A Nck-Pak1 signaling module is required for T-cell receptor-mediated activation of NFAT, but not of JNK

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The T-cell antigen receptor (TCR) triggers a signaling cascade initiated by the tyrosine kinase Lck and requiring the proto-oncogene p95^{vav}. Vav is activated by Lck and can function as a guanine nucleotide exchange factor for the Rho-family GTPases, Rac1 and Cdc42. To investigate the involvement of these GTPases in TCR signaling, we focused on their well characterized effector, Pak1. This serine/threonine kinase is activated by GTP-bound Rac1 or Cdc42. However, its role in mediating downstream signaling events is controversial. We observed rapid, TCR-dependent activation of Pak1 and TCR-inducible association of Pak1 with Nck, which was tyrosine phosphorylated following stimulation. Pak1 activation occurred independently of Ras activation or calcium flux, but was dependent on the Lck tyrosine kinase, and was downstream of Vav and Cdc42. Dominant negative Pak1 or Nck specifically inhibited TCR-mediated activation of the nuclear factor of activated T cells (NFAT) transcription factor. TCR-mediated activation of Erk2 was also inhibited by dominant negative Pak. However, Pak1 activation was neither necessary nor sufficient for TCR-dependent c-Jun N-terminal kinase (JNK) activation. Therefore, Pak1 acts downstream of Vav and is required for activation of Erk2 and NFAT by a JNK-independent pathway. This is the first demonstration of a requirement for Pak to mediate the regulation of gene expression by an extracellular ligand.

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in a rapid and sustained calcium increase. The coordinated stimulation of Ras and calcium flux is required to activate the nuclear factor of activated T cells (NFAT), a transcription factor critical for the transcriptional regulation of lymphokines such as interleukin-2.

One of the proteins critical for TCR signaling is Vav (Bustelo, 1996). *vav*^{-/-} lymphocytes have defective TCR-signal transduction (Fischer *et al.*, 1995; Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995a), whereas overexpression of Vav in Jurkat T cells augments both basal and TCR-stimulated NFAT activity (Wu *et al.*, 1995). However, the mechanism by which Vav participates in NFAT activation is still unclear.

Vav is a multi-domain protein containing a Dbl homology domain, characteristic of Rho-family guanine nucleotide exchange factors (GEFs) (Cerione and Zheng, 1996). Vav has recently been shown to catalyze guanine nucleotide exchange *in vitro* of the Rho-family G proteins (Ridley, 1996) Rac1 (Crespo *et al.*, 1997) and Cdc42 (Han *et al.*, 1997). This activity is dependent on its phosphorylation by Lck, a tyrosine kinase which initiates the events of the TCR-signal transduction pathway. Interestingly, Vav is tyrosine phosphorylated following TCR stimulation (Bustelo *et al.*, 1992; Margolis *et al.*, 1992), suggesting that its GEF activity may be activated *in vivo*. It is therefore possible that the essential role of Vav in the TCR pathway may include activation of Rac1 or Cdc42. Consistent with this hypothesis, a dominant negative allele of Rac1 has been shown to inhibit TCR-induced NFAT activation (Genot *et al.*, 1996), while a dominant negative allele of Cdc42 inhibits TCR-induced repolarization of the microtubule organizing center (Stowers *et al.*, 1995). We were intrigued by these findings, since c-Jun N-terminal kinase (JNK), which is activated by Rac and Cdc42 in some systems, is not activated by TCR stimulation alone (Su *et al.*, 1994). To address the involvement of Rac and Cdc42 in the TCR pathway, we investigated the effect of TCR stimulation on Pak1, a well characterized effector of Rac1 and Cdc42.

Pak1 belongs to a family of closely related serine/threonine kinases (Sells and Chernoff, 1997) that are activated by autophosphorylation upon binding to GTP-bound Cdc42 or Rac1 (Manser *et al.*, 1994; Martin *et al.*, 1995). Several Pak isoforms have been identified, including two in humans (Martin *et al.*, 1995; Brown *et al.*, 1996). All Pak isoforms include a C-terminal serine/threonine kinase domain, highly homologous to the kinase domain of yeast Ste20. Binding of Pak to GTP-bound Cdc42 or Rac1 is mediated by an N-terminal GTPase binding domain, known as a CRIB domain (Manser *et al.*, 1994; Burbelo *et al.*, 1995). In addition, the extreme N-terminus of Pak1 contains a conserved proline-rich sequence, which mediates binding of Pak1 to the second SH3 domain of Nck, an adaptor protein composed of a

Introduction

The T-cell antigen receptor (TCR) mediates recognition of cell-bound foreign antigens, triggering a relatively well characterized signaling pathway (Wange and Samelson, 1996; Qian and Weiss, 1997). The earliest signaling events depend on cytoplasmic tyrosine kinases which couple TCR activation to stimulation of downstream pathways. These include Ras activation, and phospholipase C activation, which leads to an increase in the second messenger inositol 1,4,5-trisphosphate (IP₃). The rise in IP₃ results

single SH2 domain and three SH3 domains (Bokoch *et al.*, 1996; Galisteo *et al.*, 1996; Lu *et al.*, 1997). Nck may recruit Pak to the membrane by binding to tyrosine-phosphorylated growth-factor receptors, which may facilitate activation of Pak by GTP-bound Rac1 or Cdc42.

