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A Novel Role for p21-Activated Protein Kinase 2 in T Cell Activation

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To identify novel components of the TCR signaling pathway, a large-scale retroviral-based functional screen was performed using CD69 expression as a marker for T cell activation. In addition to known regulators, two truncated forms of p21-activated kinase 2 (PAK2), PAK2Δ1–124 and PAK2ΔS1–113, both lacking the kinase domain, were isolated in the T cell screen. The PAK2 truncation, PAK2ΔL, blocked Ag receptor-induced NFAT activation and TCR-mediated calcium flux in Jurkat T cells. However, it had minimal effect on PMA/ionomycin-induced CD69 up-regulation in Jurkat cells, on anti-IgM-mediated CD69 up-regulation in B cells, or on the migratory responses of resting T cells to chemoattractants. We show that PAK2 kinase activity is increased in response to TCR stimulation. Furthermore, a full-length kinase-inactive form of PAK2 blocked both TCR-induced CD69 up-regulation and NFAT activity in Jurkat cells, demonstrating that kinase activity is required for PAK2 function downstream of the TCR. We also generated a GFP-fused PAK2 truncation lacking the Cdc42/Rac interactive binding region domain, GFP-PAK2Δ3–140. We show that this construct binds directly to the kinase domain of PAK2 and inhibits anti-TCR-stimulated T cell activation. Finally, we demonstrate that, in primary T cells, dominant-negative PAK2 prevented anti-CD3/CD28-induced IL-2 production, and TCR-induced CD40 ligand expression, both key functions of activated T cells. Taken together, these results suggest a novel role for PAK2 as a positive regulator of T cell activation. The Journal of Immunology, 2004, 172: 7324–7334.

Deregulation of the acquired immune response results in an attack directed against self-Ags and ultimately leads to the development of autoimmune disease. Acquired immune responses are regulated in part by T cells and involve precise control of the activation and differentiation of these cells. Consequently, the regulation of T cell activation provides an attractive basis for the development of novel therapies for the treatment of autoimmune disease and the prevention of transplant rejection. T cell activation is a complex process tightly controlled by Ag receptors, costimulatory molecules, cytokines and chemokines. T cell activation following engagement of the TCR involves the coordinated activities of a diverse set of intracellular signaling pathways, including mobilization of calcium, activation of protein kinase C, and activation of Ras and Rho family GTPases, which result in the triggering of the mitogen-activated protein kinase (MAPK)6 pathway (1). The fate of the activated T cell is ultimately determined by the set of genes activated in response to the signaling cascades that were engaged. Expression of activation markers, including CD25, CD69, and CD40 ligand (CD40L), and the production of cytokines, including IL-2, are well-defined consequences of T cell activation.

To identify novel effectors downstream of the TCR, we designed a retroviral-based functional genetic screen to select for proteins that inhibit receptor-mediated T cell activation. In addition to known regulators of Ag receptor signaling, we identified PAK2 as a regulator of TCR signaling (2). PAK2 is a member of a family of p21-activated serine/threonine protein kinases (PAKs) composed of six mammalian isoforms (3). PAKs 1–3 each contain a C-terminal catalytic domain and an N-terminal regulatory domain, which is inhibitory to the former (4). They share significant homology, particularly in the kinase domain (>90%) as well as in a 66-aa stretch within the regulatory domain (also >90% identity). The PAKs are downstream effectors of the small GTPases, Cdc42 and Rac, members of the Rho family of GTPases, which are key regulators of cytoskeletal organization (5). These GTP-binding proteins are molecular switches, which cycle between the active GTP-bound form and the inactive GDP-bound form. Binding of GTP-loaded Cdc42 or Rac to PAK family members through the Cdc42/Rac interactive binding region (CRIB) domain results in autophosphorylation and activation of the kinase (6, 7). In the case of PAK4, the fourth family member, which shares only 55% identity within the kinase domain, GTase binding does not result in catalytic activation, because it lacks the regulatory kinase inhibitory domain (8). PAK5 and PAK6 most closely resemble PAK4 but show distinct tissue expression. PAK5 is highly expressed in brain, whereas PAK6 is highly expressed in prostate and testis (3).

Despite the extensive similarities between the PAK enzymes, they appear to play different physiological functions and may be differentially regulated in vivo. PAK1 plays a role in the assembly of focal complexes, integrin-dependent sites linking the
extracellular matrix to the actin-rich cytoskeleton, and stress fibers (9, 10). This is likely mediated in part through the phosphorylation and inhibition of myosin light chain kinase by PAK1 (11). Through one of its five proline-rich elements that serve as binding sites for SH3 domains, PAK1 interacts with the PAK-interacting exchange factor (PIX), allowing it to localize to the focal complex (12). Activation and autophosphorylation negatively regulate this interaction (13). Activation of PAK1 appears to promote cell migration by facilitating the breakdown of focal complexes, which is necessary for cell migration to occur (13–15). PAK1 also interacts with one of the three SH3 domains of Nck, which localizes it to the membrane, where it promotes neurite outgrowth in PC12 cells (16). In addition, PAK1 has been reported to be activated downstream of the TCR, dependent on the tyrosine kinases η-associated protein 70 (ZAP70) and Syk (17, 18).

In contrast, PAK2 has been reported to be activated by cellular stresses such as hyperosmolarity and DNA damage (19, 20). In Jurkat T cells, PAK2 is activated after cleavage by caspase 3 and appears to mediate some of the membrane and morphological changes characteristic of programmed cell death (21). PAK2 has also been reported to play an antiapoptotic role in fibroblasts, where it suppressed stress-induced cell death (22). However, more recent evidence further implicates PAK2 in the induction of cytostasis in fibroblasts, which was shown to be dependent on its localization in the endoplasmic reticulum (23).

