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The role of p21-activated kinase in T cell receptor signaling

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The Role of p21-activated Kinase in T Cell Receptor Signaling

by

Gregory Michael Ku

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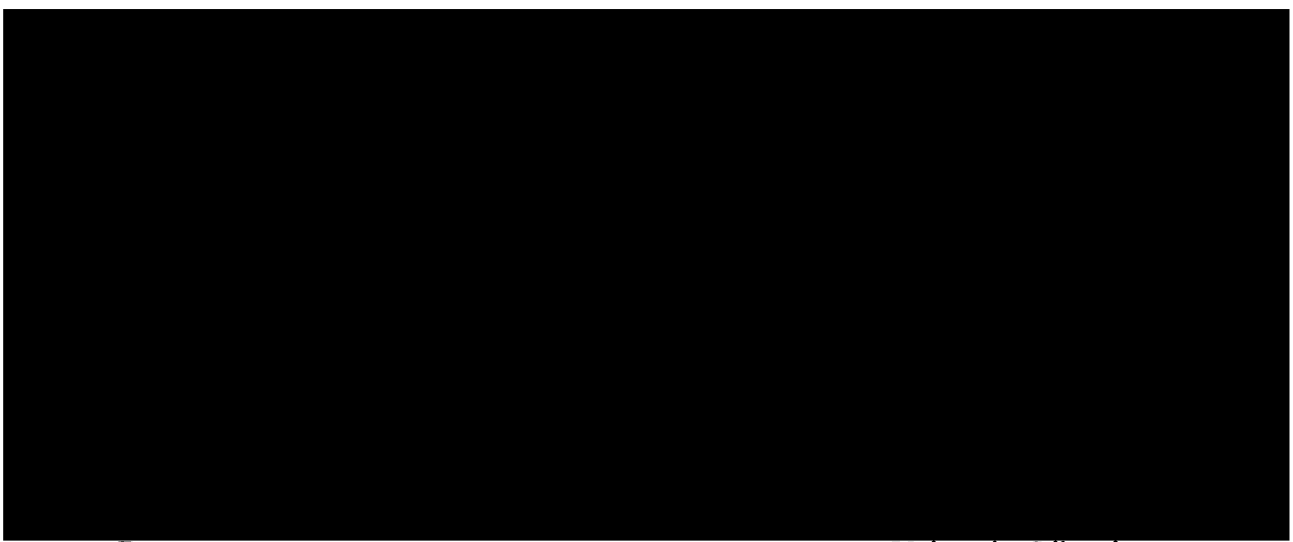

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Abstract:

p21-activated kinases (PAKs) play important roles in cytoskeletal rearrangement and signal transduction in mutiple cell types. In T cells, PAK1 is activated by T cell receptor (TCR) stimulation, but the pathway that activates PAK1 is poorly understood. We show that PAK1 is activated via a LAT- (linker of activated T cells), Slp-76-, Rac1-, and Nck-independent pathway. This pathway requires the Src and Syk family tyrosine kinases and a trimolecular complex composed of a Rac1/Cdc42 guanine nucleotide exchange factor, PIX (PAK interacting exchange factor), an Arf-GTPase activating protein, GIT, and PAK1. While overexpression of dominant negative PAK1 blocks the transcriptional activation of the nuclear factor of activated T cells (NFAT), exactly how PAK1 contributes to TCR signaling is not yet known. We show that expression of dominant negative PAK1 inhibits TCR signaling at the level of ZAP-70 tyrosine phosphorylation. Wild type PAK1 can augment ZAP-70 tyrosine phosphorylation in a heterologous system and PAK1 can serine/threonine phosphorylate ZAP-70 *in vitro*. Finally, PAK1 and ZAP-70 interact in 293T cells and in Jurkat T cells. We suggest that PAK1 and ZAP-70 might regulate each other in a positive feedback loop that is required for productive T cell activation.

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Chapter 1: Background

TCR signaling and the synapse:

Engagement of the T cell antigen receptor (TCR) triggers a complex network of signal transduction events that results in proliferation, cytokine production, and/or the acquisition of effector function. One of the most upstream nodes in this network involves the Src family kinases, Lck and Fyn. Although how receptor engagement precisely causes Src kinase activation is not known, activated Src kinases phosphorylate the immunoreceptor tyrosine based activation motifs (ITAMs) in the ζ and CD3γ, CD3δ, and CD3ε chains of the TCR. The phosphorylated ITAMs recruit the Syk family tyrosine kinases, ZAP-70 and Syk, via tandem src homology-2 (SH2) domains in their amino termini. The TCR-associated Src and Syk kinases are then able to effect, directly or indirectly, the tyrosine phosphorylation of multiple substrates (for review see(Kane et al., 2000)).

One critical substrate of the Src and Syk kinases is the transmembrane adaptor molecule LAT. The palmitoylation of LAT targets it to cholesterol-rich lipid rafts and is required for its function in the TCR signaling pathway(Lin et al., 1999a; Zhang et al., 1998b). Tyrosine phosphorylated LAT recruits additional proteins to the membrane such as Grb2 and phospholipase γ-1(PLCγ1) which are critical for the activation of the Ras and phosphatidylinositol signaling pathways (Lin and Weiss, 2001; Zhang et al., 1998a; Zhang et al., 2000). Indeed, LAT deficient Jurkat T cells are defective in the activation of both the Ras signaling pathway and the phosphatidylinositol signaling pathway (Finco et al., 1998; Zhang et al., 1999).

Yet another target of the Src and Syk kinases is the cytoplasmic adaptor Slp-76. Slp-76 can interact with multiple proteins including PLCγ1 (Yablonski et al., 2001),

Gads, LAT (via Gads) (Asada et al., 1999; Liu et al., 1999), Vav1(Wu et al., 1996), Lck(Sanzenbacher et al., 1999), Nck(Bubeck Wardenburg et al., 1998), HPK1(Sauer et al., 2001), and SLAP-130(Musci et al., 1997). Amidst this array of interactions, the precise mechanism of Slp-76 function in the context of TCR-mediated signaling remains unclear. However, a Slp-76 deficient Jurkat T cell does not optimally activate the Ras or phosphatidylinositol signaling pathways and both pathways can be reconstituted by re-expression of Slp-76(Yablonski et al., 1998b).

Besides the activation of the Ras pathway and phosphatidylinositol pathways, TCR engagement causes active reorganization of the T cell cytoskeleton. Within minutes of TCR engagement, filamentous-actin (F-actin) accumulates at the site of APC engagement and the microtubule organizing center (MTOC) reorients towards the contact site (Lowin-Kropf et al., 1998; Stowers et al., 1995). Although the contact site is initially a disorganized mixture of engaged LFA-1 and TCR complexes, the TCRs begin to coalesce into one or two clusters referred to as the c-SMAC (central supramolecular activation complex) while the LFA-1 molecules form a surrounding ring termed the p-SMAC (peripheral supramolecular activation complex). The formation of these structures collectively has been termed the immunological synapse (Monks et al., 1998) (reviewed by (Krummel and Davis, 2002)). These initial descriptions of the synapse, performed on fixed cells, are also seen by live imaging of a ζ - GFP fusion protein(Krummel et al., 2000) or of labeled MHC and ICAM-1 in artificial membrane bilayers(Grakoui et al., 1999). Besides TCR coalescence into a c-SMAC, others have described the clustering of lipid rafts at the site of TCR / APC engagement – apparently coincident with the c-SMAC(Viola et al., 1999). Futhermore, a simplified "immunological synapse" forms

between Jurkat T cells and coverslips coated with anti-TCR antibodies. As there is no LFA-1 ligand on these coverslips, there are no true c-SMACs or p-SMACs formed in this system. However, the Jurkat cells spread and polymerize actin at the outer boundary of the cell / coverslip interface(Bunnell et al., 2001).

The role of the immunological synapse in T cell activation is not clear. Although crosslinking the TCR with soluble antibodies elicits T cell activation without clear synapse formation, this mode of stimulation is clearly artificial. Since p-SMAC and c-SMAC formation takes approximately 20 minutes, whereas Lck and ZAP-70 tyrosine phosphorylation are observed in 1 minute, it would appear that c-SMAC formation is not required for Lck or ZAP-70 activation. Indeed, by staining T cell / APC couples, Lck and ZAP-70 tyrosine phosphorylation is undetectable by the time the mature immunological synapse forms(Lee et al., 2002). One caveat to this experiment is the possibility of low levels of ZAP-70 or Lck phosphorylation that are below the threshold of detection of the phospho-specific antibodies. Therefore, low levels of sustained tyrosine phosphorylation may yet depend on the synapse. In fact, T cell receptor engagement must be continuous for at least 2 hours in order to activate T cells(Lee et al., 2002; Weiss et al., 1987). Synapse formation may be required for sustained signaling over this time through signal amplification or signal discrimination by recruiting positive acting molecules or removing negative regulators from active centers of signaling (Jordan et al., 2000). In correlative support of the synapse as a signal discriminator, the ability to induce synapse formation correlates with the strength of the agonist peptide(Grakoui et al., 1999). Alternatively, the synapse may be important for effector functions such as the directed secretion of cytokines or cytotoxic products by helper or cytotoxic cells(Kupfer and

Dennert, 1984). Others believe that the synapse is important for TCR endocytosis(Lee et al., 2002).

The molecular machinery that directs synapse formation is not well understood. Both T cell receptor clustering and bulk membrane flow towards to site of APC engagement may depend on myosins (Wulfing and Davis, 1998). However, these studies rely on butanedione monoxime (BDM), a relatively non-specific inhibitor. Actin polymerization is also required as both latrunculin A and cytochalasin D block synapse formation(Krummel et al., 2000). In fact, the only substantive evidence for a particular molecule involved in synapse formation is for Vav1, a guanine nucleotide exchange factor for Rac1 and Cdc42. T cells from Vav1 deficient mice are only able to form immature, poorly condensed c-SMACs(Wulfing et al., 2000).

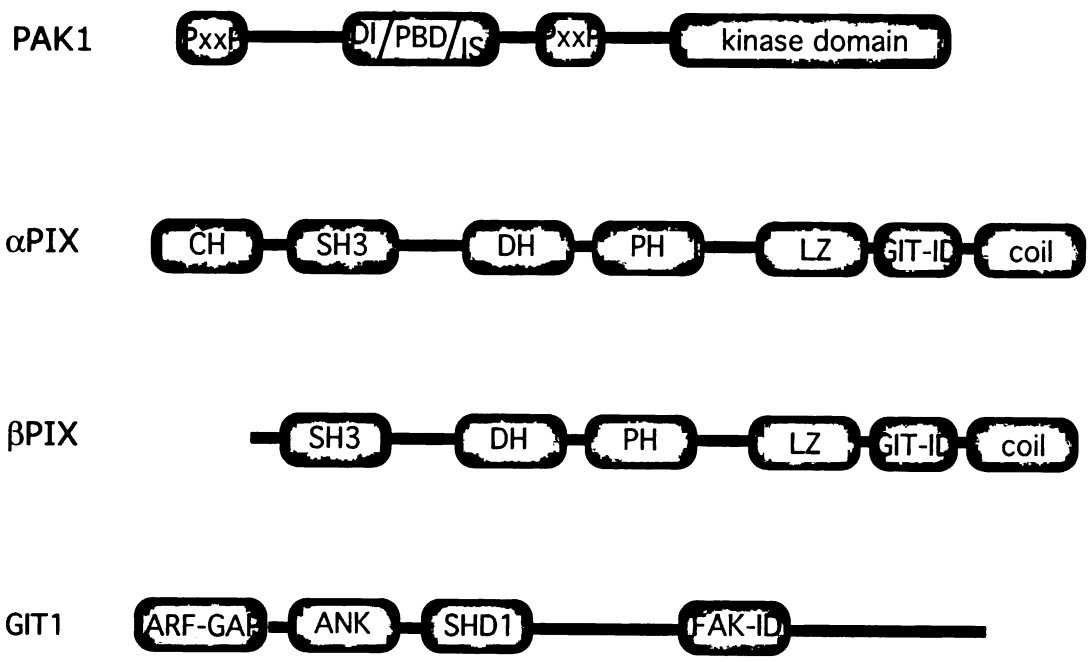
In Jurkat cell synapse formation with anti-TCR antibody coated coverslips, LAT is important for stable actin polymerization and spreading. Interestingly, in the LAT deficient J.CaM2 cells, an initial burst of actin polymerization is still seen(Bunnell et al., 2001). Among the Rho GTPases, Rho itself appears to have a unique role in the spreading of T cell on antibody coated coverslips. Expression of dominant negative Rho or C3 toxin, but not dominant negative Rac1 or dominant negative Cdc42, inhibits spreading of Jurkat cells on coverslips coated with antibodies to ITAM-containing chimeric receptors (Borroto et al., 2000). In contrast to spreading, MTOC reorientation of CD8 cells towards target cells is blocked by dominant negative Cdc42 but not dominant negative Rho or Rac1(Stowers et al., 1995). Like MTOC reorientation, Jurkat conjugate formation with Raji cells pulsed with superantigen is blocked by dominant negative Cdc42(Morgan et al., 2001).

Unlike the synapse, whose role in T cell activation is unknown, an intact actin cytoskeleton is known to be required for T cell activation. Actin depolymerizing agents such as latrunculin A or cytochalasin D inhibit calcium flux and transcriptional activation of the IL-2 promoter(Arrieumerlou et al., 2000; Fischer et al., 1998; Holsinger et al., 1998; Wulfing and Davis, 1998). Furthermore, T cells lacking the Wiskott Aldrich Syndrome protein (WASP), a critical activator of actin polymerization, have defects in TCR-induced F-actin accumulation, IL-2 production, and proliferation(Snapper et al., 1998). Notably, these drugs or genetic ablations block T cell activation in response to soluble antibody stimulations – a means of activation that does not require true synapse formation. Therefore, the actin cytoskeleton plays a role not only in synapse formation, but also in T cell activation independent of the synapse.

Vav

One molecule that is important for mature SMAC formation is Vav1 – a member of a family of Dbl guanine nucleotide exchange factors (GEFs) for Rho GTPases. Vav proteins contain not only the hallmark Dbl homology (DH) and plecktrin homology (PH) domains of Rho GEFs, but also an amino-terminal calponin homology domain, an acidic region, and an adapter-like C-terminus consisting of two src homology 3 (SH3) domains flanking a single SH2 (see figure 1). Whereas Vav1 is expressed exclusively in the hematopoietic system, Vav2 is ubiquitously expressed(Schuebel et al., 1996) and Vav3 is expressed in a number of different tissues(Movilla and Bustelo, 1999). In lymphocytes, Vav1 message is more highly expressed than Vav2 or Vav3 message (Bustelo, 2001). Besides being activated by the Syk and ZAP-70 family of tyrosine kinases, Vav proteins

Figure 1-1: Primary structures of PAK1 binding partners. PAK1, αPIX, βPIX, GIT1 are shown. PxxP = polyproline stretch; DI = dimerization segement; PBD = p21 binding domain; IS = inhibitory segment; CH = calponin homology domain; SH3 = src homology 3 domain; DH = Dbl homology domain; PH = pleckstrin homology domain; LZ = leucine zipper; GIT-ID = GIT1 interaction domain; coil = coiled-coil; ARF-GAP = Arf-GTPase activating protein domain; ANK = ankyrin repeats; SHD-1 = Spa2 homology domain; FAK-ID = FAK interaction domain.


PAK1
PxxP
PBD
PxxP
kinase domain
αPIX
CH
SH3
DH
PH
LZ
coil
βPIX
SH3
DH
PH
LZ
coil
GIT1
ANK
SHD1

are also activated by a multitude of other transmembrane receptors including tyrosine kinase receptors, receptors signaling through the Janus kinases (JAKs), and G protein-coupled receptors (reviewed by (Bustelo, 2000)).

The GEF activity of Vav proteins are regulated by tyrosine phosphorylation (Crespo et al., 1997). Vav1 GEF activity appears to be specific for Rac1, Rac2, and RhoG while the Vav2 and Vav3 appear to be specific for RhoA, RhoB, and RhoG(Crespo et al., 1997; Movilla and Bustelo, 1999; Schuebel et al., 1998). Structural nuclear magnetic resonance studies of Vav1 reveals an autoinhibitory loop which blocks binding of Rac to the catalytic Dbl domain(Aghazadeh et al., 2000). Phosphorylation of tyrosine 174 within this acidic inhibitory loop is predicted to release the inhibition of GEF activity, but other sites of regulation in the amino terminus are also possible (Katzav et al., 1989; Schuebel et al., 1998). In contrast to this structural prediction, recent mutagenesis studies indicate that tyrosine 174 and other sites in the acidic region of Vav may have inhibitory roles. In Jurkat cells, mutation of tyrosine 174 to phenylalanine causes increased GEF function and increased ability to activate NFAT (Nuclear Factor of Activated T cells), a critical transcriptional factor required for the activation of the IL-2 promoter(Billadeau et al., 2000; Kuhne et al., 2000; Lopez-Lago et al., 2000). However, this replacement of tyrosine with phenylalanine might simply disrupt the inhibitory function of the amino terminal regulatory domain, not necessarily implying a role for tyrosine phosphorylation of Y174 in negative regulation.

The ability of Vav1 to activate NFAT is not solely due to its GEF function. Overexpression of activated Rac1 or the DH-PH domains of Vav1 does not activate NFAT (Holsinger et al., 1998). Furthermore, oncogenic deletions of the amino terminus

of Vav1 that cause increased GEF activity towards Rac1 do not activate NFAT at all(Wu et al., 1995). Therefore, additional regulation of NFAT may come from the regulatory amino terminus as well as from the tandem SH3, SH2, SH3 domains in the carboxy terminus. The targets of this adapter (or GEF independent) function of Vav1 are not yet known (for review see (Bustelo, 2001)). Based on genetic data, others have also suggested that Vav1 controls two distinct pathways – one that controls calcium flux and one that controls receptor clustering. However, these knockout studies do not relate these two pathways to GEF dependent or independent functions of Vav1(Krawczyk et al., 2000).

Vav1 is critical for T cell development. Vav1 knockout mice have a defect in thymic positive and negative selection (Turner et al., 1997). The block in positive selection in the $CD4^-$ $CD8^-$ (double negative) stage can be rescued to a block in the $CD4^+CD8^+$ (double positive) stage by the transgenic expression of activated Rac1. These data show that the major function of Vav1 is to activate Rac1, at least in the double negative to double positive transition(Gomez et al., 2000). Formation of mature single positive T cells is not rescued by activated Rac1 expression since activated Rac1 expression alone blocks thymocyte development at the double positive stage. Therefore, these experiments cannot rule out Rac1-independent functions of Vav1 in double positive to single positive development or in the periphery. Biochemically, in double positive thymocytes, Vav1 is required for Itk kinase phosphorylation, PLCγ1 phosphorylation, inositol trisphosphate (IP3) production, and calcium flux. Also, Vav1 deficiency causes a defect in Akt phosphorylation and Rac1 activation by the TCR(Reynolds et al., 2002).

Vav1 is also critical for peripheral T cell function. Peripheral T cells from $Vav1^{-/-}$ mice have reduced IL-2 production, calcium flux, and actin capping, while Erk, JNK, PAK, p38, NF-kB pathways are normal (Fischer et al., 1998; Holsinger et al., 1998). One caveat to these observations is the possible compensation by other Vav family members or by other signaling pathways that may have allowed these cells to escape the thymus.

Rac1 in TCR signaling

Initial hints of the importance of the Rho family GTPase, Rac1, in TCR signaling came from its identification as a Vav1 substrate. Indeed, the overexpression of a dominant negative mutant of Rac1 (Rac1 N17), blocks TCR-induced IL-2 promoter activation in Jurkat cells(Genot et al., 1996). Detailed studies of the biochemical nature of this block reveal that Rac1 N17 or activated Rac1 (Rac1 V12) but not wild type Rac1 expression blocks the tyrosine phosphorylation of multiple substrates including ζ chain, ZAP-70, LAT, and PLCγ1. Looking downstream, calcium flux and IP3 production are modestly decreased by Rac1 N17 or Rac1 V12 expression. With regards to the cytoskeleton, Rac1 N17 reduces both basal and CD3-induced bulk F-actin content whereas Rac1 V12 increases both basal and CD3-induced bulk F-actin content(Arrieumerlou et al., 2000).

How either Rac1 N17 or Rac1 V12 blocks tyrosine phosphorylation of the TCR is not known. That both activating and inactivating alleles of Rac1 can produce the same phenotype might suggest that these constructs are non-specifically toxic to the signaling pathway. Indeed, Rac1 N17 blocks PMA and ionomycin induced IL-2 promoter activation albeit to a lesser extent than the blockade on TCR-induced IL-2 reporter

constructs(Genot et al., 1996). However, a second possibility is that cycling between GDP and GTP bound forms of Rac1 is required for the earliest events of T cell activation. Cycling appears to be important for Cdc42-mediated transformation in that Cdc42 V12 does not transform but a fast cycling mutant of Cdc42 or an oncogenic Cdc42 GEF do(Lin et al., 1999b). Exactly how Rac1 cycling might be required is unclear but the mechanism probably does not involve actin polymerization since actin depolymerizing drugs do not affect tyrosine phosphorylation of the TCR or the tyrosine phosphorylation of downstream signaling proteins(Holsinger et al., 1998).

Besides actin, another specific target of Rac1 in T cells is Akt. Rac1 V12 activates Akt in Jurkat cells. Furthermore, Rac1 N17 blocks TCR-induced activation of Akt. This activation is dependent on phosphatidyl inositol-3 kinase (PI-3 kinase) as Rac1-induced Akt activation is blocked by the pharmacological inhibitors of PI-3 kinase LY294002 and wortmannin (Genot et al., 2000). These data placing Rac1 upstream of Akt are consistent with the defect in Akt activation and Rac1 activation in Vav1 knockout thymocytes.

Whereas Rac1 knockout mice die in utero due to a failure in gastrulation(Sugihara et al., 1998), Rac2 knockout mice are viable. In part, this may be due to the fact that although Rac1 is ubiquitous, Rac2 is only expressed in hematopoietic cells. The primary sequence of Rac2 is highly similar to that of Rac1 (92% identical) with differences occurring in the hypervariable region at the C-terminus of the protein. These modest differences are thought to confer distinct substrate specificities(Didsbury et al., 1989).

Despite these small differences, Rac1 cannot compensate fully for the loss of Rac2 in a number of cell types. Rac2 knockout animals have defects in the neutrophil compartment with regards to chemotaxis, superoxide production, and L-selectin binding.

Rac2 knockout bone marrow derived mast cells have reduced survival and almost complete absence of stem cell factor-induced Akt phosphorylation, again confirming a role for Rac proteins upstream of Akt. Unfortunately, PI-3 kinase activity from the mast cells was not convincingly measured. Rac2 knockout cells have decreased phosphorylation of Bad (an Akt target) and unexpectedly have 10 fold higher levels of Bad and lower levels of Bcl-XL. Levels of Bcl-2 were not affected(Yang et al., 2000). In T cells, Rac2 deficiency causes mildly reduced proliferation, modestly decreased activation of Erk, p38 and calcium flux(Yu et al., 2001). The relevant Rac effectors to explain these phenotypes, apart from Akt, are not known but could include the p21-activated kinase (PAK) or the actin cytoskeleton.

The two GEF family members, αPIX and βPIX

Another family of GEFs for Rac1 are the PAK interacting exchange factors (PIXs). Appropriately named, PIXs use an amino terminal SH3 domain to bind to a polyproline sequence in PAK. This SH3/polyproline interaction is unusual in sequence and also in its high affinity(Manser et al., 1998). Like Vav1, both PIX family members (αPIX and βPIX) contain the tandem DH and PH domains characteristic of Rho GEFs. Like Vav1, αPIX (but not βPIX) also contains an amino terminal calponin homology domain. Unlike Vav1, αPIX and βPIX contain a region for binding to the GIT1 family of proteins (Zhao et al., 2000a), a potential coiled coil region which appears to be important in localization to the membrane(Koh et al., 2001), and a leucine zipper which is required for homodimerization (Kim et al., 2001) (see figure 1). In HeLa cells, βPIX localizes to focal complexes and the overexpression of βPIX with a mutation in the SH3 domain

(W43P, W44G) prevents wild type PAK1 from localizing to these focal complexes. Conversely, a PAK1 allele which does not bind PIX (P191G, R192A) also fails to localize to focal complexes(Manser et al., 1998). However, PAK1 kinase activity negatively regulates this association by autophosphorylation of residues adjacent to the PIX-binding polyproline sequence in PAK1(Zhao et al., 2000b).

Whereas neither αPIX nor βPIX knockout mice have been described, a mutation in αPIX has been described in human patients with an X-linked form of mental retardation. The mutation causes skipping of exon 2 resulting in the loss of 28 amino acids in the calponin homology domain(Kutsche et al., 2000). Suggestive that PIX function in the same pathway as PAK, PAK3 deficiency is also associated with mental retardation in humans (Allen et al., 1998; Bienvenu et al., 2000). Drosophila PIX is necessary along with dPAK for the proper formation of post-synaptic densities(Parnas et al., 2001). A role for PIX has not been defined in the immune system.