A number of extracellular ligands have been reported to induce rapid activation of Pak catalytic activity, including EGF, interleukin-1 α , fMLP, thrombin and insulin (Knaus *et al.*, 1995; Teo *et al.*, 1995; Zhang *et al.*, 1995b; Galisteo *et al.*, 1996; Tsakiridis *et al.*, 1996). Nonetheless, the physiological importance of Pak activation remains controversial. Pak has been suggested to mediate several of the downstream effects of Cdc42 and Rac, including activation of JNK (Bagrodia *et al.*, 1995; Minden *et al.*, 1995; Brown *et al.*, 1996) and reorganization of the actin cytoskeleton (Manser *et al.*, 1997; Sells *et al.*, 1997). Other researchers have disputed these conclusions, demonstrating that effector-domain mutants of Rac1 that fail to bind Pak are still capable of inducing JNK activation as well as cytoskeletal changes (Lamarche *et al.*, 1996; Westwick *et al.*, 1997). At present, it is clear that GTP-bound Rac1 and Cdc42 can activate Pak; however, the functional consequences of this event are not fully understood.

In this work, we have found that Pak1 is activated robustly upon TCR stimulation and undergoes TCR-inducible association with the Nck adaptor, which is tyrosine phosphorylated following stimulation. Dominant negative alleles of Pak1 or Nck specifically block TCR-induced NFAT activation. Interestingly, Pak1 is required for TCR- or Vav-mediated signaling to the nucleus by a pathway which is independent of JNK. This work, for the first time to our knowledge, demonstrates a requirement for Pak1 in a signaling pathway mediating the regulation of gene expression by an extracellular ligand.

Results

Stimulation of Pak1 by the T-cell receptor

First, we examined the effect of TCR stimulation on Pak1 catalytic activity. An *in vitro* kinase assay was performed on anti-Pak1 immune complexes isolated from untransfected Jurkat cells (Figure 1A). Pak1 activity was significantly increased following TCR stimulation, as visualized by phosphorylation of the exogenous substrate, histone H4. In addition, a phosphorylated band of the same mobility as Pak1 was visible on the autoradiograms (data not shown). To ensure that the activity measured is that of Pak1, a control immunoprecipitation was performed in the presence of an excess of the peptide epitope recognized by the anti-Pak1 antibody. Under these conditions, no Pak1 was detected in the immune complexes and no kinase activity was detected (Figure 1A). Furthermore, a TCR-inducible kinase activity could be immunoprecipitated using anti-hemagglutinin (HA) antibodies from cells transfected with HA-tagged Pak1, but not from untransfected cells nor from cells transfected with HA-tagged, kinase-dead Pak1 (see Figure 3B, and data not shown). We are therefore confident that the activity measured represents Pak1.

The time course of Pak1 activation was examined using a quantitative assay (Figure 1B). We found Pak1 activity to be increased within 2 min of TCR stimulation and to remain above basal levels for ~10 min. Similarly, Pak1

Fig. 1. Regulation of Pak1 catalytic activity by the T-cell receptor.

(A) Jurkat T cells were stimulated for 2 min with the anti-TCR monoclonal antibody C305 (1:500) or with a buffer control, lysed, and anti-Pak1 immune complexes were assayed for kinase activity as described in Materials and methods, using histone H4 as an exogenous substrate where indicated. To control for specificity, 1 μ g/ml of the anti-Pak1 epitope blocking peptide was included during immune complex formation where indicated. Reaction products were resolved on SDS-PAGE, transferred to PVDF, and phosphorylation was visualized by autoradiography (upper panel). The PVDF filter was then probed with anti-Pak1 antibodies followed by ECL (lower panel). (B) Time course of TCR-mediated Pak1 activation. Jurkat cells were stimulated with C305 for 0–15 min as indicated and anti-Pak1 immune complexes were assayed for histone H4 phosphorylation activity using a quantitative assay, as described in Materials and methods. Duplicate determinations were performed, with error bars indicating the range of the results. The data are representative of two independent experiments.

was transiently activated following anti-TCR stimulation of mouse peripheral T cells (data not shown). However, this activation occurred 30 s after stimulation and the fold activation was lower than that seen in Jurkat cells. This rapid time course suggests that Pak1 activation may be a relatively proximal consequence of TCR stimulation.