In this study, we demonstrate that PAK2 is a positive regulator of T cell activation. Dominant-negative forms of PAK2 block TCR-induced CD69 up-regulation, NFAT activation, and calcium flux. However, dominant-negative PAK2 has minimal effect on PMA/ionomycin-stimulated T cell activation, and no effect on receptor-mediated B cell activation. We also show that the effect of PAK2 is dependent on its kinase activity, which is increased in response to TCR engagement. Finally, we demonstrate that dominant-negative PAK2 can also block IL-2 production and CD40L expression in primary T cells.

Materials and Methods

Cell culture

Human BJAB B cells, Jurkat T cells (clone N), and Jurkat TAg cells (expressing SV40 large T Ag) were routinely cultured in RPMI 1640 medium supplemented with 10% FCS (JRH Biosciences, Lenexa, KS), penicillin, and streptomycin. Phoenix A cells (24) were grown in DMEM supplemented with 10% FCS, penicillin, and streptomycin. To produce the Tet transactivator (tTA)-Jurkat cell line, Jurkat cells were infected with a retroviral construct that constitutively expresses the tetracycline transactivator protein and a reporter construct that expresses Lyt2 driven by a tetracycline-responsive element (TRE) (25). The tTA-Jurkat cell population was optimized by sorting multiple rounds for high TRE-dependent expression of Lyt2 in the absence of doxycycline (Dox) and strong repression of Lyt2 expression in the presence of Dox. The cells were also sorted for maximal anti-TCR-induced expression of CD69. Dox was used at a final concentration of 10 ng/ml for at least 6 days to down-regulate expression of cDNAs from the TRE promoter.

Transfection and infection

Phoenix A packaging cells were transfected using retroviral vectors using calcium phosphate for 6 h (24). After 24 h, supernatant was replaced with complete RPMI 1640 medium, and virus was allowed to accumulate for an additional 24 h. Viral supernatant was collected, filtered through a 0.2-μm filter, and mixed with BJAB/Jurkat cells at a density of 2.5 × 10⁶ cells/ml. Cells were spun at room temperature for 3 h at 3000 rpm, followed by overnight incubation at 37°C. Transfection and infection efficiencies were monitored by GFP expression, and functional analysis was conducted 2–4 days after infection. For transient transfection, various amounts of indicated plasmids, plus 10 μg of NFAT-Luciferase plus 2 μg of a second control TK Luc luciferase construct (Promega, Madison, WI) in the case of the reporter assay, were electroporated into 10⁶ BJAB or Jurkat TAg cells, as previously described (26). Cells were cultured for 40 h after transfection and assayed as indicated.

Libraries and constructs

RNA extracted from human lymph node, thymus, spleen, and bone marrow was used to produce two cDNA libraries: one random primed and directionally cloned and the second nondirectionally cloned and provided with three exogenous ATG in three frames. cDNAs were cloned into the pTRE-exs vector giving robust Dox-regulatable transcription of cDNAs from the TRE promoter. The total combined library complexity was 5 × 10⁶ independent clones.

Dominant-negative PAK2ΔN 224 and PAK2ΔS 113 were cloned into the retroviral pTRE-ires-β-gal (27, 28) and the mammalian expression vector pCDEF3 (26). The full-length wild-type PAK2–V5-His6 was subcloned from a Research Genetics (Huntsville, AL) PAK2 clone into the pCDEF3 vector. Difficulties were encountered when attempting to express the full-length wild-type kinase. PAK1 has previously been shown to be toxic to bacteria (9), and we found that PAK2 was also toxic to bacteria. Nine of nine untagged wild-type PAK2 clones we isolated had independent frameshift mutations. An alternative subcloning approach to generate an untagged wild-type PAK2 yielded 125 of 126 inserts in opposite orientation and one parental frameshift. Although the smaller PAK2 HA tag remained toxic to bacteria, wild-type PAK2–V5-His6 was successfully isolated. To generate the kinase-inactive point mutant (K1-PAK2), Lys278 was substituted with Ala using standard PCR mutagenesis methods. The isolated kinase-inactive kinase domain construct (K1-kinase-V5-His6) (residues 162–524 of PAK2) was generated by a standard PCR procedure using the K1-PAK2–V5-His6 as template, and expressed in the pCDEF3 plasmid. The transdominant PAK2ΔN 140 fragment and the isolated CRIB domain (aa 74–87) were also generated by a standard PCR procedure and expressed as GFP fusions in the pCDEF3.GFP vector.

CD69 functional screen

Phoenix A packaging cells were transfected using calcium phosphate with a tet regulated retroviral pTRA cDNA library. tTA Jurkat cells were spin-infected with the supernatant from the Phoenix A cells for 3 h at room temperature. After 4 days of CD69 expression, library-infected cells were stimulated with anti-TCR (0.3 μg/ml C305) for 20–24 h, and stained with anti-CD69-allophycocyanin (Caltag, Burlingame, CA) for CD69 surface expression. Cells expressing the lowest levels of TCR-induced CD69 were isolated using a MoFlo (DakoCytomation, Carpinteria, CA). Sorting was repeated over multiple rounds with 6-day rest periods between stimulations until significant enrichment of nonresponsive clones was achieved. The nonresponders were single-cell sorted and reanalyzed for TCR-induced CD69 expression in the presence (cDNA off) and absence (cDNA on) of Dox. Clones were ranked by a ratio of C305-induced CD69 mean fluorescence in the presence and absence of Dox. Clones with Dox ratios of >1.5–2.0 were RT-PCR subcloned to pTRE-ires-β-gal for phenotype confirmation.