GIT

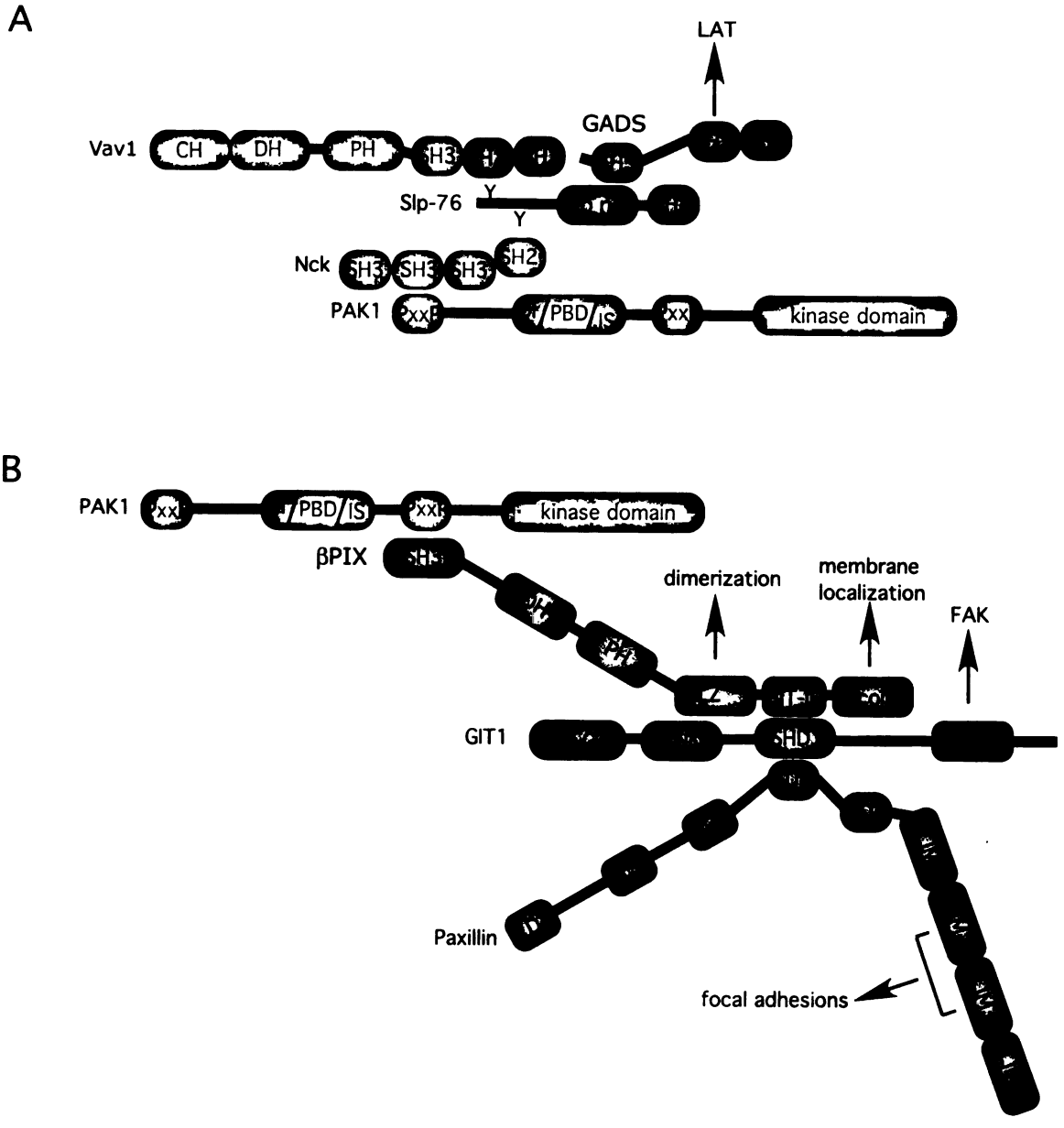
The recruitment of the PAK / PIX complex to focal adhesions requires the GIT family of proteins. GIT proteins, which currently include GIT1, GIT2, and PKL, contain an amino terminal ARF-GTPase activating protein (Arf-GAP) domain, four ankyrin repeats, an SHD-1 (Spa2 homology domain) and a carboxy terminal paxillin binding site (PBS2) (see figure 1). By sequence homology, there is an is an additional paxillin binding site within the ankyrin repeats whose functionality is controversial(Mazaki et al., 2001; Premont et al., 2000). GIT1 and PKL simultaneously bind to PIX and to paxillin, an adapter protein which localizes to focal complexes(Turner et al., 1999). However, it is

controversial whether GIT1 recruits PAK and PIX to focal complexes or if PIX activates GIT1 binding to paxillin(Zhao et al., 2000a). Although GIT2 is very similar to GIT1, it does not bind to paxillin(Premont et al., 2000). In addition, GIT1 can bind to focal adhesion kinase (FAK) and this can occur simultaneously with PIX binding(Zhao et al., 2000a). For a summary of these interactions, see figure 2B.

Although GIT1 links paxillin to PIX and PAK, GITs were first cloned as GRK (G protein coupled receptor kinase) interactors and can prevent internalization of G-protein coupled receptors via their Arf-GAP activity. ADP-ribosylation factors (ARFs) are small G proteins that regulate membrane and vesicle traffic. Arf-GTPase activating proteins like GIT1 have been implicated recently in the regulation of focal adhesions (reviewed by (Turner et al., 2001). Additionally, GIT1 overexpression prevents downregulation of G-protein coupled receptors that utilize clathrin coated pits and are sensitive to dominant negative β-arrestin and dynamin mutants(Claing et al., 2000; Premont et al., 1998). *In vitro*, the Arf-GAP activity of GIT proteins appears to be fairly promiscuous among Arf family members (Vitale et al., 2000), however, more indirect *in vivo* experiments suggest that Arf1 is the primary target of the GIT1 Arf-GAP activity(Mazaki et al., 2001).

The link between GIT1's role as an adapter between PIX and paxillin and its ARF-GAP activity is not yet clear. The overexpression of a short alternative splice form of GIT2 (GIT2-short) that lacks the carboxy terminal PBS2 paxillin binding site causes redistribution of perinuclear paxillin to the cell periphery and disruption of focal adhesions. This activity is dependent on the Arf-GAP domain of GIT2-short(Mazaki et al., 2001). Interestingly, GIT2 short appears to be expressed preferentially in immune cells(Premont et al., 2000). Unlike GIT2 short, the activity of the full length GIT1 may

Figure 1-2: Complexes of PAK1 and its binding partners (A) Putative Vav1, Slp-76, GADS, Nck, PAK1 complex. Note that Vav1, Slp-76, and Nck have been co-immunoprecipitated, Nck and PAK1 have been co-immunoprecipitated, and LAT, Slp-76, and Vav1 have been co-immunoprecipitated. (B) Putative PAK1, PIX, GIT1, Paxillin, and FAK complex. Note that only PAK1, PIX, and GIT1 have been co-immunoprecipitated in Jurkat cells. PAK1, PIX, GIT1, and Paxillin have been co-immunoprecipitated and GIT1 and FAK have been co-immunoprecipitated in non-immune cells.


A
LAT
GADS
Vav1
CH
DH
PH
SH3
Slp-76
Y
Y
Nck
SH3
SH3
SH3
SH2
PAK1
PBD
kinase domain
B
PAK1
PBD
kinase domain
βPIX
dimerization
membrane localization
FAK
GIT1
Paxillin
focal adhesions

not completely depend on its Arf-GAP activity. In NIH3T3 cells, overexpression of full length GIT1 causes loss of paxillin, but not vinculin or phosphotyrosine from focal complexes. This activity mapped to the FAK binding site at the carboxy-terminus of GIT1 and not the Arf-GAP domain(Zhao et al., 2000a).

p21 activated kinases

The p21 activated kinases are serine/threonine kinases of the STE20 family. There are currently six PAK family members that are classified into Group I PAKs (PAK1, PAK2, PAK3) and Group II PAKs (PAK4, PAK5, and PAK6). While PAK2 and PAK4 mRNAs are ubiquitous, PAK1 mRNA is present in brain, muscle, and spleen. PAK3 mRNA is only present in brain. PAK5 appears also to be brain specific while PAK6 is expressed highly in brain, testis, prostate, kidney and placenta. The PAKs are characterized by an amino terminal regulatory region containing a PBD (p21 binding domain) and two polyproline stretches followed by a carboxy terminal kinase domain(see figure 1). The primary structure of the group I PAKs are quite similar to each other but are only about 40% identical to the group II PAKs. These differences are significant in the kinase domains, suggesting that substrates for group I and group II PAKs may be quite different(Jaffer and Chernoff, 2002).

Many group I PAK substrates have been identified. These include: myosin II (Zeng et al., 2000), myosin light chain kinase(Goeckeler et al., 2000), LIM kinase-1 (Edwards et al., 1999), Raf-1 (King et al., 1998; King et al., 2001; Sun et al., 2000), MEK1, Bad (Eblen et al., 2002; Schurmann et al., 2000; Wolf et al., 2001), desmin (Ohtakara et al., 2000), and vimentin(Goto et al., 2002).

As is clear from this diverse list of substrates, PAK1 participates in multiple pathways. PAK1 phosphorylation of Bad promotes survival(Schurmann et al., 2000). PAK1 phosphorylation of S338 in Raf-1 (at least in response to certain growth factors) is required for cell survival (King et al., 1998; Sun et al., 2000). PAK1 phosphorylation of MEK1 is required for Raf-1-mediated MEK1 activation(Frost et al., 1997) and MEK1's ability to couple to Erk2(Eblen et al., 2002). Overexpression of active PAK1 causes loss of stress fibers and focal adhesions in HeLa or fibroblasts (Frost et al., 1998; Manser et al., 1997). Overexpression of dominant negative PAK1 blocks endothelial cell motility (Kiosses et al., 1999), macropinocytosis (Dharmawardhane et al., 2000) and angiogenesis(Kiosses et al., 2002).

Just as some substrates for PAK1 have not yet been validated *in vivo*, the targets responsible for many PAK-associated phenomena are not yet known. In Drosophila, dPAK is important for axon guidance (Hing et al., 1999; Newsome et al., 2000) although the relevant targets are not known. Constitutively active PAK activates the NF-κB pathway and dominant negative PAK blocks LPS-induced NF-κB activation. IKKα and IKKβ are not affected(Frost et al., 2000) and the relevant substrate(s) is not known.

The mechanism of group I PAK activation has become quite a bit clearer due to the cystallization of the PAK1 kinase domain with a portion of the amino terminus (70-149). This amino terminal fragment (70-149), termed the autoinhibitory segment, is sufficient to inhibit PAK kinase activity in trans(Zhao et al., 1998). The crystal structure shows a kinase inhibitory segment (126-149) positioned in the cleft between the amino and carboxy kinase lobes. It is stabilized in this region by an inhibitory switch region (87-136) which overlaps with the PBD (70-113). Similar to the regulation of WASP, binding

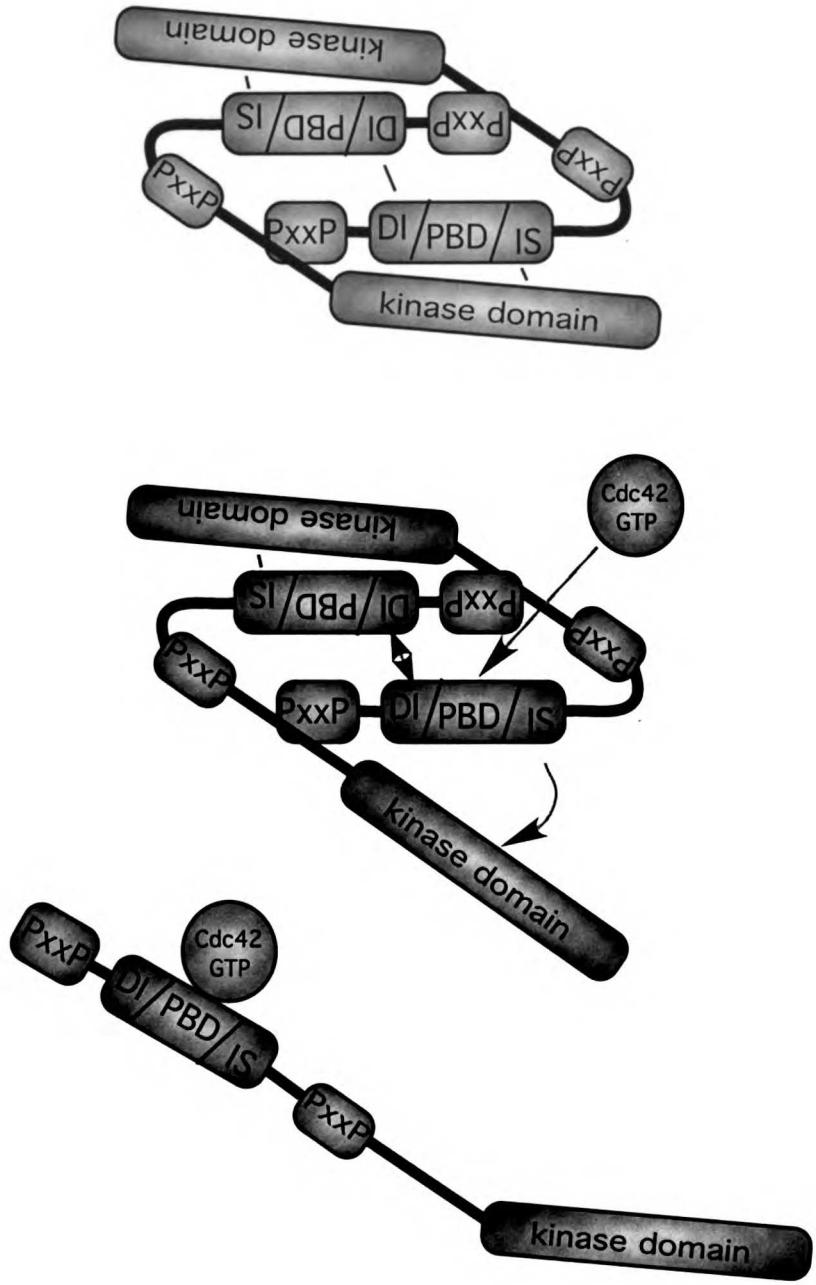

kinase domain
DI/PBD/IS
PxxP
PxxP
PxxP
DI/PBD/IS
kinase domain
Cdc42
GTP
kinase domain
DI/PBD/IS
PxxP
PxxP
PxxP
DI/PBD/IS
kinase domain
PxxP
Cdc42
GTP
DI/PBD/IS
PxxP
kinase domain

Figure 1-3: Schematic of PAK1 activation. (Top) Inactive PAK1 inhibits its homodimeric partner *in trans* via the inhibitory segment (IS). Dimerization is mediated by the dimerization segment (DI). (Middle) Binding of activated Cdc42 or Rac1 to the p21 binding domain (PBD) disrupts the IS and the DI. (Bottom) Monomeric PAK1 is then active.

of GTP-bound Cdc42 or Rac disorders the inhibitory switch and causes the kinase inhibitory segment to pull out of the kinase domain(see figure 3). Interestingly, this partial PAK1 in the crystal structure and full length PAK1 in 293T cells is detected as a dimer (Lei et al., 2000). Dimerization is mediated by a dimerization segment located at the amino terminus of the autoinhibitory segement. Rational mutagenesis of the contact surfaces between the kinase domain and the inhibitory switch demonstrates that PAK dimers are inhibited in trans. However, dimers of PAK1 do not trans phosphorylate when activated by binding to GTP-bound Cdc42(Parrini et al., 2002).

The regulation of the group II PAKs is distinct from that of the group I PAKs. Although group II PAKs bind to GTP-bound Cdc42, their catalytic activities do not appear to be enhanced by this binding. Consistent with this observation is the lack of the autoinhibitory segment and dimerization domain in the group II PAKs (reviewed by (Jaffer and Chernoff, 2002)).

Even for the group I PAKs, simple binding of activated Rac or Cdc42 to cause activation may be an oversimplification. In adherent cells that are kept in suspension, growth factors can stimulate GTP loading but not membrane localization of Rac1 or activation of PAK1. Adhesion or forced membrane localization restores Rac1 induced PAK1 activation(del Pozo et al., 2000). Therefore, adhesion contributes an additional signal which is required for coupling of Rac1 to PAK1.

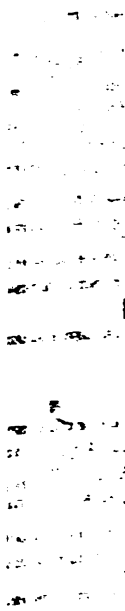
Besides the requirement for membrane localization of Rac1 and Cdc42 to the membrane, PAK1 activation may also require threonine phosphorylation at position 423 in its activation loop. PAK1 immunoprecipitated from cells and phosphatase treated *in vitro* cannot autophosphorylate at T423. PDK1 is one candidate kinase that can

phosphorylate this site in PAK1(King et al., 2000). Taken together with the crystallization and mutagenesis studies, these data suggest that PAK1 may require binding of Rho GTPases and PDK1 to become catalytically activated. Alternatively, T423 is constitutively phosphorylated in trans-inhibited PAK1 and when active Rac or Cdc42 releases the trans-inhibition, PAK1 becomes fully active.

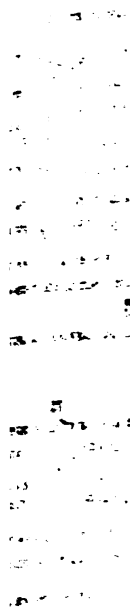
There is some evidence suggesting a Rho GTPase independent mechanism for PAK1 activation. Activated Ras (Ras V12) or activated PI-3 kinase (p110 CAAX) induced PAK1 activation could not be blocked by co-expression of Rac1 N17. However, it could be blocked by overexpression of kinase dead Akt (Tang et al., 2000). Unfortunately, these data rely exclusively on super-physiological signals to activate PAK1 and other studies do not confirm the Rac/Cdc42 independence of Ras-induced activation of PAK1. Finally, Ras mediated activation of PAK1 is not a general phenomenon in that it does not occur in Jurkat cells or in NIH3T3 cells (Tang et al., 1999; Yablonski et al., 1998a).

In T cells, PAK1 associates with the cytoplasmic adaptor Nck and becomes catalytically activated after TCR engagement (Bubeck Wardenburg et al., 1998; Yablonski et al., 1998a). Furthermore, a dominant negative allele of PAK1 blocks the TCR-induced activation of Erk, a downstream effector of the Ras pathway, and of NFAT. The activation of Rac1/Cdc42 and PAK1 is believed to require the association of Vav1, Slp-76, Nck, and PAK1 (Bubeck Wardenburg et al., 1998) (see figure 2A). In a model to explain these observations, Slp-76 recruits Vav1 to a complex containing Nck and PAK1. Vav1 catalyzes GTP loading of Cdc42 or Rac1 which, in turn, activates the Nck-associated PAK1.

Although these interactions suggest a relatively cogent model for the TCR-induced activation of PAK1, there is a relative lack of functional data to confirm this model. Using somatic mutants of the Jurkat T cell line that lack critical components of the TCR signaling pathway, we set out to confirm the requirements for PAK1 activation by the TCR. We also set out to extend studies of dominant negative PAK1 that had showed previously that blocking PAK1 activation potently inhibited NFAT reporter activation by the TCR. We found that PAK1 is activated via a novel LAT, Slp-76, Nck, and Rac1 independent pathway and that ZAP-70 is a potential target of PAK1 function in T cells.



Chapter 2: A PAK1-PIX-PKL complex is activated by the T-cell receptor independent of Nck, Slp-76 and LAT.



Introduction:

Although it is known from the overexpression of dominant negative mutants that PAK1 and Rac1 are important in the TCR signaling pathway, how these molecules are activated is poorly understood. PAK1 activation by the TCR is downstream of the Src kinase Lck. Neither phorbol esters plus calcium ionophores nor the G protein-coupled muscarinic receptor can activate PAK1 indicating that PAK1 is activated upstream of the phosphatidyinositol pathway and the Ras pathway. Given that PAK1 is known to be activated by Rac1 in overexpression systems and that the activation of Rac1 depends on Vav1 tyrosine phosphorylation, many have hypothesized a Vav1 → Rac1 → PAK1 pathway. If one assumes that Vav1's tyrosine phosphorylation is an indicator of its activity, then Vav1, Rac1, and PAK1 are predicted to require LAT, as Vav1 tyrosine phosphorylation is decreased in the LAT deficient J.CaM2 cells. The complex of Vav, Slp-76, and Nck would predict that, in addition to LAT, Slp-76 would also be required for Vav1 phosphorylation and activity. However, the Slp-76 deficient J14 Jurkat clone does not appear to have decreased Vav1 phosphorylation. In order to resolve these conflicting data on the activation of this putative Rho GTPase pathway, we set out to measure the activation of PAK1 and Rac1 directly using biochemical assays.

As expected, the activation of Rac1 by the TCR requires the transmembrane adapter LAT. The cytoplasmic adapter Slp-76 is not required for Rac1 activation – demonstrating that a Vav1 / Slp-76 complex is dispensible for Vav1 and Rac1 activation. Surprisingly, PAK1 can be activated in the absence of LAT as well as Slp-76. These data indicate that PAK1 might function independently of Rac1 and that the TCR activates PAK1 utilizing a unique LAT- and Slp-76- independent pathway.

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A PAK1–PIX–PKL complex is activated by the T-cell receptor independent of Nck, Slp-76 and LAT

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Given the importance of the Rho GTPase family member Rac1 and the Rac1/Cdc42 effector PAK1 in T-cell activation, we investigated the requirements for their activation by the T-cell receptor (TCR). Rac1 and PAK1 activation required the tyrosine kinases ZAP-70 and Syk, but not the cytoplasmic adaptor Slp-76. Surprisingly, PAK1 was activated in the absence of the transmembrane adaptor LAT while Rac1 was not. However, efficient PAK1 activation required its binding sites for Rho GTPases and for PIX, a guanine nucleotide exchange factor for Rho GTPases. The overexpression of βPIX that either cannot bind PAK1 or lacks GEF function blocked PAK1 activation. These data suggest that a PAK1–PIX complex is recruited to appropriate sites for activation and that PIX is required for Rho family GTPase activation upstream of PAK1. Furthermore, we detected a stable trimolecular complex of PAK1, PIX and the paxillin kinase linker p95PKL. Taken together, these data show that PAK1 contained in this trimolecular complex is activated by a novel LAT- and Slp-76-independent pathway following TCR stimulation.

Keywords: PAK/PIX/PKL/signal transduction/TCR

Introduction

Specific T-cell recognition of antigen triggers a cascade of diverse intracellular signaling events that includes protein phosphorylation, lipid hydrolysis, intracellular calcium flux, lipid phosphorylation, small GTPase activation, cytoskeletal rearrangements and many others. This complex series of events is required for productive T-cell activation as defined by either T-cell proliferation, differentiation or activation of effector functions (reviewed by van Leeuwen and Samelson, 1999; Kane *et al.*, 2000). Among the earliest events in T-cell receptor (TCR) signal transduction is the activation of the Src tyrosine kinase family members Lck and FynT, which directly phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in the ζ and CD3γ, δ and ε chains. The phosphorylated ITAMs recruit the Syk family tyrosine kinases ZAP-70 and Syk via tandem SH2 domains in their N-termini. The TCR-associated Src and Syk kinases are then able to effect, directly or indirectly, the tyrosine phosphorylation of multiple substrates.

One critical substrate of the Src and Syk kinases is the transmembrane adaptor molecule LAT. The palmitoylation of LAT targets it to cholesterol-rich lipid rafts and is required for its function in the TCR signaling pathway (Zhang *et al.*, 1998b; Lin *et al.*, 1999). Tyrosine-phosphorylated LAT recruits additional proteins to the membrane such as Grb2 and PLCγ1, which are critical for the activation of the Ras and phosphatidylinositol signaling pathways (Zhang *et al.*, 1998a). Indeed, LAT-deficient Jurkat T cells are defective in the activation of both the Ras signaling pathway and the phosphatidylinositol signaling pathway (Finco *et al.*, 1998; Zhang *et al.*, 1999).

Yet another target of the Src and Syk kinases is the cytoplasmic adaptor Slp-76. Slp-76 can interact with multiple proteins including PLCγ1 (D.Yablonski, manuscript in preparation), Grb2 (Jackman *et al.*, 1995), Gads, LAT (via Gads) (Asada *et al.*, 1999; Liu *et al.*, 1999), Vav1 (Wu *et al.*, 1996), Lck (Sanzenbacher *et al.*, 1999) and Nck (Bubeck Wardenburg *et al.*, 1998). Amidst this array of interactions, the precise mechanism of Slp-76 function in the context of TCR-mediated signaling remains unclear. However, it is clear from the study of a Slp-76-deficient Jurkat T cell that Slp-76, like LAT, is required for the optimal activation of the Ras and phosphatidylinositol signaling pathways (Yablonski *et al.*, 1998b).

