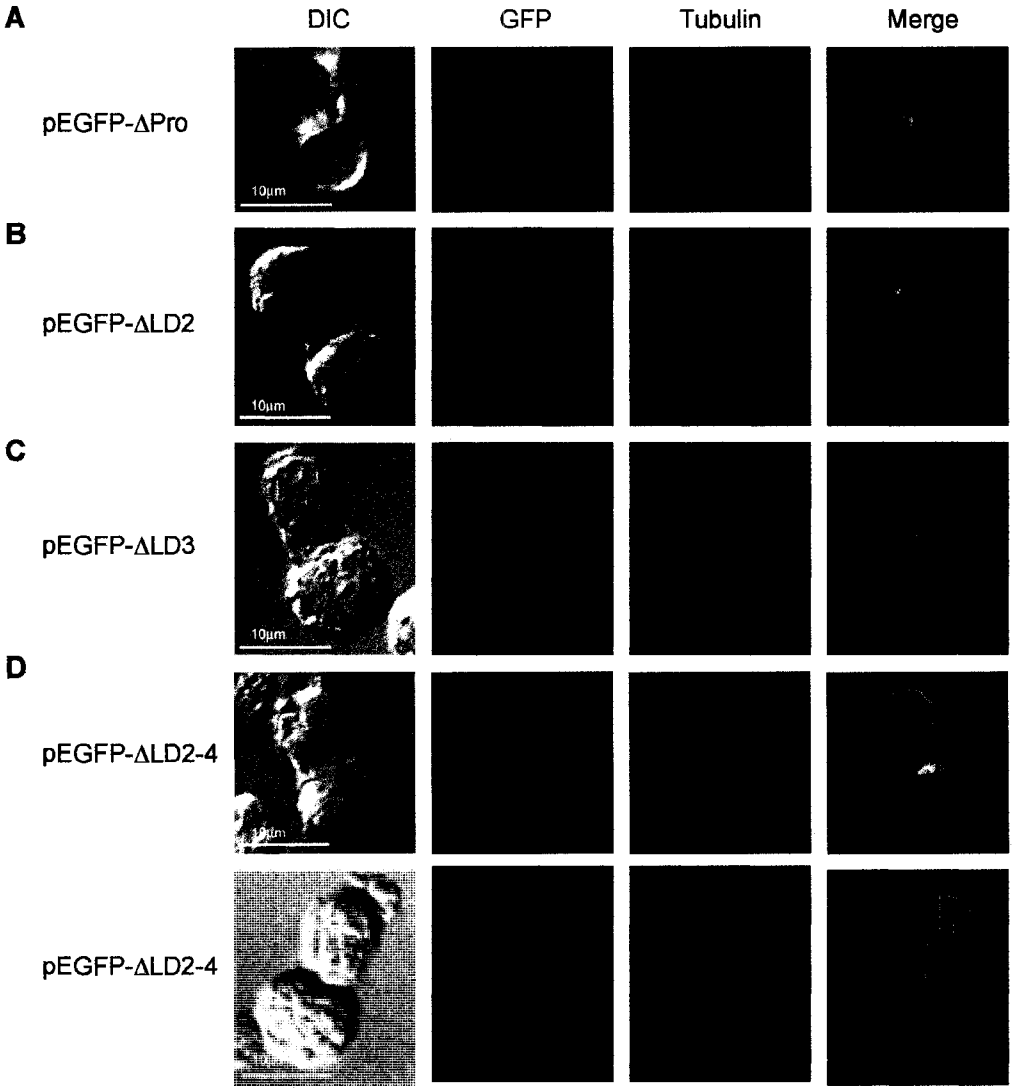


FIGURE 4-7. Truncation of LD1 and LD2 has no effect upon paxillin localization. CTL were transfected with constructs in which (A) LD1 and (B) LD1 and LD2 were truncated from full-length paxillin. The transfected CTL were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.



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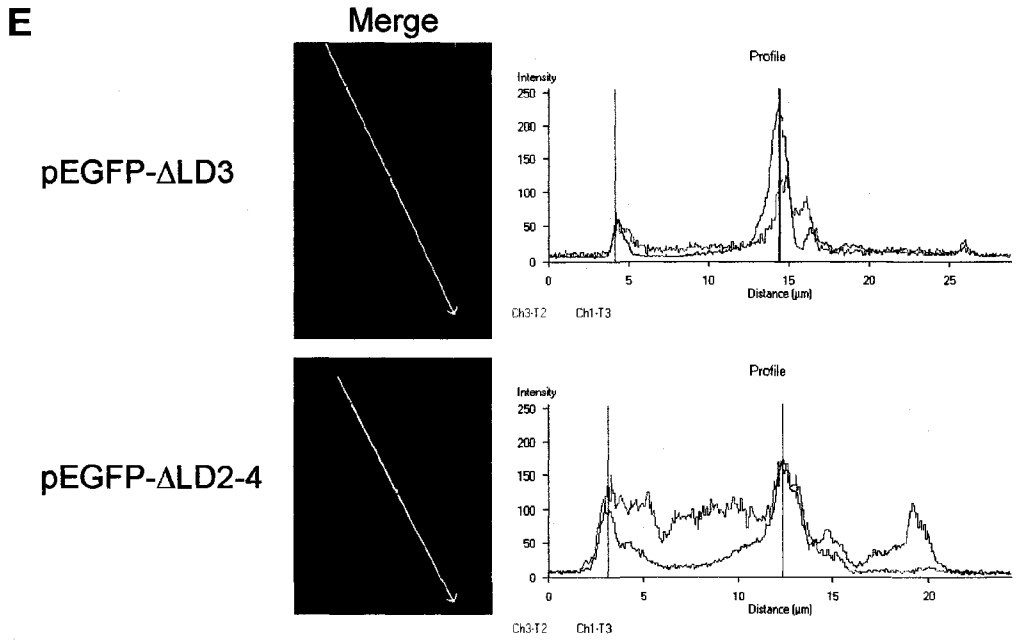

FIGURE 4-8. Deletion of a segment containing LD2, LD3 and LD4 partially disrupts paxillin localization. Full length EGFP-paxillin was modified by deletion mutations of (A) the proline-rich region (ΔPro, Δ45-54), (B) the LD2 domain (ΔLD2, Δ143-154), (C) the LD3 domain (ΔLD3, Δ220-226), and (D) the LD2, LD3, and LD4 domains (ΔLD2-4, Δ55-274). The transfected CTL were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary. Part E demonstrates the colocalization of EGFP with the MTOC for both the ΔLD3 (top) and ΔLD2-4 (bottom) constructs. The profiles on the right illustrate the staining intensity for tubulin (red) and EGFP (green) along the white arrow line shown. The red and blue marker lines orient the profile to the image itself, with the blue line marking the MTOC.

ΔLD3 construct (**Figure 4-8E**). This partial disruption of localization was similar to that observed for the small LD1-2 construct. Though the truncation mutations had established that neither LD1 nor LD5 were required for paxillin localization, it is clear that these domains are capable of interacting with the MTOC and SMAC. This binding seemingly has lesser stability than when LDs 2, 3 or 4 are present, as suggested by the significant fraction of EGFP-ΔLD2-4 distributed throughout the cytoplasm and nucleus.

To determine whether the deleted LD2-4 segment was sufficient for paxillin localization, I created an EGFP fusion protein containing only a small LD2-4 fragment (aa 136-296) and assessed its cellular distribution. The piece containing three LD domains was bound to the MTOC and microtubules and recruited to the target interface (**Fig 4-9**), demonstrating that the region of paxillin encompassing the LD2, LD3, and LD4 motifs is sufficient for normal localization, though not absolutely required.

Since Pyk2 associates with paxillin through the LD2 and LD4 domains and is itself found at the MTOC of CTL (Lysechko 2007), I next asked whether Pyk2 might play a role in recruiting paxillin to the microtubule cytoskeleton. Though it has been demonstrated that FAK does not target paxillin to the focal adhesions of adherent cells (Brown, Perrotta et al. 1996), given the different subcellular distribution of paxillin in CTL this question was addressed experimentally. In place of the larger ΔLD2-4 deletion, in which amino acids 55-274 were deleted, I engineered a paxillin construct with two small deletions at LD2 (Δ143-154) and LD4 (Δ265-274). Immunoprecipitates of EGFP were prepared from transfected cells and blotted for Pyk2 to ensure that this construct did not interact with the kinase. Surprisingly, some residual Pyk2 association was detected even when LD2 and LD4 were removed (**Fig 4-10A**), which is inconsistent with published reports. Also, it appeared that the deletions reduced the level of paxillin serine phosphorylation (as measured by its molecular weight shift) after anti-CD3 stimulation. The EGFP-ΔLD2/ΔLD4 construct was observed to localize normally in CTL (**Fig 4-10B**).

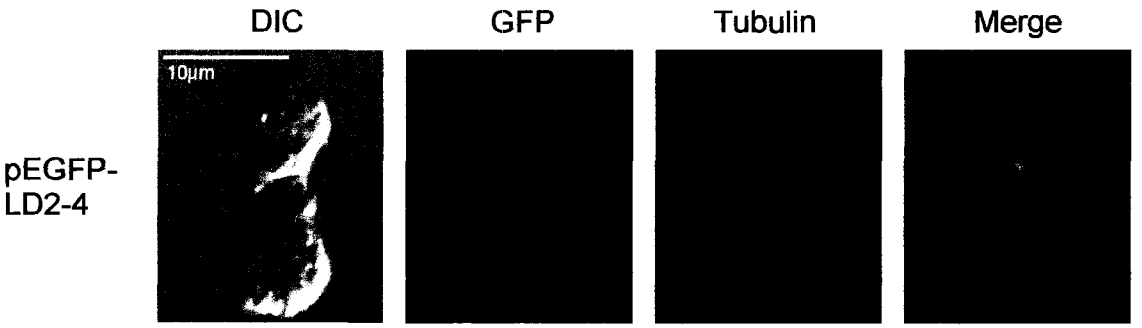

FIGURE 4-9. A fragment of paxillin containing only the LD2, LD3, and LD4 domains is sufficient for localization to both the MTOC and target contact point. CTL were transfected with a construct containing a segment encompassing LDs 2, 3, and 4. The transfected CTL were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

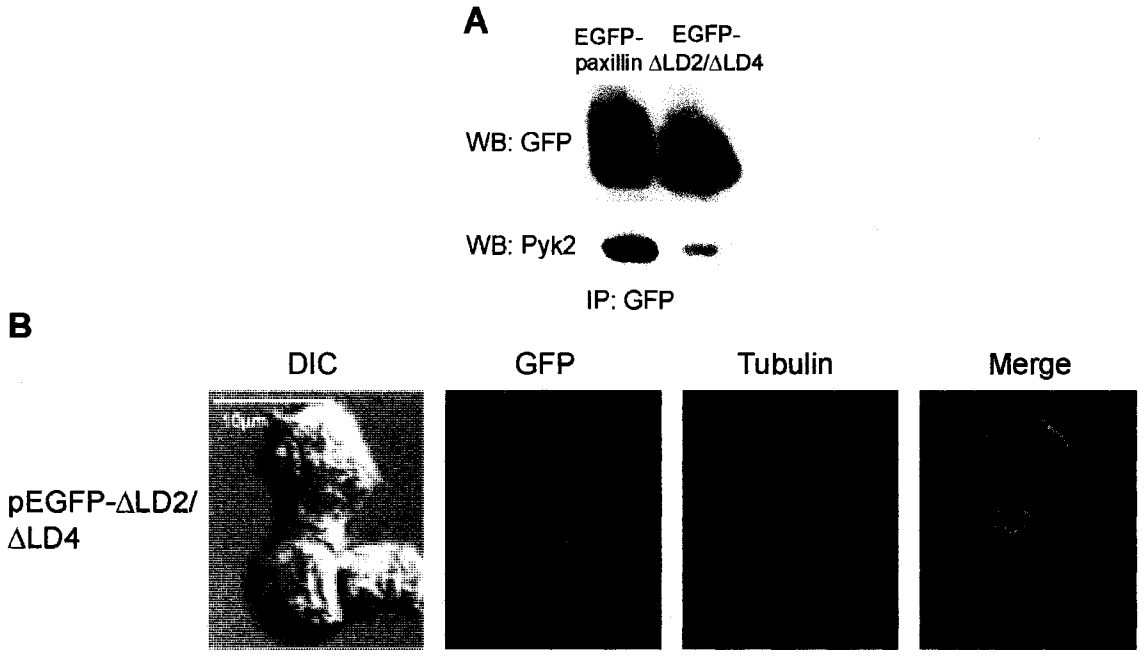

FIGURE 4-10. Deletion of LD2 and LD4 does not completely eliminate Pyk2 binding and has no effect upon paxillin localization. (A) CTL transfected with either EGFP-paxillin or EGFP-ΔLD2/ΔLD4 were stimulated with immobilized anti-CD3 and lysed. Post-nuclear lysates were subjected to immunoprecipitation with anti-GFP and analyzed by SDS-PAGE and Western blot for GFP and Pyk2. (B) CTL transfected with EGFP-ΔLD2/ΔLD4 were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

Paxillin and Fyn are both localized to the MTOC and synapse

I have demonstrated by co-immunoprecipitation that paxillin and Fyn form an association in CTL (**Fig 3-7B**), and I followed up on that result by examining whether paxillin and Fyn localized to the same subcellular structures. The N-terminus of Fyn was fused to an EGFP tag and transfected into CTL. The CTL were conjugated to L1210 K^bD^d target cells and imaged by confocal microscopy. Fyn was co-localized with the microtubule cytoskeleton and was also recruited to the immune synapse (**Fig 4-11A**). Microtubule association of Fyn in T cells has been published before, as has synapse recruitment (Ley, Marsh et al. 1994; Brown, Perrotta et al. 1998; Monks, Freiberg et al. 1998), but I wished to confirm this in our cells. Given that Fyn is known to interact with some microtubule-associated proteins (MAPs) (Lee, Newman et al. 1998; Zamora-Leon, Lee et al. 2001) and with tyrosine-phosphorylated tubulin (Marie-Cardine, Kirchgessner et al. 1995) I examined paxillin localization in CTL clones derived from a Fyn-deficient mouse (1F10 CTL). In these cells, paxillin tyrosine phosphorylation is markedly reduced despite near normal TCR signaling (**Fig 3-8**). Endogenous paxillin was seen to localize as normal to both the synapse and MTOC (**Fig 4-11B**) in 1F10 CTL. Though it cannot be ruled out that Lck may compensate for some Fyn function, this result implies that although Fyn is important for maximal TCR-induced tyrosine phosphorylation of paxillin, it is not required for its localization within the cell.