Cell surface marker analysis

tTA-Jurkat, Jurkat-N, Jurkat-TAg, or BJAB cells were stained with an allophycocyanin-conjugated mouse monoclonal anti-human CD69 Ab (Caltag) at 4°C for 20 min and analyzed using a FACSCalibur instrument (BD Biosciences, Mountain View, CA) with CellQuest software. Cell sorts were performed on a MoFlo (DakoCytomation).

CD69 up-regulation assay

tTA-Jurkat, Jurkat TAg, or BJAB cells were split to 2.5 × 10⁶ cells/ml 24 h before stimulation. Cells were spun and resuspended at 5 × 10⁶ cells/ml in fresh complete RPMI 1640 medium in the presence of 1 μg/ml C305 (anti-TCR β-chain specific to Jurkat (29)), 0.3 μg/ml anti-IgM F(ab')₂ (Jackson ImmunoResearch, West Grove, PA), or 50 ng/ml PMA for 20–26 h at 37°C, and then assayed for surface CD69 expression.

PAK1 and PAK2 TagMan

RT-PCR was performed by real-time quantitative PCR (TaQMan) using the following primers and probes: PAK1, forward, TTGAGCTCTCATTC CCTTTTCC, reverse, TGGATGCCGGACGAGGT, probe, CCCCG CGCTTTGCTGAGCC; and PAK2, forward, GAATGGAAGAGCTGTAAGCCTACT, reverse, GCCATAAAGCTTCCGTTGTAACC, probe, TCACCCCTGAGCCGAACCCG.C.

Human placenta total RNA (Clontech, Palo Alto, CA) was used for the standard curve and human RIP gene was used for normalization of total RNA. Samples were tested in triplicate. RT-PCR was performed on an Applied Biosystems (Foster City, CA) PRISM 7700.
NFAT Dual-Luciferase assay

Transfected Jurkat-TAg cells (1 × 10⁶) were aliquoted into a 96-well plate (Corning, Corning, NY) 40 h after transfection and cultured in a final volume of 100 μl of RPMI 1640 growth medium. Cells were stimulated at 37°C in the growth medium containing either 1 μg/ml C305 hybridoma supernatant or 50 ng/ml PMA and 1 μM ionomycin. After 12-h stimulation, cells were lysed in 5× lysis buffer (Promega) and assayed using the luciferase kit purchased from Promega. Luciferase activity was read on the luminometer (Dynex Technologies, Chantilly, VA) and determined in triplicate for each experimental condition.

Calcium mobilization

Jurkat cells were washed in modified Tyrode’s (MT) buffer (137 mM NaCl, 2.7 mM KCI, 1.8 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES (pH 7.4), 5.6 mM glucose, and 0.1% BSA), resuspended in MT at 10⁶ cells/ml, and incubated with 4 μg/ml indol-1 (Molecular Probes, Eugene, OR) and 4 mM Probenecid (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min. Cells were centrifuged and resuspended in MT. Analysis was performed on an LSR flow cytometer (BD Biosciences) at 37°C. For stimulation, C305 was used at 300 ng/ml and ionomycin at 5 μM.

Jurkat chemotaxis assay

Jurkat cells were cultured in RPMI 1640 with 10% charcoal-stripped FBS for 24 h before the assay. Cells were washed and resuspended in serum-free medium. A total of 10⁵ cells was added to the upper chamber of a Costar (Cambridge, MA) Transwell apparatus (5-μm pore), and allowed to migrate for 2.5 h into the lower chamber containing either 100 nM sphingosine-1-phosphate (SPP), 100 nM stromal cell-derived factor (SDF)-1, or serum-free medium alone. The percentage of GFP-positive cells of the migrated population (lower chamber) was analyzed by FACS.

Results

An N-terminal PAK2 fragment lacking a kinase domain was identified as a functional hit in a CD69 T cell screen

A functional retroviral-based T cell screen was performed to select for proteins that inhibit TCR-mediated T cell activation, using CD69 expression as the readout for T cell activation (30). ITA Jurkat cells (25) stably transfected with pTTRA CDNA library were stimulated with anti-TCR, and stained for CD69 surface expression. Cells expressing the lowest levels of TCR-induced CD69 were sorted, expanded, and resorted until significant enrichment of nonresponsive clones was achieved. Individual clones were isolated by single-cell sorting and were reanalyzed for TCR-induced CD69 expression in the presence (cDNA off) and absence (cDNA on) of Dox (Fig. 1A). Over 2800 single-cell clones were expanded, grown in the presence or absence of Dox, stimulated with anti-TCR, and analyzed for CD69 surface expression. Of these, 46.8% showed a Dox-regulatable phenotype. The cDNAs were successfully identified from ~200 clones, representing ~50 distinct genes and including known regulators of the TCR signaling pathway, such as ZAP70, Syk, Lck, and phospholipase C (PLC)γ1 (2). The fourth most frequently represented cDNA was PAK2. Two kinase domain-truncated forms of PAK2 were isolated multiple times as functional hits in the CD69 screen (Fig. 1B). The phenotype of one of the PAK2 screen clones is shown (Fig. 1C). The second clone, PAK2ΔL, which lacks the PIX binding domain, gave a similar, albeit less potent, phenotype (2), suggesting that the PIX binding domain is not completely required for the inhibitory effect. PIX directly interacts with PAK proteins and is responsible for their recruitment to focal complexes (9).

Although PAK1 has been implicated previously in TCR signaling (17, 18), this is the first report suggesting that PAK2 may play a role in signaling downstream of the TCR. A number of lines of evidence support a role for PAK2 in T cell signaling. Rudel and Bokoch (21) showed that Jurkat T cells predominantly express PAK2. Furthermore, Fas ligation of T cells induces caspase-mediated cleavage of PAK2, but not PAK1, to generate a constitutively active PAK2 fragment (21). Finally, a highly conserved HIV protein, Nef, interferes with CD3 signaling in T cells, thereby blocking up-regulation of CD69 expression, and induction of IL-2 and IFN-γ mRNAs (31, 32). Interestingly, Nef specifically associates with and activates PAK2, but not PAK1, in T cells (33, 34).