While both Ras activation and calcium flux are necessary for T-cell activation, Rho family GTPases (Rac1, Cdc42 and Rho) also appear to play an important role. The expression of a dominant-negative Rac1 allele inhibits TCR-induced interleukin-2 (IL-2) promoter activation (Genot *et al.*, 1996) and dominant-negative Cdc42 blocks T-cell polarization towards antigen presenting cells (Stowers *et al.*, 1995). Furthermore, Vav1, a guanine nucleotide exchange factor (GEF) for Rac1 and Cdc42, is required for efficient positive and negative selection of T cells. In mature T cells, Vav1 is important for TCR-induced calcium flux and perhaps also Ras pathway activation (Turner *et al.*, 1997; Fischer *et al.*, 1998; Holsinger *et al.*, 1998; Costello *et al.*, 1999).

The study of PAK1, an effector of Rac1/Cdc42, further demonstrates the importance of the Rho family of GTPases in T-cell activation. After TCR engagement, PAK1 associates with the cytoplasmic adaptor Nck and becomes catalytically activated (Bubeck Wardenburg *et al.*, 1998; Yablonski *et al.*, 1998a). Furthermore, a dominant-negative allele of PAK1 blocks the TCR-induced activation of Erk, a downstream effector of the

Ras pathway, and of the nuclear factor of activated T cells (NFAT), a critical transcriptional element in the IL-2 promoter. The activation of Rac1/Cdc42 and PAK1 is believed to require the association of Vav1, Slp-76, Nck and PAK1 (Bubeck Wardenburg *et al.*, 1998). In a model to explain these observations, Slp-76 recruits Vav1 to a complex containing Nck and PAK1. Vav1 catalyzes GTP loading of Cdc42 or Rac1, which, in turn, activates the Nck-associated PAK1.

The study of PAK1 activation in other cell types has revealed a possible role for the PAK-interacting exchange factor or PIX (also known as Cool) (Bagrodia *et al.*, 1998; Manser *et al.*, 1998; Daniels *et al.*, 1999; Yoshii *et al.*, 1999). Cloned as a PAK-interacting protein, PIX is a GEF for Rac1 and possibly for Cdc42. Thus far, α and β genes have been cloned and each appears to have multiple splice forms. Interestingly, αPIX, but not βPIX, has an N-terminal calponin homology domain similar to that of Vav1. However, distinct from Vav1, both αPIX and βPIX contain an SH3 domain that binds with unusually high affinity to a polyproline stretch in PAK1. Finally, PAK1 activation in fibroblasts and neuronal cells may involve the p95PKL family of PIX-interacting proteins (p95PKL, Cat and GIT), which link PAK1 to the integrin-associated adaptor paxillin (Bagrodia *et al.*, 1999; Turner *et al.*, 1999; Zhao *et al.*, 2000).

In the present study, we examined the requirements for PAK1 and Rac1 activation by TCR signaling. PAK1 activation required ZAP-70, but not LAT, Slp-76 or Nck. Similar to PAK1, Rac1 activation also required ZAP-70 but not Slp-76. However, unlike PAK1, Rac1 was not activated in the absence of LAT. Finally, we showed that PIX and p95PKL are critical for the activation of PAK1 by the TCR. These data demonstrate that PAK1 and Rac1 are activated by novel mechanisms that are distinct from the TCR-mediated activation of the Ras and phosphatidylinositol signaling pathways.

Results

Activation of PAK1 requires the tyrosine kinase ZAP-70

We have shown previously that PAK1 is activated by TCR stimulation and that this activation requires the Src kinase Lck (Yablonski *et al.*, 1998a). In order to identify additional requirements for PAK1 activation by TCR stimulation, we measured the activation of endogenous PAK1 in the P116 Jurkat mutant cell line, which does not express ZAP-70 or Syk (Williams *et al.*, 1998). Parental Jurkat cells, the P116 cells or P116 cells stably transfected with a wild-type ZAP-70 cDNA were stimulated with an anti-TCR antibody for 3 min or a buffer control. Endogenous PAK1 was immunoprecipitated and *in vitro* kinase activity, using histone H4 as an exogenous substrate, was measured, as has been described previously (Yablonski *et al.*, 1998a). Fold activation of PAK1 kinase activity after TCR stimulation was normalized to that of wild-type Jurkat cells in the same experiment. The typical fold activation of wild PAK1 kinase activity in wild-type Jurkat cells ranged between 6- and 8-fold. Baseline kinase activity was the same in all cells and with all mutant PAK1 constructs (data not shown). As shown in Figure 1, P116 cells failed to induce PAK1 kinase activity following TCR stimulation. However, P116 cells stably reconstituted with a wild-type ZAP-70 cDNA induced PAK1 kinase activity nearly as well as parental Jurkat cells after TCR stimulation. Therefore, TCR-induced PAK1 activation requires ZAP-70.

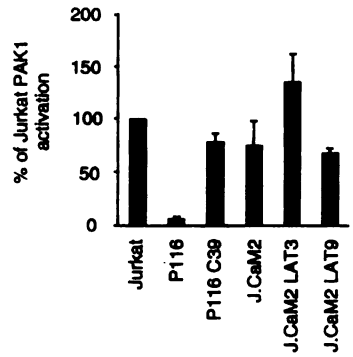

Fig. 1. PAK1 activation requires ZAP-70 but not LAT. The cells indicated were stimulated for 3 min with C305 (+) or a buffer control (–) at 37°C. Endogenous PAK1 was immunoprecipitated with an anti-PAK1 antibody and subjected to an *in vitro* kinase assay as described in Materials and methods. The fold increase in H4 phosphorylation after TCR stimulation was normalized to the fold activation of parental Jurkat cells in the same experiment. This percentage of wild-type Jurkat PAK1 activation was averaged over *n* experiments and plotted above. Error bars indicate the range or standard error over *n* experiments. P116 ($n = 2$) is a ZAP-70-deficient Jurkat cell. P116 C39 ($n = 2$) is a ZAP-70 reconstituted P116 cell. J.CaM2 ($n = 11$) is a LAT-deficient cell. J.CaM2 LAT3 ($n = 3$) and J.CaM2 LAT9 ($n = 2$) are two J.CaM2 cells independently reconstituted with LAT.

PAK1 activation occurs in the absence of LAT

One important downstream target of ZAP-70 is the lipid raft-anchored adaptor LAT. LAT is a critical link between the TCR-associated Src and Syk tyrosine kinases and the activation of the Ras and phosphatidylinositol pathways (Finco *et al.*, 1998; Zhang *et al.*, 1999). Since PAK1 activation was strictly dependent on ZAP-70 and Lck, we asked whether LAT is also required for PAK1 activation by measuring PAK1 kinase activity in the LAT-deficient Jurkat cell line, J.CaM2. As shown in Figure 1, J.CaM2 cells reproducibly induced PAK1 kinase activity after 3 min of TCR stimulation. At time points of up to 15 min of TCR stimulation, the activation of PAK1 in J.CaM2 was similar to that in wild-type Jurkat cells (data not shown). The induction of PAK1 kinase activity in two stable clones of J.CaM2 reconstituted with a wild-type LAT cDNA was similar to that of J.CaM2 and Jurkat. These data show that PAK1 can be activated independently of LAT.

TCR-mediated PAK1 activation is independent of Slp-76 and Nck

That PAK1 could be activated independently of LAT demonstrated a fundamental difference between the activation of the Ras and phosphatidylinositol pathways and the activation of PAK1. To confirm this difference, we asked whether the cytoplasmic adaptor Slp-76, which functions in concert with LAT to activate the Ras and phosphatidylinositol pathways, was also dispensable for PAK1 activation by TCR stimulation. We measured the TCR induction of PAK1 kinase activity in a Jurkat mutant

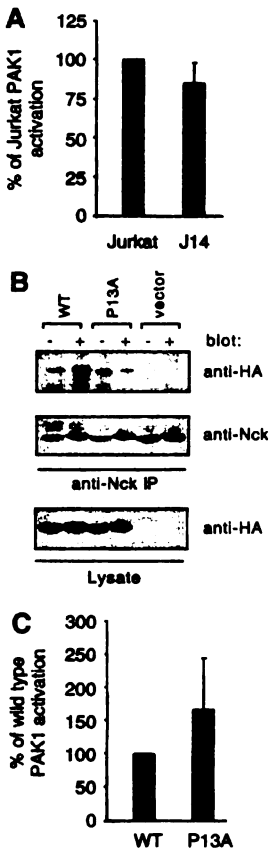

Fig. 2. TCR-mediated PAK1 activation is independent of Slp-76 and Nck. (**A**) Parental Jurkat cells and the Slp-76-deficient Jurkat subline, J14, were tested for PAK1 kinase activity as in Figure 1 ($n = 4$). (**B**) Jurkat T cells were transfected with either 5 μg of pEF-HA-PAK1 (WT), 12 μg pEF-HA-PAK1P13A (P13A) or 5 μg pEFBos (vector) to achieve equal expression levels. Fourteen hours later, the cells were stimulated for 3 min with C305 (+) or a buffer control (–) at 37°C. Anti-Nck immunoprecipitates were analyzed by 7.5% SDS–PAGE and blotted with an anti-HA antibody (top panel) or an anti-Nck antibody (middle panel). Whole cell lysates were blotted with an anti-HA antibody (bottom panel) showing equivalent expression of HA-PAK1. The experiment shown is representative of two independent experiments. (**C**) As in (B), but anti-HA immunoprecipitates were tested for kinase activity ($n = 4$). The increased mean fold activation and large range of PAK1 P13A was due to a single experiment out of four in which PAK1 P13A was uncharacteristically activated 3.5-fold above wild-type PAK1.

line, J14, which does not express Slp-76 (Yablonski *et al.*, 1998b). The TCR-inducible activation of endogenous PAK1 immunoprecipitated from J14 was essentially identical to that of wild-type Jurkat T cells (Figure 2A), showing that PAK1 can be activated by the TCR in the absence of Slp-76.

Previously, a complex of Nck/Slp-76/Vav1 was proposed to be necessary for PAK1 activation by the TCR (Bubeck Wardenburg *et al.*, 1998). Although Slp-76-independent PAK1 activation was inconsistent with such a model, we asked whether Nck might still be required for PAK1 activation. We generated a mutant of PAK1 (P13A) described previously that is unable to interact with Nck *in vitro* and in fibroblasts (Bokoch *et al.*, 1996). We first confirmed that in Jurkat cells, P13A PAK1 failed to associate inducibly with Nck. Either hemagglutinin (HA)-tagged wild-type PAK1 or HA-tagged P13A PAK1 was transfected into Jurkat cells. Nck was immunoprecipitated with an anti-Nck antiserum and its association with PAK1 was detected with an anti-HA antibody. Whereas wild-type PAK1 associated inducibly with Nck as previously reported (Yablonski *et al.*, 1998a), the mutant P13A PAK1 failed to do so (Figure 2B, lanes 3 and 4). The inducible interaction of the polyproline stretch in PAK1 and the second SH3 domain of Nck is likely to be regulated by conformational changes in one or both proteins. The kinase activity of HA-tagged wild-type or P13A PAK1 was then measured by an *in vitro* kinase assay of anti-HA immune complexes. In spite of its failure to bind Nck, P13A PAK1 kinase activity was induced by TCR stimulation to a level comparable to that of wild-type PAK1 (Figure 2C). These data, taken together, demonstrate that PAK1 activation requires neither Slp-76 nor direct binding to Nck.

Rac1 activation by the TCR requires LAT and ZAP-70 but not Slp-76

Since we found that PAK1 activation does not require LAT or Slp-76, we asked whether the activation of Rac1, a potential activator of PAK1, had similar requirements. Endogenous, GTP-bound Rac1 was specifically precipitated using glutathione *S*-transferase (GST) fused to the G-protein binding domain (GBD) of PAK1 (Manser *et al.*, 1998) and then detected with a Rac1-specific antibody (Figure 3A, top panels). TCR stimulation alone was sufficient to activate Rac1 in Jurkat cells as described previously (Kuhne *et al.*, 2000). A specific increase in the amount of GTP-bound Rac1 was detected after TCR stimulation in the Slp-76-deficient J14 cell line but not the LAT-deficient J.CaM2 or ZAP-70-deficient P116 cell lines. Although J14 cells did not activate Rac1 as robustly as Jurkat cells, the J14 cell line, generated by random mutagenesis, is likely to have additional deficiencies that could explain this partial defect. Indeed, in multiple experiments, the reconstitution of the J14 cell line with Slp-76 (J14 76-11) did not markedly enhance the activation of Rac1, demonstrating that the presence of Slp-76 does not have an effect on Rac1 activation. However, reconstitution of the J.CaM2 cell line with a wild-type LAT cDNA (J.CaM2 LAT3) restored Rac1 activation. Two independently reconstituted J.CaM2 cells showed similar results (data not shown). Reconstitution of P116 cells with a wild-type ZAP-70 cDNA (P116 C39) also restored Rac1 activation by the TCR. Similar trends in the mutant cell lines were observed at later time points as Rac1 activation declined (data not shown). These data show that Rac1 activation can occur in the absence of Slp-76 but not in the absence of ZAP-70 or LAT.

Because PAK1 was activated in the absence of Rac1 activation in the LAT-deficient J.CaM2 cell, we asked whether Rho family GTPase binding is dispensable for PAK1 activation in response to TCR stimulation. To do so, we generated the H83,86L mutant of PAK1 described previously, which does not bind to Rac1 or Cdc42 (Manser *et al.*, 1997). We transfected the wild type or the H83,86L mutant of HA-epitope-tagged PAK1 into Jurkat cells and

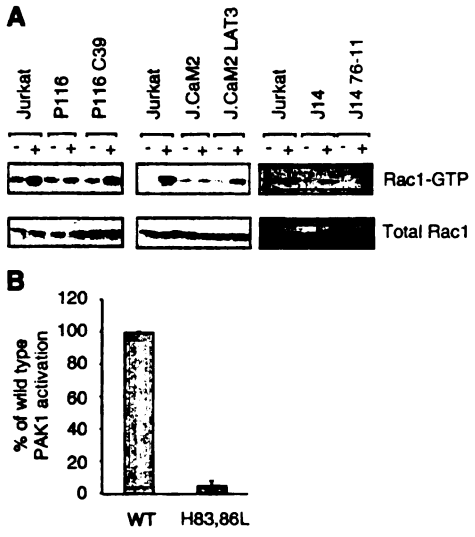

Fig. 3. Rac1 activation by the TCR requires ZAP-70 and LAT, but not Slp-76. (**A**) The cells indicated were stimulated for 2 min with C305 (+) or a buffer control (–) and then lysed. Rac1-GTP was specifically precipitated by incubation with GST–PAK1–CRIB domain for 15 min followed by a rapid wash with lysis buffer. Bound Rac1 was detected by SDS–PAGE and a Rac1-specific antibody (top panel) and compared with total Rac1 (bottom panel). These results are representative of at least three independent experiments. The cells tested were: the ZAP-70-deficient P116 cell, or the ZAP-70 reconstituted P116 C39, the LAT-deficient J.CaM2 cell, a LAT reconstituted J.CaM2 LAT3, the Slp-76-deficient J14 cell and a Slp-76 reconstituted J14 76-11. (**B**) Jurkat T cells were transfected with either 5 μg of pEF-HA-PAK1 (WT) or 5 μg of pEF-HA-PAK1H83L,H86L (H83,86L) to achieve equal expression. Kinase activity of the HA-tagged PAK1 was measured as in Figure 2C ($n = 3$).

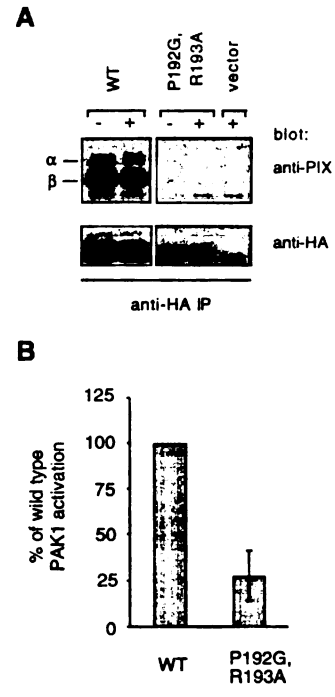

Fig. 4. PIX binding is required for PAK1 activation. (**A**) Jurkat T cells were transfected with either 5 μg of pEF-HA-PAK1 (WT) or 3 μg of pEF-HA-PAK1P192G,R193A (P192G, R193A) to achieve equal expression. The cells indicated were stimulated for 2 min with C305 (+) or a buffer control (–) and then lysed. Anti-HA immunoprecipitates were analyzed for PIX content by 7.5% SDS–PAGE and western blotting with an anti-PIX antibody (top panel) or an anti-HA antibody (bottom panel). Results are representative of three independent experiments. (**B**) PAK1 kinase assays were performed on anti-HA immunoprecipitates prepared as in (A) ($n = 3$).

assayed their catalytic activation following TCR stimulation. In contrast to the inducible activation of the P13A mutant PAK1, the H83,86L PAK1 was not activated by TCR stimulation (Figure 3B). These data demonstrate that an intact GTPase binding site is required for PAK1 activation in response to TCR stimulation.

Association with PIX is required for PAK1 activation

Previous studies from our laboratory and others had suggested that PAK1 might function downstream of the GEF Vav1, which was thought to require interaction with Slp-76 and LAT for function (Bubeck Wardenburg *et al.*, 1998; Yablonski *et al.*, 1998a; van Leeuwen and Samelson, 1999). In view of the LAT and Slp-76 independence of PAK1 activation, we asked whether another GEF, PIX, is important for PAK1 activation by the TCR. First, we confirmed that PIX could interact with the N-terminus of PAK1 in T cells, as has been described in fibroblasts (Manser *et al.*, 1998). Immunoprecipitates from Jurkat cells of transfected wild-type PAK1 contained multiple isoforms of α and β PIX as detected by an anti-PIX antibody (Figure 4A). Similar results were seen with the immunoprecipitation of endogenous PAK1 (Figure 6B). The interaction between PAK1 and PIX was not enhanced by TCR stimulation although a portion of the PAK1-associated αPIX shifted to a lower-mobility form after stimulation. This mobility shift might indicate phosphorylation or other post-translational modification. Although there appears to be a reduction in PAK1-associated αPIX after TCR stimulation, this was not reproducible in multiple experiments. The interaction between PAK1 and PIX is mediated by the SH3 domain of PIX and a polyproline region in the N-terminus of PAK1. In agreement with previous results (Manser *et al.*, 1998), mutation of residues P192 and R193 in PAK1 to glycine and alanine, respectively, abrogated the co-immunoprecipitation of the multiple PIX isoforms with PAK1 in Jurkat cells (Figure 4A). We then tested the ability of the TCR to activate this P192G,R193A PAK1. As shown in Figure 4B, this allele of PAK1 was poorly activated by TCR stimulation in Jurkat cells.

In order to confirm the requirement for PIX binding for PAK1 activation by the TCR, we stably transfected epitope-tagged wild-type (WT), Dbl homology mutant (DH*) or SH3 mutant (SH3*) βPIX into Jurkat cells. Several stable clones expressing each βPIX allele were isolated and screened for approximately equivalent over-expression of βPIX. Stable clones expressed at least 5-fold more βPIX than non-transfected parental cells as detected by an anti-PIX antibody that recognizes the SH3 domain of PIX (Figure 5A, top panel). The disrupted SH3 domain of

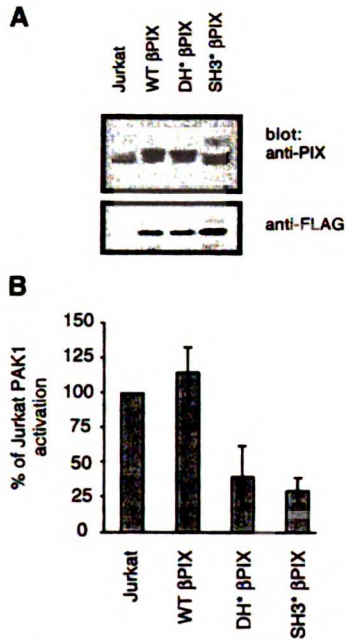

Fig. 5. Overexpression of βPIX mutants blocks PAK1 activation. (**A**) Anti-PIX immunoblot of 1×10^6 cells from parental Jurkat, representative clones overexpressing either wild-type βPIX (WT βPIX), L238R, L239S βPIX (DH* βPIX) or W34P, W44G βPIX (SH3* βPIX). (**B**) Endogenous PAK1 kinase assays performed as in Figure 1B on representative clones expressing the indicated βPIX allele ($n = 3$). Results are representative of least three independent clones expressing each PIX allele (data not shown).

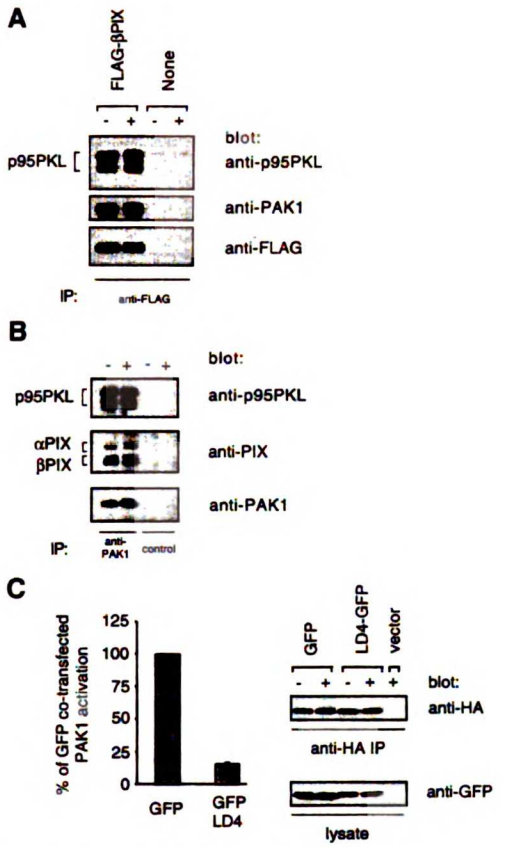

Fig. 6. p95PKL–PIX–PAK1 is required for efficient PAK1 activation by the TCR. (**A**) Either Jurkat cells stably expressing wild-type FLAG-βPIX or untransfected Jurkat cells were stimulated for 2 min with C305 (+) or a buffer control (–) and then lysed. Anti-FLAG immunoprecipitates were analyzed by 7.5% SDS–PAGE and blotting with an anti-p95PKL antibody (top panel) or an anti-PAK1 antibody (middle panel). The blot was then stripped and reprobed with an anti-FLAG antibody (bottom panel). These data are representative of two independent experiments. (**B**) Jurkat cells were stimulated for 2 min with C305 (+) or a buffer control (–) and then lysed. Endogenous PAK1 was immunoprecipitated from Jurkat cells with a C-terminally directed anti-PAK1 antibody (lanes 1 and 2) or the same antibody pre-incubated with a PAK1-blocking peptide (lanes 3 and 4). The immunoprecipitates were analyzed by 7.5% SDS–PAGE and western blotting with an anti-p95PKL antibody (top panel) and anti-PAK1 antibody (bottom panel). The top panel was stripped and reprobed with an anti-PIX antibody (middle panel), which explains the relatively weaker PIX signal. (**C**) Jurkat T cells were co-transfected with 15 μg of the GFP parental vector and 5 μg of pEF-HA-PAK1 or with 15 μg of LD4–GFP and 20 μg of pEF-HA-PAK1 to equalize expression of PAK1. Six hours after transfection, the cells were stimulated for 2 min with C305 or a buffer control at 37°C and PAK1 kinase activity was measured (left panel, $n = 3$). Equivalent immunoprecipitation of HA-PAK1 was confirmed by an anti-HA western blotting (right top panel) and expression of GFP and LD4–GFP was confirmed by an anti-GFP western blot from whole cell lysate (right bottom panel).

SH3* βPIX is not detected by the anti-PIX antibody and equivalent expression was confirmed using the FLAG epitope tag (Figure 5A, bottom panel). Endogenous PAK1 activation was measured from these βPIX-overexpressing clones. While clones overexpressing WT βPIX activated PAK1 as well as the Jurkat parental line, clones expressing DH* or SH3* mutant βPIX were significantly impaired in their ability to activate PAK1 (Figure 5B). In contrast to the impaired PAK1 activation, the overall pattern of tyrosine phosphorylation following TCR stimulation in all clones was similar (data not shown). Taken together, these data show that the PAK1–PIX interaction is critical for TCR-mediated PAK1 activation.