The Lck tyrosine kinase is required for TCR-induced Pak1 activation

TCR signaling in Jurkat cells is initiated by tyrosine phosphorylation of receptor ITAMs by the Src-family tyrosine kinase Lck. Subsequent recruitment of the ZAP-70 tyrosine kinase to the phosphorylated ITAMs and phosphorylation of downstream substrates leads to activation of two major pathways, a Ras-dependent pathway and a calcium-dependent pathway. To determine whether Pak1 activation is mediated by elements of the known TCR-signaling pathway, we investigated the involvement of Lck, Ras and calcium flux in Pak1 activation.

The requirement for Lck was investigated using the Lck-deficient cell line JCaM1.6. This cell line fails to activate Pak1 upon TCR stimulation, whereas Pak1 was efficiently activated in JCaM1.6 cells stably transfected with wild-type Lck (Figure 2A). These results demonstrate

Fig. 2. (A) TCR-mediated Pak1 activation is dependent on Lck. Pak activity was measured following stimulation of the TCR for 5 min, as in Figure 1B. Cells were: Jurkat (wild-type), the Lck–Jurkat derivative, JCaM1.6, or JCaM1.6 cells stably reconstituted with wild-type Lck. The results are representative of two independent experiments. (B) Pak1 is not activated by PMA or by ionomycin. Jurkat cells were stimulated for 5 min with C305, ionomycin (1 μ M) and/or PMA (50 ng/ml), and Pak1 catalytic activity was measured as in Figure 1B.

that TCR-stimulated tyrosine phosphorylation is required for Pak1 activation.

To test whether Pak1 activation is triggered proximally or more distally in the TCR-regulated signaling pathway, we examined the effects of the pharmacological agents PMA and ionomycin. These compounds are used to mimic experimentally downstream responses to TCR stimulation, while bypassing the requirement for proximal events such as receptor phosphorylation. PMA is sufficient to activate Ras (Downward *et al.*, 1990), while ionomycin mimics TCR-induced calcium flux. However, neither of these stimuli, alone or in combination, activated Pak1 (Figure 2B). This result suggests that Pak1 is not activated downstream of either Ras or calcium flux, but may be activated upstream or independently of these pathways.

Inhibition of TCR signaling by dominant negative Pak1

To evaluate the functional importance of TCR-induced Pak1 activation, we constructed a dominant negative allele of Pak1. It has been demonstrated previously that the truncated N-terminus of Pak2/hPak65, including the CRIB domain but lacking the kinase domain, can act as a dominant negative allele to block activation of JNK by Cdc42 (Minden *et al.*, 1995). We constructed an analogous dominant negative allele of Pak1 by terminating the open reading frame immediately following residue 265 (Figure 3A).

To assess whether this allele would inhibit TCR-mediated activation of wild-type Pak1, we transfected Jurkat cells transiently with full-length, epitope-tagged Pak1 along with the truncated Pak1 or a vector control. Cotransfection of truncated Pak1 completely blocked TCR-induced Pak1 activation (Figure 3B). In contrast, cotransfection of a dominant negative allele of Raf did not affect Pak1 activation. Western blot analysis of the immune complexes confirmed that equal amounts of full-length Pak1 were immunoprecipitated in each case (Figure 3C). This experiment demonstrates that the Pak1 N-terminus (Pak1DN) can act as a dominant negative inhibitor of TCR-induced Pak1 activation.

Next, we examined the effect of Pak1DN on signaling

events downstream of the TCR, using an NFAT-luciferase reporter plasmid. The NFAT transcription factor can be activated by TCR stimulation, or by a combination of PMA and ionomycin, which activates the requisite Ras and calcium pathways. Cotransfection of Jurkat cells with Pak1DN, but not wild-type Pak1, potently blocked TCR-induced NFAT activation (Figure 4A, left panel). In contrast, activation of NFAT by PMA and ionomycin was not reduced by dominant negative Pak1, but was consistently increased (Figure 4A, right panel). These results demonstrate that Pak1DN is not toxic, but specifically inhibits the TCR-signal transduction pathway at a step upstream of the points at which PMA and ionomycin act.

While overexpression of Pak1DN inhibited TCR-mediated NFAT activation over a wide range of plasmid doses, overexpression of wild-type Pak1 failed to inhibit at all doses (Figure 4B, left panel). Notably, wild-type Pak1 was expressed at a higher level than the dominant negative allele at every dose tested (Figure 4B, right panel). Since both wild-type and dominant negative Pak1 include the CRIB domain, this result suggests that the inhibition of NFAT is not caused by nonspecific sequestration of Rac1 and Cdc42 by overexpression of a CRIB domain.