PAK1 and PAK2 have previously been reported to be ubiquitously expressed (4, 35). However, when we examined the expression of PAK1 and PAK2 in a panel of 15 tissues by quantitative PCR (TagMan), we found PAK2 to be broadly expressed, whereas PAK1 was expressed predominantly in brain (Fig. 2).

PAK2ΔL specifically inhibits anti-TCR, and not PMA-induced CD69 up-regulation, in T cells

To determine whether the effect of the PAK2 truncation was restricted to T cells, Jurkat N32H cells and BJAB cells, infected with the PAK2ΔL construct or vector alone, were stimulated with anti-TCR and anti-BCR, respectively (Fig. 3A). TCR cross-linking increased CD69 expression 5- to 8-fold in both the GFP-positive and GFP-negative vector-infected Jurkat cells. Introduction of PAK2ΔL reduced CD69 expression to the level before stimulation. Stimulation of vector-infected BJAB cells with anti-IgM increased CD69 surface expression ~4-fold in both GFP-negative and GFP-positive cells. However, in BJABs, introduction of PAK2ΔL had no effect on anti-IgM-induced CD69 up-regulation.

To gain further insight into the role played by PAK2 in TCR signaling, we looked at whether PAK2ΔL could also block PMA-induced CD69 expression. The phorbol ester, PMA, bypasses early...
signaling events by directly activating protein kinase C and downstream signaling molecules such as ERK1/2. To obtain a brighter GFP signal and facilitate gating of the GFP-positive cells, we switched to a transient expression system, using the pCDEF3-IRES/GFP plasmid. Although PAK2/L/H9004 blocked anti-TCR-stimulated CD69 expression in Jurkat TAg cells, it did not prevent the more potent CD69 up-regulation (30-fold) induced by PMA (Fig. 3B). The slight effect of PAK2/L/H9004 on PMA-induced CD69 up-regulation was minor compared with the effect on anti-TCR stimulation and was less pronounced in other experiments. Taken together, this suggests that PAK2 plays a role in early signaling events after Ag receptor stimulation in T cells, but not B cells.

PAK2/L inhibits TCR-mediated Ca2+ flux in Jurkat cells

To determine the effect of this inhibitory PAK2 construct on calcium mobilization, Jurkat TAg cells were again transiently transfected with either pCDEF3-PAK2L-IRES/GFP or empty vector, labeled with the calcium indicator, indo-1, and stimulated with anti-TCR, and the calcium signals of the GFP-negative and GFP-positive cells were analyzed simultaneously. In comparison with cells transfected with vector alone, or GFP-negative cells, decreased Ag-induced calcium mobilization was observed in the PAK2L-expressing cells (Fig. 4A). However, calcium influx induced by the ionophore, ionomycin, was unaffected, confirming

FIGURE 1. CD69 cell surface marker screen in Jurkat cells. A, Screening strategy. tTA Jurkat cells stably transfected with pTRA cDNA library were stimulated with anti-TCR, and stained for CD69 surface expression. Cells expressing the lowest levels of TCR-induced CD69 were sorted, expanded, and resorted until significant enrichment of nonresponsive clones was achieved. The cells were single-cell sorted and were reanalyzed for TCR-induced CD69 expression in the presence (cDNA off) and absence (cDNA on) of Dox. B, Two kinase domain-truncated forms of PAK2, PAK2L/H9004 and PAK2ΔS, were isolated multiple times as functional hits in the CD69 screen. Schematic of the structure of PAK2 and the hits from the CD69 screen. C, TCR-induced CD69 expression inhibited by switching on PAK2L/H9004. A Jurkat TAg screen clone stably expressing PAK2L/H9004 cultured in the presence or absence of Dox, and stimulated with anti-TCR (1 μg/ml C305) for 20–24 h and stained for CD69 expression.

FIGURE 2. TaqMan analysis of PAK1 and PAK2 tissue expression. mRNA expression was measured by quantitative RT-PCR using either PAK1 or PAK2 primers and total RNA from a panel of 15 tissues (PBL). To normalize for mRNA input, human ribosomal protein (Hu RIP) mRNA was measured for each tissue type. Results were expressed as the ratio of PAK1 or PAK2 mRNA level to Hu RIP.
that these cells retained the capacity to mobilize calcium. These data suggest that PAK/H9004L negatively regulates signaling events proximal to the TCR, which lead to mobilization of calcium, and consequent activation of NFAT. There is evidence that inhibitors of PLC and calmodulin block activation of PAK in neutrophils, suggesting that PAK is activated downstream of PLC (36). However, in mast cells, Cdc42/Rac has been placed upstream of inositol 1,4,5-triphosphate production and calcium mobilization, possibly interacting directly with PLC/H92531 (37). It would be interesting