A PKL–PIX–PAK1 complex is important for PAK1 activation by the TCR

The ability of the SH3* mutant of βPIX to block PAK1 activation suggested that a βPIX–PAK1 complex might be recruited to upstream components for PAK1 activation. Thus, the overexpressed SH3* mutant βPIX may compete with wild-type PIX for binding to these upstream components, thereby preventing endogenous PAK1 from being recruited and activated. One possible candidate that might link PIX to the TCR was the recently cloned p95PKL, which interacts with PIX and paxillin (Turner *et al.*, 1999). Indeed, anti-FLAG immunoprecipitates from Jurkat cells stably expressing FLAG-tagged wild-type βPIX contained multiple isoforms of p95PKL as identified by western blotting with an anti-p95PKL antibody (Figure 6A). Like the interaction between PAK1 and PIX, the association between PIX and p95PKL was not induced by TCR stimulation. As expected, endogenous PAK1 was also present in these anti-PIX immune com-

plexes. Importantly, anti-PAK1 immunoprecipitates from untransfected Jurkat cells contained both p95PKL and PIX (Figure 6B). These data taken together demonstrate that p95PKL, PIX and PAK1 form a trimolecular complex constitutively *in vivo*.

In a previous study, p95PKL was shown to link PIX to the integrin-associated adaptor paxillin by binding directly to both proteins (Turner *et al.*, 1999). Having demonstrated that p95PKL interacts with βPIX and PAK1 in T cells, we asked whether p95PKL was functionally important for PAK1 activation. To interfere with p95PKL function, we fused 20 amino acids spanning the LD4 region of paxillin to the N-terminus of green fluorescent protein (GFP), analogous to an approach used in a previous study (Turner *et al.*, 1999). By binding directly to the paxillin binding site in p95PKL, the LD4–GFP competitively inhibits interactions between p95PKL and paxillin. LD4–GFP or GFP alone was co-transfected into Jurkat T cells with HA-tagged wild-type PAK1. The kinase activity of the HA-tagged PAK1 was measured with an *in vitro* kinase assay on anti-HA immune complexes. Relative to the expression of GFP alone, expression of LD4–GFP potently inhibited PAK1 activation induced by TCR stimulation (Figure 6C). These data suggest that p95PKL may be important in PAK1 activation by the TCR and may function by recruiting PIX–PAK1 to upstream components of the PAK1 activation pathway.

Discussion

We have further elucidated the TCR signaling pathways that activate PAK1 and Rac1. A previous study implicated Vav1, Nck and Slp-76 in TCR-mediated PAK1 and Rac1 activation. In this model, Slp-76 served as a bridge between an Nck–PAK1 complex and Vav1. Mutant forms of these molecules were demonstrated to block PAK1 activation by the TCR (Bubeck Wardenburg *et al.*, 1998). We have taken advantage of the known P13A mutation in PAK1 and the Slp-76-deficient Jurkat cell to test this model without overexpressing dominant-negative proteins. Surprisingly, we show that Nck binding to PAK1 is dispensable for PAK1 activation by the TCR. Moreover, PAK1 is activated in a Slp-76-deficient Jurkat cell. While a Nck–Slp-76–Vav1 complex may play some role in the activation of PAK1, our data suggest that this complex is not necessary for PAK1 activation following TCR stimulation.

In order to characterize this Slp-76/Nck-independent pathway, we measured the activation of PAK1 in the LAT-deficient J.CaM2 cell. Surprisingly, we observed reproducible activation of endogenous PAK1 in the absence of LAT. The activation of PAK1 in J.CaM2 contrasts with the almost complete absence of Erk1/2 activation and calcium flux in these cells (Finco *et al.*, 1998). These results are consistent with our previous data that PAK1 activation is independent of Ras (Yablonski *et al.*, 1998a). Taken together, they demonstrate a fundamental difference between the requirements for PAK1 activation and the requirements for the activation of the Ras and phosphatidylinositol pathways. Furthermore, we believe that these are among the first descriptions of a LAT- and Slp-76-independent pathway. Our finding that Lck and ZAP-70

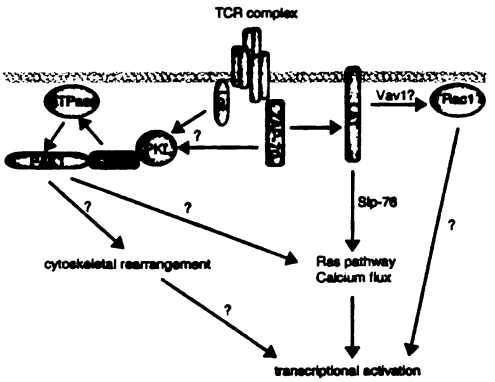

Fig. 7. Proposed model for PAK1 and Rac1 activation by the TCR. PAK1 activation requires Lck and ZAP-70, but occurs independently of LAT and Slp-76. Rac1 activation requires ZAP-70 and LAT, but is independent of Slp-76. These contrast the requirement of ZAP-70, LAT and Slp-76 for Ras and phosphatidylinositol pathway activation. Each of these divergent pathways is required to activate the transcriptional responses of T-cell activation. PAK1 activity most likely contributes to cytoskeletal reorganization as well. Undoubtedly, cytoskeletal rearrangements require contributions from many other components besides PAK1, but these have been omitted for clarity.

are absolutely required for PAK1 activation demonstrates that this novel LAT- and Slp-76-independent pathway diverges at the level of the Src and Syk kinases (Figure 7).To confirm that PAK1 is activated by a LAT- and Slp-76-independent pathway, we asked whether Rac1, a Rho GTPase that activates PAK1 in many systems, is activated by a similar mechanism. Like PAK1, Rac1 activation by the TCR did not require Slp-76, but did require ZAP-70. However, unlike PAK1, Rac1 was absolutely dependent on LAT for its activation by the TCR. The surprising Slp-76 independence but LAT dependence of Rac1 activation shows that Rac1 is activated by a mechanism that is distinct from both the PAK1 pathway and Ras/phosphatidylinositol pathways. Consistent with this conclusion, we have observed that doses of phorbol 12-myristate 13-acetate and ionomycin that strongly activate the Ras pathway and the NFAT transcriptional element are poor activators of Rac1 (data not shown). One possible caveat to these studies is the constitutive activation of the PI-3 kinase pathway in Jurkat cells caused by deficiency of PTEN, the tumor suppressor lipid and protein phosphatase (Shan *et al.*, 2000). This constitutive activation may bypass requirements for PAK1 and Rac1 activation.

Previous studies had implicated Vav1 as a GEF upstream of PAK1 by binding a complex of Nck/Slp-76/Gads/LAT (Bubeck Wardenburg *et al.*, 1998; van Leeuwen and Samelson, 1999). Our observation that PAK1 activation was independent of LAT, Slp-76 and Nck suggests that Vav1 may not play an important role in PAK1 activation by the TCR. In the light of this surprising result, we asked whether another GEF, PIX, is important in TCR-mediated PAK1 activation. We first showed that PAK1 constitutively interacts with multiple isoforms of PIX in Jurkat cells. Furthermore, a mutant PAK1 that cannot bind PIX (P192G,R193A) is significantly impaired in its activation by the TCR. These data suggest two

simple models of PIX function: (i) PIX might be important for the recruitment of PAK1 to the membrane for activation or (ii) PIX might be important as a GEF to allow GTPase-mediated PAK1 activation.

We used the overexpression of mutants of βPIX to address these possibilities. Overexpression of a βPIX mutant that does not have GEF function (DH*) inhibited PAK1 activation by the TCR. This is consistent with the second model, which suggested that βPIX is required for the localized activation of a Rho GTPase, which then activates PAK1 (Figure 7). Indeed, this model is also consistent with our finding that PAK1 requires Rho GTPase binding for activation by TCR stimulation. However, this GTPase is probably not Rac1 in view of Rac1's dependence on LAT for activation. In support of this hypothesis, Rac1 activation was not affected by the overexpression of wild-type, DH* or SH3* βPIX (data not shown). Cdc42 is an excellent candidate GTPase for PAK1 activation since we have shown previously that a dominant-negative allele of Cdc42 inhibits PAK1 activation by the TCR (Yablonski *et al.*, 1998a). However, we have been unable to reproducibly observe endogenous Cdc42 activation in Jurkat cells.

That the SH3* βPIX could block PAK1 activation is consistent with our first model, which suggested that PIX is responsible for recruiting PAK1 to appropriate sites for activation by binding to additional upstream components of this cascade. One candidate for such an upstream component is p95PKL, recently identified in fibroblasts as a 95 kDa, tyrosine-phosphorylated protein that can interact with PIX and the integrin-associated adaptor paxillin (Bagrodia *et al.*, 1999; Turner *et al.*, 1999). In Jurkat cells, we showed that p95PKL, PIX and PAK1 form a trimolecular complex *in vivo*. We asked whether p95PKL is important for TCR-mediated PAK1 activation in T cells by overexpressing the paxillin LD4 repeat, which binds to p95PKL, as a GFP fusion protein. Indeed, compared with overexpression of GFP alone, LD4–GFP potently blocked PAK1 activation by the TCR. These data, to our knowledge, are the first demonstration of a role for p95PKL and PIX in a receptor-mediated activation of PAK1.

While the platelet-derived growth factor and EphB2 receptor tyrosine kinases appear to utilize PI-3 kinase and PIX to activate PAK1 (Yoshii *et al.*, 1999), the mechanism by which the TCR and its associated tyrosine kinases, Lck and ZAP-70, activates p95PKL/PIX/PAK1 is unclear. The interactions between PKL, PIX and PAK1 described here appear to be unaffected by TCR stimulation and we have not observed obvious tyrosine phosphorylation of any of the three proteins. Based on work in fibroblasts, we might predict a complex containing paxillin/p95PKL/PIX/PAK1 in T cells. However, we have been unable to detect paxillin in complex with p95PKL or PIX and vice versa in T cells (data not shown). Nonetheless, interactions between a paxillin superfamily member and p95PKL are intriguing in view of reported observations that paxillin and Lck inducibly associate in T cells (Ostergaard *et al.*, 1998). This inducible interaction could bring a paxillin–p95PKL–PIX–PAK1 complex to the TCR complex where PAK1 could be activated. Another possible mechanism for p95PKL–PIX–PAK1 recruitment to the TCR complex may be its association with FAK as suggested by a GIT1–FAK interaction detected in fibroblasts (Zhao *et al.*, 2000). Since others have reported that FAK or the FAK-like Pyk2 can interact with Lck (Berg and Ostergaard, 1997), Fyn (Ganju *et al.*, 1997; Qian *et al.*, 1997) and ZAP-70 (Katagiri *et al.*, 2000), this interaction might also link the p95PKL–PIX–PAK1 complex to the TCR. These possibilities are currently being investigated.

The relevant downstream targets of PAK1 in TCR signaling are not yet known. We have shown previously that a dominant-negative allele of PAK1 blocks TCR-mediated activation of NFAT. Surprisingly, this block could not be overcome by either pharmacological activation of the Ras pathway or calcium mobilization alone, suggesting that PAK1 acts upstream of Ras pathway activation and calcium flux (Yablonski *et al.*, 1998a). One possible mechanism for these observations may be PAK1's activation of Raf1 (Chaudhary *et al.*, 2000; Sun *et al.*, 2000), although this alone does not explain PAK1's effect on the calcium-dependent pathway. Another possible mechanism may be PAK1's effects on the cytoskeleton (reviewed by Bagrodia and Cerione, 1999). The regulation of actin polymerization specifically may explain PAK1's influence on NFAT activation since cytochalasin D, an actin depolymerizing agent, can block TCR-induced NFAT and IL-2 reporter activation (Holsinger *et al.*, 1998). However, it should be emphasized that many molecular events besides PAK1 activation may be required to orchestrate the cytoskeletal rearrangements of T-cell activation.

The role of Vav1 in PAK1 and Rac1 activation remains unclear. The data presented here do not exclude a role for Vav1 in Rac1 or PAK1 activation by the TCR. However, our findings do contradict the simple model that Rac1 and PAK1 are activated by a Vav1–Slp-76–Gads–LAT complex. Consistent with this, others have shown that the Slp-76–Vav1 interaction is not required for their synergistic activation of the IL-2 promoter and that Slp-76 and Vav1 can function in distinct pathways (Raab *et al.*, 1997; Fang and Koretzky, 1999). Nevertheless, Vav1 may yet be responsible for the activation of Rac1 downstream of LAT but independent of Slp-76. Although Vav1 is hypophosphorylated in the LAT-deficient J.CaM2 cell line (Finco *et al.*, 1998), recent studies demonstrate that both negative and positive regulatory sites of tyrosine phosphorylation appear to be present in Vav1 (Kuhne *et al.*, 2000; Lopez-Lago *et al.*, 2000). These recent observations and our data showing Slp-76-independent Rac1 activation suggest that the link between LAT, Vav1 and Rac1 requires additional investigation.

In this study, we have defined requirements for the activation of PAK1 by the TCR. Surprisingly, PAK1 activation does not require the adaptors LAT or Slp-76. For optimal activation by the TCR, PAK1 requires its binding site for Rho GTPases and its binding site for a PIX–p95PKL complex. These data imply that molecules originally identified as focal adhesion signaling molecules are important for TCR signaling. This overlap between elements of focal adhesions and elements of the TCR complex as well as recent descriptions of the highly ordered and dynamic T cell–APC interface (van der Merwe *et al.*, 2000) suggest that the TCR organizes a complex structure that resembles, in part, a

focal adhesion at the T cell–APC interface. We propose that the relatively upstream activation of the PAK1 pathway helps orchestrate the cytoskeletal rearrangements that assist in the formation and maintenance of this dynamic signaling machine.

Materials and methods

Reagents

The cell lines used were the Jurkat T cell line J.E6-1 (Weiss *et al.*, 1984), the Slp-76-deficient Jurkat subline J14, the Slp-76 reconstituted J14 76-11 (Yablonski *et al.*, 1998b), the LAT-deficient Jurkat subline J.CaM2 (Goldsmith *et al.*, 1988), the LAT reconstituted J.CaM2 LAT3 and J.CaM2 LAT9 cell lines (Finco *et al.*, 1998), the ZAP-70-deficient P116 Jurkat subline and the ZAP-70 reconstituted P116 C39 (Williams *et al.*, 1998). Cells were grown in RPMI-1640 with 5% fetal calf serum (FCS) supplemented with penicillin, streptomycin and glutamine at 37°C in humidified 5% CO_2. TCR stimulations were performed with the anti-Jurkat TCR Vβ C305 mAb (Weiss and Stobo, 1984). Prior to any stimulation, CD3 surface expression was confirmed by fluorescence-activated cell sorter (FACS) analysis. The M2 anti-FLAG antibody was obtained from Sigma. The anti-PAK1 antibodies, sc882 and sc881, were obtained from Santa Cruz Biotechnology. The 12CA5 anti-HA antibody was obtained from Boehringer Mannheim. The polyclonal anti-Nck antibody was provided by Joseph Schlessinger (New York University). The anti-Rac1 antibody and the anti-p95PKL antibody were both obtained from Transduction Laboratories. The anti-GFP antibody was obtained from Clontech. The anti-PIX antibody was described previously (Manser *et al.*, 1998).

Transfections

Jurkat T cells (2×10^7) in 0.4 ml of serum-free RPMI-1640 were transiently transfected by electroporation using the Gene Pulser (Bio-Rad) at 250 V and 960 μF. Following transfection, cells were incubated in RPMI containing 10% FCS. Stable transfectants were isolated by performing the above protocol, but 48 h after transfection, cells were plated under limiting dilution conditions in 2 mg/ml G418 (Calbiochem). Approximately 4 weeks later, clonal cell populations were isolated, screened for equivalent surface expression of CD3 by FACS analysis and equivalent expression of the FLAG epitope-tagged βPIX by western blotting.

Constructs

PAK1 was mutated by overlapping PCR to generate a proline to alanine change at codon 13, histidine to leucine change at codons 83 and 86, a proline to glycine change at codon 192 and an arginine to alanine change at 193. Briefly, sense and antisense oligos were synthesized that contained the desired mutation and flanking sequence. PCR was performed with an oligo in the polylinker of pcDNA hPAK1 and an antisense oligo containing the desired mutation. A second PCR was performed with the sense oligo containing the mutation and an oligo 3′ to the *ClaI* site internal to the hPAK1 cDNA. These overlapping pieces were assembled with a final PCR to generate the mutated PAK1 N-terminus and then placed into the full-length pEF-hPAK1 vector (Yablonski *et al.*, 1998a) using the polylinker *Asp*718 site and the internal *ClaI* site. All PAK1-containing cDNAs were grown at 30°C to minimize spontaneous mutation of the kinase. All mutants were transfected into 293 HEK cells and PAK1 kinase assays were performed to confirm kinase activity. The LD4–GFP construct was constructed by ligation of annealed oligos encoding the human paxillin LD4 sequence (underlined) MATRELDELMASLSDFKFMAQGGG preceded by a 5′ Kozak ACC sequence into the pEGFP-N1 vector (Clontech) between the *Eco*RI and *Pin*AI sites. Bacterially expressed GST–CRIB was prepared as described previously (Manser *et al.*, 1998). Wild-type βPIX, DH domain mutant βPIX (L238R, L239S), SH3 domain mutant βPIX (W34P, W44G) (Manser *et al.*, 1998) were inserted into the pcdef3 vector for efficient expression in Jurkat T cells. All constructs were confirmed by sequencing.

Cell stimulation, immunoprecipitation and western blotting

Cells were harvested and washed in phosphate-buffered saline (PBS) and resuspended at 10^8 cells/ml of PBS and incubated at 37°C for 10 min. Cells were stimulated with a 1:500 dilution of C305 for 2–3 min followed by rapid centrifugation and resuspension of the pellet by vigorous vortexing in a lysis buffer appropriate for the experiment. For immunoprecipitations, the lysis buffer was identical to that used for the PAK1 kinase assays. After clarification of the lysate by centrifugation in a microfuge at 13 000 r.p.m. for 10 min at 4°C, the supernatant was spun at 55K for 15 min in a tabletop ultracentrifuge. The supernatant was tumbled with 30 μl of protein A-expressing pansorbin bacteria (Calbiochem) for 20 min at 4°C. The supernatant was then tumbled with the relevant antibody prebound to the relevant beads for 1.5 h at 4°C. The beads were washed four times with lysis buffer and resuspended in reducing sample buffer for SDS–PAGE. Gels were transferred to Immobilon P (Millipore Corporation) and probed with primary antibodies as described in the text and the appropriate secondary antibodies coupled to horseradish peroxidase. Detection was performed with enhanced chemiluminescence (Amersham).

Rac1 activation assay

Specific isolation of Rac1-GTP was performed as described previously (Manser *et al.*, 1998; Kuhne *et al.*, 2000). Briefly, cells were stimulated as described above and lysed in lysis buffer containing 0.5% Triton X-100, 25 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM EGTA, 20 mM β-glycerophosphate, 4% glycerol, 10 mM NaF, 2 mM sodium orthovanadate, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin. After 10 min at 4°C, the lysates were clarified in a microfuge for 10 min at 13 000 r.p.m. The supernatant was incubated with 20 μg of GST–CRIB for 15 min at 4°C with rotation. The pellets were washed once with 500 μl of lysis buffer and resuspended in 40 μl of sample buffer. GST–CRIB-associated Rac1 was identified by SDS–PAGE and western blotting with a Rac1-specific antiserum.

PAK1 kinase assay

PAK1 kinase assays were performed as described in Yablonski *et al.* (1998a). In all experiments equal amounts of immunoprecipitated kinase were confirmed by western blotting (data not shown). Quantitation of histone H4 phosphorylation was performed by Fuji Multimager analysis of ^{32}P-labeled histone H4 identified after SDS–PAGE or by Cherenkov counting of 40 μl of the 50 μl kinase reaction spotted onto Whatman 3 mm paper after extensive washing in buffer containing 10% trichloroacetic acid and 10 mM sodium pyrophosphate. Background measurements were taken either by immunoprecipitation in the presence of the sc882 blocking peptide or by anti-HA immunoprecipitations on untransfected cells. Typical activation of PAK1, either endogenous or transfected, in Jurkat was 6- to 8-fold. To allow averaging of kinase assays performed on separate days, fold activation for each cell line or PAK1 allele was normalized to the activation of wild-type PAK1 measured in the same experiment. This percentage was averaged over n experiments as reported in the figure legend. Error bars show the standard error of the mean when $n > 2$ or the range when $n = 2$. In all experiments, the background PAK1 activity was similar.

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Chapter 3: Discussion

An adapter independent pathway activates PAK1

Previous data suggested that PAK1 was activated by the TCR via a LAT, GADS, Slp-76, Vav, Nck pathway(Bubeck Wardenburg et al., 1998). In particular, the trimolecular complex of Slp-76 bound to both Vav and Nck was suggested to recruit PAK1 via its most amino terminal polyproline sequence. ZAP-70- or Lck-phosphorylated Vav1 would activate Rac1 that would in turn activate Nck-associated PAK1.

Several lines of evidence described here suggest that this model is not the only way PAK1 can be activated by the TCR. First, Rac1 can be activated in the absence of Slp-76, suggesting that a Vav1 / Slp-76 complex is not necessary for Rac1 activation. Second, PAK1 can be catalytically activated in the absence of both Slp-76 or LAT. Third, a mutant allele of PAK1 which cannot bind to Nck still becomes catalytically activated. Taken together, these three independent pieces of data suggest that PAK1 can be activated in the absence of a LAT, Vav1, Slp-76, Nck complex.

One possible caveat to the Nck binding experiment is that the mutant PAK1 that cannot bind to Nck (P13A PAK1) might be able to dimerize with wild type PAK1 resulting in apparent activity. We believe that this is not the case since immunoprecipitation performed under identical conditions to that of the kinase assay shows that P13A PAK1 does not bind Nck. If heterodimers of wild type and P13A mutant PAK1 were being formed, we would have expected a 50% reduction in PAK1/Nck co-immunoprecipitation. However, we observed no inducible P13A PAK1/Nck association. Also, the PIX binding mutant and p21 binding mutant would also be predicted for form heterodimers and give false activity and they did not.

Instead of requiring a Vav, Slp-76, Nck complex, PAK1 requires binding to a GIT/PKL and PIX complex. Notably, the p95PKL antibody used in Chapter 2 is now believed to recognize multiple GIT family members as can be seen from the multiple bands detected in Figure 2-6A. A GIT1 specific antibody detects a single band which also coimmunoprecipiates specficially with PAK1 and PIX (data not shown). As both p95PKL and GIT1 are likely both functionally important for PAK1 activation, we will refer to them generically as GIT proteins. This trimolecular complex is present in unstimulated Jurkat cells and is not affected by TCR stimulation. Functionally, the GEF activity of PIX and its ability to associate with PAK1 are important for the activation of PAK1 as overexpression of a GEF inactive βPIX or an SH3 mutant of βPIX blocks endogenous PAK1 activation by the TCR. Overexpression of βPIX that cannot bind to PAK1 might sequester the upstream components that are required to activate PAK1, whereas overexpression of DH mutant βPIX might prevent activation of the relevant Rho GTPase in the vicinity of PAK1. These data suggest a model in which a GIT, PIX, PAK1 complex is activated by Lck and ZAP-70. This Lck and ZAP-70 dependent activation somehow triggers the GEF function of PIX towards an unknown GTPase, other than Rac1, which would then activate PAK1.

Regulation and role of GIT, PIX, PAK complex in T cells

How might the GIT, PIX, PAK complex become activated downstream of activated Lck and ZAP-70? Another protein might recruit the complex to the TCR, triggering PIX GEF function, thereby activating PAK1.

One candidate could be Nck as this adapter inducibly associates with CD3ε(Gil et al., 2002). Interestingly, Nck also inducibly associates with PAK1, suggesting that Nck itself might also undergo a conformational change after TCR engagement. However, this Nck-PAK1 association is not required for PAK1 activation; a mutant allele of PAK1 that cannot bind to Nck is still activated by TCR stimulation. Nonetheless, Nck might still play a role in the localization of activated PAK1.

The integrin adapter protein paxillin is another candidate since it interacts with both Lck and GIT1. Whereas overexpression of the LD4 repeat of paxillin fused to GFP is able to potently block PAK1 activation by the TCR, paxillin is not detected specifically in βPIX or PAK immunoprecipitates from Jurkat cells (not shown). It is possible that the overexpression of LD4-GFP perturbs the function of GIT1 or binding of the GIT1, PIX, PAK1 complex to another partner. One candidate for this GIT1 binding partner is ZAP-70 as I have recently detected a ZAP-70 / PAK1 interaction (see appendix B). Indeed, ZAP-70 could be functioning as an adapter that recruits the PAK1, PIX, GIT complex to the TCR (see below).

Besides recruitment, GIT1 and PIX may be regulated by their tyrosine and serine/threonine phosphorylation, respectively. GIT1 tyrosine phosphorylation appears to be regulated with the cell cycle(Bagrodia et al., 1999) and FAK is a potential GIT1 kinase(Zhao et al., 2000a). Bands comigrating with GIT1 from βPIX immunoprecipitates from unsynchronized Jurkat cells are tyrosine phosphorylated with or without TCR stimulation (data not shown), but the function of this tyrosine phosphorylation is not known. Both αPIX and βPIX undergo mobility shifts upon TCR stimulation – presumably reflecting serine / threonine phosphorylation, perhaps by associated PAK1.