To test further whether Pak1DN acts by sequestration of Rac1 and Cdc42, we mutated two critical histidine residues (83 and 86) required for binding of the Pak1 CRIB domain to Rac1 and Cdc42 (Manser *et al.*, 1997; Sells *et al.*, 1997). This construct, Pak1DN83,86L, was a less efficient inhibitor of TCR-mediated NFAT activation than Pak1DN (Figure 4C), although both were expressed at equal levels (Figure 4C, right panel). It is therefore likely that part of the inhibitory effect of Pak1DN is due to blocking Cdc42- and Rac1-dependent pathways. However, Pak1DN83,86L retained significant inhibitory activity. This activity is probably due to blocking the function of other proteins that normally interact with the Pak1 N-terminus. This result suggests that Pak1DN does not function solely by sequestering activated Rac and Cdc42, but may be a more specific inhibitor of Pak1 function, perhaps by binding to more than one protein that regulates Pak. This claim is supported further by the

Fig. 3. Characterization of a dominant negative Pak1. (A) The structure of wild-type and dominant negative Pak1 (Pak1DN). (B) Effect of Pak1DN on TCR-mediated activation of wild-type Pak1. Jurkat cells were cotransfected with 10 μ g/cuvette of pEFhPak1WT, encoding HA-tagged Pak1, along with 30 μ g of pEF-BOS (vector), pEFhPak1DN or pEFhRafDN as indicated. Twenty-seven hours later, the cells were stimulated for 5 min with C305 or buffer control, lysed, and anti-HA (12CA5) immune complexes were assayed for kinase activity as in Figure 1B. The results are representative of at least two independent experiments. (C) Lysates from B were resolved on SDS-PAGE, transferred to PVDF, and probed with anti-HA antibodies to compare the expression of full length and Pak1DN. Lane 1, untransfected Jurkat cells; lane 2, Pak1 + vector; lane 3, Pak1 + Pak1DN; lane 4, Pak1 + RafDN.

observation that the effects of Pak1DN are more specific than those of dominant negative Rac or Cdc42, which inhibit activation of NFAT by PMA and ionomycin as well as by the TCR (Genot *et al.*, 1996; data not shown).

Pak1DN inhibits a proximal event specific to the TCR signaling pathway

To investigate further the specificity of inhibition by Pak1DN, we examined its effect on the signal transduction pathway initiated by the human muscarinic receptor (HM1R). The Jurkat-derived cell line JHM1.2.2, which

Fig. 4. Effect of Pak1DN on TCR signaling. (A) Inhibition of TCR-mediated NFAT activation by Pak1DN. Jurkat cells were transfected with 15 μ g of pNFAT-Luc along with 30 μ g of pEF-BOS (vector), Pak1WT or Pak1DN. Twenty-four hours later the cells were stimulated for 6 h with medium (unstimulated), C305 (1:1000), or PMA (50 ng/ml) and ionomycin (1 μ M) and luciferase activity was assayed as described in Materials and methods. This experiment is representative of at least three independent experiments. (B) Dose dependence of NFAT inhibition by Pak1DN. Jurkat cells were transfected with 15 μ g of pNFAT-Luc along with 0–30 μ g of Pak1WT or Pak1DN. Vector DNA was added to each transfection as needed to equalize the amount of DNA. Twenty-four hours later, the cells were stimulated and assayed for luciferase activity as in (A). The results are expressed as a fraction of the activity obtained upon stimulation with PMA and ionomycin. Lysates prepared from the transfected cells were resolved on SDS-PAGE, transferred to PVDF, and probed with anti-HA antibodies to compare the expression of full-length and dominant negative Pak1 (right panel). This experiment is representative of two independent experiments. (C) The role of the Pak1DN CRIB domain in mediating inhibition of signaling. Jurkat cells were cotransfected with 20 μ g of pNFAT-Luc, along with 15 μ g of control vector, Pak1WT or Pak1DN, or 40 μ g of Pak1DN83,86L, all in pEF-BOS, and NFAT luciferase reporter activity was measured as in (A). These amounts of plasmid produced equivalent expression of Pak1DN and Pak1DN83,86L as assessed by Western blotting (right panel). The results are expressed as a fraction of the activity obtained upon stimulation with PMA and ionomycin. This experiment is representative of five independent experiments.

stably expresses the HM1R, was used for these studies (Goldsmith *et al.*, 1989). The HM1R is a seven-transmembrane domain receptor, which, when expressed in Jurkat cells, can mediate activation of NFAT (Wu *et al.*, 1995). Unlike the TCR pathway, the HM1R