Serine phosphorylation is not required for paxillin localization

Mass spectrometry analysis of chicken paxillin revealed that in the N-terminal LD region there are five phospho-tyrosine and twenty-seven possible phospho-serine residues that translate to mouse paxillin, though it is unknown which of these are modified in response to TCR signaling (Webb, Schroeder et al. 2005). I have established that engagement of the TCR in CTL triggers paxillin serine and tyrosine phosphorylation (**Fig**

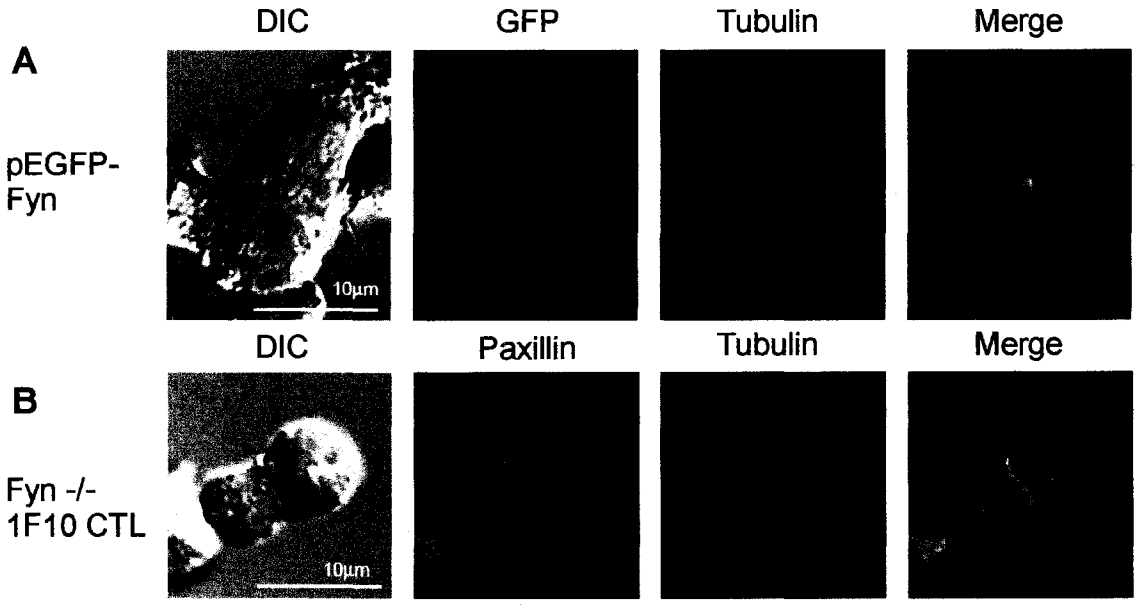

FIGURE 4-11. Fyn localizes to the microtubule cytoskeleton and the immune synapse, but localization of paxillin is not dependent on Fyn. (A) CTL transfected with pEGFP-Fyn or were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. (B) Fyn-deficient 1F10 CTL were stained for paxillin and tubulin. Anti-GFP and anti-paxillin were detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

3-2), and since many of these sites are in the region I have determined important for localization, I investigated whether paxillin phosphorylation influenced its localization.

Published reports have established that paxillin is a target of extracellular-regulated kinase (ERK) in epithelial and macrophage cells and have identified two serine residues as ERK targets: Ser^{83} and Ser^{130} (Liu, Yu et al. 2002; Ishibe, Joly et al. 2004; Cai, Li et al. 2006). In CTL, I have shown that TCR-induced serine, but not tyrosine, phosphorylation of paxillin is dependent upon ERK activity (Robertson, Mireau et al. 2005). Site-directed mutagenesis was used to mutate serines 83 and 130 of full-length pEGFP-paxillin into alanine residues (S83A/S130A). Wild type EGFP-paxillin was serine phosphorylated in response to anti-CD3, as measured by the molecular weight shift in paxillin. Mutation of Ser^{83} partially inhibited the molecular weight shift, while mutation of both Ser^{83} and Ser^{130} almost completely inhibited the shift (**Fig 3-9**). These two residues are therefore likely to be key sites permissive of downstream serine phosphorylation events. The mutants EGFP-S83A and EGFP-S83A/S130A were visualized in CTL to assess their localization. Both EGFP-S83A and EGFP-S83A/S130A were seen to localize normally both to the microtubule cytoskeleton and to the target cell interface (**Fig 4-12**), suggesting that serine phosphorylation of paxillin is not necessary for its subcellular localization.

Only the low-molecular weight form of paxillin is present in the nuclear/cytoskeletal pellet fraction

A comparison of the biochemical characteristics of paxillin at the microtubules and target interface became desirable for the insights into function that such information might provide. Unfortunately, no protocol for the biochemical isolation of the microtubules was available, and instead an attempt was made to examine microtubule cytoskeletal material by analyzing the pellet remaining after centrifugation of cell lysates. The pellet material contains both the nuclei and cytoskeletal components such as polymerized actin and microtubules. As such, it is not a pure representation of

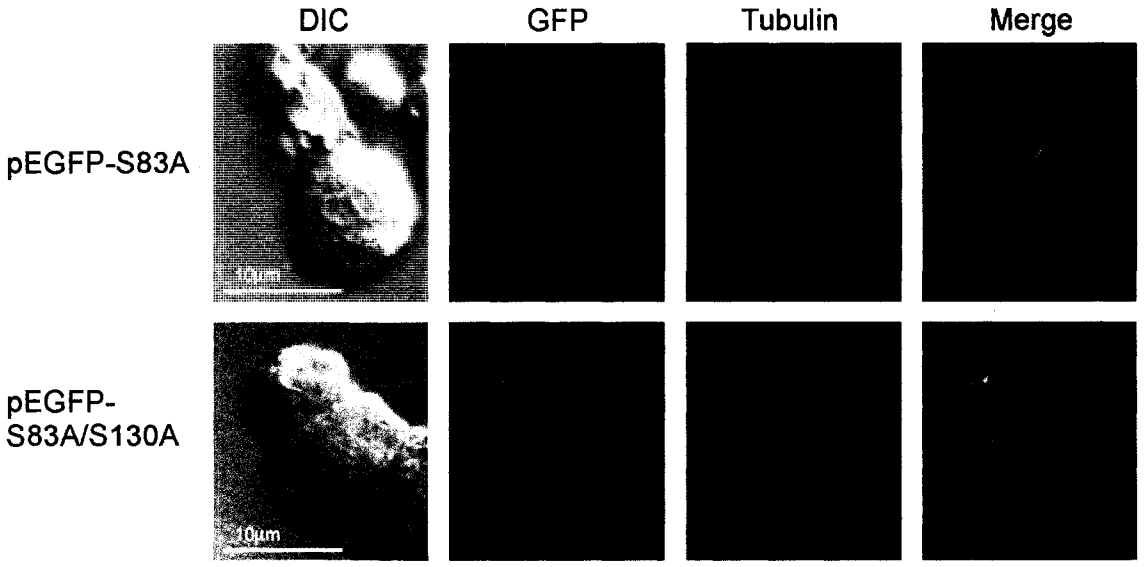

FIGURE 4-12. Mutation of Ser^{83} and Ser^{130} does not affect paxillin localization in CTL. CTL transfected with (A) pEGFP-S83A and (B) pEGFP-S83A/S130 mutants were conjugated to target cells and stained with anti-GFP and anti-α-tubulin as in Figure 4-3. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

microtubule-associated proteins, though analysis of the pellet can be expected to give a more accurate sample than would immunoprecipitation of free tubulin from the cell lysate. Pellet material was analyzed by SDS-PAGE electrophoresis and shown by Western blot to contain tubulin (**Fig 4-13B**). Phosphorylated ERK was present in the pellet fraction, though this could have been due to the presence of nuclear material (**Fig 4-13A**). Paxillin, which has never been observed in the nucleus by microscopy, was detected in the pellet but only in the low-molecular weight form. After anti-CD3 or phorbol myristyl acetate (PMA) stimulation of CTL the paxillin in the lysate was clearly serine phosphorylated, but the paxillin in the pellet material remained at a low molecular weight. This result suggests that only paxillin that is not serine phosphorylated can associate with the microtubule cytoskeleton.

Tyrosine phosphorylation of Y31 and Y118 is not required for paxillin localization

In addition to a large amount of serine phosphorylation TCR engagement triggers tyrosine phosphorylation of paxillin, though the latter is not dependent on ERK as is the former (**Fig 3-4**). A previous study which dissected the tyrosine phosphorylation sites of paxillin in chicken embryo cells showed sites Tyr^{31} and Tyr^{118} to be key residues. Their individual removal resulted in significant reductions in overall phospho-tyrosine, and a double mutation of both residues almost completely abolished tyrosine phosphorylation (Schaller and Schaefer 2001). Given that phosphotyrosine residues act as docking sites for molecules containing Src homology 2 (SH2) domains and thus mediate protein interactions, I investigated whether modification of Tyr^{31} and Tyr^{118} was important for paxillin localization after TCR activation. I produced EGFP-coupled mutants of paxillin containing the tyrosine to phenylalanine Y31F and Y118F mutations, in addition to a double Y31F/Y118F mutant. All three of the variants were shown to be serine phosphorylated to the same degree as wild type paxillin in previous experiments (**Fig 3-11**). Analysis of the localization of the tyrosine mutants demonstrated no discernible change in paxillin recruitment to either the target interface or the microtubules, showing

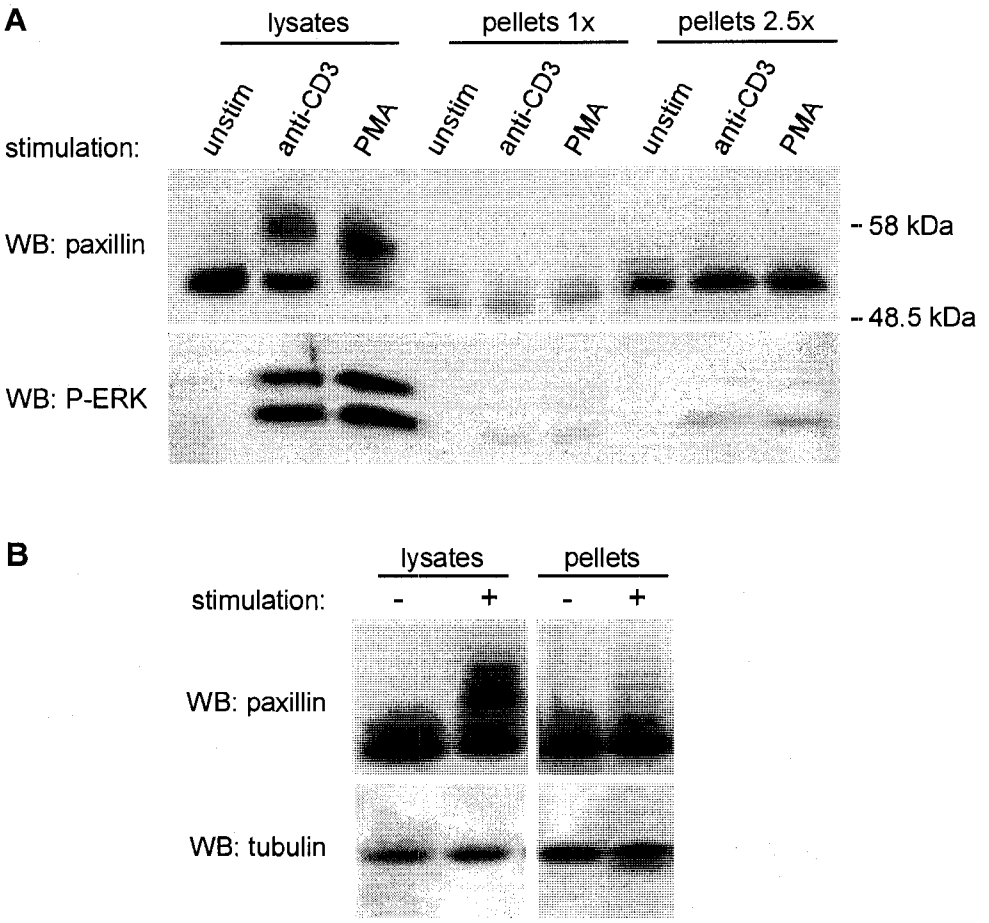

FIGURE 4-13. Only the low-molecular weight form of paxillin is present in the microtubule-containing pellet fraction. (A) CTL were left unstimulated or stimulated either on immobilized anti-CD3 for 25 minutes or with 100ng/mL phorbol myristyl acetate (PMA) for 10 minutes. The cells were then lysed and centrifuged to pellet the nuclei and cytoskeletal material. Either 20μL (1x) or 50μL (2.5x) of the resuspended pellet material was loaded onto an SDS-PAGE gel for analysis. Western blots were detected with anti-paxillin (top) and anti-phospho-ERK (bottom). (B) CTL were stimulated, lysed, and the pellets extracted as described in (A), though in this case 50μL of pellet material was loaded on the gel. Lysates and pellets were analyzed for the presence of paxillin (top) and tubulin (bottom) to show the presence of tubulin in the pellet fraction.

that any protein interactions permitted by these residues are not critical for localization **(Fig 4-14).**

As described in Methods and Materials, the images shown in this chapter were selected based on whether the cells had clearly a distinguishable MTOC and target interface, in order to facilitate an analysis of paxillin localization. In many cases this was best achieved by showing cells in which the MTOC had not fully reoriented toward the target cell membrane, as can be seen in many of the images shown. The partial MTOC reorientation may have one of two explanations: either the conjugate in question is at an early stage of maturity and the MTOC is captured at mid-translocation, or, the transfected EGFP fusion proteins are affecting MTOC movement. Regarding the latter possibility, a comparison of untransfected CTL and CTL transfected with EGFP alone (empty vector) showed that EGFP alone significantly reduced MTOC translocation, from 80% (after a ten minute incubation with targets) to 44%. The reason for this is unknown; however, as a consequence, no conclusions can be drawn from these images with regard to the effect of the EGFP constructs on MTOC reorientation.