FIGURE 3. Kinase domain-truncated PAK2 inhibits T cell activation. A, PAK2ΔL inhibits Ag receptor signaling in T cells, but not in B cells. PAK2ΔL in the pTRA-IRES/GFP vector or vector alone was infected into Jurkat N32H cells or BJAB cells. After 48 h, the Jurkat and BJAB cells were stimulated with anti-TCR (1 μg/ml C305) and anti-BCR (0.3 μg/ml anti-IgM F(ab')2), respectively, for 20–24 h, and CD69 expression of the GFP-positive and -negative cells was compared by analytical gating. The geometrical means of allophycocyanin-CD69 fluorescence are shown for GFP-negative and GFP-positive cells. B, PAK2ΔL blocks TCR-, but not PMA-induced CD69 up-regulation. Jurkat TAg cells were transiently transfected with pCDEF3-PAKΔL-IRES/GFP (lower panel) or empty vector (upper panel) were labeled with the calcium indicator, indo-1, and stimulated with anti-TCR followed by ionomycin at 37°C on an LSR flow cytometer with UV laser. Calcium influx, measured as a ratio of bound to unbound indo-1, was monitored over a 10-min time course. The calcium signals of the GFP-negative (untransfected) (hatched line) and GFP-positive (transfected) (bold line) cells were analyzed simultaneously. In the upper panel showing the vector control, the lines representing the GFP-positive and GFP-negative cells are overlapping. B, PAK2ΔL has no effect on T cell chemotaxis. Jurkat TAg cells were transiently transfected with pCDEF3-PAKΔL-IRES/GFP, pCDEF3-EDG5-IRES/GFP, or empty vector, and cultured in the presence of charcoal-stripped FBS. A total of 10⁶ cells was added to the upper chamber of a Costar Transwell apparatus (5-μm pore), and allowed to migrate for 2.5 h into the lower chamber containing either 100 nM SPP, 100 nM SDF, or serum-free medium alone. GFP expression of the migrated population (lower chamber) was analyzed by FACS.
PAK2 kinase activity is enhanced following TCR stimulation

The PAK2 hits obtained from the CD69 T cell screen were all kinase domain truncations, suggesting that PAK2 kinase activity may be critical for the signaling events mediated by PAK2 in response to TCR stimulation. We therefore cloned full-length PAK2 to examine the effect of TCR cross-linking on its kinase activity. Jurkat TAg cells transiently expressing wild-type PAK2-V5-His\(^6\) were stimulated with anti-TCR for 0–10 min. PAK2 kinase activity was increased >3-fold after 5 min of anti-TCR cross-linking (Fig. 5A). Immunoprecipitation from Jurkat TAg cells transfected with vector alone or a kinase-inactive version of PAK2 resulted in no histone H4 phosphorylation (data not shown).

Because activation of PAK1 downstream of the TCR has been reported previously (17, 18), we sought to confirm that the endogenous PAK2 protein was also activated. Jurkat TAg cells were stimulated with anti-TCR for 0–20 min, and PAK1 and PAK2 proteins were immunoprecipitated. Both PAK1 and PAK2 proteins were activated in response to anti-TCR stimulation (Fig. 5B). T cell activation was further confirmed by measuring induction of linker for activation of T cells (LAT) and p42/44 MAPK phosphorylation in the cell lysates (Fig. 5C).

PAK2 signaling downstream of the TCR is dependent on its kinase activity

One of the outcomes of T cell activation is the production of a variety of cytokines such as IL-2 (40). In Jurkat cells, cross-linking the TCR is sufficient to activate the NFAT transcription factor, which binds and activates the IL-2 promoter. NFAT activation requires inputs from both the calcium and the Ras/MAPK pathways, activated in response to Ag receptor stimulation (40–42).

We wanted to test the effect of PAK2\(\Delta L\) on TCR-triggered NFAT activation. To further confirm the importance of PAK2 kinase activity in transmitting signals downstream of the TCR, we also constructed a kinase dead-point mutant of PAK2. Based on homology with PAK1 and other serine/threonine kinases, Lys\(^{278}\), a critical lysine residue in the active site of the kinase domain, was mutated to alanine. The effect of the full-length wild-type and kinase-inactive PAK2 constructs on TCR-stimulated CD69 up-regulation (Fig. 5D) and NFAT activation (E) was tested. The kinase-inactive point mutant inhibited both TCR-mediated CD69 up-regulation and NFAT activation, albeit not as potently as the PAK2 truncation, PAK2\(\Delta L\), whereas the wild-type kinase had little or no effect.

Taken together, these data suggest that stimulation of the TCR leads to enhanced PAK2 kinase activity, resulting in the activation of target genes.

The PAK2 fragment PAK2\(_{83–149}\) has transdominant kinase inhibitory activity

PAKs are known to be critical effectors of Cdc42/Rac (4). Although Cdc42/Rac binding results in PAK activation, there is a report of PAK1 mediating GTPase function independent of PAK kinase activity (43). Furthermore, activation of PAK kinase activity independent of GTPase binding cannot be ruled out. Both dominant-negative Ras and Cdc42 inhibit TCR-induced NFAT activation, but, as expected, only the latter has an effect on PAK activity (17). Our studies so far have shown that both a kinase-domain truncation and a kinase-inactive point mutant form of PAK2 can block TCR-mediated signals. However, both of these dominant negatives retain the ability to bind Cdc42/Rac and are perhaps blocking downstream of the TCR the function of Cdc42/Rac, PAK1, and PAK2. Although we showed that PAK2 kinase activity is increased in response to TCR cross-linking, this does not prove that PAK2 is a critical link between TCR stimulation and T cell activation. To assess the relevance of PAK2 in TCR signaling, we wanted to examine the effect of specifically blocking PAK2 kinase activity without affecting Cdc42/Rac.

To do this, we referred to insights gained from mechanistic studies of PAK1. N-terminal deletion of PAK1 dramatically activates its kinase activity, suggesting some form of autoregulation (44). Interestingly, the PAK1 fragment (70–149) inhibits PAK1 activation in vivo and in vitro and binds directly to the PAK1 kinase domain. This inhibitory activity is not dependent on binding to Cdc42/Rac.