Fig. 5. Specificity of inhibition by Pak1DN. J.HM1.2.2 cells, which stably express the human muscarinic receptor, were cotransfected with 15 μ g of pNFAT-Luc and 30 μ g of control vector, Pak1WT or Pak1DN. Twenty-four hours later, the cells were stimulated for 6 h with medium (unstimulated) C305 (1:1000), carbachol (500 μ M), or PMA (50 ng/ml) and ionomycin (1 μ M), and luciferase activity was measured as in Figure 4. Results for each transfection were normalized to the activity obtained upon stimulation with PMA and ionomycin. The results shown are the average of five independent experiments.

signal is transduced by a heterotrimeric G protein which activates PLC β (Desai *et al.*, 1990). Although the HM1R ultimately activates the Ras and calcium pathways required for NFAT activation, it does this through distinct proximal events. Overexpression of Pak1DN in J.HM1.2.2 inhibited NFAT activation by the TCR, but did not inhibit NFAT activation by the HM1R (Figure 5). This result suggests that Pak1DN specifically inhibits the TCR pathway at a step upstream of the point at which the TCR and HM1R pathways converge.

Role of the Nck adaptor protein in TCR signal transduction

To obtain independent confirmation of the involvement of Pak1 in the TCR pathway, we investigated the role of Nck, an adaptor protein which has been found to associate via its second SH3 domain with the first proline-rich region of Pak1 (Bokoch *et al.*, 1996; Galisteo *et al.*, 1996; Lu *et al.*, 1997). As has been reported in fibroblasts, we found that endogenously expressed Nck and Pak1 associate in Jurkat T cells although the association was not constitutive, but was induced following TCR stimulation (Figure 6A). Inducible association of Pak1 and Nck could be demonstrated by coimmunoprecipitation of Nck with anti-Pak1 (Figure 6A) or by the reverse experiment in which Pak1 is coimmunoprecipitated by anti-Nck (data not shown). Furthermore, we found that Nck becomes tyrosine phosphorylated and undergoes a mobility shift upon TCR stimulation (Figure 6B). Together with our observation of catalytic activation of Pak1 following TCR stimulation, these observations suggest that the Nck-Pak1 complex interacts functionally with the TCR-signal transduction machinery.

To confirm the functional involvement of Nck in the TCR pathway, we utilized a dominant negative allele of Nck, containing a W143K mutation which inactivates the second SH3 domain of Nck and abolishes its interaction with Pak1 (Lu *et al.*, 1997). Like Pak1DN, dominant negative Nck specifically inhibited TCR-mediated NFAT activation, but did not inhibit NFAT activation by the HM1R (Figure 6C). Taken together, our data suggest that an Nck-Pak1 signaling complex is required to mediate activation of NFAT by the TCR.

Fig. 6. Involvement of Nck in the TCR pathway. (A) Stimulation-dependent association of Nck with Pak1 in T cells. Jurkat T cells were stimulated for 1–10 min with C305, and lysates were immunoprecipitated with a rabbit polyclonal antibody directed against the C-terminal portion of Pak1. To control for specificity, 1 μ g/ml of the anti-Pak1 epitope blocking peptide was included during immune complex formation where indicated. Immune complexes were analyzed by immunoblotting with anti-Pak (top) or with anti-Nck (bottom) polyclonal antisera. (B) Tyrosine phosphorylation of Nck following TCR stimulation. Jurkat T cells were stimulated for 2 min with C305 or buffer control, lysed, and Nck was immunoprecipitated with an anti-Nck mAb. Immune complexes were resolved on SDS-PAGE, transferred to PVDF, and analyzed by immunoblotting with anti-phosphotyrosine (upper panel). The blot was then stripped and reprobed with anti-Nck (lower panel). The tyrosine phosphorylated band seen in the upper panel corresponds to the slower migrating band in the anti-Nck blot. (C) Inhibition of TCR signaling by dominant negative Nck. NFAT-luciferase activity was determined in J.HM1.2.2 cells cotransfected with 15 μ g of pNFAT-Luc and 30 μ g of control vector or NckDN, in which a W143K mutation inactivates the second SH3 domain of Nck. Twenty-four hours later, the cells were stimulated and luciferase activity was measured as in Figure 5. Results for each transfection were normalized to the activity obtained upon stimulation with PMA and ionomycin. The results shown are the average of four independent experiments.