Indeed, phosphorylation of PIX by PAK1 has been observed previously but PAK1 phosphorylation of PIX does not appear to affect GEF function(Manser et al., 1998).

GIT1's Arf-GAP activity might also mediate TCR-induced activation of the complex. Arf GTPases have been implicated in vesicle traffic to and from the plasma membrane. Overexpression of GIT1 is able to decrease G protein-coupled receptor internalization from the plasma membrane(Premont et al., 1998). This activity was dependent on its Arf-GAP domain. GIT1 overexpression also causes focal complex dissocation(Zhao et al., 2000a). It is yet not known how these activities relate to GIT's adapter function for PIX and paxillin or if Arf-GAP activity is required to activate PAK1. Furthermore, it is likely that GIT proteins have functions other than the activation of PAK1 – one candidate process is TCR downregulation since the TCR is internalized via clathrin coated pits. Studies of GIT1 overexpression or GIT2short (the predominant isoform in Jurkat) could be undertaken to probe the role of the Arf-GAP domain in T cell function – both microscopically and biochemically. Constitutively active or dominant negative Arf GTPases could be used to complement these studies. As Arf GTPases are now believed to function upstream of Rac for membrane ruffling(Santy and Casanova, 2001), Arf and GIT might play very early roles in polarization and signaling in T cells.

PIX also likely has functions in T cells that are independent of PAK1. In Drosophila postsynaptic densities, dPIX has additional functions independent of dPAK. These functions could be related to its GEF function and the activation of different Rho GTPase effectors. Preliminarily, we have overexpressed βPIX in Jurkat cells and observed suppression of NFAT responses by wild type, SH3 mutant or DH mutant

PIX(data not shown). These effects could indicate a PIX function independent of PAK1 activation since these mutants had distinct effects on PAK1 kinase activity.

The PAK1 and ZAP-70 interaction

Our recent data suggests that the PAK1 / PIX / GIT complex may be recruited to the TCR complex by PAK's association with ZAP-70 (see appendix B). Surprisingly, this association does not appear to require PAK1's interaction with PIX and GIT as the P192G, R193A PAK1 mutant, which does not interact with PIX, co-immunoprecipitates ZAP-70 as well as wild type PAK1. The interactions of PAK1 with Nck or with Rho GTPases also appear to be dispensible. As this co-immunoprecipitation was performed in 293T cells, the interaction may be indirect. If so, PAK1 and ZAP-70 must be bridged by endogenous 293T cell proteins specific proteins. A direct interaction should be tested using GST-PAK1 and recombinant ZAP-70.

What regions of PAK1 might interact with ZAP-70? There are two additional polyproline regions and undoubtedly other unidentified regions within PAK1 that might interact with ZAP-70 directly or via an intermediate protein. Deletion studies of PAK1 are warranted to elucidate which region(s) of PAK1 is required to interact with ZAP-70. We have recently detected a complex of PAK1 and ZAP-70 in Jurkat cells when PAK1 is overexpressed. Interestingly, co-immunoprecipitation of PAK1 and ZAP-70 is observed only in the unstimulated state(data not shown).

These data suggest that ZAP-70 interacts with PAK1 prior to TCR stimulation and that ZAP-70 might recruit PAK1 to the TCR complex upon T cell activation. Although the activation of PAK1 is defective in ZAP-70-deficient P116 cells and is

restored by re-expression of ZAP-70, it is possible that ZAP-70 kinase activity is not required to activate PAK1 and that ZAP-70 merely recruits the complex to the TCR. Therefore, it should be determined whether the kinase activity of ZAP-70 is required for PAK1 kinase activation using kinase dead ZAP-70 to reconstitute P116 cells. This experiment could be confounded by the fact that kinase dead ZAP-70 does not localize to the subcortical region of Jurkat cells like wild type ZAP-70, suggesting a failure to interact with an unknown protein. Therefore, the kinase inactive mutation might not recapitulate the potential adapter function of ZAP-70. However, one could still determine whether a kinase inactive mutant of ZAP-70 can interact with PAK1 in 293T cells.

Identification of additional factors in the ZAP-70 and PAK1 complex may help elucidate how the complex becomes activated. We have identified α and β PIX in a large complex of approximately 1-2 MDa from resting Jurkat cells by gel filtration of 1% NP-40 lysates (data not shown). This complex should be confirmed and tested for the presence of GIT1, ZAP-70, and PAK1. Specifically, the size of the complex should be measured before and after TCR stimulation. Additional components could be identified by careful immunoprecipitation of the individual components of the complex from size fractionated lysates by silver staining.

Besides looking biochemically at the components of this large complex, localization of PAK1, PIX, GIT1, and ZAP-70 should be studied in Jurkat cells. Although conventional microscopy does not have the resolution to see individual protein / protein interactions, imaging would provide valuable temporal and spatial information about these complexes before and after stimulation. For example, since ZAP-70 becomes transiently recruited to the TCR's ITAMs, PAK1 and ZAP-70 might co-localize at the c-

SMAC. Alternatively, since PAK, PIX, and GIT can associate with integrins and ZAP-70 and Syk can directly bind to β3 integrin tails(Woodside et al., 2001; Woodside et al., 2002), PAK1 and ZAP-70 might colocalize only with the LFA-1 containing p-SMAC. Induced interactions among components could be studied by colocalization studies and fluorescence resonance energy transfer.

Are integrins the link between the TCR and PAK1?

The activation of PAK1 downstream of the TCR might depend on integrin activation. TCR-induced upregulation of LFA-1 affinity, as measured by binding to plates coated with ICAM-1, has requirements that are strikingly similar to TCR-induced PAK1 activation. Each requires ZAP-70 and Lck but not LAT(Epler et al., 2000). However, if integrins do mediate PAK1 activation downstream of the TCR, it must occur through integrins other than LFA-1; the LAT deficient J.CaM2 activates PAK1 but does not express LFA-1. One way to test whether integrin engagement is required for PAK1 activation is to increasingly dilute the Jurkat cells during the stimulation to prevent cell-cell contact. Increasing the dilution of cells during stimulation should decrease PAK1 activation if integrin engagement is required to activate PAK1 downstream of the TCR. Alternatively, removal of divalent cations would prevent integrin activation. PAK1 phospho-specific antibodies or kinase assays could then be used to measure PAK1 activity. Paradoxically, although TCR-induced LFA-1 activation requires ZAP-70, ZAP-70 deficient P116 cells are still able to form LFA-1 / ICAM-1 dependent conjugates with superantigen pulsed Raji B cells(Morgan et al., 2001) . However, the formation of cell to

cell contacts is clearly a more complex phenomenon and could be compensated for by other molecules.

Vav1 and PAK1 – a possible feedback loop?

The role of Vav1 in the activation of PAK1 is still not yet clear. In double positive thymocytes, Vav1 is essential for the activation of Rac1 downstream of the TCR(Reynolds et al., 2002). However, PAK1 activation in Vav1-deficient thymocytes was not reported. In Vav1 deficient peripheral T cells, PAK1 activation is reportedly normal while Rac1 activation was not reported(Fischer et al., 1998). That the PIX-binding deficient P192G, R193A PAK1 allele is not activated by the TCR suggests that most PAK1 activity is dependent on PIX binding. However, this does not completely exclude a role for Vav1 in PAK activation in combination with PIX. Studies of a Vav1 deficient Jurkat cell(Cao et al., 2002) are in progress to examine the relative role of Vav1 in PAK1 activation.

It is likely that there are multiple ways to activate PAK1. One way might be through a GIT and PIX complex that can occur independently of LAT. Once LAT and Vav1 are activated, additional PAK1 activation might take place through a Vav, LAT, Slp-76 pathway. These two modes of PAK1 activation could be distinct quantitatively, spatially, or temporally. Obvious quantitative or temporal differences have not been detected; total PAK1 kinase activity from LAT deficient J.CaM2 cells is very similar to parental Jurkat cells and LAT reconstituted J.CaM2 cells over the 20 minute TCR-induced burst of PAK1 kinase activity(data not shown). In constrast, while the Slp-76 deficient J14 Jurkat cell activates PAK1 equally well as Slp-76 positive parental Jurkat

cells, the Slp-76 reconstituted J14 cell line activates PAK1 to a greater extent than that seen in Jurkat cells. Normal PAK1 activation in J14 cells could reflect compensatory changes in a Slp-76-independent pathway that can be augmented by the re-expression of Slp-76. These data could indicate that there exists a Slp-76-independent and a Slp-76-dependent pathway to activate PAK1.

There is some evidence that actin polymerization may also have distinct modes of regulation. Actin polymerization occurs very transiently in the LAT-deficient J.CaM2 Jurkat cell but is only sustained with functional LAT(Bunnell et al., 2001) – consistent with a LAT-independent intial phase of actin polymerization and a LAT-dependent sustained phase.

What is the Rho GTPase that activates PAK1?

That the GEF function of PIX may be important for PAK1 activation is consistent with PAK's requirement of an intact p21 binding domain (PBD). Mutation of the PBD using the previously characterized H83,86L mutation renders it completely inactive in response to TCR stimulation. Because this mutation also disrupts the autoinhibitory segment, PAK1 H83,86L kinase activity is increased in fibroblasts. However, in Jurkat cells, we observed no increase in basal activity of the H83,86L mutant. This discrepancy could suggest a negative regulator of PAK1 kinase activity that is present in T cells. As the PAK1 immunoprecipitates used in the kinase assays are undoubtedly quite complex – containing at least PIX and GIT family members – this negative regulator could be present throughout the kinase assay or could have modified PAK prior to the kinase assay preventing the autoinhibitory region from being dysregulated by the H83,86L mutation.

Our data suggest that Rac1 may not be the Rho GTPase that activates PAK1. In support of our data, Vav1-deficient peripheral T cells have normal PAK1 activation (Fischer et al., 1998) while they presumably do not activate Rac1 as has been shown in double positive thymocytes(Reynolds et al., 2002). However, in J.CaM2 cells, a PAK1 localized pool of Rac1 may still be activated but is below the limits of detection of the CRIB pulldown assay. It is also possible that PAK1 activation in the J.CaM2 cell line has different requirements for activation from the PAK1 in the parental Jurkat cell line. This possibility could be excluded by transfecting the PAK1 H83,86L allele into J.CaM2 and measuring PAK1 activation. Pharmacological inhibitors of the Rho GTPases, such as lethal toxin, could be used to confirm that PAK1 activation in J.CaM2 is dependent on a Rho GTPase, although it is unclear whether these toxins inhibit the novel Rho GTPases that could potentially activate PAK1. Given the relative completion of the human genome, a candidate approach for finding this LAT independent GTPase is possible. One could use the CRIB pulldown assay to look for activation of each candidate in J.CaM2 cells and confirm that overexpression of a dominant negative mutant of the candidate blocks PAK1 activation in J.CaM2 cells. Alternatively, siRNA could be used to specifically reduce the activity of the candidate GTPae.

The activation of Rac1 by the TCR and CD28

Our studies indicate that Rac1 can be activated by TCR stimulation alone. CD28 ligation neither activates Rac1 nor does it augment anti-TCR-induced Rac1 activation in Jurkat cells (data not shown). Our data contradict published reports that Rac1 is activated synergistically by both CD3 and CD28 stimulation (Krawczyk et al., 2000). The

discrepancy could reflect a difference between Jurkat and mouse primary cells with regards to CD28 signaling. One possible molecular explanation for this difference is the lack of PTEN in Jurkat cells. Thus, these experiments could be repeated in the PTEN inducible Jurkat subclone(Xu et al., 2002). A final question about the activation of Rac1 is how LAT phosphorylation allows Vav1 tyrosine phosphorylation and presumed GEF activation. Since Rac1 activation appears normal in Slp-76-deficient J14 cells, Vav1 is not likely recruited to LAT via a Slp-76, GADS complex. A thorough site directed mutagenesis of the tyrosines in LAT and their effects on complex formation has already been reported (Lin and Weiss, 2001; Zhang et al., 1998a; Zhang et al., 2000). One could use these mutants to analyze the structural basis for LAT recruitment of Vav1 and consequent activation of Rac1.

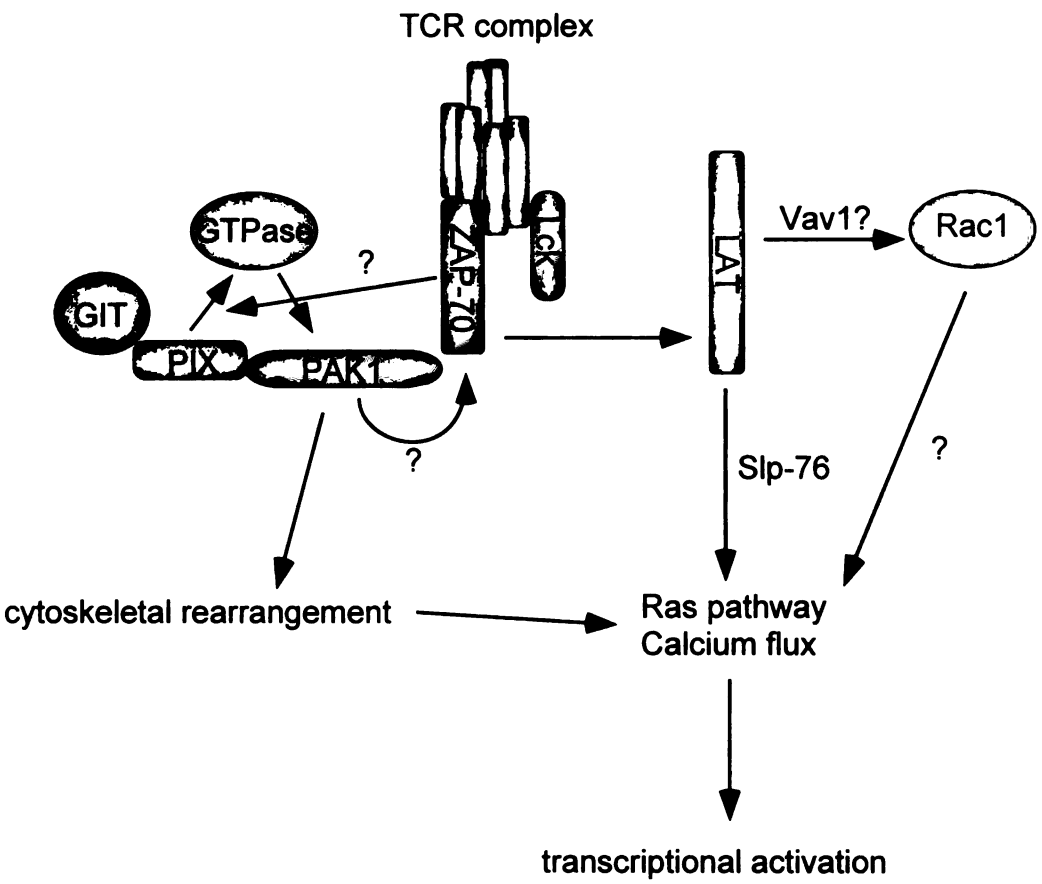
A functional role for PAK1 in ZAP-70 activation?

What is the function of PAK1 in T cells? Expression of dominant negative PAK1 disrupts the cytoskeleton of T cells (see appendiix A). F-actin levels are decreased in the basal state and MTOC reorientation towards an anti-TCR antibody coated coverslip is inhibited. Also, dominant negative PAK1 blocks conjugate formation between Jurkat cells and superantigen-pulsed Daudi B cells. These effects could be mediated through a disruption in the actin cytoskeleton. Defects in the actin cytoskeleton could also explain the defect in NFAT activation as latrunculin A also blocks an NFAT-driven reporter(Arrieumerlou et al., 2000). However, defects in actin polymerization cannot explain dominant negative PAK1's effect on Erk2 activation as neither latrunculin A nor cytochalasin D block Erk2 phosphorylation.

I would suggest that besides the actin cytoskeleton, another target of PAK1 is ZAP-70. Dominant negative PAK1 blocks ZAP-70 tyrosine phosphorylation in Jurkat cells (see appendix B for the data and figure 4 for a model). While I believe that reduced ZAP-70 tyrosine phosphorylation will correlate with reduced kinase activity, ZAP-70 kinase activity should be directly measured. Furthermore, in COS cells that express CD8-ζ, Lck, and ZAP-70, the overexpression of wild type PAK1 augments ZAP-70 tyrosine phosphorylation. Although we have not observed wild type PAK1 overexpression augmenting ZAP-70 tyrosine phosphorylation or NFAT responses in Jurkat cells, PAK1 potentiation of signaling in Jurkat cells may be masked by heavy TCR crosslinking or tonic signaling through the TCR(J. Roose, unpublished observations). The COS-18 system allows the study of Lck and ZAP-70 tyrosine phosphorylation with suboptimal stimulation since CD8 is not ligated.

How PAK1 might affect ZAP-70 tyrosine phosphorylation is not clear. One possibility is that PAK1 modifies ZAP-70 by direct serine/threonine phosphorylation. Indeed, we show that, *in vitro*, PAK1 can directly phosphorylate ZAP-70. While the PAK1 *in vitro* kinase assay demonstrates that ZAP-70 is a potential substrate of PAK1, ^{32}P-orthophosphate labeling of Jurkat cells transfected with or without an activated PAK1 allele is required to assess PAK1 phosphorylation of ZAP-70 *in vivo*. Alternatively, serine or threonine phosphorylation of ZAP-70 could be assessed using anti-phospho-serine or anti-phospho-threonine antibodies. The effect of dominant negative PAK1 on inducible serine or threonine phosphorylation could also be checked. If PAK1 phosphorylation of ZAP-70 can be demonstrated *in vivo*, one could determine the actual sites of serine/threonine phosphorylation by phosphopeptide mapping or mass

Figure 3-1: Proposed model of the PAK1 pathway in TCR signaling. Lck and ZAP-70 are activated by TCR signaling. By an unknown mechanism, these tyrosine kinases activates a GIT, PIX, PAK1 complex which might be associated with ZAP-70 in the basal state. Activated PAK1 allows further activation of ZAP-70 which can phosphorylate downstream substrates such as LAT leading to Rac1 activation, Ras pathway activation and calcium flux. The effects of PAK1 on the cytoskeleton may be an independent pathway.


TCR complex
GTPase
?
GIT
PIX
PAK1
ZAP-70
LCK
LAT
Vav1?
Rac1
?
?
Slp-76
cytoskeletal rearrangement
Ras pathway
Calcium flux
transcriptional activation

spectroscopy. Mutation of the corresponding phosphorylation sites to alanine, glutamate, or aspartate would allow assessment of the biological function, if any, of these sites in ZAP-70 by reconstitution of ZAP-70 deficient P116 cells.

It is possible, however, that PAK1's effect on ZAP-70 is indirect -- PAK1 may augment ZAP-70 tyrosine phosphorylation via other targets or even simply via a kinase independent mechanism. ZAP-70 localizes to the subcortical region of both T cells and fibroblasts and this localization appears to require a functional kinase domain(Huby et al., 1997). This localization may be important for efficient ZAP-70 function as it would be localized quite close to the membrane bound ITAMs of ζ. Perhaps, ZAP-70 is localized to this region as a result of its interaction with PAK1. As mentioned above, localization of PAK1 and ZAP-70 in T cells or COS cells could be performed by fluorescence deconvolution micropscopy to test this possibility.

We have previously shown that ZAP-70 is required to activate PAK1 in T cells. If PAK1 does have functional effects on ZAP-70 activation, the two proteins would regulate each other in a positive feedback loop. Initially, ZAP-70 would be catalytically activated and cause the activation of a GIT, PIX, PAK complex. Then, PAK1 would phosphorylate ZAP-70 and allow its further activation or the activation of additional ZAP-70 molecules in the cell. Increasing ZAP-70 activation would then result in further PAK1 activation, the tyrosine phosphorylation of LAT and the activation of transcriptional activation(see figure 4).

The role of the PAK and ZAP-70 interaction could be tested in the context of this positive feedback loop and the interaction between the two molecules. Once the interaction sites have been identified, mutation of these sites in PAK1 and ZAP-70 could

help identify whether the interaction is important for PAK1 activation of ZAP-70 or is important for PAK1 function on ZAP-70, or, in the case of a positive feedback loop, perhaps both.

Future experiments examining PAK1 function in T cells should focus on loss and gain of function. Loss of function experiments could utilize siRNA to reduce expression of PAK1 and PAK2 in Jurkat cells. Use of dominant negative PAK1 should be avoided until the smaller inhibitory pieces of PAK1, shown to block PAK1 activation in other systems, are tested in T cells. These dominant negatives should be more specific in that they cannot sequester Rho GTPases, Nck or PIX. These techniques could be used to confirm a role of PAK1 in the activation of NFAT, ZAP-70 and the NF-kB pathways.

Understanding how PAK1 functions in T cells will require identification of its relevant substrate or substrates. Since so many substrates are already known, these could be used in a candidate approach to see which of the molecules can become phosphorylated by TCR engagement in a PAK1 dependent fashion. Unknown molecules could be identified by using in vitro kinase assays with a mutant PAK1 which can only accept an ATP analogue(Shah et al., 1997). Such substrates would then be mutated at the appropriate phosphorylation site and tested for similar inhibitory effects on NFAT and TCR signaling.

Through these studies on the activation and function of PAK1, I have identified a novel signaling pathway that functions independently of LAT and Slp-76. This TCR-activated pathway utilizes a complex of PIX and GIT to activate PAK1. The function of this pathway may involve regulation of the cytoskeleton as well as direct effects on ZAP-70 through a possible positive feedback loop.

Appendix A: Dominant negative PAK1 blocks both basal and TCR-induced cytoskeletal rearrangements

Summary:

It has been previously demonstrated that dominant negative PAK1 blocks TCR-induced NFAT activation. Given the role of PAK1 in the regulation of the cytoskeleton and the emerging role of the cytoskeleton in T cell activation, we hypothesized that PAK1 might be required for T cell cytoskeletal rearrangement. As expected, dominant negative PAK1 expression in Jurkat T cells reduced their F-actin content. Furthermore, these cells were unable to form conjugates with Daudi B cells prepulsed with Staphylococcal enterotoxin E and also failed to reorient their microtubule organizing centers towards a polarized anti-TCR stimulus.

Introduction:

Many known PAK1 targets influence the actin and myosin based cytoskeleton, including LIM kinase-1, myosin light chain kinase, and myosins themselves(reviewed by (Bagrodia and Cerione, 1999)). Since actin, myosin, and the microtubule cytoskeleton are important for TCR-induced cytoskeletal rearrangements, we hypothesized that PAK1 might be required for TCR-induced cytoskeletal rearrangements including F-actin accumulation, MTOC reorientation, synapse formation, and p/c-SMAC formation. Blocking inducible PAK1 activation might disrupt these complex phenomena.

We further hypothesized that dominant negative PAK1's ability to suppress NFAT transcriptional activation might be due to its effects on the cytoskeleton. Actin depolymerizing agents block both clustering of the ζ chain of the TCR(Krummel et al., 2000) as well as calcium flux(Arrieumerlou et al., 2000). Dominant negative PAK1 also blocks TCR signaling above the level of calcium flux – TCR stimulation and phorbol ester treatment cannot induce NFAT transcriptional activation whereas addition of calcium ionophore and phorbol ester together restores normal signaling(Yablonski et al., 1998a).

Here, we demonstrate that dominant negative PAK1 overexpression had profound effects on the cytoskeleton. Dominant negative PAK1 expressing Jurkat T cells had reduced F-actin content even without TCR stimulation. Furthermore, dominant negative PAK1 expressing cells could not form stable conjugates with superantigen pulsed Daudi B cells and surprisingly, this effect was independent of ZAP-70. Finally, dominant negative PAK1 expression prevented reorientation of the MTOC towards an anti-TCR

Results:

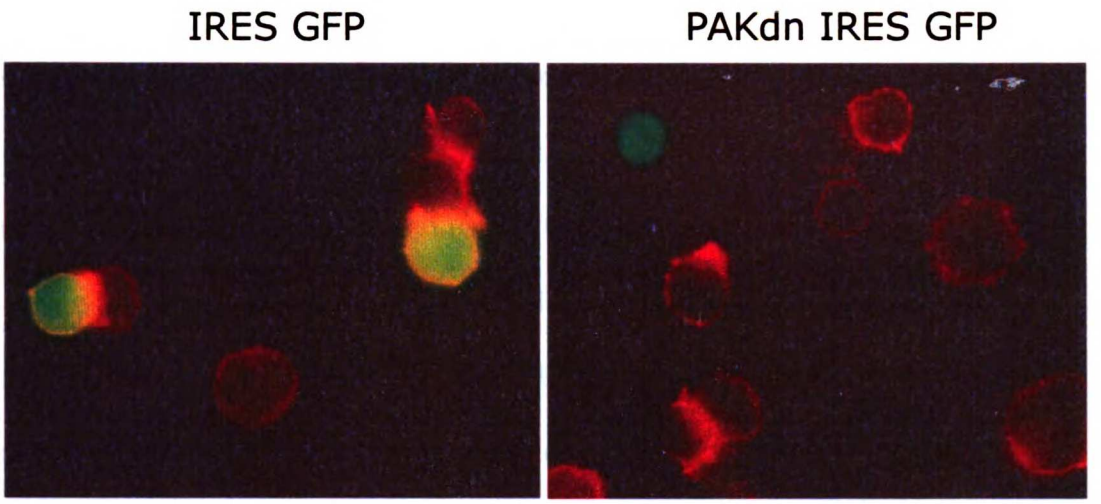
Previous research on PAK1 in fibroblasts and neurons suggested a role for PAK1 in the actin cytoskeleton. We therefore examined how the overexpression of dominant negative PAK1 would affect the actin cytoskeleton.