An elegant study of the crystal structure of PAK1 provided further insights into the mechanism of PAK activation (45). PAK1 appears to exist as a dimer in an autoinhibited conformation. The autoregulatory fragment (70–149) contains a dimerization domain (80–87), an inhibitory switch domain (87–136), and a kinase inhibitory domain (137–149). Two PAK1 monomers associate through the dimerization domains, and the inhibitory switch domain tightly associates with the C-terminal lobe of the kinase domain, whereas the kinase inhibitory domain occupies the cleft of the kinase domain and thereby stabilizes a disabled catalytic site. Binding of GTP-loaded Cdc42 or Rac to the CRIB domain (75–90) disrupts the dimer, and unfolds the inhibitory switch domain, removing the kinase inhibitory domain from the cleft of the kinase domain. This releases the activation loop, allowing phosphorylation of Thr\(^{213}\), which results in activation of the kinase. Once activated, the kinase autophosphorylates at several Ser residues, preventing reversion to an inactive conformation.

To generate a dominant-negative PAK2 that does not bind Cdc42/Rac, a GFP-PAK2\(_{83–149}\) fusion was constructed (Fig. 6A). Based on the PAK1 studies, this short PAK2\(_{83–149}\) fragment should act as a dominant negative by directly occupying the cleft to look at the effect of PAK2\(\Delta L\) expression on PLC\(\gamma\) phosphorylation in T cells to determine whether PAK2 plays a role in regulating the activity of PLC\(\gamma\) or its potential interaction with Cdc42/Rac.

Jurkat cell migration toward SPP and SDF is unaffected by PAK2\(\Delta L\)

Having established that PAK2 is involved in signaling downstream of the TCR, we wanted to determine whether PAK2 played a role in activation-independent T cell functions. Lymphocyte traffic is controlled by chemokines, which can be broadly classified into inflammatory and homeostatic. Inflammatory chemokines are responsible for recruiting activated lymphocytes to sites of inflammation. In contrast, inflammation-unrelated chemokines are constitutively produced and are involved in maintaining physiological traffic, guiding precursor cells to correct sites in bone marrow and thymus to ensure proper progenitor cell development (38). SDF-1 and its receptor CXCR4 play a critical role in lymphopoiesis and myelopoiesis. Thymocytes of all developmental stages migrate to SDF-1. The lysosphingolipid SPP, thought to act both as an extracellular mediator and an intracellular second messenger, is also a potent chemotactrant for resting T cells (39). SPP binds members of the endothelial differentiation gene (EDG) family of plasma membrane G protein-coupled receptors. We looked at the ability of Jurkat cells expressing PAK2\(\Delta L\) to migrate toward SDF-1 and SPP. Migration of Jurkat T cells expressing PAK2\(\Delta L\) to SPP and SDF-1 was comparable with that of cells expressing control vector (Fig. 4B). In contrast, overexpression of EDG-5, a receptor for SPP, blocked T cell migration toward SPP (Fig. 4B), but not SDF-1 (data not shown). This suggests that PAK2 specifically regulates receptor-mediated activation of T cells, but plays no role in other activation-independent T cell functions, such as migration of resting T cells toward SPP or SDF-1.
**FIGURE 5.** PAK2 kinase activity is required for its role in T cell signaling. A. Exogenous PAK2 kinase activity is enhanced following TCR stimulation. Jurkat TAg cells transiently expressing wild-type PAK2-V5-His<sup>6</sup> were stimulated with anti-TCR for 0–10 min. The cells were lysed, and the exogenous PAK2 was immunoprecipitated through the V5 tag. The activity of the immunoprecipitated kinase at the different time points after stimulation was determined by in vitro kinase assay using histone H4 as substrate. The γ<sup>32</sup>P-labeled products of the kinase reaction were separated by SDS-PAGE and visualized by phosphor imager (upper panel). The amount of PAK2-V5-His<sup>6</sup> immunoprecipitated was determined by anti-V5 Western blot (lower panel).

B. Endogenous PAK1 and PAK2 kinase activities are enhanced following TCR stimulation. Jurkat TAg cells were starved for 2 h and stimulated with anti-TCR for 0–20 min. The cells were lysed, and endogenous PAK1 and PAK2 were immunoprecipitated through their N terminus (SC no. 882 and CST no. 2608, respectively). The activity of the immunoprecipitated kinase at the different time points after stimulation was determined as in A. The fold of induction of PAK1 and PAK2 kinase activities was quantitated in multiple experiments and averaged (upper panel). The amount of PAK1 and PAK2 proteins immunoprecipitated was determined by anti-PAK1 (CST no. 2602) and anti-PAK2 (CST no. 2608) Western blot.

C. Jurkat activation was confirmed by probing Western blots of the lysates for phosphorylation of LAT and p42/44 MAPK. PAK1 and PAK2 levels are shown as loading controls.

D. Full-length kinase-inactive PAK2 blocks TCR-mediated CD69 up-regulation. Jurkat TAg cells were transiently transfected with 40 μg of WT (wild type)-PAK2-V5-His<sup>6</sup>, KI-PAK2-V5-His<sup>6</sup>, PAK2ΔL-V5-His<sup>6</sup>, or vector alone, and cotransfected with 4 μg of pCDEF3.IRES/GFP. The cells were stimulated with anti-TCR (1 μg/ml C305) for 20–24 h. CD69 expression of the GFP-positive and -negative cells was compared by analytical gating. The geometrical means of CD69-allophycocyanin fluorescence of the GFP-positive cells and the GFP-negative cells are shown in the respective gates.