The position of Pak1 in the TCR pathway relative to Ras and Rho-family G proteins

It has been established previously that the catalytic activity of Pak1 is regulated by the Rho-family G proteins Rac and Cdc42 (Sells and Chernoff, 1997). However, the role of Rho-family G proteins in TCR-signal transduction is

Fig. 7. Pak1 acts downstream of Vav and Rho-family G proteins, but is upstream of Ras and calcium flux. **(A)** Effect of dominant negative G proteins on TCR-mediated Pak1 activation. TAG Jurkat cells were cotransfected with 5 μ g/cuvette of pEFhPak1WT and 10 μ g/cuvette of pNFAT-Luc, along with 40 μ g of vector, RasN17 or Cdc42N17, as indicated. Twenty-four hours later, one aliquot of the cells was stimulated for 5 min with C305 or buffer control, lysed, and anti-HA (12CA5) immune complexes were assayed for kinase activity as in Figure 1B. Results are expressed as fold-activation of Pak1 following TCR stimulation (left panel). A second aliquot was stimulated for 6 h and assayed for luciferase activity (right panel). The results are representative of at least two experiments. **(B)** NFAT-luciferase activity was determined in JHMI.2.2 cells cotransfected with 20 μ g of pNFAT-Luc and 15 μ g of control vector, Pak1WT or Pak1DN, and stimulated with C305 alone or in combination with PMA (50 ng/ml) or ionomycin (1 μ M). Results for each transfection were normalized to the activity obtained upon stimulation with PMA and ionomycin. The results shown are the average of two independent transfections. **(C)** Inhibition of TCR-mediated Erk2 activation by Pak1DN. Jurkat cells were cotransfected with 10 μ g of myc-tagged Erk2 along with 40 μ g of vector, Pak1DN or Pak1WT. Twenty-four hours later, the cells were stimulated for 5 min with anti-TCR (C305; 1:500), PMA (50 ng/ml) or buffer control, lysed, and anti-myc immune complexes were resolved by SDS-PAGE and analyzed by immunoblotting with anti-phospho-Erk (top) and anti-Erk2 (bottom). These results are representative of two experiments. **(D)** Inhibition of Vav-mediated NFAT activation by Pak1DN. An NFAT-luciferase assay was performed in Jurkat cells cotransfected with three different plasmids (20 μ g of each): pNFAT-Luc, along with vector (pEF-BOS), Vav (pEF116N) or activated Ras (pEFv-H-Ras), as well as vector, Pak1WT, Pak1DN or RafDN, all in pEF-BOS, as indicated. Twenty-four hours later the cells were stimulated for 6 h with medium (unstimulated) C305 (1:1000), or ionomycin (1 μ M), and luciferase activity was measured as in Figure 4. These results are representative of two experiments.

less well established than that of Ras. It has been suggested that Rac may act downstream of Ras in fibroblasts (Rodriguez-Viciana *et al.*, 1997) as well as in the TCR pathway (Genot *et al.*, 1996). In contrast, our experiments suggest that Pak1 does not act downstream of Ras in the TCR pathway. These considerations suggested that the relationship within the TCR pathway between Pak1 and Ras and Rho-family small G proteins should be investigated further.

To test the dependence of Pak1 activation on Ras, epitope-tagged Pak1 was coexpressed with dominant negative Ras, RasN17. While RasN17 efficiently inhibited NFAT activation (Figure 7A, right), it had no effect on activation of Pak1 (Figure 7A, left). In contrast, dominant negative Cdc42 inhibited TCR-mediated Pak1 activation, as would be expected if Pak1 were regulated by Rho-family G proteins. The effect of dominant negative Rac on Pak1 activation could not be assessed owing to nonspecific effects on the level of Pak1 expression (data not shown). From this experiment we conclude that Pak1 activation is mediated by Cdc42 (perhaps together with other Rho-family GTPases), but occurs upstream or independently of Ras.

Since Pak1 is activated upstream of Ras, its activity may be required for TCR-mediated Ras activation. To test this hypothesis, we measured the effect of Pak1DN on NFAT-luciferase activity induced by various combinations of stimuli (Figure 7B). Pak1DN inhibited NFAT activation in response to anti-TCR alone, or a combination of anti-

TCR and ionomycin. Ionomycin bypasses the requirement for TCR stimulation of calcium flux. Therefore, under these conditions, TCR-mediated Ras activation is the sole requirement for NFAT activation. This result suggests that Pak1DN inhibits stimulation of Ras by the TCR. Similarly, Pak1DN inhibited NFAT activation by a combination of anti-TCR and PMA. Since PMA bypasses the requirement for stimulation of Ras by the TCR, this experiment suggests that Pak1DN blocks TCR-mediated calcium flux. We propose therefore that Pak1 acts at a very proximal position in the pathway, upstream of the point at which the Ras and calcium pathways diverge.