C. Discussion

I have demonstrated that paxillin exists in at least two distinct pools within the cell: one associated with the microtubule cytoskeleton, including the MTOC and the tubules themselves, and the other recruited to the contact point between a CTL and an antigen-bearing target cell. The two pools of paxillin are plainly distinguished by their sub-cellular distribution, though it is not clear whether they are also distinguished by other factors such as serine or tyrosine phosphorylation. Phosphorylation is not required for paxillin localization to the MTOC or SMAC, but it is possible that such modifications of paxillin may be distinct at each location. Phospho-specific paxillin antibodies have proven ineffective both for Western blotting and for immunofluorescence, and therefore I

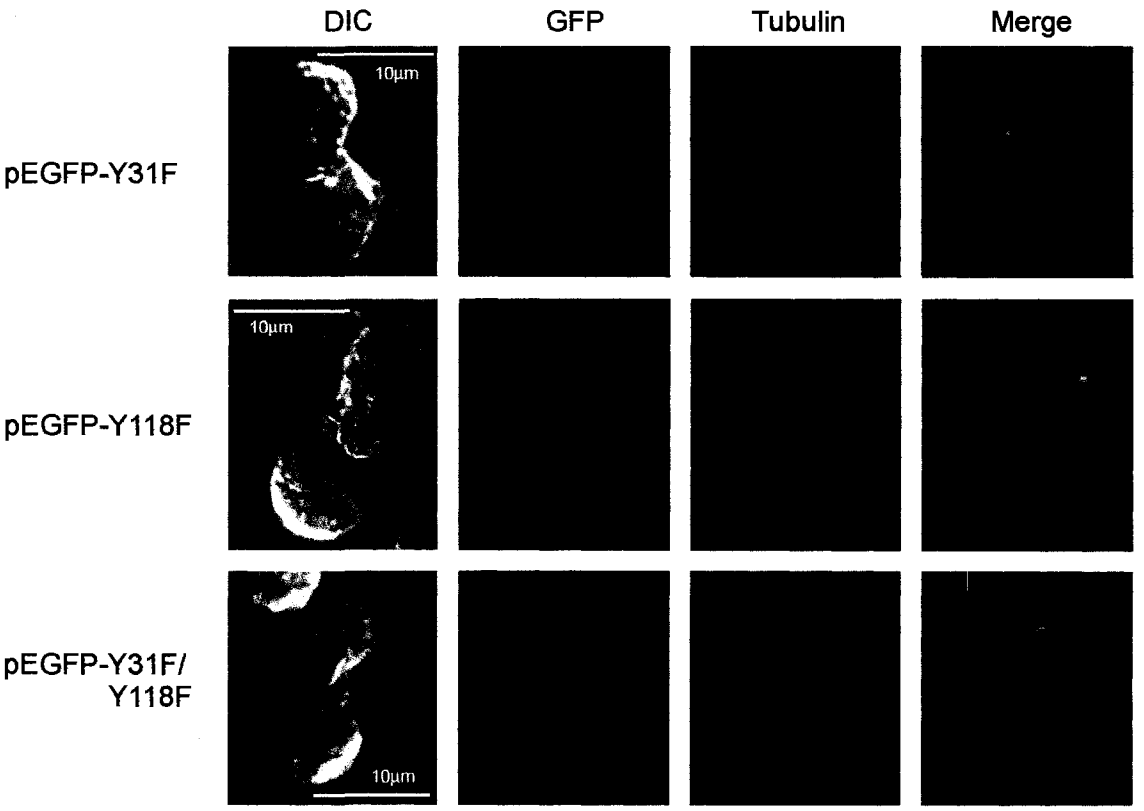

FIGURE 4-14. Mutation of tyrosine residues does not affect normal paxillin localization. Two principal phospho-tyrosine sites, Tyr^{31} and Tyr^{118}, were mutated to phenylalanine by PCR mutagenesis, either singly or together. CTL were transfected with the resulting pEGFP-Y31F, pEGFP-Y118F, or pEGFP-Y31F/Y118F plasmid vectors and conjugated to allogeneic target cells. The conjugates were prepared as in Figure 4-3 and stained with anti-GFP and anti-α-tubulin. Anti-GFP was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

have been unable to assess the phosphorylation status of paxillin in each location. While paxillin resides constitutively at the MTOC and microtubules, it appears to be actively recruited to the immune synapse and leading edge of migrating cells but does not appear to be associated with the membrane outside of these adhesive zones. It is likely then that paxillin is mobilized to sites of adhesion from either the microtubule pool or the cytoplasm. It is not known whether the microtubule-associated paxillin is static or constantly cycling on and off into the cytoplasm, though live-cell photobleaching experiments may be able to address this question in the future. It is difficult to speculate about the dynamics of paxillin recruitment, regulation, and cycling given that it is not yet known what binding interactions and protein binding partners are involved in effecting localization.

The influence of paxillin phosphorylation on its localization was investigated by examining serine and tyrosine point mutants by microscopy. Though mutation of serine residues 83 and 130 almost completely prevented serine phosphorylation and the concomitant molecular weight shift, paxillin localization was not affected (**Fig 4-12**). Analysis of the pellet fraction of cell lysates, which contains both nuclei and the polymerized microtubule cytoskeleton, demonstrated that even after TCR signaling paxillin present in the pellet is in the low-molecular weight, un-serine phosphorylated form (**Fig 4-13**). This result raises the interesting possibilities that serine phosphorylation events may either cause detachment paxillin from the microtubules or prevent the association in the first place. The triggering of detachment by serine phosphorylation is a common theme in microtubule biochemistry and applies to microtubule-associated proteins such as tau and the MAP2 family (Ozer and Halpain 2000; Dehmelt and Halpain 2005). Also, phosphorylation of paxillin at Ser^{273} in LD4 has recently been shown to weaken binding to FAK (Bertolucci, Guibao et al. 2008).

It is not yet known where in the cell phosphorylation of paxillin actually takes place, though ERK was originally characterized as a microtubule-associated protein (Reszka,

Seger et al. 1995; Morishima-Kawashima and Kosik 1996) and it is tempting to hypothesize that paxillin may be serine phosphorylated by ERK on the microtubule scaffold and then dissociate, thus becoming available for recruitment to the target interface. Attempts to determine the localization of ERK in CTL were unsuccessful due to limitations of the antibodies available. Further investigation of the validity this model is needed, though it may prove technically challenging. It is hoped that a mutant of paxillin in which Ser^{83} and Ser^{130} are replaced with phosphomimetic aspartic acid residues will be constitutively serine phosphorylated and therefore a useful tool for answering these outstanding questions.

In addition to serine phosphorylation I also addressed the importance of tyrosine phosphorylation events on paxillin recruitment. Previous work had identified tyrosines 31 and 118 as key targets for kinases such as FAK and Pyk2 (Schaller and Schaefer 2001), and I have previously established that removal of these residues has no effect upon serine phosphorylation (**Fig 3-11**). Mutation, either singly or together, of Tyr^{31} and Tyr^{118} also had no disruptive effect upon paxillin localization (**Fig 4-14**). This result is consistent with the other data presented here, as paxillin recruitment is likely to be mediated by the LD domains and not by interactions between phospho-tyrosine and SH2 domain-containing proteins.

By examining the effect of paxillin domain mutations on localization I discovered that removal of a region encompassing LD2, LD3, and LD4 reduced the binding of paxillin to the MTOC and SMAC and resulted in more paxillin in the cytoplasm and nucleus (**Fig 4-8D**). A similar staining pattern was observed when a small segment containing only LD1 and LD2 was transfected into CTL (**Fig 4-6D**). Though normal paxillin localization was destabilized, it was not completely prevented. The only modification made which completely abolished paxillin binding to the MTOC and its recruitment to the target interface was the total removal of the LD-containing N-terminal half of the molecule. My results suggest a model in which paxillin LD domains cooperate to bind to target

proteins and in which no one LD domain is essential. As the data demonstrates, even when large truncations and deletions are made, the remaining LD domains are able to maintain some degree of MTOC and interface localization. In the case of EGFP-ΔLD2-LD4, LD1 and LD5 remain, or LD1 and LD2 in the case of the EGFP-LD1-2 fusion protein.

A similar binding pattern to that of paxillin with the MTOC was recorded when the interaction between paxillin and the α_4 integrin subunit was examined (Liu, Kiosses et al. 2002). GST fusion proteins of paxillin segments were passed through a column bearing the α_4 integrin cytoplasmic tail and the retention of the GST fusions proteins was used as a measure of binding. All individual LD domains were able to bind α_4 integrin, though in some cases at much reduced levels. A fragment containing LDs 3 and 4 was sufficient yet not completely necessary for the interaction, much as I found a segment containing LDs 2, 3, and 4 to be sufficient but not absolutely required for paxillin localization to the MTOC and SMAC. The LD3-LD4 segment (Ala^{176} – Asp^{275}) was found to act as a dominant negative for α_4-paxillin function and the LD2-LD4 segment I produced may behave in a similar way in CTL; this is something that will be tested in future. In light of the findings relating to α_4 integrin it is perhaps not surprising that I was unable to fully disrupt paxillin localization by deleting combinations of LD domains.

A certain level of redundancy has also been observed in the binding of the LDs to other target proteins: vinculin is able to bind to LDs 1, 2, and 4, actopaxin to LDs 1 and 4, and FAK and Pyk2 to LDs 2 and 4 (Tumbarello, Brown et al. 2002). Other paxillin binding partners, however, are very specific to one LD domain or another, indicating that the LDs are not fully interchangeable. As yet, no partners have been identified for LDs 3 and 5. The flexibility of the LD interactions may be advantageous in allowing the amalgamation of larger protein complexes when binding partner proteins are competing for docking sites on paxillin. The co-operative and adaptable nature of the LD motifs was made apparent in the course of my mutagenesis studies of the LD region and is likely

the result of common biochemical characteristics, which in this case permit the LDs of paxillin to interact with binding partners at the immune synapse and MTOC in a number of different configurations.

Though not identical in amino acid sequence, the LD domains all share biochemical properties and conform to a general consensus sequence of LDXLLXXL, with the exception of LD3, in which the LD residues are replaced by VE (**Table 4-1**).

LD1:	M	D	D	**L**	**D**	**A**	**L**	**L**	**A**	**D**	**L**	E	S	T
LD2:	L	S	E	**L**	**D**	**R**	**L**	**L**	**L**	**E**	**L**	N	A	V
LD3:	R	P	S	**V**	**E**	**S**	**L**	**L**	**D**	**E**	**L**	E	S	S
LD4:	T	R	E	**L**	**D**	**E**	**L**	**M**	**A**	**S**	**L**	S	D	F
LD5:	G	S	Q	**L**	**D**	**S**	**M**	**L**	**G**	**S**	**L**	Q	S	D

Table 4-1. Alignment of the amino acid sequences of murine paxillin LD domains.

LD domains form amphipathic α-helices with the leucine residues aligned on one face of the helix to form a hydrophobic patch flanked by negatively charged aspartic and glutamic acid residues. A particular type of protein module capable of binding to LD motifs has been characterized and named the PBS, or paxillin-binding subdomain. The PBS is present in both Pyk2 and FAK kinases, as well as the actin-linking proteins vinculin and actopaxin and other binding proteins. Though the primary amino acid sequences are poorly conserved between the binding partners, the secondary structures are predicted to be similar (Tumbarello, Brown et al. 2002). The nature of the molecular interaction between the LD and PBS domains was revealed when NMR analysis was used to solve the structure of the FAK PBS in contact with an LD2 peptide (Gao, Prutzman et al. 2004). The hydrophobic face of LD2 interacts with one of two similar hydrophobic patches on the PBS, while negatively charged aspartic and glutamic acid residues of the LD pair with the positively charged histidine, lysine, and arginine residues that encircle the hydrophobic patches. It is thought that the residues flanking the LD domain may account for the binding specificity observed for some protein interactions. LD domain

protein interactions do not appear to be restricted to the PBS, however, as the α_4 integrin cytoplasmic domain does not contain such a motif and binds paxillin instead via a small, 9 amino acid sequence. The phosphorylation of a serine residue within this sequence prevents paxillin association (Liu and Ginsberg 2000; Han, Liu et al. 2001). The biochemical nature of the interaction has not been examined.

My results suggest that although all LD domains may contribute to localization there is likely a preferred configuration, determined by the differential affinities of each LD domain for the binding protein. LD domains with an amino acid sequence more suited to bind the relevant target proteins will make a greater contribution to localization, and consequently their removal will disrupt paxillin recruitment to a greater degree. Such a model could account for the combined LD1 and LD2 domains (pEGFP-LD1-2) being insufficient for optimal MTOC and SMAC recruitment compared to the construct containing the LD2, 3, and 4 domains (pEGFP-LD2-4). Analysis is complicated by the fact that more than one LD domain may interact with a target protein at once, such as in the case of Pyk2 and FAK, where LDs 2 and 4 cooperate (Brown, Perrotta et al. 1996; Thomas, Cooley et al. 1999). A more detailed dissection of LD domain binding preferences can be made once the protein(s) responsible for retaining paxillin at the microtubules and target interface are identified.

The recruitment of paxillin to two distinct subcellular locations such as the structural microtubule network and the adhesive immune synapse of CTL raises the possibility that paxillin may perform different functions at each location. A segment of paxillin encompassing the LD2, LD3, and LD4 domains is sufficient for localization both to the microtubules and to the target cell interface, and it is worth noting that no mutant of paxillin was recruited to only one of the two locations; MTOC and synapse localization were always seen coincidentally. Since the same region of paxillin is responsible for its recruitment to both sites, it is possible that paxillin recruitment is being effected by the same binding partner. However, the flexibility of binding inherent in the LD region

opens the possibility that paxillin is in fact retained at the microtubules by one protein and marshaled to the synapse by another. Another possibility is that localization to the microtubules and synapse are sequential, and that an event at one site allows recruitment to the other.

The immune synapse of CTL, with its adhesive and signaling functions, may seem analogous to focal adhesions though in adhesive cells it is the LIM3 domain that is required for focal adhesion localization of paxillin, and not the LDs (Brown, Perrotta et al. 1996). The mechanism used by paxillin to incorporate itself into focal adhesions has never been demonstrated, though paxillin may do so by interacting directly with some integrins, as it does with α_4 and possibly β_1 integrins (Schaller, Otey et al. 1995). If this is the case in CTL, integrin engagement at the target interface and the leading edge of migrating cells may result in the recruitment of paxillin to these sites. Future experiments could address the ability of such integrins as very late antigen-4 (VLA-4) ($\alpha_4\beta_1$) or LFA-1 ($\alpha_L\beta_2$) to interact with paxillin, either directly or indirectly, and whether such interactions play a role in bringing paxillin to the immune synapse. It is also possible that paxillin is recruited to the SMAC by TCR-related signaling proteins. The serine and tyrosine phosphorylation of paxillin downstream of the TCR, as well as its association with such TCR signaling molecules as Lck, Fyn, and Pyk2 (Ostergaard, Lou et al. 1998) places it in the context of antigen-triggered signaling events. Ultimately, identification of paxillin binding partners at the SMAC may provide clues as to the role of paxillin in this location, be it integrin regulation or the gathering of signaling complexes.

Microtubule association of paxillin has not as yet been recorded in studies using adherent cell types, but in lymphocytes only (Herreros, Rodriguez-Fernandez et al. 2000). If it proves that paxillin binds microtubules only in hematopoietically-derived cells or lymphocytes, it is unlikely that paxillin interacts directly with tubulin itself, but rather with another microtubule-associated binding partner. Another argument against a direct association between paxillin and tubulin appears upon close examination of paxillin

staining: microtubule-associated paxillin appears to be punctate in distribution as compared to tubulin itself. This pattern has been observed before, with c-Jun N-terminal kinase (JNK), a kinase of paxillin (Nagata, Puls et al. 1998). Well-characterized microtubule-associated proteins (MAPs) such as tau and the MAP2 family interact with microtubules via repeats of an 18 amino acid sequence containing a KXGS motif. Murine paxillin contains two KXGS sequences, but their spacing and surrounding sequence is not in any way similar to that of the MAPs and it seems unlikely that they function in this manner.