E. Full-length kinase-inactive PAK2 blocks TCR-induced NFAT activation. Jurkat TAg cells were transiently transfected with WT-PAK2-V5-His<sup>6</sup>, KI-PAK2-V5-His<sup>6</sup>, PAK2ΔL-V5-His<sup>6</sup>, or vector alone, as well as NFAT-luciferase reporter and a second control TK luciferase construct. After 48 h, the cells were stimulated with anti-TCR (1 μg/ml C305) for 12 h or left untreated. The cells were lysed and assayed for luciferase activity in triplicate. Fold induction of luciferase activity over the unstimulated, vector-transfected sample is shown. The data are representative of several independent experiments.
of the kinase domain and preventing activation of the kinase. However, it should not interfere with Cdc42 or Rac binding to the CRIB domain. PAK2 - 149 lacks five of eight of the core residues of the CRIB motif, including one of the two histidines, critical for Cdc42/Rac binding (46). A short GFP fusion containing the CRIB domain (74 – 87) was generated as a control. To determine whether the PAK2 - 149 fragment could bind directly to the kinase domain of PAK2, the PAK2 KI-kinase-V5-His was transiently coexpressed in Jurkat cells with either GFP-PAK2 - 149 or GFP-CRIB. The cells were lysed, and the GFP fusions were immunoprecipitated through the GFP tag. Any associated PAK2 KI-kinase-V5-His was detected by Western blot using an anti-V5 Ab. C. PAK2 - 149 blocks TCR-mediated CD69 up-regulation. Jurkat TAg cells were transiently transfected with GFP-CRIB, GFP-PAK2 - 149, pCDEF3-PAKΔL-IRE/GFP, or vector alone. The cells were stimulated with anti-TCR (1 μg/ml C305) for 20–24 h. CD69 expression of the GFP-positive and -negative cells was compared by analytical gating. The geometrical means of CD69-allophycocyanin fluorescence of the GFP-positive cells and the GFP-negative cells are shown in the respective gates.

Having shown that GFP-PAK2 - 149 can bind directly to the kinase domain of PAK2, we next examined whether this fragment could block TCR-induced CD69 up-regulation in Jurkat cells. Jurkat TAg cells were transiently transfected with GFP-CRIB, GFP-PAK2 - 149, pCDEF3-PAKΔL-IRE/GFP, or vector alone, and stimulated with anti-TCR for 20–24 h (Fig. 6C). Overexpression of GFP-PAK2 - 149, but not GFP-PAK2 CRIB, inhibited TCR-induced CD69 up-regulation in Jurkat cells. Taken together, these results strongly suggest that GFP-PAK2 - 149 acts as a transdominant inhibitor of PAK2 kinase activity by directly interacting with the kinase domain. Direct inhibition of PAK2 kinase activity, independent of Cdc-42/Rac binding, blocks TCR-induced T cell activation, indicating that PAK2 is a critical mediator of signals originating from the TCR.

The inhibitory effect of GFP-PAK2 - 149 is weaker than that of PAKΔL. This could simply be due to expression levels, given that
A 10-fold excess of the inhibitory PAK1 fragment (75–132) was required for maximal inhibition of PAK1 kinase activity in vitro (44). In addition, PAKΔL could block both PAK1 and PAK2 activation by potentially blocking Rac/Cdc42 function, whereas GFP-PAK2Δ149-L would block only PAK2 activation. Furthermore, the presence of the Nck binding domain in PAK2ΔL may also contribute to its greater inhibitory activity. Dominant-negative Nck has previously been shown to block NFAT activation in Jurkat cells (17). Collectively, it is likely that the role of PAK2 in TCR signaling is mediated by its kinase activity and its multiple interactions with regulatory proteins.

PAK2ΔL inhibits T cell signaling in primary T cells

Having placed PAK2 downstream of the TCR, we wanted to confirm that this novel role assigned to PAK2 is indeed physiologically relevant. We therefore examined the effect of dominant-negative PAK2 on two primary T cell functions, IL-2 secretion and CD40L up-regulation. Primary T cells purified from PBMCs were infected with PAK2ΔL in the pTRA-IRESCFP vector or vector alone, sorted, and stimulated with either anti-TCR/anti-CD28, or PMA/ionomycin. IL-2 secretion was determined for both GFP-positive and GFP-negative primary T cells (Fig. 7A). PAK2ΔL inhibited IL-2 secretion stimulated by anti-TCR/anti-CD28, but not in response to PMA/ionomycin. CD40L is up-regulated in response to T cell stimulation through the TCR. Expression of PAK2ΔL in primary T cells caused a significant decrease in the total number of CD40L-expressing cells compared with primary T cells expressing GFP alone or GFP-negative cells (Fig. 7B, left panel). In addition, in those PAK2ΔL-expressing cells displaying surface CD40L, a 2-fold reduction in the amount of CD40L expressed was observed (Fig. 7B, right panel). These results suggest that PAK2 plays an important role in the activation of primary T cells.

Discussion

In this report, we describe the use of a functional retroviral-based genetic screen to identify novel components of the TCR signaling pathway. We identified a novel role for PAK2 as a positive regulator of T cell activation. We have shown that a kinase domain truncation of PAK2 behaves as a dominant negative and blocks up-regulation of cell surface expression of the activation marker CD69, NFAT promoter activation, and calcium flux in Jurkat cells (Figs. 4A, and 5, D and F). Simply mutating a single residue in the kinase domain of PAK2, thereby rendering it inactive, mimics the effect of complete removal of the kinase domain, illustrating that the function of PAK2 is dependent on its kinase activity (Fig. 5). However, dominant-negative PAK2 did not significantly block phorbol ester-induced T cell activation, T cell chemotaxis, or receptor-mediated B cell activation (Figs. 3 and 4B). Given the similarity between the PKA family members, particularly PAK1 and PAK2, and given their universal ability to bind Cdc42/Rac, we wanted to confirm that PAK2 plays a role in linking TCR stimulation with T cell activation. Therefore, we generated a PAK2 fragment (PAK2Δ149) that bound directly to the PAK2 kinase domain, thereby inhibiting its activation (Fig. 6, A and B). Transdominant PAK2Δ149 inhibited TCR-mediated CD69 up-regulation, illustrating that PAK2 forms an essential link between TCR engagement and T cell activation (Fig. 6C). Furthermore, we demonstrated that endogenous and transfected PAK2 kinase activity is enhanced following TCR cross-linking (Fig. 5, A–C). Finally, we showed that dominant-negative PAK2 had similar effects in preventing primary T cell activation (Fig. 7). Taken together, these results indicate that PAK2 is a critical regulator of the T cell activation.