Jurkat cells were transfected with either an internal ribosomal entry site – green fluorescent protein (IRES-GFP) vector or a kinase domain deleted form of PAK1 (dominant negative PAK1) IRES GFP that has been previously shown to block TCR-inducible PAK1 kinase activity(Yablonski et al., 1998a). Transfected cells were incubated with staphylococcal enterotoxin-pulsed Daudi lymphoblastoid B cells. F-actin levels were measured by staining with phalloidin-rhodamine. By fluorescence microscopy, vector transfection did not affect F-actin content in resting Jurkat cells, but transfection of dominant negative PAK1 reduced F-actin content. The difference in F-actin levels was confirmed by FACS analysis (see figure A-1). While this decrease was reproducible, it was not nearly as large as the precipitous decrease in F-actin content when cells were treated with latrunculin A, a known actin depolymerizing agent. Importantly, untransfected cells in the same experiment did not have decreased levels of F-actin as compared to vector transfected cells. In this experiment, we also noticed that vector transfected cells were often seen in conjugates with superantigen pulsed Raji B cells while dominant negative PAK1 expressing cells were not.

The reorientation of the MTOC is a later event in T cell activation and appears to be required for directional secretion in T cells(Kupfer and Dennert, 1984). We examined the reorientation of the MTOC in Jurkat cells transfected with either empty IRES-GFP vector or dominant negative PAK1 IRES-GFP. After transfection, cells were allowed to

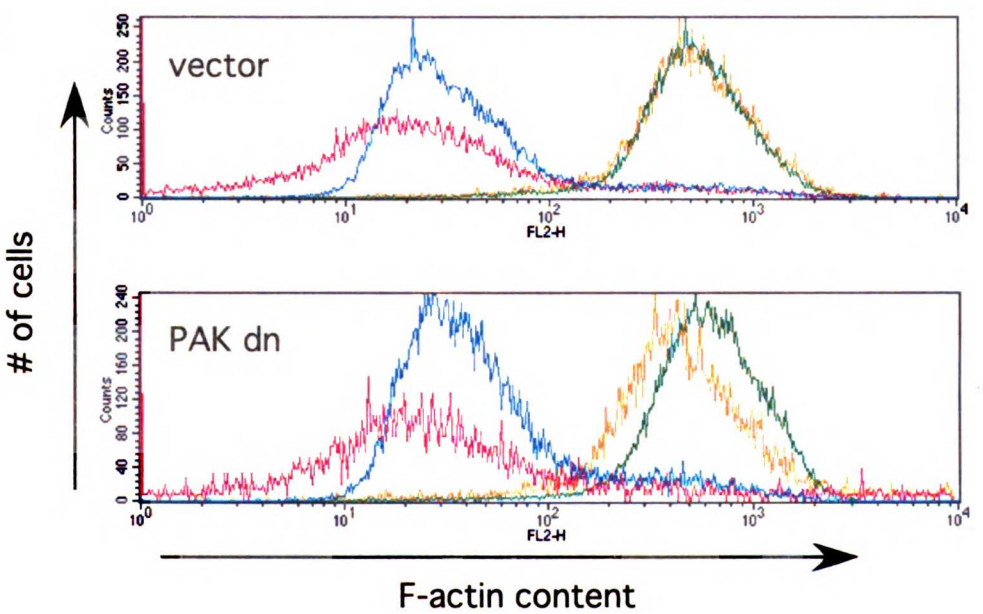
Figure A-1: Expression of a dominant negative form of PAK1 reduces F-actin content. (A) Jurkat cells were transiently transfected with 30 μg of MSCV EF1a PAK dominant negative IRES GFP or 30 μg of MSCV EF1a IRES GFP. 16 hours after transfection, conjugates were formed with Raji B cells prepulsed with staphylococcal enterotoxin E and the couples were fixed in 4% paraformaldehyde onto coverslips, permeabilized with 0.1% Triton X-100 and stained with 10 μg/ml of phalloidin TRITC. Representative images are shown. (B) Performed as in A, but Jurkat cells were transfected with 20 μg of pEF6B PAK dominant negative and 10 μg of pEF CD25T or 20 μg of pEF6B and 10 μg of pEF CD25T. 16 hours after transfection the cells were either immediately fixed or treated with Latrunculin A for 15 minutes. Cells were fixed in 4% paraformaldehyde and permeabilized in FACS tubes. The cells were then stained wth an anti-CD25 FITC antibody and 10 mg/ml of phalloidin TRITC. Representative FACS scans are shown.

A



red = phalloidin
green = GFP

B



Key:

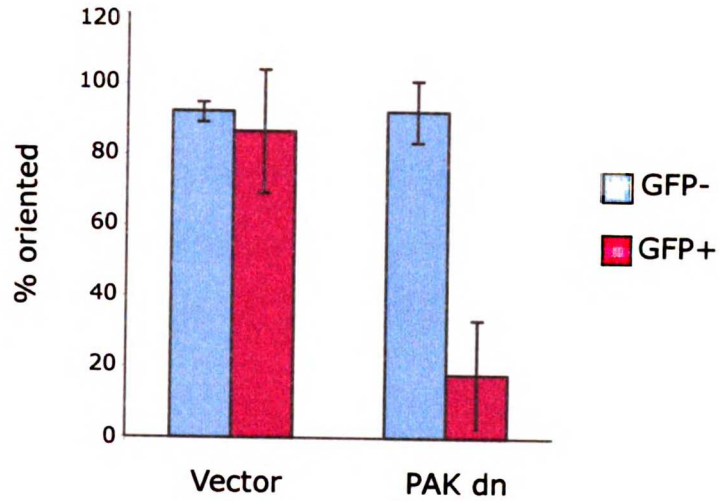
- transfected cells (by CD25 T)
- untransfected cells
- transfected cells + Lat A
- untransfected cells + Lat A

settle on anti-TCR antibody coated coverslips for 20 minutes at 37°C. The cells were fixed and the microtubule cytoskeleton was visualized using a rat anti-tubulin antibody and an anti-Rat Cy3 secondary antibody. Transfected cells were identified based on GFP fluorescence. The cells were imaged using fluorescence microscopy of 7-8 1 μm sections through the cells. MTOC reorientation was scored as positive if the MTOC was present in the first 3 1 μm sections and negative if the MTOC was present in any of the other sections. Over three independent transfections and analyses, dominant negative PAK1 expressing cells did not reorient their MTOCs (~20%) where as untransfected cells in the same field reoriented their MTOCs as well as vector transfected cells (~90%) (figure A-2).

Given the role of actin and myosin in the formation of the immunological synapse and the role of PAK1 in regulating actin polymerization and myosin activity, we examined the effect of dominant negative PAK1 expression on synapse formation. As we had noticed while attempting to look at F-actin accumulation in T cell-APC couples, dominant negative PAK1 expression blocked superantigen-induced synapses between Jurkat cells and Raji B cells (figure A-3). As it has been recently reported that conjugate formation does not require ZAP-70(Morgan et al., 2001), we tested the ability of PAK1 to block conjugate formation in the ZAP-70 deficient P116 cell line. Notably, dominant negative PAK1 blocked conjugate formation in the ZAP-70 deficient P116 cells, suggesting that dominant negative PAK1 effects did not require ZAP-70. These data also suggested that dominant negative PAK1 might not require inhibition of inducible PAK1 kinase activity with regards to conjugate formation since PAK1 is not activated in P116 cells.

Figure A-2: Dominant negative PAK1 blocks MTOC reorientation. Performed as in A-1A except after transfection, cell were allowed to settle onto slides coated with an anti-TCR antibody (C305) for 15 minutes at 37°C. Following fixation and permeabilization, cells were stained with an anti-tubulin antibody and a Cy3 labeled secondary antibody. (A) % reoriented cells over three independent experiments are shown. (B) Representative fields at 1 μm and 5 μm from the glass are shown.

A



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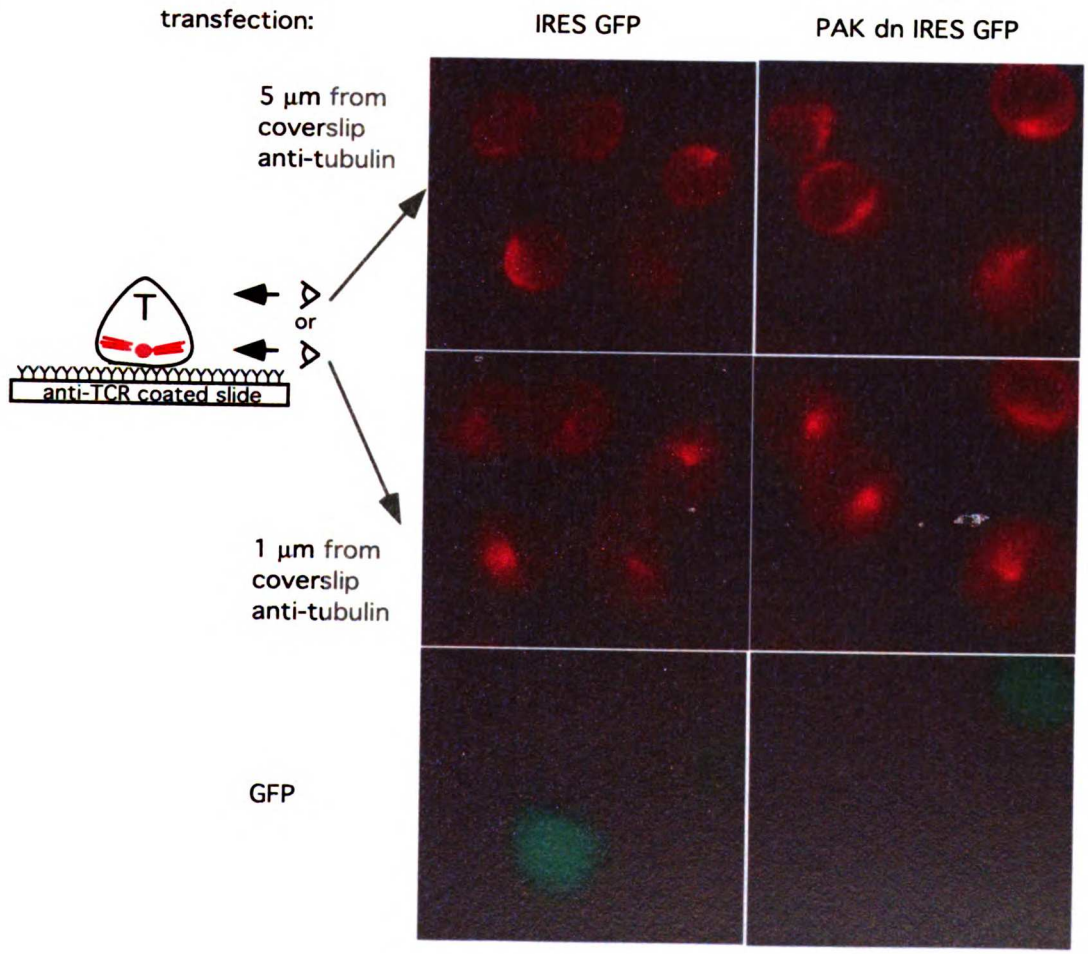
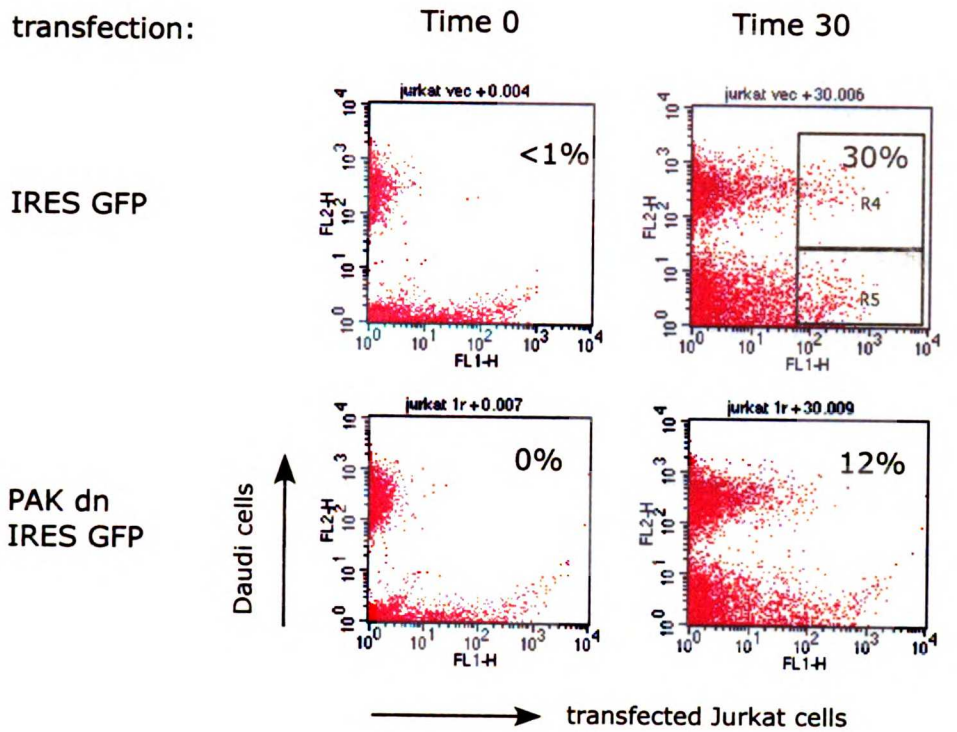


Figure A-3: Dominant negative PAK1 blocks conjugate formation. (A) Jurkat cells were transfected with either MSCV EF1a IRES-GFP or MSCV EF1a PAK dn IRES-GFP. 16-18 hours later, the cells were spun down with Daudi B cells prepulsed with Staphylococcal enterotoxin E and PKH26 for 15 minutes as described in the Materials and Methods section. The pellet was resuspended in 4% paraformaldehyde and conjugates were analyzed by fluorescence activated cell sorting. Representative FACS scans of 3 independent experiments are shown. (B) As in A except with ZAP-70-deficient P116 cells.

A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Time 30
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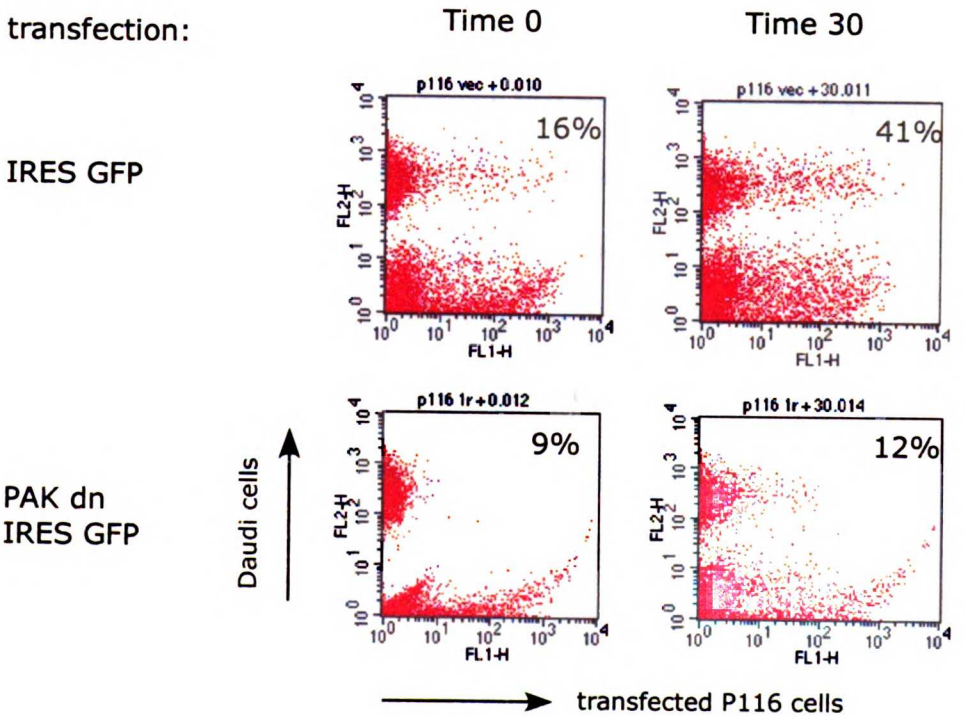
PAK dn
IRES GFP
jurkat vec +0.004
<1%
jurkat vec +30.006
30%
R4
R5
jurkat 1r +0.007
0%
jurkat 1r +30.009
12%
FL1-H
FL2-H
Daudi cells
transfected Jurkat cells

B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Time 30
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PAK dn
IRES GFP
p116 vec +0.010
16%
p116 vec +30.011
41%
p116 1r +0.012
9%
p116 1r +30.014
12%
FL1-H
FL2-H
Daudi cells
transfected P116 cells

Discussion:

PAK1 is known to regulate the actin cytoskeleton. Through triggering a LIM kinase-1 and cofilin pathway, PAK1 promotes F-actin accumulation(Edwards et al., 1999). In Jurkat T cells, expression of dominant negative PAK have effects consistent with these observations. Total F-actin content of resting Jurkat cells is decreased by expression of dominant negative PAK1 (figure A-1). Interestingly, these effects are seen in unstimulated Jurkat cells where PAK1 is not activated. It is possible that dominant negative PAK1 blocks actin polymerization by interfering with a low level of PAK1 activity present in unstimulated Jurkat cells. We attempted to stimulate cells with anti-TCR antibodies to examine inducible F-actin accumulation, but induced F-actin increases were small and not reproducible even in vector transfected cells. However, others have reported a decrease in actin capping with the TCR when dominant negative PAK1 is overexpressed(Bubeck Wardenburg et al., 1998).

Since myosin motors are important for the formation of the immunological synapse(Wulfing and Davis, 1998) and PAK1 is known to regulate myosins through direct and indirect means, we attempted to assess myosin function in conjugates of Jurkat cells expressing dominant negative PAK1 and Daudi B cells pulsed with superantigen. We were surprised to find that cells expressing dominant negative PAK1 do not form conjugates as compared to untransfected or vector transfected cells (figure A-2). This block might be due to the defect in actin polymerization in dominant negative PAK1 expressing cells. Alternatively, dominant negative PAK1 might block inside-out integrin signaling.

Interestingly, in the ZAP-70 deficient P116 cell line, dominant negative PAK1 also blocks conjugate formation. In these same cells, PAK1 does not become inducibly activated. As in the reduced level of F-actin in unstimulated Jurkats, dominant negative PAK1 in P116 cells might block a basal level of PAK1 activity cells that is required for conjugate formation. This basal PAK1 activity might be required for normal maintenance of the actin cytoskeleton and therefore for conjugate formation. However, from these studies it is difficult to rule out the possibility that dominant negative PAK1 functions independently of its ability to block PAK1 activation by the TCR. Notably, we have not examined the effects of wild type PAK1 overexpression in these assays and it is possible that the effects of dominant PAK1 on the T cell cytoskeleton are dependent on an adapter function of PAK1 such as PIX recruitment(Obermeier et al., 1998). Further investigation of PAK1 function should rely on the PAK1 inhibitory segment alone as a dominant negative or other means of reducing PAK1 expression such as siRNA(see chapter 3).

To overcome the defect in conjugate formation, we examined the reorientation of the MTOC towards coverslips coated with anti-TCR antibodies. When cells were scored for successful MTOC orientation, dominant negative PAK1 expressing cells infrequently oriented their MTOCs (~25% oriented) while untransfected cells in the same field oriented with high efficiency (~90% oriented). Perhaps, the defect in MTOC reorientation is not surprising given the reduced F-actin content of dominant negative PAK1 expressing cells. However, the link between F-actin and MTOC reorientation is not clear(Sedwick et al., 1999).

That dominant negative PAK1 overexpression has such a dramatic effect on the T cell cytoskeleton is perhaps not unexpected given previous data on PAK1 substrates.

However, confusingly, dominant negative PAK1's effects on F-actin levels and conjugate formation appear to be independent of inducible PAK1 activity – suggesting a low basal level of PAK1 activity is required for these events. This possibility calls into question the function of inducible PAK1 kinase activity. Alternatively, dominant negative PAK1 has an effect on the T cell cytoskeleton that is independent of its effect on PAK1 kinase activity – a possibility that should be initially tested by looking at wild type PAK1's effect on the T cell cytoskeleton.

We would hypothesize that there exist other PAK1 substrates that do depend on inducible PAK1 activation. As briefly highlighted in Chapter 3, these substrates could be found using *in vitro* kinase assays using a modified GST-PAK1 recombinant kinase on Jurkat cell lysates using a modified radioactive ATP analogue. Confirmation of *in vivo* phosphorylation and using non-phosphorylatable mutants of these targets to confirm functional relevance would be critical in this screening approach. Alternatively, the known list of PAK1 substrates could serve as a starting point in a hunt for relevant substrates in T cells. As will be discussed in Appendix B, we believe that ZAP-70 is an excellent candidate based on its interaction with PAK1 and dominant negative PAK1's functional effect on ZAP-70 tyrosine phosphorylation.

Materials and Methods:

Reagents

The cell lines used were the Jurkat T cell line E6-1 (Weiss and Stobo, 1984), the ZAP-70 deficient P116 Jurkat subline, and the ZAP-70 reconstituted P116 C39(Williams et al., 1998). Cells were grown in RPMI-1640 with 5% fetal calf serum supplemented with penicillin, streptomycin and glutamine at 37°C in humidified 5% CO_2. TCR stimulations were performed with the anti-Jurkat TCR V_β C305 mAb (Weiss et al., 1984). Prior to any stimulation, CD3 surface expression was confirmed by fluorescence-activated cell sorter analysis. The rat anti-tubulin antibody YOL1/34 was purchase from Harlan Sera-Laboratories (Loughborough, UK). The anti-rat Cy3 secondary antibody was purchased from Jackson Immuno Research Laboratories (West Grove, PA). Staphylococcal enterotoxin E (SEE) was purchased from Toxin Tecnology (Sarasota, FL). Phalloidin-TRITC was purchased from Sigma (St. Louis, MO). Latrunculin A was purchased from Calbiochem (San Diego, CA).

MTOC reorientation assay

Cells were washed once in PBS and rested in PBS at 37°C for 15 minutes. The cells were plated onto slides precoated with anti-TCR antibody (C305 ascites used as 1:100 dilution in PBS overnight at 4°C). The cells were allowed to settle onto the slide for 20 minutes at 37°C were fixed by the careful addition of 20% paraformaldehyde to achieve a final concentration of 4% paraformaldehyde. After 15 minutes of fixation, the liquid was removed and the cells were permeabilized with 0.1% Triton X-100 in staining buffer (0.1% non-fat powdered dry milk, 0.1% BSA). The cells were rinsed in a large volume of

staining buffer and blocked in the same buffer for 15 minutes at room temperature. A 1:100 dilution of the anti-tubulin antibody in staining buffer was added for 15 minutes at room temperature. The cells were washed for 5 minutes 3 times in staining buffer, followed by 15 minutes in 1:100 secondary antibody in staining buffer and an additional 3 washes. 11 μl of Mowiol 4-88 mounting solution (Calbiochem, San Diego, CA) plus 0.2% DABCO anti-fade (Sigma, St. Louis, MO) was carefully placed on the cells followed by a coverslip. The mount was allowed to dry for 24 hours before analysis by fluorescence microscopy.

Conjugate formation assay

Daudi B cells were prelabeled with PKH26 (red) (final concentration of $2x10^{-6}$ M for 10^7 cells/mL) according to the manufacturer's protocol (Sigma, St. Louis, MO) and then preloaded with superantigen SEE (20 minutes at 37°C, 1 μg/ml final). Jurkat cells were pelleted in a microfuge for 1 minute at low speed with an equal number of prelabeled and preloaded Daudi B cells. The undisturbed pellet was placed at 37°C for 15 minutes. To stop the reaction, the supernatant was removed and the pellet was gently disrupted by resuspending in 4% paraformaldehyde in PBS. Cells were washed in FACS wash buffer and analyzed by FACS.