The brain-specific isoform of the Src kinase Fyn has been shown to bind to MAPs, specifically tau and MAP2c (Lee, Newman et al. 1998; Zamora-Leon, Lee et al. 2001), and in T cells Fyn is recruited to the microtubules (**Fig 4-11A**, (Ley, Marsh et al. 1994). The interaction between paxillin and Fyn is therefore a possible mechanism by which paxillin could be held at the microtubules. In Fyn-deficient CTL, however, EGFP-paxillin displayed a normal localization pattern (**Fig 4-11B**), so Fyn does not appear to be performing this function, or is not exclusive in this function.

I attempted to determine whether Pyk2 is responsible for paxillin microtubule association by creating small deletions of the LD2 and LD4 domains. This construct localized normally, but when the mutant protein was immunoprecipitated from CTL it was found that some Pyk2 association remained, albeit at much reduced level (**Figure 4-10A**). One possible explanation is that the remaining LD domains are capable of binding to the Pyk2 PBS domain, though this has never been observed before (Brown, Perrotta et al. 1996; Thomas, Cooley et al. 1999). Initial work characterized the FAK binding sites by pull-down with GST-paxillin fusion proteins. It was assumed that Pyk2 interacted in the same way, though this has never actually been dissected. A second explanation is that paxillin and Pyk2 are capable of indirect association through another protein which is not dependent on LDs 2 and 4. Fyn is a candidate for the formation of such a complex, as Fyn co-precipitates with both Pyk2 and paxillin (**Figure 3-7B,** (Qian, Lev et al. 1997). In

any event, I was unable to determine whether Pyk2 plays a role in microtubule recruitment of paxillin. The question of how paxillin binds to microtubules and how this association is regulated may be addressed in the future by biochemically comparing binding partners of wild type paxillin to those of mutants lacking LD2-LD4 (EGFP-ΔLD2-4) or the N-terminal half (EGFP-CT).

The recruitment of paxillin to two distinct subcellular locations such as the structural microtubule network and the adhesive immune synapse of CTL raises the possibility that paxillin may perform different functions at each location. The importance of paxillin to the regulation of integrin-based focal adhesions of adherent cell types is well studied and may also apply to CTL, though the latter do not form classical focal adhesions. Integrin clustering and signaling are critical factors in the competence of a CTL to bind to an antigen-bearing target cell and adhere tightly enough to maintain contact over the time necessary for activation and degranulation to occur. I can speculate that paxillin may play a role in integrin-based adhesion to the target, and therefore in conjugate formation and stability. As mentioned above, the collection of signaling molecules such as Lck, Fyn, and Pyk2 at the synapse is another potential function of paxillin, which would affect TCR-triggered signaling and activation events. Such issues may be resolved by a more detailed examination of the requirements of paxillin movement to the immune synapse as well as functional studies designed to examine the effect of paxillin knockdown by small interfering RNA (siRNA) on such events as adhesion, spreading and conjugate formation.

A greater mystery is presented by the association of paxillin with microtubules and the functional implications of such an interaction. Regulation of the microtubule network and the MTOC are especially influential in CTL function as the tubules themselves are used as "guide rails" for cytolytic granules moving toward the target cell as directed by the reoriented MTOC. I can speculate that paxillin may play a role in recruiting other proteins important for microtubule regulation or possibly even in tethering the granules to the microtubules. The observed requirement for paxillin in NK lysis (Li, Ge et al. 2008),

when considered in the context of the data here, could be a result of paxillin involvement in conjugate formation, MTOC reorientation, or granule trafficking. My efforts to analyze the biochemistry, phosphorylation, and binding partners of paxillin at the microtubules and immune synapse have been hampered by the lack of a reliable means of purifying the microtubule cytoskeleton and thus of separating the two pools of paxillin. Functional studies involving the knockdown of paxillin expression will be subject to similar limitations, though the nature of any observed defects in CTL activity may point toward either microtubule or SMAC function. In any event, future studies will aim not only to fill in details relating to paxillin localization, but will aim to build upon the functional implications of the data to answer larger questions about the role of paxillin in cytotoxic T cell function.

CHAPTER 5: Paxillin and integrin adhesion in CTL

A. Introduction

The tight cellular adhesion that is required for a cytotoxic T lymphocyte (CTL) to attach to and kill an infected or tumourigenic target cell is mediated by integrins and is controlled by signals originating from T cell receptor (TCR) contact with cognate antigen. Integrins are heterodimeric transmembrane receptors consisting of α and β subunits that mediate adhesion either between two cells or between cells and the extracellular matrix. After antigen priming, effector CTL search the tissues for target cells presenting the viral or altered-self antigen on Class I major histocompatibility complexes (MHC). The first early contacts between a CTL and a potential target are made by integrins, which stabilize the weak interaction while the TCR scans for antigen. The integrin leukocyte function-associated antigen-1 (LFA-1), composed of an α_L subunit (CD11a) and a β_2 subunit (CD18), interacts with intercellular adhesion molecule-1 (ICAM-1, CD54) on potential targets and is critical both for early contact events and for prolonged, tight adhesion. Signals emanating from activated TCRs are transmitted back to the cell surface by various pathways and modify integrin activity in a mechanism defined as "inside-out" signaling. Both integrin affinity and avidity can be adjusted to modulate adhesion and both are subject to inside-out regulation by the TCR. LFA-1 exists in low, intermediate, and high-affinity conformations and shifts between them in response to ICAM-1 binding and inside-out signals. The avidity of LFA-1 is a function of its ability to coalesce into clusters and its lateral mobility in the plasma membrane; LFA-1 tethered to actin in inactive CTL is less mobile and less apt to cluster. The current model suggests that upon TCR engagement low-affinity, low-mobility LFA-1 is released from actin and merges into clusters. Inside-out signals also elicit the transition of LFA-1 into higher affinity states, and tighter adhesion to the target cell is achieved. High-affinity LFA-1 that is clustered and bound to ICAM-1 is then re-attached to actin in order to stabilize the interaction between the CTL and target cell (Dustin, Bivona et al. 2004).

Firm and stable anchoring of the CTL to its target is tightly regulated by the TCR and is a critical component of the complex and dynamic process of target cell killing.

The interface between a CTL and an antigen-bearing target cell is termed the "immunological synapse" and is a focal point both for TCR signaling and for the carefully aimed delivery of the cytolytic granules whose contents trigger target cell death. The immune synapse is not an amorphous accumulation of receptors, however, but a highly organized "bulls-eye" formation made up of adhesion and signaling components both in the membrane and in association with the membrane. These proteins are separated into two phases: the central supramolecular activation cluster (cSMAC), to which are segregated the TCR and associated signaling molecules, and, in a ring around the cSMAC, the peripheral SMAC (pSMAC), which contains integrins such as LFA-1 (Monks, Freiberg et al. 1998). The pSMAC not only contains integrins but complexes of the proteins that regulate inside-out signaling and the structural proteins that link integrins to actin fibres. Following adhesion of a CTL to a target, the microtubule organizing centre (MTOC), an anchoring point for the microtubule minus ends, and the Golgi body reorient themselves toward the SMAC (Geiger, Rosen et al. 1982; Kupfer and Dennert 1984). Granules containing cytolytic ingredients such as granzymes and perforin are trafficked along the microtubules in the direction of the target (Burkhardt, McIlvain et al. 1993; Stinchcombe, Bossi et al. 2001). Once the MTOC and granules are in close proximity to the membrane within the cSMAC the granules fuse with the plasma membrane and release their contents toward the target cell. The targeting function of the SMAC in CTL is important for the prevention of indiscriminate granule release and bystander killing of uninfected cells.

Unlike lymphocytes, which patrol the lymph nodes and tissues constantly, adherent cell types such as fibroblasts and smooth muscle cells migrate little and form tight, integrin-based contacts with the extracellular matrix. Focal adhesions are sites of firm adhesion with the extracellular matrix and consist of clustered integrins tethered to the actin cytoskeleton, particularly to actin stress fibres which anchor the cell. Focal

adhesions, like the peripheral SMAC in T cells, contain both structural components that link to actin and signaling components that regulate actin dynamics and the formation and dissolution of focal adhesions.

Paxillin is a ubiquitously-expressed adaptor protein which acts as a platform for the assembly of focal adhesion complexes in adherent cell types. The actin-binding proteins vinculin and actopaxin complex with paxillin at sites of integrin engagement (Turner, Glenney et al. 1990; Nikolopoulos and Turner 2000). Paxillin contains five N-terminal leucine-aspartic acid (LD) domains, a proline-rich domain, four C-terminal lin-11 isl-mec-3 (LIM) domains, and numerous phosphorylation sites, all of which potentially interact with binding partners. Paxillin has been documented to interact with many different proteins, both structure- and signaling-based, and the primary role of these proteins appears to be the disassembly of focal complexes to permit cell migration (Webb, Donais et al. 2004). The importance of paxillin in the migration and adhesion of cells is underscored by the embryonic lethal phenotype of paxillin-null mice (Hagel, George et al. 2002) and its broad expression pattern implies that it plays a role in the activities of many different cell types.

The function of paxillin in T cells is far less studied than its role in adherent cells, but those studies that have investigated paxillin in T cells have revealed important differences between the two cellular systems. Antibody staining for paxillin in adherent cells shows it to be localized to punctate, membrane-associated focal adhesions, most often at the ends of stress fibres (Glenney and Zokas 1989; Turner, Glenney et al. 1990). In contrast, paxillin in T cells has been shown to be associated with the MTOC (Herreros, Rodriguez-Fernandez et al. 2000), as well as the microtubules themselves and the immune synapse formed between a CTL and a target cell (Robertson, Mireau et al. 2005). Paxillin in CTL interacts with the Src family kinases Fyn (**Fig 3-7B**) and Lck, the latter of which is a quintessential TCR signaling molecule essential to the initiation of signaling (Molina, Kishihara et al. 1992; Ostergaard, Lou et al. 1998). In both lymphocytes and adherent

cells paxillin interacts with members of the focal adhesion kinase (FAK) family; the former express mainly proline-rich tyrosine kinase 2 (Pyk2), which is favoured in the hematopoietic system, and the latter express FAK itself (Ostergaard and Lysechko 2005). Tyrosine and serine phosphorylation of paxillin is a common response to a wide array of growth factors, chemokines and other stimuli, and not surprisingly TCR activation with antigen is also among this group (Ostergaard, Lou et al. 1998; Brown and Turner 2004). Phosphorylation of tyrosine and serine residues is capable of creating docking sites for other proteins or modulating binding properties, thus affecting the complement of proteins with which paxillin interacts. Paxillin has been shown to be needed for NK cell killing of target cells, but how paxillin is important and whether this applies to CTL has not yet been tested (Li, Ge et al. 2008).

Identification of the mechanism by which paxillin is recruited to integrin complexes has proven elusive. Synthetic peptides mimicking the β_1 cytoplasmic tail were capable of binding to paxillin *in vitro*, but the importance of this was not addressed in cells (Schaller, Otey et al. 1995). Paxillin has been shown to bind directly to the cytoplasmic tail of α_4 integrin (CD49d) (Liu, Thomas et al. 1999). The α_4 chain is a component of very late antigen-4 (VLA-4, $\alpha_4\beta_1$) and is principally expressed in cells of the immune system where it encourages migration and cell trafficking (Alon, Kassner et al. 1995; Berlin, Bargatze et al. 1995). Binding to α_4 integrin cannot account for paxillin focal adhesion targeting in most cells, however, especially when it is considered that LIM3 mediates focal adhesion targeting but paxillin interacts with α_4 via the LD domains (Brown, Perrotta et al. 1996; Liu, Kiosses et al. 2002). It is important to point out that the CTL clones used in my experiments do not express α_4 integrin (Ma, Lou et al. 1997). Given the questions surrounding paxillin translocation to membrane complexes in adherent cells, it is not surprising that in T cells the recruitment of paxillin to the SMAC is also poorly understood. The phosphorylation of paxillin downstream of the TCR and the interaction between paxillin and signaling molecules such as Lck suggest an involvement in TCR signaling though its traditional function in integrin regulation

implies an analogous role for paxillin in T cell adhesion and migration. This preliminary investigation has attempted to address this uncertainty by further describing the translocation of paxillin into the SMAC and its relationship to TCR activation.

In this study I have used confocal microscopy to characterize the recruitment of paxillin to the immune synapse in CTL. I found that engagement of the TCR by the target cell was not necessary for paxillin translocation to the synapse. In conjugates where TCR signaling was present paxillin was separated into the peripheral SMAC along with LFA-1. Paxillin is heavily phosphorylated in response to the TCR, yet in cases where the TCR and integrin signals were provided on different surfaces, paxillin was preferentially enriched at the site of cell contact. The results suggest that paxillin in CTL may perform a function analogous to that in adherent cells and may be involved in regulating integrin adhesion to target cells in response to inside-out signals originating at the TCR.

B. Results

Paxillin is recruited to the target cell interface even in the absence of TCR signaling

I have published that paxillin is constitutively localized to the microtubules and MTOC of CTL and is recruited to the immune synapse after target cell contact (**Fig 4-1**, or (Robertson, Mireau et al. 2005). In those experiments, non-transformed CTL clones were conjugated to L1210 (H-2^d) target cells transfected with an allogeneic Class I MHC (L1210 K^bD^d) molecule appropriate for the activation of the H-$2K^b$-specific CTL clones. Paxillin is heavily phosphorylated downstream of the TCR and though I have shown that phosphorylation is not required for localization (**Figs 4-12 and 4-14**) I wished to address whether paxillin translocation to the SMAC is dependent upon TCR activation. Two types of CTL clones, AB.1 (H-2^d) and Clone 11 (H-$2^{d/k}$), were mixed with untransfected L1210 targets, stained with antibodies against paxillin and α-tubulin, and imaged by

confocal microscopy (**Fig 5-1**). Even in the absence of allogeneic MHC conjugates did form between the L1210 and CTL, contact likely being maintained by integrin adhesion. Paxillin was recruited to the interface despite the lack of a TCR signal. MTOC reorientation did not occur. A biochemical examination of paxillin after target contact has shown that there is a brief and minimal molecular weight shift in paxillin after contact with L1210, as compared to the more prolonged and pronounced shift induced by L1210 K^bD^d (**Fig 3-1B**). Paxillin localization appears to be independent of phosphorylation, though it is possible that the small amount of phosphorylation induced by the L1210 cells is affecting its recruitment to the membrane.

Although used as an MHC-negative control, L1210 cells do express integrin ligands and other receptors that may provide signals to the CTL. Also, it cannot be declared with certainty that the self-MHC ($H\text{-}2^d$) of the L1210 cells is not providing a minor signal to the CTL, given that TCRs are screened for their ability to recognize self MHC. This signal would not trigger degranulation as would antigen-MHC or allo-MHC, but it may play a role in homeostatic proliferation of the T cell population. What is evident from this data is that contact between the TCR and antigen is unnecessary to achieve enrichment of paxillin at the membrane contact point, leaving open the possibility that integrins or other receptors may be mediating this event.