To test further whether Pak1 is required for TCR-mediated Ras activation, we examined the effect of Pak1DN on TCR-mediated activation of Erk2, a well characterized, Ras-dependent TCR response (Izquierdo *et al.*, 1993). Using an antibody specific for phospho-Erk to monitor its activation, we found that TCR-mediated activation of Erk2 was inhibited by Pak1DN, but not by Pak1WT, while activation of Erk2 by PMA was unaffected (Figure 7C). We therefore propose that Pak1 acts upstream of Ras and participates in a signaling event required for TCR-mediated Ras activation.

Next we tested whether Pak1 acts at a step which is downstream of Vav. Overexpression of Vav in Jurkat cells has been shown to activate NFAT by a Ras-dependent pathway (Wu *et al.*, 1995). Activated Ras activates NFAT if the transfected cells are also stimulated with ionomycin (Woodrow *et al.*, 1993). We found that cotransfection of

either Pak1DN or, as a control, a dominant negative allele of Raf1, inhibited Vav-mediated NFAT activation (Figure 7D, left panel). In contrast, Pak1DN did not inhibit NFAT activation induced by activated Ras and ionomycin, although RafDN does inhibit Ras-induced NFAT activation (Figure 7D, right panel). These results are consistent with our previous conclusion that Pak1DN blocks NFAT activation at a step upstream of Ras activation, but suggest that this step is downstream of Vav. The results of this analysis are consistent with a model in which Vav, upon phosphorylation by Lck, activates Rac1 or Cdc42 which, in turn, activates Pak1.

Pak1-independent regulation of JNK in Jurkat T cells

Several studies have linked Pak to the regulation of JNK activity in fibroblasts (Bagrodia *et al.*, 1995; Minden *et al.*, 1995; Brown *et al.*, 1996). However, JNK activation in T cells requires costimulation of both the TCR and CD28 receptors (Su *et al.*, 1994), whereas this study shows that TCR stimulation alone is sufficient to activate Pak1. We tested a panel of different antibodies that can activate the TCR-signal transduction pathway by engaging the TCR-CD3 complex (Figure 8A). Both the monoclonal antibodies mAb 235 and Leu4, which recognize the CD3 chains of the TCR, stimulated Pak1 activity as well as the clonotypic antibody C305, which is specific for the Jurkat Ti β chain. No crosslinking of the antibodies was required. In contrast, engagement of the costimulatory receptor CD28 failed to activate Pak1, nor did costimulation of CD28 with the TCR further augment TCR stimulation of Pak1. These results demonstrate that, in T cells, a stimulus which is insufficient for JNK activation is, nonetheless, sufficient for Pak1 activation.

Next we tested whether Pak1DN inhibits JNK activation in T cells. Transient transfection was used to introduce epitope-tagged JNK and Pak1DN or a control plasmid into Jurkat cells. Immune-complex kinase assays were used to assess JNK activity following costimulation of the TCR and CD28 receptors. As a control, we used a dominant negative allele of ZAP-70, which has been shown to bind to phosphorylated TCR ITAMs and to block TCR signaling (Qian *et al.*, 1996). In contrast to dominant negative ZAP-70, Pak1DN failed to block JNK activation significantly (Figure 8B). Relative to cells transfected with a control vector, Pak1DN transfectants exhibited a lower basal level of JNK activity. However, the fold activation induced upon TCR and CD28 costimulation was not significantly different from the vector control (Figure 8C). Since Pak1DN is sufficient to inhibit TCR-mediated activation of Pak1 (see Figure 3B), these results suggest that Pak1 is not required for JNK activation in this system. Pak1 therefore participates in a TCR-regulated signaling pathway which is distinct from the JNK pathway.

Discussion

We have identified a new component of the TCR signal transduction pathway, the p21-activated kinase Pak1 and its associated adaptor Nck. Upon TCR stimulation, Pak1 is activated catalytically and associates with Nck, which becomes tyrosine phosphorylated. These events occur within minutes of stimulation. Like most other TCR-

Fig. 8. Pak1 activation is not sufficient or necessary for JNK activation in Jurkat cells. (A) Jurkat cells were stimulated for 5 min with the indicated antibody or antibody combinations, and Pak catalytic activity was measured as in Figure 1B. The anti-CD28 antibody used in the experiment shown was 9.3; however, equivalent results were obtained using YTH913.12 (anti-CD28, Serotec; data not shown). (B) Jurkat cells were cotransfected with 15 μ g/cuvette of HA-tagged JNK along with 40 μ g/cuvette of pEFPak1DN, ZAP70 DN or vector control. Twenty hours later the cells were stimulated for 15 min with a combination of anti-TCR and anti-CD28 antibodies, and anti-HA immune complexes were assayed for JNK activity as described in Materials and methods. Reaction products were resolved on SDS-PAGE, transferred to PVDF, and phosphorylation was visualized by autoradiography (upper panel). The PVDF filter was then probed with anti-HA antibody followed by ECL (lower two panels). A representative experiment is shown. (C) Four independent experiments performed as in (B) were quantitated using a Fuji Multimager, and the average fold activation of JNK is shown.

regulated responses, Pak1 activation is dependent on the Lck tyrosine kinase, which initiates TCR-signal transduction.