PAK2, like other PAK family members, is activated upon Cdc42/Rac binding to the CRIB domain within the N-terminal regulatory region. Although Rho family GTPases are known to play important roles in the regulation of actin-based cytoskeletal organization, they also play a role in more diverse cellular functions, such as cell cycle progression, gene expression, and response to environmental stress (5). Despite the close homology of PAK1 and PAK2, these two proteins appear to fulfill quite different functions. PAK1 interacts with Nck and PIX, which localize it to the cell membrane, and more specifically to focal complexes in the case of PIX (12, 16, 47, 48). There, in response to growth factors and other stimuli, PAK1 is activated and enhances cell migration, at least in part by promoting the disassembly of focal complexes (9, 13–15). No role in these processes has been attributed to PAK2.

FIGURE 7. PAK2 is a positive regulator of primary T cell activation. A. Primary T cells were prepared from PBMCs. PAK2ΔL in the pCRU-IRESCFP vector or vector alone was infected into primary T cells. The cells were sorted 48 h after infection into pure GFP-positive and GFP-negative populations, and stimulated for 20 h with either anti-TCR/anti-CD28 or PMA/ionomycin, or left untreated. For both GFP-positive and GFP-negative cells, IL-2 secretion was measured by ELISA. B. Primary T cells were infected and stimulated as above. Both the percentage of CD40L-expressing cells in the GFP-positive and GFP-negative populations (left panel) and the geometric mean fluorescence of CD40L expression (right panel) were measured by FACS.
However, both PAK1 and PAK2 can phosphorylate myosin II regulatory light chain in vitro and have been implicated in fibroblast and endothelial cell retraction (15, 49, 50).

PAK2 has primarily been attributed cytostatic properties and is activated in response to DNA damage and different types of stress. Microinjection of PAK2 into frog embryos results in arrest of cell cleavage (51). Furthermore, the regulatory domain of PAK2 is cleaved by caspase-3 in Jurkat cells undergoing apoptosis (21). This produces a constitutively active fragment that regulates some of the morphological changes seen in apoptotic cells, which include the formation of apoptotic bodies and the exposure of phosphatidylserine. This caspase-mediated activation has been reported only for PAK2. In addition, PAK2, but not PAK1, is activated and translocates to the particulate fraction in response to hyperosmotic stress (19). However, PAK2 can phosphorylate the proapoptotic family member Bad, and protect against cell death in BALB3T3 fibroblasts (22, 52). This antiapoptotic role appears to be a function of the full-length kinase rather than the caspase-cleaved form.

In this paper, we have demonstrated a novel function of PAK2 in the activation of T cells. To date, only PAK1 has been attributed a role in TCR signaling. Yablonski et al. (17) demonstrated a role for PAK1 downstream of Vav and Cdc42 in the TCR-induced activation of ERK2 and NFAT. A requirement for a complex between SLP-76, Nck, and Vav was also demonstrated (53). Subsequently, however, TCR engagement was shown to stimulate activation of PAK1 only if in a complex with PIX and p95SKP1, in a ZAP70-, but not LAT-, SLP-76-, or Nck-dependent manner (18). Furthermore, two reports suggest that stimulation of CD28 alone is sufficient to activate PAK1 and that costimulation of CD28 with TCR enhances PAK1 activity even further than stimulation of either alone (54, 55). In contrast, Yablonski et al. (17) did not detect any effect of CD28 stimulation, either alone or in conjunction with anti-TCR, on the activity of PAK1.

We identified PAK2 in a screen designed to discover novel proteins involved in TCR-induced T cell activation. PAK2 has been reported to be activated as a result of caspase-mediated cleavage in T cells undergoing Fas-induced cell death (21). This is the first report of a requirement for PAK2 in the activation of T cells. We propose that PAK2 is a critical player in T cell activation. Several lines of evidence support this idea. Jurkat T cells predominantly express PAK2 (21). Furthermore, the highly conserved HIV protein, Nef, specifically associates with and activates PAK2, but not PAK1, in T cells (33, 34, 56). Nef plays an essential role in the pathogenesis of HIV and subsequent development of AIDS, by promoting efficient viral budding and enhancing viral particle infectivity. Nef down-regulates CD4 and MHC class I surface expression, and interferes with signaling downstream of the TCR. More recently, Nef was shown to prevent T cell apoptosis through the activation of PAK2 and phosphorylation and inactivation of the proapoptotic Bcl-2 family member, Bad (57). Taken together, these data implicate PAK2 as a critical regulator of T cell function. In a recent report, the role of the hemopoietic-specific GTPase Rac2 in T cell activation was studied using Rac2 knockout mice (58). The effects of TCR engagement, including proliferation, activation of MAPKs, calcium mobilization, and actin polymerization were reduced in Rac2-/- T cells. Rac2 acts, most likely downstream of Vav, as an important mediator of both transcriptional and cytoskeletal changes during T cell activation. Here we demonstrated the key role played by PAK2 in T cell activation, combined with the evidence that PAK2 is activated by Cdc42/Rac downstream of Vav, it is tempting to speculate that Rac2 connects signals from the TCR with cytoskeletal changes through the specific activation of PAK2. Matching different PAK effector kinases with individual Rac GTPases may help to explain how signaling specificity is achieved in a background of high protein homology.

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