Paxillin is a component of the peripheral SMAC

Given that paxillin is an adaptor protein of focal adhesions in adherent cells and appears to be recruited to sites of integrin attachment in CTL, I tested whether paxillin was segregated into the signaling-rich central SMAC or the integrin-rich peripheral SMAC upon target conjugation. Integrins such as LFA-1 are gathered into the ring of the pSMAC that surrounds the TCR and associated proteins in the cSMAC (Monks, Freiberg et al. 1998). CTL clones were conjugated to L1210 K^bD^d target cells and stained with

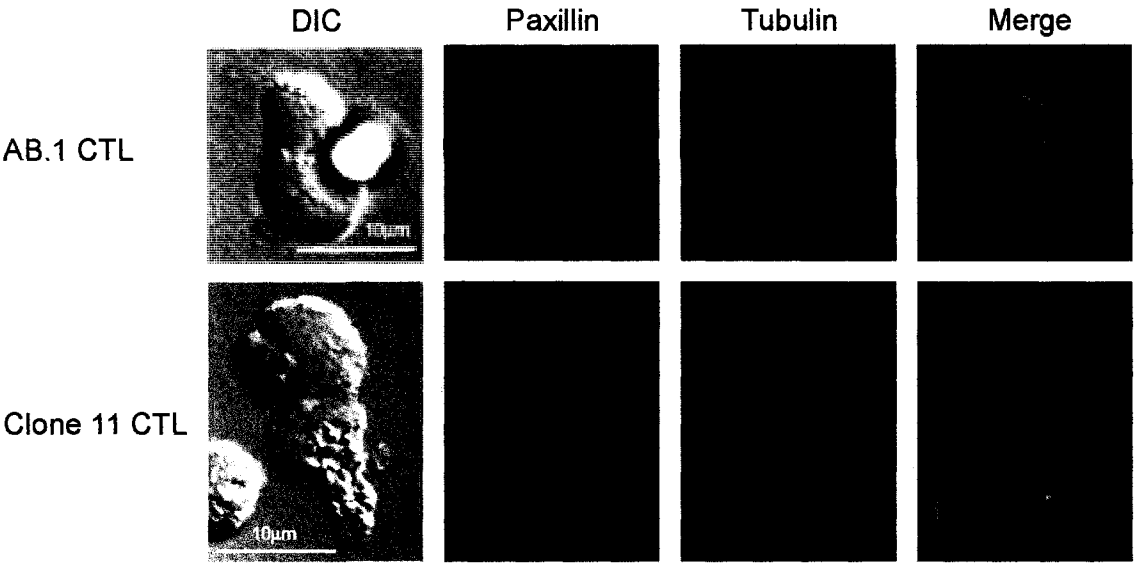

FIGURE 5-1. Paxillin is recruited to the target interface even in the absence of TCR activation. AB.1 (top) or Clone 11 (bottom) CTL clones were conjugated with untransfected target cells pre-dyed with Cell Tracker Blue. The conjugates were fixed and permeabilized and stained for paxillin and α-tubulin. Anti paxillin was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary. The bead which appears out-of-plane in the upper panel may be in contact with the target cell but does not appear to affect the conjugate.

antibodies against paxillin and the β_2 integrin subunit of LFA-1. For CTL with mature SMACs, a series of image stacks were collected in order to reconstruct the immune synapse in three dimensions. When the SMAC was rotated into a head-on projection, it was clear that paxillin co-localized with LFA-1 in the pSMAC ring (**Fig 5-2**). The co-localization was not complete, however, which suggests that LFA-1 either does not itself recruit paxillin to the pSMAC or is not the only receptor that does so. This result demonstrates that despite the interaction between paxillin and TCR signaling molecules such as Lck and Fyn (**Fig 3-7B,** and (Ostergaard, Lou et al. 1998), membrane-associated paxillin is separated into the integrin adhesion zone of the mature immune synapse where little TCR signaling occurs.

Due to the nature of the experiment and the fixation of the cells, it is difficult to establish a "time 0" for conjugation and thus all CTL-target conjugates observed may be at different points in development. As a result, some CTL display mature SMACs while most do not, being at a point either before or behind that of structured synapse formation. It is important to note that most TCR signaling occurs before the development of the mature synapse (Lee, Holdorf et al. 2002), and in most CTL observed paxillin was part of an unstructured immune synapse in which LFA-1 was evenly dispersed. The presence of paxillin in the pSMAC does however suggest that it may be involved in integrin regulation and that integrin receptors may be responsible for the translocation of paxillin to the membrane, as is the case in adherent cell types.

Paxillin is preferentially recruited to the site of cell contact

Imaging of the immune synapse demonstrated that paxillin was excluded from the cSMAC where the TCR and its associated signaling molecules congregate, and I next addressed whether paxillin was preferentially translocated to the site of integrin recruitment when the TCR signal was provided on a different surface from the adhesion signal. To isolate the TCR signal, cell-size latex beads coated in anti-CD3 antibody were

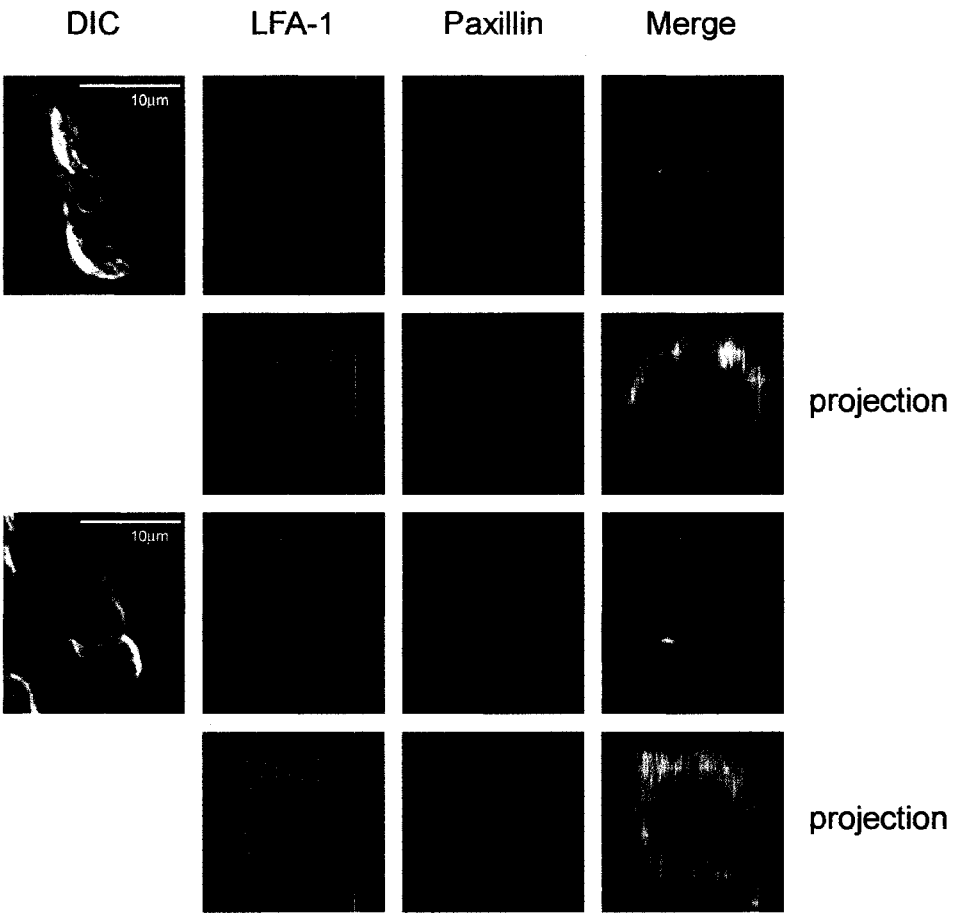

FIGURE 5-2. Paxillin is recruited to the peripheral supramolecular activation cluster (pSMAC). CTL clones were conjugated to allogeneic L1210 K^bD^d target cells (pre-dyed with CellTracker Blue) and stained for paxillin and LFA-1 after fixation and permeabilization. Anti paxillin was detected with a secondary antibody coupled to rhodamine, and anti-LFA-1 was detected with an antibody coupled to Alexa Fluor 488. 3D projections of the CTL-target interface were made using the Zeiss LSM software. The top projection was reconstructed from 15 optical slices of 0.5μm, the bottom projection from 15 optical slices of 0.64μm.

prepared. In order to demonstrate that the beads were capable of initiating TCR signaling, CTL were first mixed with beads coated either with anti-CD3 or with anti-LFA-1 (β_2) to facilitate adhesion and were then stained with antibodies against phosphotyrosine (PY) and α-tubulin. Anti-LFA-1 was insufficient to engender significant PY signaling at the contact area (**Fig 5-3**). Anti-CD3 beads did however cause PY production and staining at the contact point between the CTL and the bead, and also triggered MTOC reorientation, a phenomenon observed in previous publications (Lowin-Kropf, Shapiro et al. 1998; Sedwick, Morgan et al. 1999). This result confirmed that the anti-CD3 beads were having the desired effect of triggering TCR signaling events. CTL were then mixed with both anti-CD3 beads and L1210 target cells. In cases where a CTL was conjugated to both a bead and a target cell simultaneously paxillin was heavily enriched at the target contact site but little or no paxillin was localized the bead (**Fig 5-4**). A small amount of tubulin was usually observed to collect under the anti-CD3 bead and therefore it is difficult to definitively say whether paxillin was recruited to membrane complexes at that point or merely a passenger bound to the accumulated microtubules. The experimental result suggested that although paxillin is phosphorylated in response to TCR signals its enrichment at the SMAC is independent of TCR contact with antigen-MHC, or, in this case, with allogeneic MHC.

Paxillin is not required for TCR signaling events

Despite the finding that paxillin localization is not dependent on the TCR, paxillin does associate with Lck, Fyn and Pyk2 and in the early stages of conjugation is likely to be in proximity to the TCR before being apportioned into the pSMAC. The following experiment was designed to address whether paxillin was necessary for the initiation or maintenance of TCR signaling. Paxillin expression was knocked down using small interfering RNA (siRNA) oligos, which were transfected into the CTL clones. After 48 hours, CTL lysates were analyzed for the expression of paxillin. The knockdown achieved in the experiment shown was 74%. CTL transfected with either paxillin siRNA

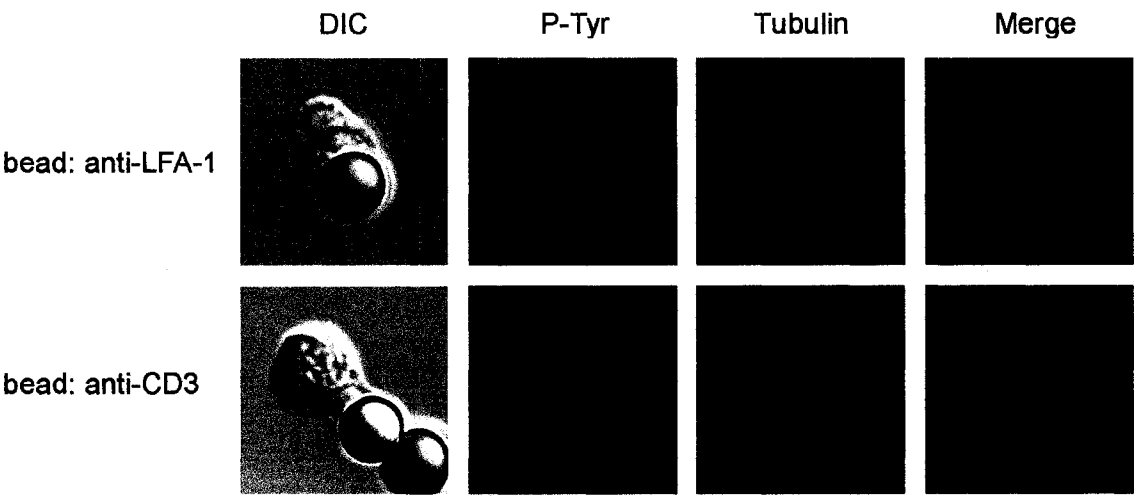

FIGURE 5-3. Latex beads coated with anti-CD3 trigger tyrosine phosphorylation and MTOC reorientation in CTL. Clone 11 CTL were mixed with beads coated with anti-LFA-1 for adhesion or anti-CD3 for TCR activation. After fixation and permeabilization, the cells were stained for phosphotyrosine (P-Tyr) and α-tubulin. Anti P-Tyr was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

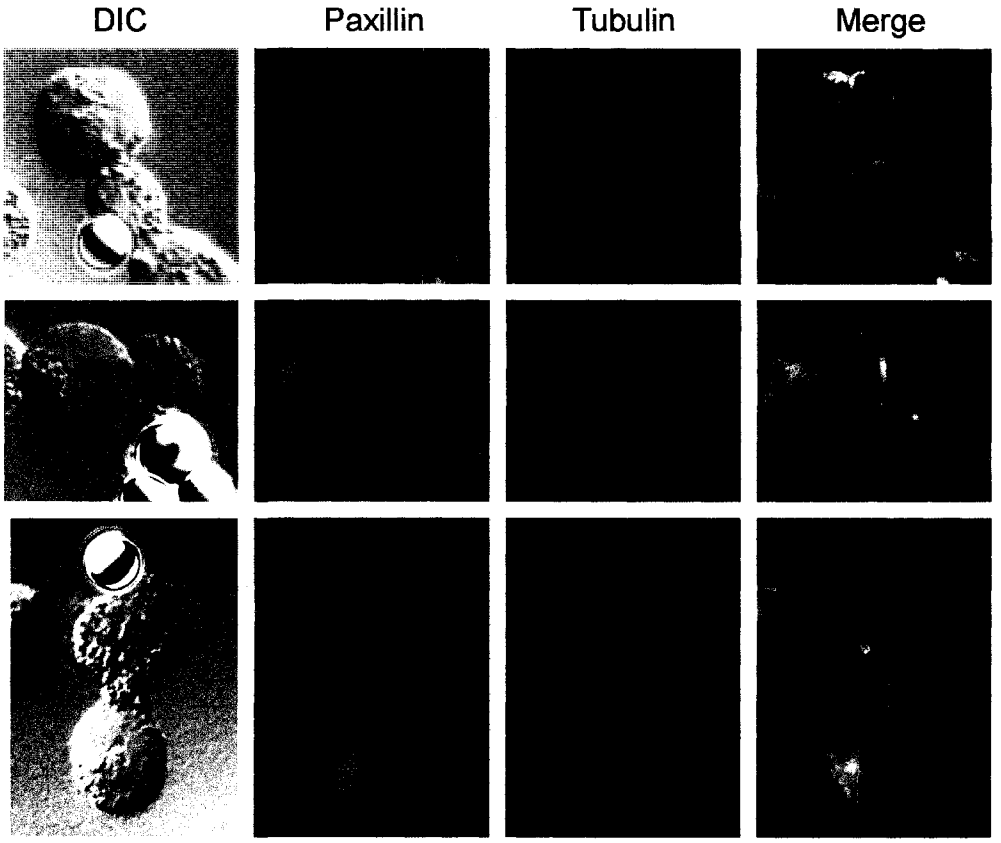

FIGURE 5-4. Paxillin is preferentially translocated to areas of integrin adhesion. Clone 11 CTL were mixed with allo-MHC-negative L1210 targets (pre-dyed with Cell Tracker Blue) and anti-CD3-coated beads. The cells were fixed, permeabilized, and stained for paxillin and tubulin. Anti paxillin was detected with a secondary antibody coupled to Alexa Fluor 488, and anti-α-tubulin was detected with an Alexa Fluor 594-coupled secondary.

or a scrambled siRNA control were stimulated on anti-CD3 for varying times and then lysed. Analysis of the lysates showed that at each time point up to 2 hours, TCR-induced global PY and phospho-ERK levels were identical whether paxillin expression was normal or reduced (**Fig 5-5**). Though knockdown was not complete, paxillin appeared to be dispensable for optimal TCR signaling. This is not wholly unexpected given that paxillin is serine phosphorylated downstream of ERK, which, being at the culmination of the Ras pathway, is a relatively late event in TCR signaling cascades.