F-actin staining by fluorescence microscopy

Cells were washed in PBS with calcium and magnesium and rested for 15 minutes at 37°C. Conjugates were formed as described in the conjugate formation assay except PKH dye was not used to label the Daudi cells and the couples were plated onto slides during

fixation and allowed to settle. After permeabilization as described above for MTOC reorientation, the cells were stained with 1 μg/ml of phallodin-TRITC for 30 minutes at room temperature. The cells were washed 3 times in staining buffer and fixed as described above. Transfected cells were identified by GFP fluorescence.

F-actin staining by FACS

Cells were washed in PBS with calcium and magnesium and rested for 15 minutes at 37°C. The cells were fixed in FACS tubes in 4% paraformaldehyde for 15 minutes on ice and then washed once in FACS staining buffer (PBS without calcium or magnesium + 1% fetal bovine serum) and permeabilized in 0.1% Triton X-100 in FACS staining buffer. Following a wash with FACS staining buffer, the cells were stained with 1 μg/ml of phallodin-TRITC and anti-CD25 FITC (1 μl / 1 million cells / 100 μl of FACS buffer) for 30 minutes at room temperature. The cells were washed 3 times in FACS buffer and analyzed by FACS.

Appendix B: A potential functional interaction between PAK1 and ZAP-70

Summary:

Whereas dominant negative PAK1 blocks TCR-induced NFAT and Erk activation, the precise nature of the block is not known. Here, we demonstrate that dominant negative PAK1 overexpression blocks the TCR-induced tyrosine phosphorylation of ZAP-70. Furthermore, in a heterologous system that recapitulates ZAP-70 recruitment to ζ and its subsequent tyrosine phosphorylation, wild type PAK1 overexpression potentiates ZAP-70 tyrosine phosphorylation. Finally, we demonstrate that PAK1 can serine or threonine phosphorylate ZAP-70 *in vitro* and that the two molecules can interact in 293T cells.

Introduction:

The overexpression of a kinase domain deleted allele of PAK1 functions as a dominant negative for PAK1 activation by the TCR. In addition, dominant negative PAK1, but not wild type PAK1, blocks NFAT activation by the TCR. This block is specific to TCR signaling as dominant negative PAK1 overexpression does not block the muscarinic G protein coupled receptor's ability to activate NFAT through PLCβ. Furthermore, the block appears to be upstream of both the Ras and phosphoinositide pathways in that either calcium ionophore or phorbol ester alone with TCR stimulation cannot restore normal TCR-induced NFAT activation. Dominant negative PAK1 does not appear to generally disrupt the NFAT response since calcium ionophore and phorbol ester together can restore NFAT activation. Consistent with this effect on NFAT reporter activation, the phosphorylation of Erk2, one downstream component of the Ras pathway is reduced by the overexpression of dominant negative PAK1(Yablonski et al., 1998a). How dominant negative PAK1 blocks the TCR signaling pathway and how normal PAK1 function is important for TCR signaling is not yet known.

In this appendix, we further the study of PAK1 function in T cells. First, we attempted to assess the block in TCR signaling caused by dominant negative PAK1 overexpression. Second, we attempted to understand how this block was induced by blocking PAK1 function. We demonstrate that overexpression of dominant negative PAK1 in Jurkat cells blocks TCR-induced ZAP-70 tyrosine phosphorylation but wild type PAK1 does not. Conversely, in a COS cell system reconstituted with CD8-ζ, Lck, and ZAP-70, the overexpression of wild type PAK1 augments the tyrosine phosphorylation of ZAP-70. Furthermore, *in vitro*, PAK1 is capable of directly

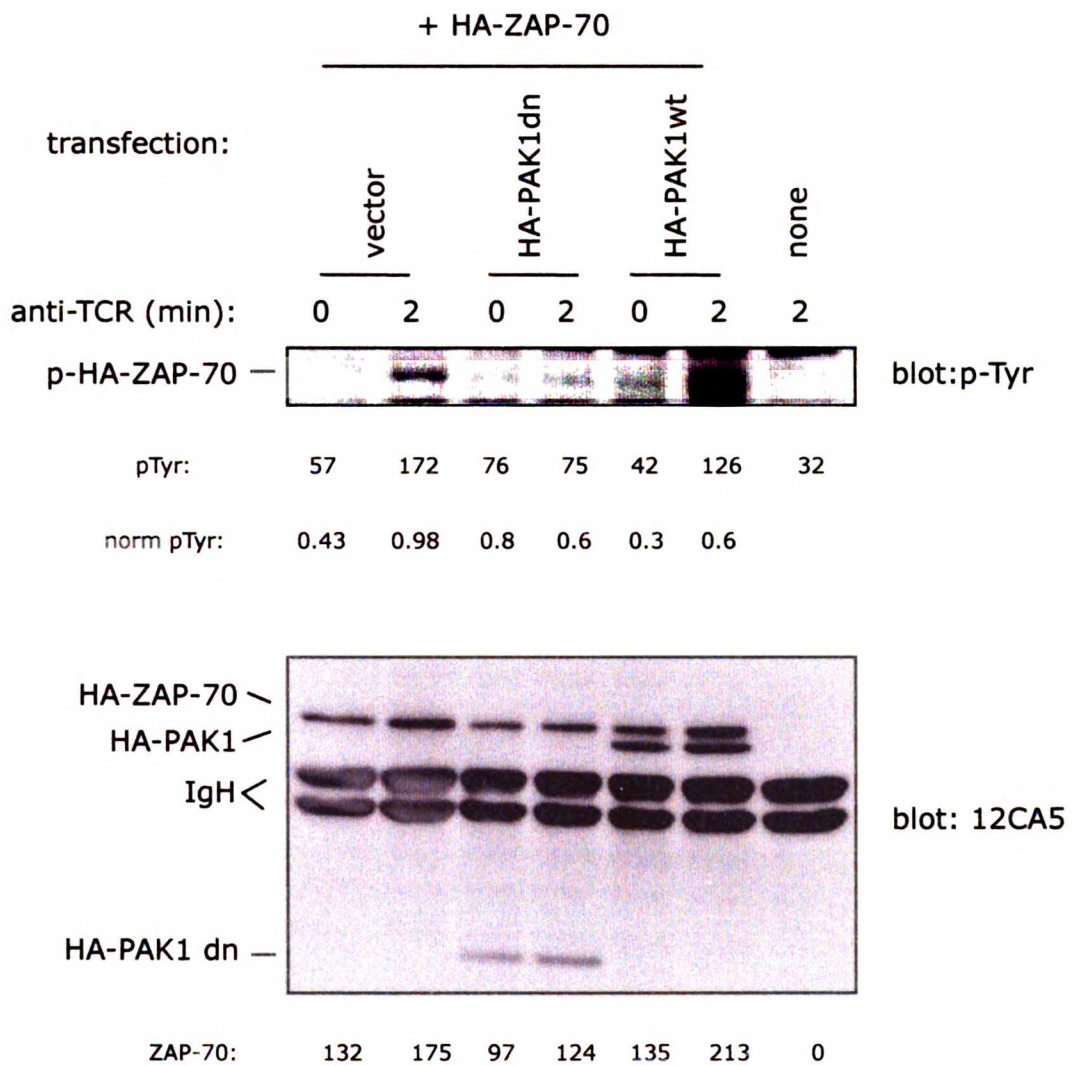
phosphorylating ZAP-70 and the two proteins specifically associate in 293T cells. These data suggest a possible role for PAK1 in the normal function of ZAP-70 in a positive feedback loop between the two molecules.

Results:

Since dominant negative PAK1 blocks signaling upstream of both the Ras and phosphatidylinositide hydrolysis pathways, we examined the effects of dominant negative PAK1 on the most proximal events of TCR signaling. Gross patterns of tyrosine phosphorylation from Jurkat cells overexpressing dominant negative PAK1 suggested a defect in ZAP-70 tyrosine phosphorylation. To confirm this, we transiently transfected Jurkat cells with haemagluttinin (HA) tagged-wild type ZAP-70 and either vector, HA-tagged dominant negative PAK1 or HA-tagged wild type PAK1. The cells were stimulated with an anti-TCR antibody or a buffer control and ZAP-70 was immunoprecipiated using an anti-HA antibody. SDS-PAGE and western blotting were used to determine the tyrosine phosphoryation status of ZAP-70. As shown in figure B-1, TCR-induced ZAP-70 tyrosine phosphorylation was blocked by the expression of dominant negative PAK1, but not by vector or wild type PAK1 expression. Notably, in these experiments, PAK1 was not detectably tyrosine phosphorylated(not shown). To rule out an effect on Lck function, we examined the effect of dominant negative PAK1 overexpression on the tyrosine phosphorylation of a chimeric CD25-ζ receptor in Jurkat T cells. CD25 was either crosslinked or stimulated with a PBS control and CD25 was immunoprecipitated. As seen in figure B-2, expression of dominant negative PAK1 did not block tyrosine phosphorylation of CD25-ζ, suggesting that Lck kinase activity is not affected by dominant negative PAK1 expression.

We then utilized a heterologous system to study ζ, Lck, and ZAP-70 activation and tyrosine phosphorylation. The COS-18 derivative of COS-7 cells stably expresses a CD8-ζ chimeric receptor. This CD8-ζ chimera can be tyrosine phosphorylated by

Figure B-1: Dominant negative PAK1 blocks ZAP-70 tyrosine phosphorylation. Jurkat cells were transiently transfected with the indicated plasmids to achieve equal expression. Cells were stimulated with an anti-TCR antibody or a buffer control for 2 minutes. Anti-HA immunoprecipitates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. Note that under these conditions, PAK1 is not tyrosine phosphorylated as detected by the 4G10 anti-phosphotyrosine antibody. Densitometry of ZAP-70 is shown below both gels. Normalized pTyr was calculated by dividing the pTyr signal by the ZAP-70 signal. Similar results were seen in 4 independent experiments.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pTyr:
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norm pTyr:
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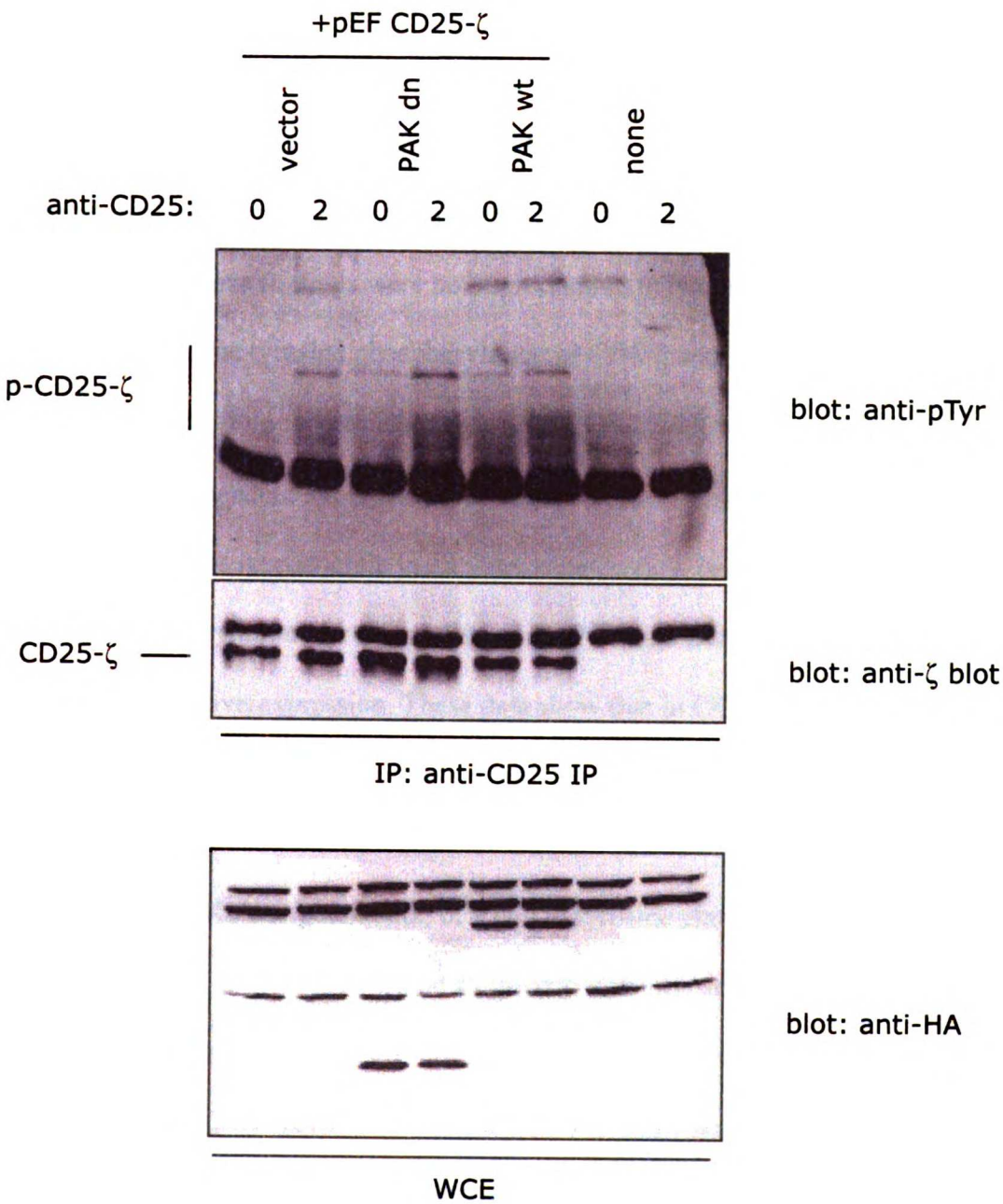
HA-PAK1
IgH
HA-PAK1 dn
blot: 12CA5
ZAP-70:
132 175 97 124 135 213 0

Figure B-2: Dominant negative PAK1 does not block CD25-ζ tyrosine phosphorylation. Jurkat cells were transfected with the indicated constructs to achieve equal expression. Cells were stimulated with a mouse anti-CD25 antibody and crosslinked with a goat anti-mouse secondary antibody for 2 minutes. Anti-CD25 immunoprecipitates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. The lower panel shows the expression of HA-tagged dominant negative PAK1 and HA-tagged wild type PAK1 from whole cell extracts. Similar results were seen in 3 independent experiments.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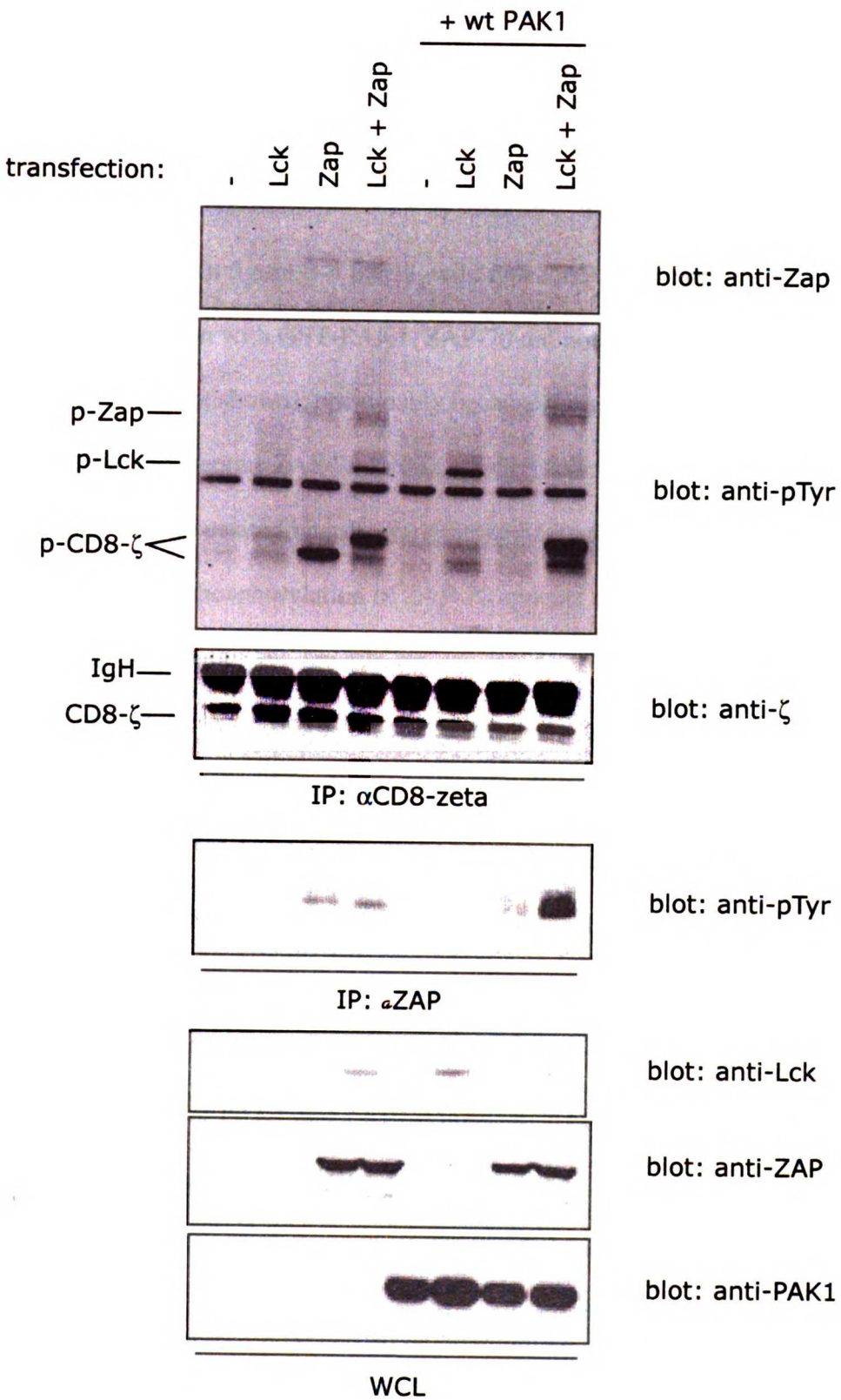
CD25-ζ
blot: anti-pTyr
blot: anti-ζ blot
IP: anti-CD25 IP
blot: anti-HA
WCE

expression of lck. Co-expression of Lck and ZAP-70 allows ZAP-70 recruitment to tyrosine phosphorylated CD8-ζ(Iwashima et al., 1994). When wild type PAK1 was overexpressed with Lck and ZAP-70 in COS-18 cells, ZAP-70 tyrosine phosphorylation was augmented, while Lck phosphorylation or CD8-ζ phosphorylation were not dramatically increased (figure B-3). Although in this particular experiment it appears that Lck phosphorylation is augmented by wild type PAK1 expression (figure B-3, lane 6) and ZAP-70 tyrosine phosphorylation is not enhanced by Lck co-transfection (compare figure 3B, lanes 3 and 4), these findings were not reproducible in two other independent experiments. Furthermore, the tyrosine phosphorylation of CD8-ζ was also variable in these experiments – there was no reproducible change in CD8-ζ with wild type PAK1 overexpression in two independent experiments. In constrast, PAK1-induced ZAP-70 tyorsine phosphorylation was reproducible in all three experiments. In agreement with unchanged CD8-ζ phosphorylation, the association of ZAP-70 and CD8-ζ was also not altered by wild type PAK1 overexpression. These data show that in COS cells, wild type PAK1 augments ZAP-70 tyrosine phosphorylation without a reproducible effect on Lck phosphorylation of CD8-ζ.

While wild type PAK1's augmentation of ZAP-70 tyrosine phosphorylation could be mediated by any number of mechanisms, the simplest possibility would be direct serine or threonine phosphorylation of ZAP-70 by PAK1 that might promote enhanced tyrosine phosphorylation of ZAP-70. We tested ZAP-70 as an *in vitro* substrate of PAK1 using recombinant GST-PAK1. GST-PAK1 is constitutively active when purified from bacteria both in autophosphorylation and in phosphorylation of histone H4(Chong et al., 2001). 293T cells were transfected with cDNAs encoding vector, wild type ZAP-70 or

Figure B-3: PAK1 potentiaites ZAP-70 tyrosine phosphorylation in COS cells. COS-18 cells, that stably express CD8-ζ, were transiently transfected with the indicated plasmids. Cells were lysed and ZAP-70 or CD8-ζ was immunoprecipitated. SDS-PAGE and western blotting were performed as shown. Changes in CD8-ζ and Lck tyrosine phosphorylation were not reproducible in 3 independent experiments while increased ZAP-70 phosphorylation due to PAK1 overexpression was seen in all 3 experiments.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transfection:
-
Lck
Zap
Lck + Zap
-
Lck
Zap
Lck + Zap
blot: anti-Zap
p-Zap
p-Lck
p-CD8-ζ
blot: anti-pTyr
IgH
CD8-ζ
blot: anti-ζ
IP: αCD8-zeta
blot: anti-pTyr
IP: αZAP
blot: anti-Lck
blot: anti-ZAP
blot: anti-PAK1
WCL

ΔSH2 ZAP-70 in which both SH2 domains and interdomain A are deleted. Lysates from these cells were denatured by boiling for 15 minutes in 1% SDS to disrupt any ZAP-70 nucleated complexes. After cooling on ice, the lysates were diluted with 1% NP-40 lysis buffer to 0.5% SDS and anti-HA immunoprecipitates were subject to a PAK1 kinase assay using 0.5 μg of GST-PAK1 and γ^{32}P-ATP as described in the Materials and Methods section. As shown in figure B-4 lane 2, wild type ZAP-70 became phosphorylated by incubation with GST-PAK1. ZAP-70 did not become phosphorylated without GST-PAK1 (data not shown), presumably because there is no manganese in the kinase buffer to allow endogenous ZAP-70 kinase activity(Isakov et al., 1996) and because interactions with associated kinases were disrupted by denaturation before immunoprecipitation. This phosphorylation of ZAP-70 appears to localize to the amino terminus as the deletion of both SH2 domains and interdomain A greatly reduced PAK1-induced phosphorylation. Notably, ΔSH2 ZAP-70 was not phosphorylated under conditions of equal loading in the kinase assay in an independent experiment(data not shown), suggesting that the failure of PAK1 to phosphorylate ΔSH2 ZAP-70 in the experiment shown was not due to excessive levels of ΔSH2 ZAP-70.

Finally, given that ZAP-70 can be a substrate for PAK1 in vitro, we asked if PAK1 could interact with ZAP-70 in cells. 293T cells were transfected with either vector, PAK1 alone, ZAP-70 alone or PAK1 and ZAP-70 together. Anti-PAK1 immunoprecipitates were then blotted for ZAP-70. As shown in figure B-5, anti-PAK1 antibodies specifically immunoprecipitated ZAP-70 from 293T cells. This was repeated using anti-HA immunoprecipitates of PAK1, confirming that this co-immunoprecipitation was not due to crossreactivity of the PAK1 antibody to ZAP-70. We

Figure B-4: PAK1 can phosphorylate ZAP-70 *in vitro*. 293T cells were transfected with the indicated constructs. 2 days after transfection, the cells were lysed in standard immunoprecipitation buffer, denatured and boiled to disrupt protein-protein interactions, and subject to anti-HA immunoprecipitation. PAK1 kinases were performed on these immunoprecipitates using 0.5 μg of recombinant GST-PAK1, prepared as described in the Materials and Methods section. Autoradiography and western blotting were performed after SDS-PAGE. Densitometry measurements in arbitrary units are shown below each lane for the revelant band. Normalized phosphorylation to the amount of ZAP-70 is also shown. This experiment was performed 2 times with similar results.