C. Discussion

In other studies I have investigated the TCR signaling pathways that regulate paxillin phosphorylation and have demonstrated that paxillin in CTL is localized to the microtubule cytoskeleton and the immune synapse. The series of experiments presented here, though preliminary, has incorporated both of the previous avenues and attempted to characterize the importance of the TCR in the enrichment of paxillin at the immune synapse. Localization of paxillin to the point of contact with an associated target occurred regardless of whether allogeneic MHC was present on the target or whether the anti-CD3 signal was provided by an attached bead (**Figs 5-1 and 5-4**). Paxillin enrichment at the membrane does not therefore require the occurrence of antigen-specific TCR signals within the cell, nor is it subject to a particular location of TCR contact. The first conclusion is consistent with mutagenesis studies which established that TCR-triggered phosphorylation of serine and tyrosine (Y31, Y118, Y31/Y118 in tandem) residues did not disrupt paxillin localization to either the microtubules or the SMAC (**Figs 4-12 and 4-14**). Again, it must be noted that some low-level TCR signaling may occur as a result of the recognition of self (H-2^d) MHC by the TCRs of the CTL clones used.

As mentioned, paxillin has been shown to interact with Lck and Fyn and its presence in the pSMAC would seem to be at odds with these findings. Lck and its family member

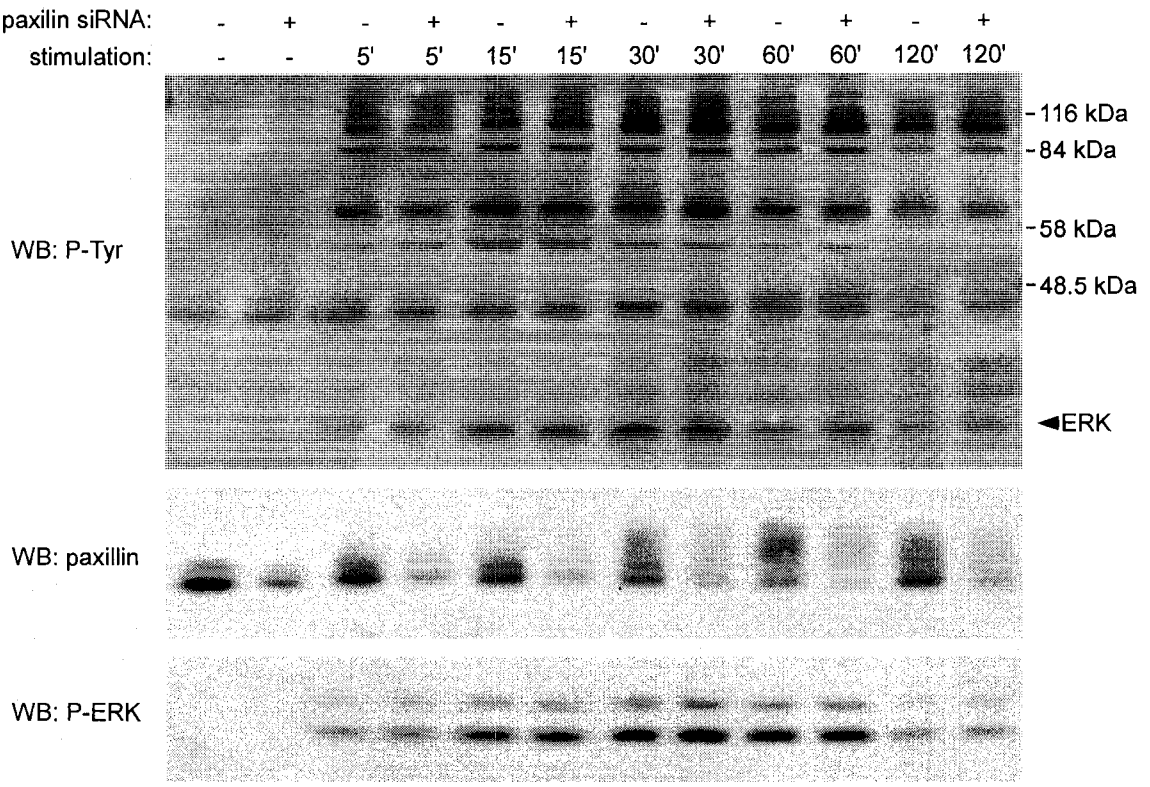

FIGURE 5-5. Paxillin knockdown does not disrupt TCR signaling. Clone 11 CTL were transfected with either paxillin siRNA oligos or a non-targeting siRNA. After 48 hours, the cells were stimulated with immobilized anti-CD3 for the times indicated. The cells were lysed in Laemmli reducing sample buffer and analyzed by SDS-PAGE. Western blotting was performed with the antibodies indicated. The degree of knockdown for the experiment shown was determined by densitometry to be 74%, relative to actin.

Fyn are both located at the centre of the mature immune synapse in $CD4^+$ T cells, but there are visible clusters of the TCR and Src kinases dispersed outside of the cSMAC (Monks, Freiberg et al. 1998). Microscopy of T cells in contact with planar lipid bilayers has better illustrated that "microclusters" of the TCR and active signaling molecules such as Lck, linker for activation of T cells (LAT), and zeta-associated protein-70 (ZAP-70) form at the periphery of the immune synapse and migrate toward the centre (Campi, Varma et al. 2005; Yokosuka, Sakata-Sogawa et al. 2005). It is therefore possible that paxillin might associate with Lck in the pSMAC. Unlike Fyn, Lck has not been observed at the microtubules (Ley, Marsh et al. 1994) and it can be expected that paxillin bound to Lck is at the target interface. Knockdown of paxillin expression by 74% was shown to have no effect upon the initiation of TCR signaling or its maintenance over 2 hours after activation with anti-CD3 (**Fig 5-6**). Antibody stimulation is a particularly strong stimulus, however, and may not be reflective of physiological situations in which antigen in limited. Future studies may employ different modes of antigen stimulation, including target cells, in order to address this issue. The functional significance of the association between paxillin and Lck and Fyn will also be the subject of further enquiries.

In the case of CTL, integrin adhesion occurs both during migration, allowing the CTL to patrol tissues searching for targets, and during conjugation, holding a target cell in place while degranulation occurs. That paxillin synapse enrichment is independent of the TCR (**Fig 5-1**) is in agreement with the result of previous imaging experiments showing paxillin to be concentrated at the leading edge of migrating, unstimulated CTL (**Fig 4-1A**). The leading edge is an actin-rich site of active integrin attachment and clustering. This attachment provides sufficient anchorage to generate the tension needed for motility. In the microscopy images available paxillin does not appear to be associated with the membrane outside of adhesive zones such as the SMAC and leading edge, and if this is the case it seems that active clustering or signaling of integrin receptors is what is needed to achieve its translocation to the membrane. It cannot be ruled out, however, that some paxillin constitutively associates with membrane integrins and then appears to be

recruited to the membrane when clustering of integrins creates a concentration effect. Additional experiments will be required to identify which elements are associating with paxillin at the SMAC, and such findings would facilitate a more detailed study of the dynamics of paxillin membrane association.

The mechanism by which paxillin translocates into focal adhesion complexes remains in doubt even after much study, and the same uncertainty applies to my studies of SMAC recruitment in CTL. An early publication determined that the LIM3 domain was required for paxillin to associate with focal adhesions (Brown, Perrotta et al. 1996), but the relevant binding partner of LIM3 has not been identified. A direct association between paxillin and synthetic peptides mimicking the β_1 integrin cytoplasmic tail was observed *in vitro,* and a similar assay detected direct binding of paxillin to the cytoplasmic tail of α_4 (Schaller, Otey et al. 1995; Liu, Thomas et al. 1999). Interestingly, paxillin interacts with α_4 not through LIM3 but through the LD domains (Liu, Kiosses et al. 2002). Characterization of binding revealed that no one LD domain was required for the interaction and that all LDs could bind α_4 albeit in some cases at a minimal level. A fragment encompassing LDs 3 and 4 was sufficient for binding but was not absolutely required. Similar results were observed in my examination of the requirements for paxillin microtubule and SMAC localization; a segment containing LDs 2, 3, and 4 was sufficient but not completely required for localization to both sites and only the complete removal of the LDs could disrupt localization (**Figs 4-5 through 4-9**). Lymphocytes express α_4 integrins and VLA-4 is known to partition into the pSMAC of helper $CD4^+$ T cells, though this recruitment event was independent of its ligands, vascular cell adhesion molecule-1 (VCAM-1, CD106) and fibronectin, which were not expressed on the APCs used (Mittelbrunn, Molina et al. 2004). It is known that the CTL clones I have used do not express the α_4 chain (Ma, Lou et al. 1997), which rules it out as a means of paxillin pSMAC enrichment.

Over the course of my mutagenesis studies, paxillin microtubule and synapse localization were never observed separately; any mutation that altered localization always affected both simultaneously. This outcome suggested that the same protein may be recruiting paxillin to both subcellular sites. In light of the data presented here, it seems increasingly likely that this is not the case and that paxillin SMAC recruitment is effected by surface integrins or their associated proteins. Given the flexibility inherent in the LD binding properties it is not unrealistic to propose that two different proteins may interact through the same domain(s) but with divergent outcomes for localization and function. Though challenged by the inherent difficulty in biochemically separating the microtubule- and membrane-associated populations of paxillin, additional work will be required to characterize the two pools of paxillin and to identify the means of recruitment for each.

The preferential association of paxillin with integrin-rich areas of CTL is in accordance with its extensively studied involvement in focal adhesion regulation in adherent cells, though it must be recognized that lymphocytes differ significantly in terms of paxillin localization and integrin dynamics. Study of paxillin in T cells has revealed a novel subcellular localization for paxillin: the microtubule cytoskeleton and MTOC (Herreros, Rodriguez-Fernandez et al. 2000), and **Fig 4-1**). It is currently unknown what the function of microtubule-associated paxillin might be, though it is likely to be a role not yet described for this protein. The accumulation of paxillin at the leading edge and the peripheral SMAC would suggest that it is also participating in integrin regulation in lymphocytes. Lymphocytes utilize integrins differently from adherent cells in that they do not form focal adhesions or actin stress fibres and are far more motile within the extracellular matrix. In CTL integrin-mediated tight adhesion to a target cell is regulated by the TCR and allows time for signaling and degranulation to occur. The finding that NK cell killing is dependent upon paxillin (Li, Ge et al. 2008) may be indicative of a role for the adaptor protein in conjugate formation or stabilization. Given that paxillin is phosphorylated downstream of the TCR and is enriched at the pSMAC, there is potential

for this adaptor protein to operate as an inside-out intermediary between the TCR and cell-surface integrins. The antigen-independent membrane enrichment of CTL does not preclude its involvement in inside-out signaling, as its binding partners may vary according to TCR-induced phosphorylation and signaling. In view of the fact that paxillin is essential for focal adhesion turnover and migration in adherent cells, it is also possible that it facilitates detachment of the CTL from the target after granule delivery. In acknowledgement of all these possibilities, upcoming studies will address whether paxillin promotes adhesion and migration of CTL or affects the dynamics of conjugate formation.

CHAPTER 6: General Discussion

A. Summary of Results

Paxillin is phosphorylated in response to many cell stimuli and the first aim of this study was to characterize the regulation of paxillin phosphorylation after engagement of the TCR. TCR activation has been shown to elicit tyrosine phosphorylation and a shift in the molecular weight of paxillin (Ostergaard, Lou et al. 1998). I demonstrated that the gel mobility shift of paxillin is in large part due to extensive serine phosphorylation; a cascade of events initiated by phosphorylation of Ser^{83} and Ser^{130} (Chapter 3). These residues have been identified as key targets of ERK (Cai, Li et al. 2006), and though I could not detect co-immunoprecipitation of paxillin and ERK the data I obtained was consistent with the published model. Both PI3K and ERK are required to achieve the maximal TCR-induced serine modifications, but neither is required for tyrosine phosphorylation. This result provided evidence that the post-translational modification of paxillin tyrosine and serine residues is regulated by distinct mechanisms.

Paxillin phosphotyrosine can be detected at a minimal basal level in resting CTL but is much enhanced upon TCR stimulation. Both Src kinases and FAK/Pyk2 have been identified as potential kinases for paxillin (Schaller and Parsons 1995; Schaller and Schaefer 2001) and their modification of Tyr^{31} and Tyr^{118} is a critical step in focal adhesion turnover (Webb, Donais et al. 2004). Previous studies in T cells found interactions between paxillin and Lck and Pyk2 (Li and Earp 1997; Ostergaard, Lou et al. 1998) and I was able to detect an association with Fyn. Both serine and tyrosine phosphorylation of paxillin are sensitive to treatment with the Src kinase inhibitor PP2, but tyrosine phosphorylation could not be restored by PMA treatment as could serine phosphorylation. Serine and tyrosine point mutations of paxillin suggested that serine and tyrosine phosphorylation are not only separately regulated, but are independent of one another. By integrating my data with earlier studies I can propose a model in which

paxillin associates constitutively with Pyk2 and Fyn and inducibly with Lck and that the existence of this complex, upon TCR activation, facilitates the activation of Pyk2 by Src kinases and the tyrosine phosphorylation of paxillin. My studies in CTL have expanded upon existing knowledge and have characterized the regulation of paxillin phosphorylation by TCR signaling cascades. Paxillin is a target of two independent TCR signaling pathways: a PI3K-ERK pathway and a Fyn/Lck/Pyk2 pathway. The former is obligatory for paxillin serine phosphorylation; the latter for tyrosine phosphorylation (**see Figure 6-1**).

The association of paxillin with Lck, Fyn, and Pyk2 has interesting implications for TCR signaling and I attempted to determine whether paxillin was needed for optimal TCR activation. Using an siRNA-based knockdown approach, I was able to reduce paxillin expression by approximately 75%. Comparison between CTL transfected with either paxillin siRNA or a control showed no difference in their responses to anti-CD3 stimulation: global tyrosine phosphorylation and ERK activation are unperturbed by paxillin knockdown. In future this experiment may be repeated with target cells and low antigen or anti-CD3 concentrations to assess the effect suboptimal stimulation conditions. This is important as, in a normal immune response, pathogen-derived antigen peptides may be limited or may not be optimal for binding to a given TCR. The signaling strength may then be attenuated and may require the involvement of signaling-enhancing proteins not needed under the optimal conditions of anti-CD3 stimulation. So far, the association between Lck or Fyn and paxillin appears to occupy a place in the T cell activation program that is distinct from the TCR-proximal events of CD3 phosphorylation and Ras pathway initiation.

My biochemical examination of paxillin led to questions regarding the subcellular localization of paxillin in CTL and its relationship to the TCR and its binding partners. These questions formed the basis of the second main goal of my studies: to examine

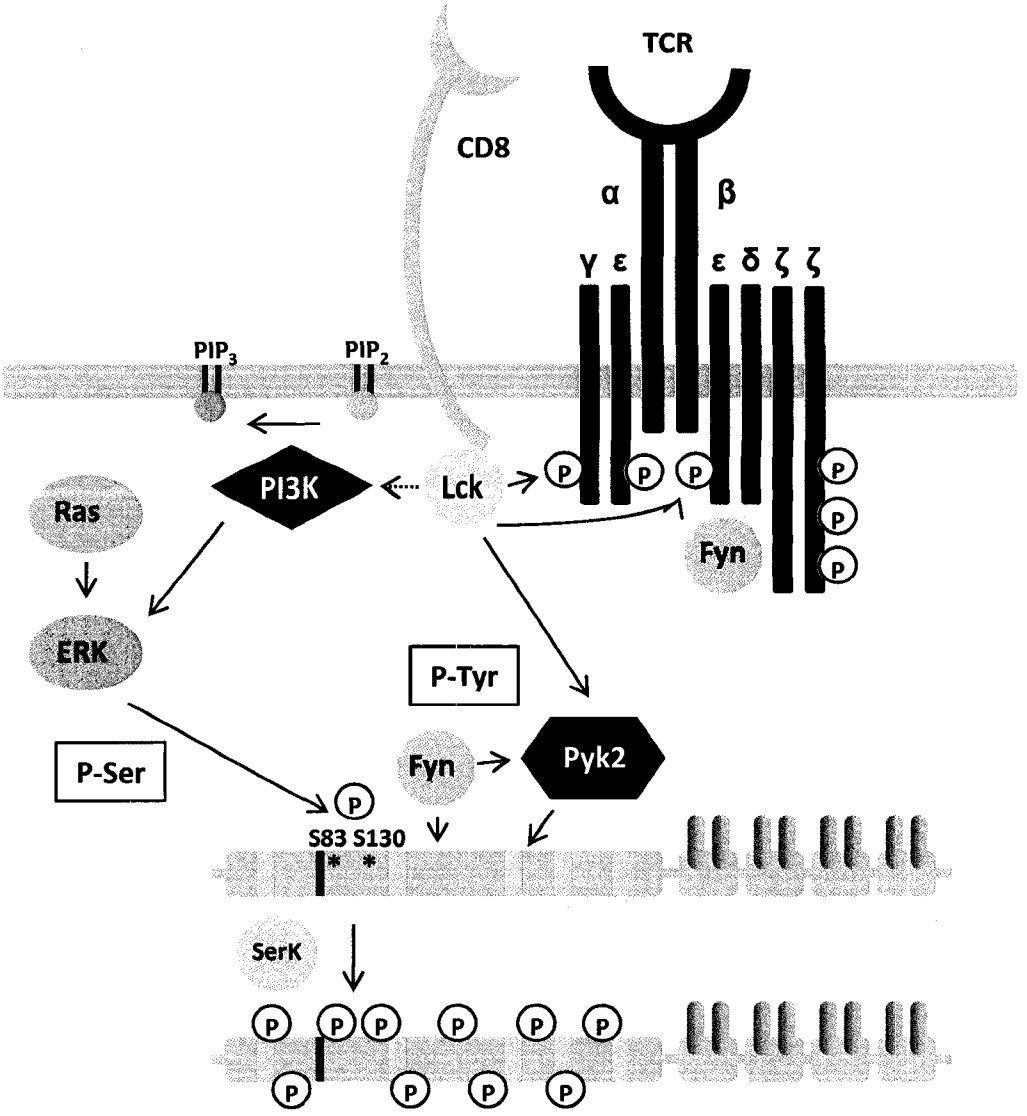

FIGURE 6-1. Model for the phosphorylation of paxillin in response to TCR activation. TCR engagement results in activation of Src kinases Lck and Fyn, which in turn activate Pyk2. These kinases phosphorylate tyrosine residues on paxillin. In parallel, the activation of ERK results in phosphorylation of serines 83 and 130, which initiates a cascade of serine phosphorylation by other kinases (SerK).

paxillin distribution in CTL by microscopy and to identify those domains which control its localization. I have shown that paxillin is located at the MTOC and microtubules and is enriched at the leading edge of crawling CTL and at the immune synapse between a CTL and target cell. Extensive mutagenesis of EGFP-paxillin identified the N-terminal LD region as containing the localization domain. No one LD domain is essential for microtubule and synapse binding but a segment encompassing LDs 2, 3, and 4 is sufficient for normal localization. Serine phosphorylation is dispensable for localization, as are tyrosines 31 and 118. My studies showed that at least two pools of paxillin exist in CTL, distinguished by their localization. The mechanism by which paxillin is translocated to the immune synapse was naturally of interest and the next series of experiments addressed the regulation of paxillin SMAC enrichment.

It was initially assumed that paxillin translocation to the SMAC of CTL was dependent upon TCR engagement, though this proved to be incorrect as conjugates formed between CTL and target cells lacking the allogeneic MHC needed to trigger a lytic response display an enrichment of paxillin at the contact point. A closer examination of the immune synapse between a CTL and a cognate target showed that paxillin is segregated into the peripheral SMAC with LFA-1. This result, together with my earlier observation that paxillin is concentrated at the leading edge of migrating CTL, suggested that paxillin was being actively recruited to sites of integrin adhesion. Further experiments presented the CTL with the anti-CD3 stimulus on a bead surface while integrin engagement was supplied by a non-cognate target cell. In this circumstance, paxillin was preferentially recruited to the target cell. Given the well-described link between paxillin and integrin regulation these results may indicate an analogous role for paxillin in T cells.

My experiments did not establish a function for paxillin in CTL killing but took important steps toward understanding paxillin regulation in this cell type. The results, when considered together with the literature, carry functional implications for paxillin, and these will be discussed in the following section.

B. Discussion of Potential Functions for paxillin in CTL

PAXILLIN AND CYTOLYTIC MECHANISMS

During the writing of this thesis, a paper was published showing that target cell killing by the NKL NK cell line was reduced after siRNA knockdown of paxillin (Li, Ge et al. 2008), a finding which is understandably very interesting to us and which implies the possibility of paxillin being involved in CTL degranulation. Unfortunately the publication in question provided no exact numbers for the decrease in killing (appears from the graph to be ~ 50% reduction with paxillin siRNA) nor for the percent of paxillin knockdown achieved. Only target cell killing was measured, and no supporting experiments were presented. The principal aim of the paper was to investigate the importance of JNK (c-Jun N-terminal kinase) in NK cell polarization and degranulation (those results will be discussed further below). The published outcome will first have to be reproduced in the CTL system. Assuming that CTL killing displays a similar dependence on paxillin, the next step will be to identify the stage(s) upon which paxillin acts. A few possibilities can be derived from my data: paxillin may be important for signaling leading to degranulation, for integrin adhesion and thus for conjugate formation and stabilization, or, at the microtubules, it may facilitate MTOC reorientation or granule trafficking. Based on its position in the pSMAC, it seems improbable that paxillin participates in the membrane-proximal process of granule fusion, and likewise there is no reason to suspect any involvement in the packaging of perforin or granzymes into the granules. In the following paragraphs, I will expand upon these possibilities as they relate to the literature and the results I have presented.

Signaling leading to MTOC polarization

Polarization of the MTOC is one process that is fundamental to cytotoxicity and in which paxillin might play a part, given its association with the microtubule cytoskeleton. In CTL and NK cells the translocation of the MTOC to the immune synapse is critical for the targeting and delivery of cytolytic granules (Stinchcombe, Majorovits et al. 2006). TCR signaling is necessary and sufficient for MTOC reorientation; examination of Jurkat T cells deficient in particular signaling molecules showed that intact ITAMs, Lck, ZAP-70, its substrate LAT, and the adaptor SLP-76 are all required (Lowin-Kropf, Shapiro et al. 1998; Kuhne, Lin et al. 2003). Cdc42 and its GEF (GTPase exchange factor) Vav are also involved in MTOC movement (Stowers, Yelon et al. 1995; Ardouin, Bracke et al. 2003). There remains much that is unknown about the physical mechanism by which MTOC repositioning occurs and how it is controlled by the TCR.

Pyk2, paxillin, and Src kinases such as Fyn are all familiar players in focal adhesion regulation and cell migration but in T cells their singular localization to the MTOC creates the potential for a novel function. Indeed, both Pyk2 and Fyn have been implicated in the signaling of MTOC reorientation (Sancho, Nieto et al. 2000; Martin-Cofreces, Sancho et al. 2006). Fyn is necessary for MTOC reorientation under sub-optimal conditions, but Fyn deficiency can be compensated for when stimulation is optimal (Martin-Cofreces, Sancho et al. 2006). Both Fyn and Pyk2 appear to regulate the MTOC via Vav and Cdc42 activation (Ren, Du et al. 2001; Martin-Cofreces, Sancho et al. 2006). As paxillin is an adaptor protein with no catalytic activity, it can be inferred that any involvement it may have in MTOC dynamics takes the form of recruiting relevant enzymes. It remains to be seen whether paxillin has any role in tethering Pyk2 and Fyn to the microtubules, or vice versa. My imaging of Fyn-deficient CTL clones showed normal paxillin distribution. I attempted to disrupt Pyk2 binding by deleting the LD2 and LD4 regions, but this was not successful as some Pyk2 remained bound to the

EGFP-ΔLD2/ΔLD4 paxillin mutant (see Chapter 4). Changes in the microtubule-associated pools of Fyn and Pyk2 might be monitored under condition of paxillin knockdown, but such experiments are challenging given the lack of a reliable method for microtubule purification and the difficulties of microscopic quantification.

Conjugate formation and detachment

Productive conjugates between CTL and target cells are formed and sustained by integrin adhesion regulated by inside-out signals originating at the TCR. Equally as important but much less studied is the disengagement of the CTL from the target cell as it withdraws to continue its search for antigen. I have demonstrated that paxillin membrane enrichment occurs independently of TCR signaling but this does not eliminate the possibility of paxillin acting as an inside-out regulator of integrins; though its localization is maintained its binding partners may vary according to its phosphorylation state and other factors determined by the TCR.

Inhibition of PI3K with wortmannin has been shown to delay conjugate formation and prevent signaling leading to degranulation (Fuller, Ravichandran et al. 1999; Robertson, Mireau et al. 2005). The pathway downstream of PI3K which governs these events is unknown. Paxillin is a candidate to be a component of this pathway as PI3K is needed for maximal paxillin serine phosphorylation (see Chapter 3). It is also worth noting that the prevention of degranulation that accompanies PI3K inhibition is not likely to be a result of the delay in conjugate formation, as anti-CD3 triggered degranulation does not involve LFA-1 but is sensitive to wortmannin.

Paxillin separation into the peripheral immune synapse could indicate either an association with integrin complexes or, as will be discussed in the following segment, with microtubule regulators, or both of the above. Without knowing which protein(s) is responsible for paxillin pSMAC distribution it is difficult to speculate about how paxillin

might influence conjugate formation. Only by knocking down paxillin and performing conjugate assays can we assess its role. Given that paxillin and its binding partners are primarily associated with focal complex detachment and turnover, it is possible that they are important for disintegration of pSMAC adhesion structures and thus for CTL-target dissociation. PTP-PEST, for example, has been shown to down-regulate B cell antigen receptor signaling and to promote the breakdown of focal adhesions (Angers-Loustau, Cote et al. 1999; Davidson and Veillette 2001). Furthermore, PTP-PEST and Fyn can act in opposition to regulate actin polymerization at the immune synapse of T cells. PTP-PEST phosphatase activity favours breakup of the SMAC and antagonizes TCR-induced actin polymerization (Badour, Zhang et al. 2004). Detachment is more difficult to measure than conjugate formation as it is an asynchronous process and cannot be measured using standard adhesion assays. Flow cytometry might be used to monitor conjugates over time, but as successful conjugates end in target cell death this may prove unfeasible.

The mechanics of MTOC reorientation

Proteins involved in the mechanics of MTOC reorientation have been identified, and though a complete picture has yet to materialize it is clear that the peripheral immune synapse plays a key role. Microscopic studies of CTL performed by the Poenie group illustrated that the MTOC is pulled toward the SMAC until it is in apposition to the membrane. Most of the microtubules spanning the distance between the MTOC and the target cell do not shorten, but rather bend laterally outward at the pSMAC to leave a conical space near the SMAC devoid of tubules (Kuhn and Poenie 2002) (see **Figure 1-2B**). The microtubule bends coincide with LFA-1 staining, while the MTOC itself oscillates within the margins of the cSMAC. MTOC reorientation proceeds in the absence of LFA-1 however, so it does not appear that the integrin is functionally involved (Lowin-Kropf, Shapiro et al. 1998; Combs, Kim et al. 2006).

An interesting 2006 publication, also from the Poenie group, demonstrated that the inside-out integrin signaling molecule ADAP is required for MTOC reorientation and furthermore identified the microtubule motor protein dynein as a component of the pSMAC (Combs, Kim et al. 2006). Fyn associates with and phosphorylates ADAP, resulting in its recruitment to the LAT adaptor in the membrane (da Silva, Li et al. 1997; Raab, Kang et al. 1999) and ultimately to integrin adhesion sites. Microscopy showed that ADAP is localized to the pSMAC, consistent with its presence in integrin complexes, but this localization occurred even in the absence of LFA-1. In resting Jurkat cells ADAP could also be seen at the MTOC and microtubules. Dynein, which effects MTOC movement in fibroblasts (Palazzo, Joseph et al. 2001), co-precipitated with ADAP and moved into the pSMAC in an ADAP-dependent manner (Combs, Kim et al. 2006). The data could be distilled into a model in which dynein at the pSMAC attaches to microtubules and "reels" in the MTOC, resulting in the sharp tubule bends observed at the pSMAC in the 2002 paper from the Poenie group.

The acetylation state of microtubules also appears to be a factor in MTOC reorientation as overexpression of the histone deacetylase HDAC6, capable of deacetylating α-tubulin, reduced MTOC movement and disturbed mature synapse formation in Jurkat (Serrador, Cabrero et al. 2004). The latter observation is interesting in its illustration of a link between microtubule dynamics and SMAC organization. The molecular mechanism underlying these observed effects remains uncharacterized. HDAC6, like ADAP and dynein, is a component of the pSMAC ring. It is clear that despite recent developments there remain gaps in our knowledge of how T cell MTOC polarization is executed and how it might affect seemingly unrelated events in the cell.