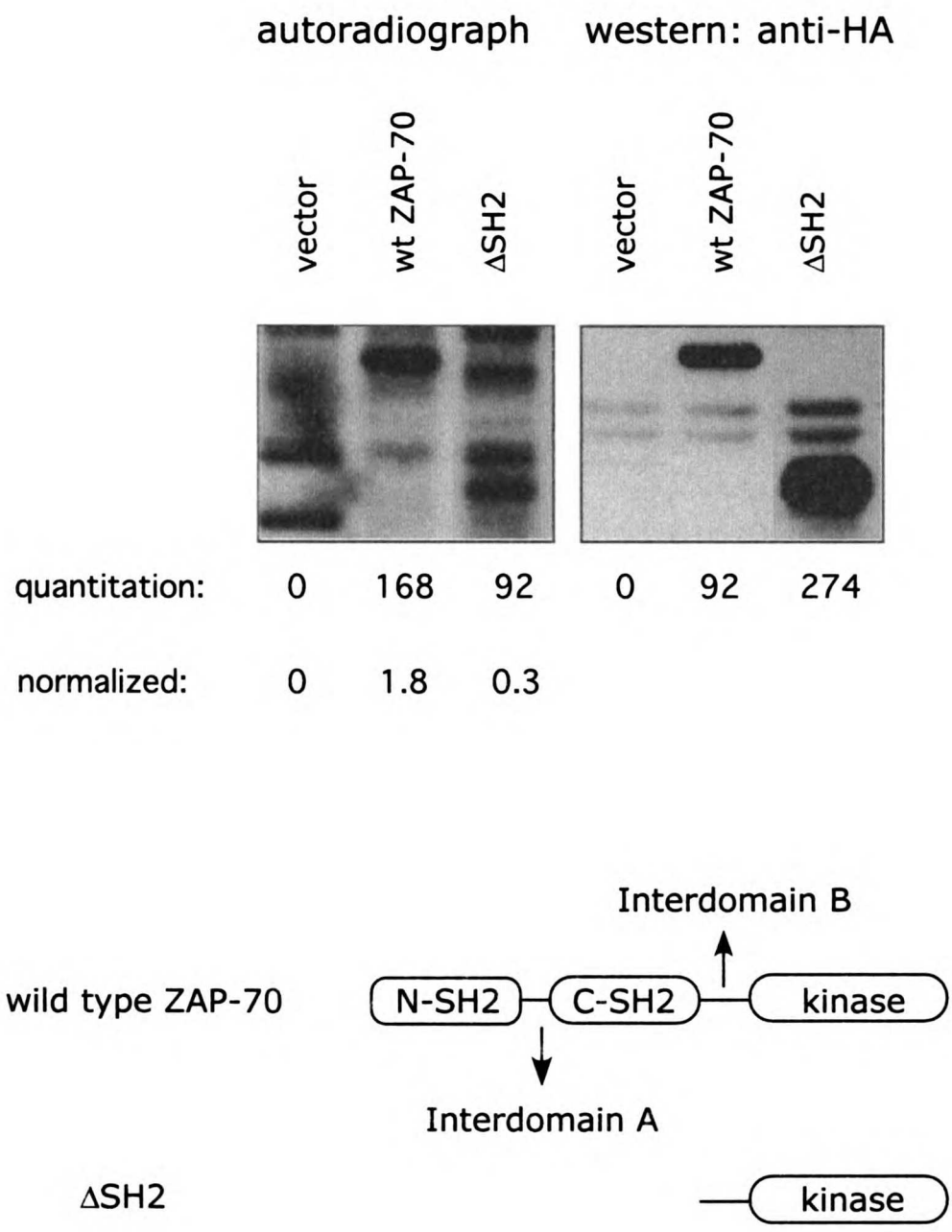

autoradiograph
western: anti-HA
vector
wt ZAP-70
ΔSH2
vector
wt ZAP-70
ΔSH2
quantitation: 0 168 92 0 92 274
normalized: 0 1.8 0.3
Interdomain B
wild type ZAP-70
N-SH2
C-SH2
kinase
Interdomain A
ΔSH2
kinase

Figure B-5: PAK1 associates with ZAP-70 in 293T cells. 293T cells were transfected with the indicated plasmids. Cells were lysed and PAK1 was immunoprecipitated using an anti-PAK1 antibody. Immunoprecipitates were analyzed for the presence of ZAP-70 and PAK1 by western blotting in the upper two gels. Levels of PAK1 and ZAP-70 from whole cell lysates are shown in the lower two gel. This experiment was repeated 4 times with similar results.

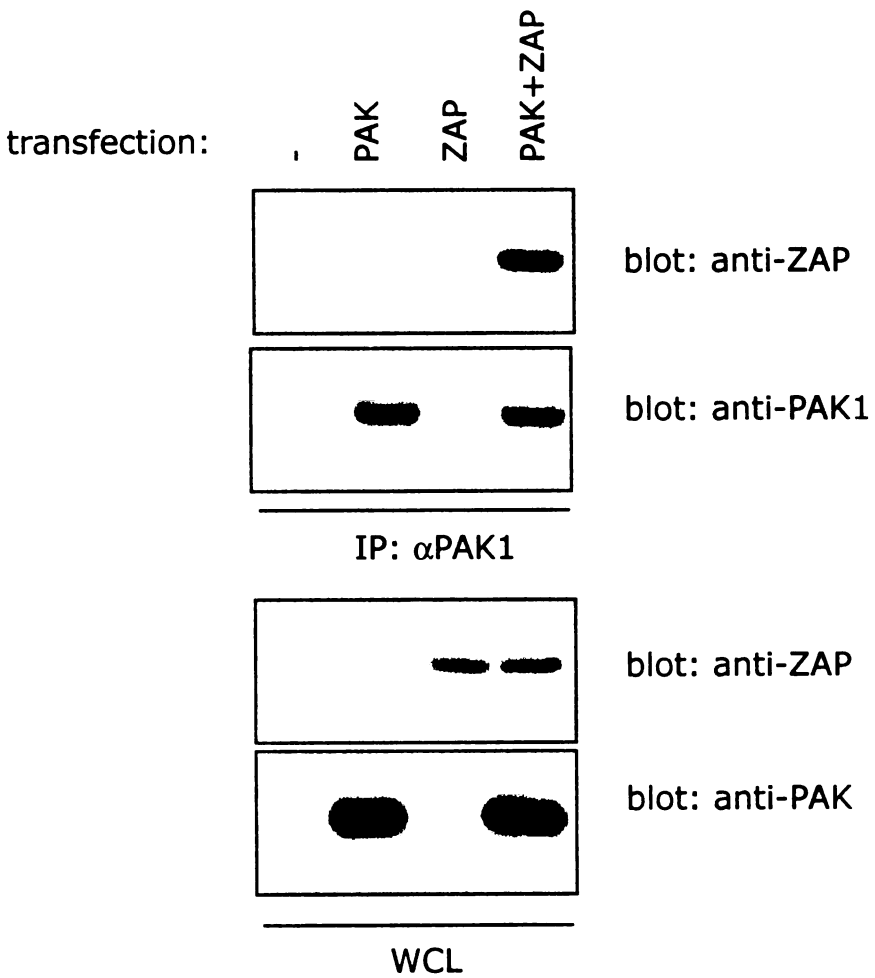
transfected

itated using

of ZAP-70

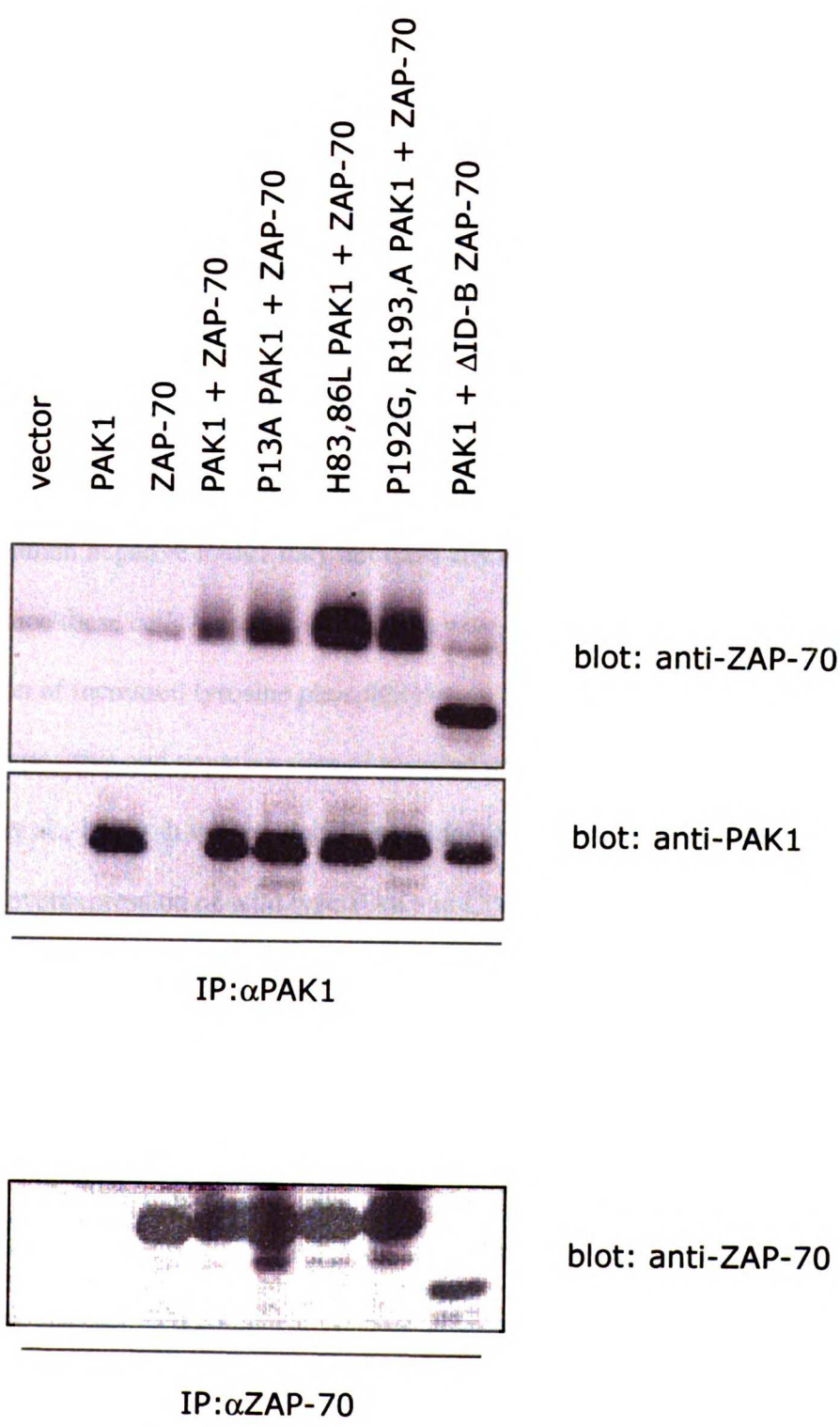
AP-70 from

ated 4 times



then asked in the interaction between PAK1 and ZAP required binding sites for PIX, Nck, or Rho GTPases. Surprisingly, none of the sites mutated alone abrogated co-immunoprecipiation of ZAP-70 and PAK1 (figure B-6). Furthermore, deletion of interdomain B in ZAP-70 had no effect on PAK1 and ZAP-70 association.

Figure B-6: Neither Nck, Rho GTPase, of PIX binding sites are required for PAK1 association with ZAP-70. As in Figure B5 except the indicated PAK1 mutants and ZAP-70 mutants were transfected. This experiment was performed twice with similar results.


vector
PAK1
ZAP-70
PAK1 + ZAP-70
P13A PAK1 + ZAP-70
H83,86L PAK1 + ZAP-70
P192G, R193,A PAK1 + ZAP-70
PAK1 + ΔID-B ZAP-70
blot: anti-ZAP-70
blot: anti-PAK1
IP:αPAK1
blot: anti-ZAP-70
IP:αZAP-70

Discussion:

Overexpression of dominant negative PAK1 in Jurkat T cells blocks TCR-induced ZAP-70 tyrosine phosphorylation. Furthermore, overexpression of wild type PAK1 in COS cells that stably express CD8-ζ causes augmented ZAP-70 tyrosine phosphorylation. ZAP-70 recruitment to CD8-ζ is relatively unaffected. However, wild type PAK1 does not augment ZAP-70 tyrosine phosphorylation in Jurkat cells and dominant negative PAK1 does not block ZAP-70 tyrosine phosphorylation in COS-18 cells(data not shown). In the case of Jurkat cells, wild type PAK1 overexpression might not significantly increase the already basally activated pool of PAK1 in these cells. In the COS-18 cells, dominant negative PAK1 may not have any endogenous PAK1 kinase activity to block since these cells were not stimulated.

The function of increased tyrosine phosphorylation of ZAP-70 could be positive or negative as both positive and negative sites of tyrosine phosphorylation have been described(Wange et al., 1995). It will be important to define which phosphorylation sites are augmented by overexpression of wild type PAK1 in COS-18 cells and which sites are blocked by the overexpression of dominant negative PAK1 in Jurkat cells (see chapter 3). Furthermore, measuring kinase activity and perhaps adapter function of ZAP-70 will be required to define the functional effect of PAK1 on ZAP-70.

How could wild type PAK1 cause increased tyrosine phosphorylation of ZAP-70? One possibility is that Lck kinase activity is increased by PAK1. However, dominant negative PAK1 does not block the inducible tyrosine phosphorylation of a CD25-ζ chimeric molecule in Jurkat cells. Also, in COS-18 cells, we did not observe reproducible increases in total Lck tyrosine phosphorylation or in the Lck substrate, CD8-ζ when

PAK1 was overexpressed. However, Lck has both positive and negative regulatory sites of tyrosine phosphorylation and these should be looked at individually. Also, induction of CD25-ζ phosphorylation by secondary antibody crosslinking may not be equivalent to IgM mediated-TCR crosslinking. Finally, CD25-ζ is also an indirect readout of Lck function. Therefore, effects on Lck should be further examined.

A second possibility is that PAK1 serine or threonine phosphorylation of ZAP-70 somehow allows ZAP-70 to be more efficiently tyrosine phosphorylated. Several potential PAK1 phosphorylation sites can be found in ZAP-70. One potential PAK1 phosphorylation site by sequence homology to known PAK1 substrates (S312) is located in interdomain B where it might play a role in relieving the negative regulatory activity of this region towards ZAP-70 function(T. Kadlecek, unpublished observations). Threonine phosphorylation is also possible; inducible threonine phosphorylation of ZAP-70 has been reported with TCR stimulation(Chan et al., 1995).

We investigated this possibility by performing PAK1 *in vitro* kinase assays using GST-PAK1 purified from bacteria and HA-epitope tagged ZAP-70 constructs expressed in 293T cells. Indeed, PAK1 could phosphorylate ZAP-70 *in vitro*, but contrary to our initial hypothesis, these potential phosphorylation sites appear to be predominantly located in the SH2 domains and interdomain A region of ZAP-70. Consistent with the fact that PAK may not play a role in regulating interdomain B, dominant negative PAK1 is able to block interdomain B-deleted ZAP-70's ability to reconstitute NFAT responses in P116 cells(not shown). Furthermore, mutation of the serine 312 in ZAP-70 to alanine or aspartate has no effect on ZAP-70's ability to reconstitute NFAT activation in P116 cells(T. Kadlecek, unpublished observations). If these PAK1 phosphorylation sites in the

SH2 domains or interdomain A are truly phosphorylated *in vivo*, how they might function in regulating ZAP-70 tyrosine phosphorylation is not known.

Since these experiments demonstrate that ZAP-70 is a possible substrate of PAK1 *in vivo*, we looked to see if there was an association between ZAP-70 and PAK1. Indeed, in 293T cells, PAK1 can specifically immunoprecipitate ZAP-70. This interaction does not require interdomain B of ZAP-70 or the Nck, Rho GTPase, or PIX binding sites in PAK1. The domains that mediate this interaction are not yet known. These data, taken together, suggest that ZAP-70 might be a downstream target of PAK1.

Given that ZAP-70 is required to activate PAK1, these data, taken together, suggest that PAK1 and ZAP-70 could regulate each other in a positive feedback loop. In this model, an initially limited amount of ZAP-70 activation by the TCR would trigger PAK1 activation. In turn, PAK1 activation would allow the additional activation of ZAP-70 thereby additional PAK1. This amplification circuit would add sensitivity to the activation of ZAP-70 and downstream T cell activation by TCR signals.

Materials and Methods:

Reagents

The additional cell lines used were COS-18 cells (Iwashima et al., 1994) and 293T cells (ATCC). The anti-PAK1 antibody used to co-immunoprecipitate PAK1 and ZAP-70 was sc-881, obtained from Santa Cruz Biotechnology. The 12CA5 anti-HA antibody was obtained from Boehringer Mannheim. The anti-ZAP-70 antibodies used for western blotting were 2F3.2(for PAK1 co-immunoprecipitation)(Iwashima et al., 1994) and R1225, a polyclonal rabbit serum directed against human ZAP-70 amino acids 326-341, was a gift from Dr. Nicolai VanOers. The anti-human CD25 antibody (clone M-A251) was purchased from Pharmingen. The goat anti-mouse crosslinking antibody was purchased from Sigma(St. Louis, MO). The ZAP-70 deletion constructs have been previously decrsibed(Iwashima et al., 1994) and (Zhao et al., 1999). The pEF-CD25-ζ was a gift from Dr. Nicolai VanOers.

CD25 stimulations

Cells were washed 1x in PBS containing calcium and magnesium. After 30 minutes at 37°C, the cells were placed on ice for 3 minutes and 1 μg of anti-CD25 antibody was added to 15 million cells in 250 μL and the antibody was allowed to bind for 15 minutes on ice. The cells were then washed 2x with 4°C PBS with calcium and magnesium. Stimulation was initiated by the addition of 10 μg of rabbit anti-mouse IgG in 250 μL PBS with calcium and magnesium. After 2 minutes at 37°C, the cells were spun down and lysed in 500 μL of standard immunoprecpipitation buffer. 1 μg of anti-CD25 antibody was used per immunoprecipitation of 15 million cells.

Purification of recombinant GST-PAK1

A single colony of BL21(DE3)pLysS (Stratagene, Cedar Creek, TX) transformed with pGEX-full length PAK1(Chong et al., 2001) was inoculated into 50 mL of Luria Broth and grown from 16 hours overnight at 32°C with shaking. This 50 mL culture was diluted into 500 mL Luria Broth and grown at 32°C for one hour. IPTG was added to a final concentration of 100 μM to induce expression and the culture was allowed to grow for an additional 5 hours at 32°C. Bacteria were spun down and lysed in buffer containing 1% NP-40, 20 mM Tris HCl pH 7.6, 150 mM NaCl, 2 mM EDTA pH 8.0, 1 mM 2-mercaptoethanol, 0.5 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. The resuspended bacteria were sonicated for 3 times for 30" at 40% duty cycle, 4 output level, rested on ice for 20 minutes and precleared by spinning at 15K in an SS-34 rotor. GST-PAK1 was specifically precipitated using 150 μl of washed (in lysis buffer) glutathione agarose beads. The mixture was tumbled at 4°C for 30 minutes and then washed 5x with 5 ml of lysis buffer, then 2 times with 1 ml of lysis buffer and finally 2 times with once with lysis buffer without detergent. The GST was eluted with 2x1.5 ml of wash buffer + 5 mM reduced glutathione (Sigma, St. Louis, MO) for 15 minutes each tumbling at 4°C. These proteins were desalted using a PD-10 column (Pharmacia) into 10 mM Tris pH 8.0 with 2 mM EDTA and 100 mM NaCl. The sample (3 mL) was loaded onto a 50 mL Mono Q (Pharmacia) column run on a Akta FPLC purification system. A linear gradient from 150 mM to 1 M NaCl was run and fractions that contained GST-PAK were identified by Coomassie blue staining (eluting at ~200 mM NaCl). These fractions were

pooled, concentrated, and buffer exchanged into PAK1 kinase buffer using Millipore Centricon 50 columns (Bedford, MA).

GST-PAK1 kinase assay on ZAP-70

PAK1 kinase assays were performed as previously described(reference my own paper here). 293T cells transfected with various ZAP-70 expression constructs were lysed in standard immunoprecipitation lysis buffer and then denatured by addition of 10% SDS to a final concentration of 1%. The lysates were mixed and boiled for 15 minutes. After being placed on ice, the lysate was renatured by the addition of standard immunoprecipitation lysis buffer (without SDS) to a final concentration of 0.5% SDS. Anti-HA (12CA5) antibody preincubated with protein G was added and immunoprecipitated for 1.5 hours at 4°C. The beads were washed 3 times in RIPA buffer (1% NP-40, 0.1% SDS, 150 mM NaCl, 20 mM TrisHCl pH 7.5) and then washed 2 times in PAK1 kinase buffer. 20 μl of recombinant GST-PAK (~0.5 μg) was added followed by addition of the radioactive ATP mix (1 μM cold ATP, 66 nM or 10 μCi γ^{32}P-ATP) for 20 minutes at 30°C. Reactions were stopped by the addition of 1 ml of RIPA lysis buffer containing 80 mM EDTA. The beads were spun down and the lysis buffer was removed. 1x reducing sample buffer was added to the beads and they were boiled for 5 minutes. The samples were analyzed by SDS-PAGE and autoradiography or anti-HA western blots.

Transfections

293T and COS-18 cells were transfected using the CalPhos transfection kit (Stratagene). Each transfection was done on 1 10 cm plate. Cells were harvested on the third day after transfection. All constructs were transfected at 10 μg / plate with empty vector added to normalize total DNA used per plate.

Cell Stimulation, Immunoprecipitation, and Western Blotting

Cells were harvested and washed in phosphate-buffered saline and resuspended at 10^8 cells/ml of PBS and incubated at 37°C for 10 minutes. Jurkat cells were stimulated with a 1:500 dilution of C305 for 2-3 minutes followed by rapid centrifugation and resuspension of the pellet by vigorous vortexing in a lysis buffer appropriate for the experiment. 293T or COS-18 cells were lysed by scraping a 10 cm plate into 1 mL of phosphate-buffered saline. The cells were spun down and lysed in 1 mL of standard immunoprecipitation lysis buffer. This lysis buffer is identical to that used for the PAK1 kinase assays except without sodium pyrophosphate or β-glycerophosphate and with only 10 mM sodium fluoride. After clarification of the lysate by centrifugation in a microfuge at 13,000 rpm for 10 minutes at 4°C, the supernatant was then tumbled with the relevant antibody pre-bound to the relevant beads for 1.5 hours at 4°C. The beads were washed three times with lysis buffer and resuspended in reducing sample buffer for SDS-PAGE. Gels were transferred to Immobilon P (Millipore Corporation) and probed with primary antibodies as described in the text and the appropriate secondary antibodies coupled to horseradish peroxidase. Detection was performed with the luminol substrate based Western Lightning Kit(Perkin Elmer, Boston, MA).

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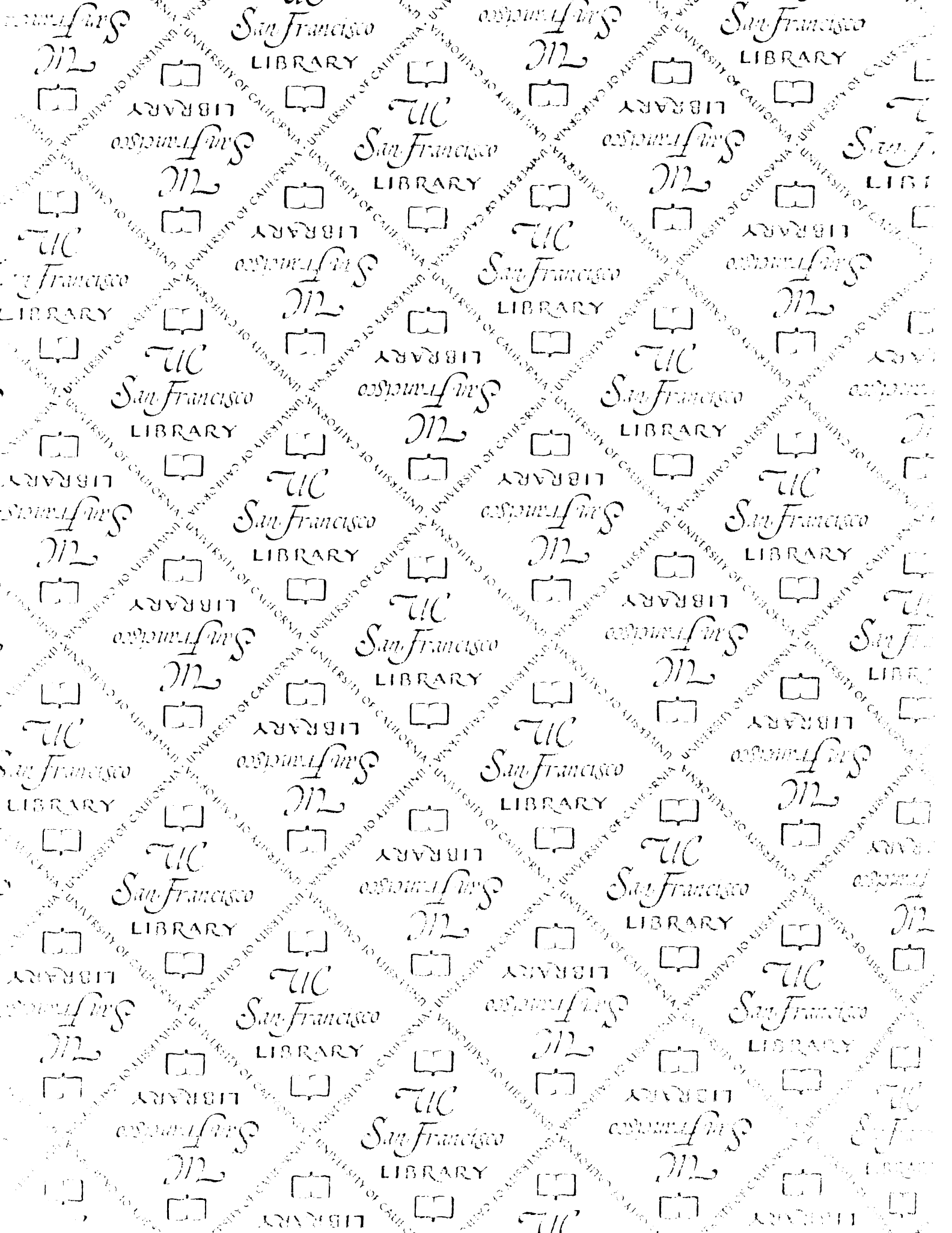
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