Whether paxillin participates in the redirection of the MTOC remains to be tested, though it is intriguing that proteins such as ADAP, dynein, and HDAC6 share the same subcellular distribution as paxillin. The pSMAC, long known to be a region of integrin adhesion, also appears to be a site of microtubule anchorage and regulation. It might be

that paxillin acts as a scaffold for Pyk2, Fyn, or any number of other relevant proteins. The enrichment of paxillin at the immune synapse is mediated by the same domains as microtubule localization; that is, LDs 2, 3, and 4 are sufficient but not absolutely required. The LIM3 domain of paxillin recruits the protein into nascent focal complexes (Brown, Perrotta et al. 1996); this difference in membrane-targeting domains, together with microtubule association in CTL, may indicate a functional divergence.

Future experiments will make use of paxillin siRNA knockdown to address whether deficiency of the adaptor protein affects MTOC reorientation. Unfortunately, knockout animals or cells are not available due to the embryonic lethality of paxillin deficiency. Varying TCR activation conditions such as a titration of anti-CD3/antigen concentrations will be applied to CTL. The EGFP-LD2-LD4 fragment, which localizes normally but has abbreviated protein-binding capabilities, may work as a dominant-negative and this possibility will also be explored. A more laborious task will be the characterization of pertinent binding partners should paxillin prove to play a role in microtubule dynamics. The large number of protein-binding domains in paxillin, the flexible nature of LD binding properties, and the tendency of paxillin to attract aggregate complexes such as the PKL-PIX-PAK complex are all factors that challenge such an endeavor.

Granule polarization

In CTL the microtubule network serves not only to mark the location of target cell binding but provides a physical scaffold for the movement of cytolytic granules (Burkhardt, McIlvain et al. 1993; Stinchcombe, Majorovits et al. 2006). CTL granules slide toward the MTOC-anchored minus ends of microtubules by virtue of dynein motors, but it is not known how the vesicles are attached to the tubules in the first place. Also, there appear to be additional regulatory steps as not all granules are polarized at once; some are held in reserve for subsequent target cells. This has made quantification of granule polarization particularly difficult in our CTL clones.

To date, only a few proteins have been implicated in granule polarization, one of them being the AP-3 (adaptor protein 3) adaptor. CTL clones derived from a patient with AP-3 deficiency (Hermansky-Pudlak syndrome type 2) are incapable of target killing due to defective microtubule granule transport (Clark, Stinchcombe et al. 2003). The established function of AP-3 is to sort transmembrane proteins into lysosomes, and the authors point out that although other proteins function alongside AP-3 and can readily compensate for its absence, it is possible that a granule component essential for trafficking is being incorrectly sorted.

Two paxillin kinases have been associated with granule transport in NK cells: ERK and JNK (Wei, Gamero et al. 1998; Li, Ge et al. 2008). ERK has also been shown to be required for CTL degranulation, but granule movement has not been verified as the ERK-regulated step in this system (Berg, Puente et al. 1998; Robertson, Mireau et al. 2005). JNK can be activated downstream of FAK and Rac1, and, once active, is a component of focal adhesions (Minden, Lin et al. 1995; Oktay, Wary et al. 1999; Almeida, Ilic et al. 2000). JNK phosphorylates Ser^{178} of paxillin to promote focal complex turnover and migration (Huang, Rajfur et al. 2003). In NK, JNK was also necessary for MTOC polarization (Li, Ge et al. 2008). Both ERK and JNK have been observed to bind to microtubules (Reszka, Seger et al. 1995; Morishima-Kawashima and Kosik 1996; Nagata, Puls et al. 1998). The JNK and ERK substrates responsible for their effects on degranulation are unknown, though we can assume them to be cytosolic or cytoskeletal proteins since the rapidity of target cell killing obviates the possibility of gene transcription and translation. Pinpointing the ERK-dependent step(s) in CTL degranulation may provide a clue as to its effector protein(s), but certainly paxillin and proteins associated with paxillin are viable candidates. There is no direct evidence to advocate paxillin involvement in microtubule trafficking of granules, but nevertheless its association with ERK and JNK is suggestive of such a role and may account for the observed effect of paxillin knockdown on NK cytotoxicity.

Defective granule transport and membrane fusion is a characteristic of several autosomal recessive immune deficiency diseases such as Griscelli syndrome type 2 (Rab27a mutation), familial hemophagocytic lymphohistiocytosis subtypes 3 and 4 (Munc 13-4 and syntaxin 11 mutations, respectively), Chediak-Higashi syndrome (mutation in Lyst, a lysosome sorting protein), and the aforementioned Hermansky-Pudlak syndrome type 2 (AP-3 mutation) (de Saint Basile and Fischer 2001). Many of these have accompanying mouse models. Such immunodeficiencies occurring in the human population are proof of the effectiveness of cytotoxicity as a means for eliminating compromised self cells, preserving immune homeostasis, and preserving the health of the organism.

PAXILLIN DISTRIBUTION AND TRAFFICKING

My work has determined that paxillin exists in at least two cellular pools: microtubule-associated and membrane-associated, but if and how the protein is cycled between these locations is the subject of conjecture. The imaging I have done is not sufficient to declare with certainty whether paxillin associates with the membrane in the absence of integrin clustering or whether paxillin exists free in the cytoplasm. Certainly these are both plausible situations.

Microtubule-bound paxillin may be relatively stagnant or it may cycle continuously into the cytoplasm. A preliminary result presented in Chapter 4 favours the latter: paxillin present in the microtubule-containing post-lysis pellet was exclusively the low-molecular weight, non-phosphorylated form. Given that serine phosphorylation is dependent upon ERK and that ERK can be associated with microtubules, it is tempting to propose a model in which paxillin phosphorylation by ERK on the microtubule scaffold is followed by its detachment, potentially freeing the protein for membrane recruitment (**Figure 6-2**). Taking advantage of my identification of Ser^{83} and Ser^{130} as key early

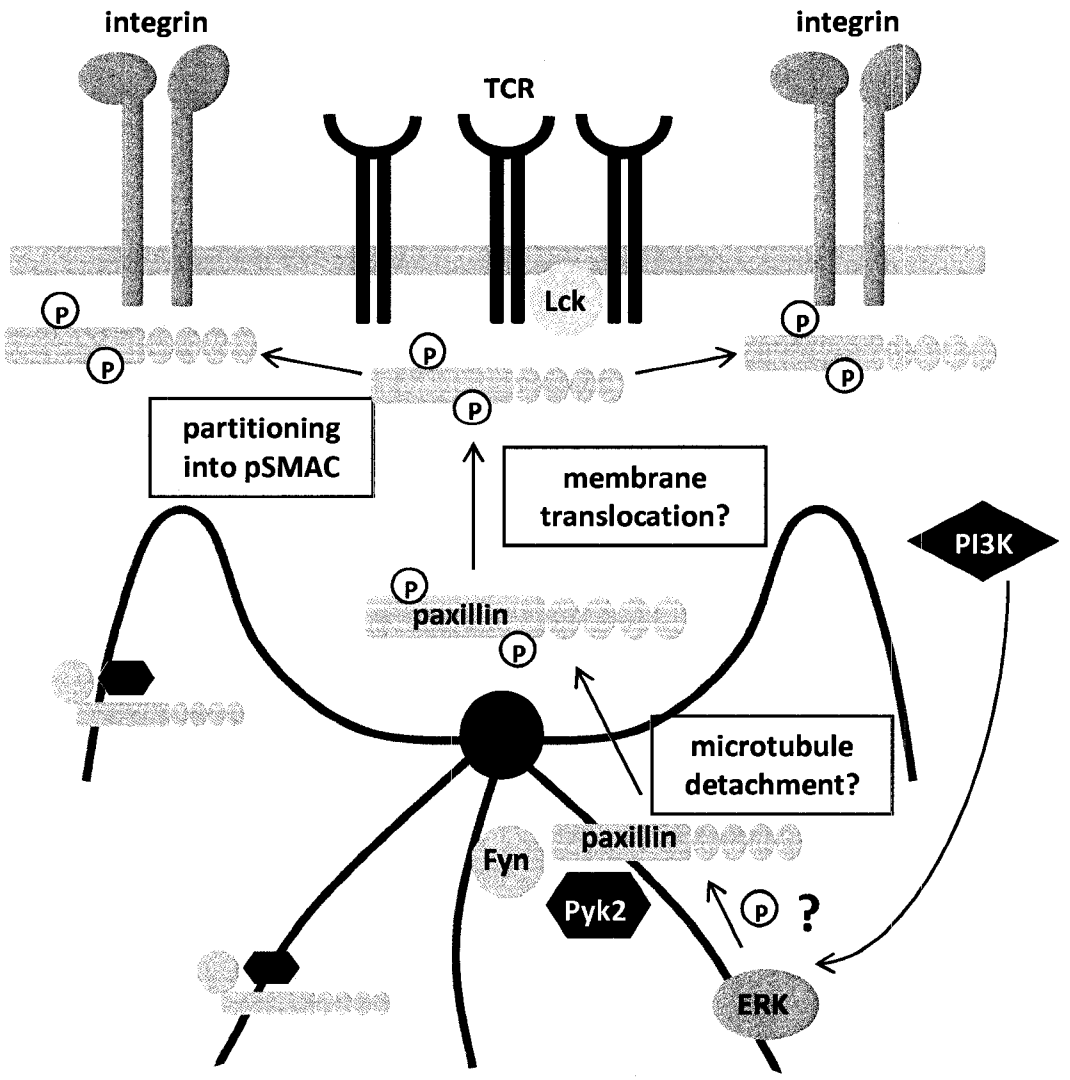

FIGURE 6-2. Potential model for paxillin dynamics in CTL. Paxillin at the microtubules, possibly complexed with Pyk2 and Fyn. TCR stimulation leads to PI3K and ERK activation, and the resulting serine phosphorylation of paxillin, which may or may not occur on the microtubule scaffold. Paxillin then translocates to the membrane, where it is eventually partitioned into the integrin-rich peripheral SMAC (red arrows). It remains unknown which proteins hold paxillin at the microtubules and bind to it at the pSMAC.

phosphoserine residues, a phosphomimetic S83D/S130D mutant of paxillin is in preparation which hopefully will be constitutively serine phosphorylated and allow me to determine whether microtubule association is affected. Live-cell photobleaching experiments may also be useful in observing the turnover rate of the paxillin at the microtubules.

In addition to focal adhesions of adherent cell types, paxillin is maintained in a peri-nuclear area and appears to cycle between the two sites. Whether paxillin is actively shuttled is unknown, though there is some evidence to suggest that the ARF (ADP ribosylation factor) GTPases may be involved in localizing paxillin. The ARF GTPases and the proteins that regulate them (ARF-GEFs and ARF-GAPs) are known for their roles in vesicular trafficking in the secretory and endocytic pathways and the sorting of vesicle contents (D'Souza-Schorey and Chavrier 2006). ARF1 has been shown to promote the movement of paxillin into focal contacts (Norman, Jones et al. 1998); however the mechanism is unknown as subcellular staining for paxillin has never indicated a vesicular distribution. Paxillin interacts with ARF-GAP proteins such as PKL and its family members Git1 and Git2 (Turner, Brown et al. 1999; Turner, West et al. 2001). An alternately spliced form of Git2, termed Git2short, is expressed mainly by immune cells and also appears to regulate aspects of paxillin localization (Mazaki, Hashimoto et al. 2001). Our understanding of ARF and ARF-GAP proteins as they relate to immune cell functions is limited; they are known to participate in phagocytosis, but whether they are involved in the trafficking of secretory lysosomes or other organelles such as LFA-1-containing vesicles is unknown.

C. Future Directions

In my study of paxillin in CTL, I have addressed questions of regulation and localization but few questions directly pertaining to function. Our ability to transfect CTL with plasmid DNA and siRNA oligos was acquired rather recently, and it remains a

challenge to obtain large numbers of transfected cells. Combining knockdown and transfection strategies in order to reconstitute cells treated with paxillin siRNA with paxillin mutants would allow many interesting experiments but at present such an approach is hindered by the low viability of transfected cells and the different time frames required for gene expression and knockdown. In this section I will discuss some experimental approaches that may prove to be fruitful in refining the model of paxillin regulation and localization and in identifying the function of paxillin in CTL.

It is evident that in order to elucidate paxillin function it is imperative to first establish the identity of its binding partners and a means of disrupting the associations by mutation. Given that the focus of our interest is T cells, the associations between paxillin and Fyn and Lck are particularly interesting. By serially mutating phosphotyrosine residues and testing for association with Lck by immunoprecipitation the SH2 binding site of Lck could be determined. The existing tyrosine mutants, Y31F, Y118F, and Y181F, have not yet been tested and other PY sites such as Tyr^{40} and Tyr^{88} could be mutated.

Pyk2 is known to bind to paxillin LD2 and LD4, though evidently in my system this is not the only means of association between the two proteins. The pitfall of working with a protein like paxillin, which possesses many protein-interaction sites, some of which are redundant, is that disruption protein binding by mutation can be difficult. My localization studies with LD mutants are a case in point. The question of whether Pyk2 might recruit paxillin to the microtubules or vice versa is still open. Our laboratory can reliably knock down Pyk2 with siRNA, and cells treated in this way could be examined for paxillin localization.

Given that paxillin appears to occupy a unique niche in T cells, it is not unreasonable to suppose that it may interact with a distinct group of proteins, and ultimately a more biochemical proteomics approach may be needed to identify the binding partner(s)

responsible for paxillin microtubule association. Comparison of binding proteins between wild-type or the ΔLD2-LD4 mutant from Chapter 4 (partially disrupted localization) may be done either by immunoprecipitation or by GST pull-down, though the latter is not always suitable for phosphorylation-based interactions. GST pull-downs were successful in identifying association of the PKL-PIX-PAK complex with LD4 (Turner, Brown et al. 1999).

Another avenue which might prove interesting but which is somewhat tangential from the work described here is the study of the paxillin family member leupaxin. Leupaxin is expressed by CTL and appears in paxillin immunoprecipitates (my own preliminary observation). Little is known of its function. Its localization pattern is identical to that of paxillin (my own observation) and examining its influence on paxillin function could be could be worthwhile both on its own merits and as a means of better understanding paxillin.

As discussed above, the importance of paxillin in CTL effector function will have to be addressed experimentally, especially in light of the recent result in NK cells (Li, Ge et al. 2008). There are many functional experiments that might be done to test different steps of CTL killing. It might be most logical to begin by testing overall target cell killing, as was done for NK cells. If the results are promising, we can pursue more specific assays to test conjugation, MTOC polarization and degranulation. More problematic to measure are granule polarization and target cell detachment. The functional experiments will then guide the ensuing lines of enquiry. Whether paxillin proves to regulate integrin adhesion, microtubule dynamics, or other functions entirely, it is likely to be a novel role not previously described for this protein, given the nature of CTL and the distinct distribution and regulation of paxillin in these cells. Pursuing a microscopic study of paxillin in CTL will ultimately, it is hoped, lead to an improved macroscopic understanding of CTL cytolysis as these lymphocytes work within the immune system to purge the body of compromised and infected cells.

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