To investigate the functional relevance of the Nck-Pak1 complex in TCR signaling, we have utilized dominant negative alleles. Pak1DN, consisting of the N-terminal regulatory domain of Pak1, potentially blocked activation of wild-type Pak1 by the TCR. Furthermore, Pak1DN inhibited TCR-mediated activation of the NFAT transcription factor. We observed similar inhibition of NFAT using a dominant negative allele of Nck, which is mutated in the second SH3 domain and fails to bind to Pak1 (Lu *et al.*, 1997). Inhibition by both Pak1DN and NckDN is specific to the TCR-signal transduction pathway, as they did not

inhibit activation of the same NFAT reporter when mediated by the HM1R or by PMA and ionomycin. Our results therefore demonstrate that the activity of an Nck–Pak1 complex is required for TCR signaling. To our knowledge, this is the first demonstration of a requirement for Pak1 to mediate the regulation of gene expression by an extracellular ligand.

Inhibition of TCR-mediated NFAT activation by Pak1DN correlates with its inhibition of Pak1 activation, suggesting that Pak1 activity is required to mediate activation of NFAT by the TCR. However, Pak1DN may also inhibit the activity of other key Rac and Cdc42 effectors, since the CRIB domain of Pak1DN, by binding to the effector domains of GTP-bound Rac1 and Cdc42, may inhibit their interactions with all effectors, including but not limited to Pak1. It is notable therefore that overexpression of wild-type Pak1 failed to inhibit NFAT activation at all doses, even though wild-type Pak1 was expressed at a higher level than the dominant negative allele at every dose tested. Since both wild-type and dominant negative Pak1 include the CRIB domain, this result suggests that the inhibition of NFAT by Pak1DN is not caused by nonspecific sequestration of Rac1 and Cdc42 by an overexpressed CRIB domain.

To further exclude the possibility that Pak1DN acts solely by sequestration of Rac1 and Cdc42, we mutated two critical histidine residues (83 and 86) required for the binding of the Pak1 CRIB domain to Rac1 and Cdc42 (Manser *et al.*, 1997; Sells *et al.*, 1997). This construct was a less efficient inhibitor of TCR-mediated NFAT activation than Pak1DN, although it still retained significant inhibitory activity (~60%). The remaining inhibitory activity suggests that Pak1DN83,86L blocks the function of other proteins that normally interact with the Pak1 N-terminus, such as Nck. We therefore suggest that the unusual potency of Pak1DN as a dominant negative may be caused by its interactions with multiple target proteins that normally regulate the activity of Pak1. Such multivalent interactions would decrease the amount of Pak1DN required to inhibit Pak1 activation, relative to a monovalent inhibitor. Furthermore, multiple interactions with proteins that regulate Pak1 would tend to increase the specificity of the inhibitor for Pak1, relative to other targets of Rac or Cdc42. These considerations suggest that Pak1 is likely to be the main target inhibited by Pak1DN, and lead us to conclude that Pak1 activity is itself required for TCR signaling leading to NFAT activation.

In investigating the relative position of Pak1 within the TCR pathway, we have found that TCR-dependent Pak1 activation occurs independently of Ras. This conclusion is based on several lines of evidence. First, PMA, which efficiently activates Ras, failed to activate Pak1. Similarly, the HM1R, which also activates Ras, failed to activate Pak1 (data not shown). Furthermore, expression of dominant negative Ras at a level sufficient to inhibit signal transduction did not affect Pak1 activation.

Additional evidence suggests that Pak1 acts upstream of Ras and is required for TCR-mediated Ras activation. Pak1DN efficiently blocked TCR-mediated NFAT activation, even if the anti-TCR stimulus was supplemented with PMA or with ionomycin. However, Pak1DN did not inhibit NFAT activation by a combination of PMA and ionomycin, or by activated Ras and ionomycin, suggesting

that it blocks a step upstream of the activation of Ras and calcium flux. Furthermore, Pak1DN blocked TCR-mediated (but not PMA-mediated) activation of Erk2. Collectively, these observations suggest that Pak1 is required for TCR-mediated activation of the Ras pathway. This is in contrast to other systems in which Pak activation has been suggested to occur downstream of Ras, resulting from activation of Rac by Ras (Coso *et al.*, 1995; Minden *et al.*, 1995), and suggests that Pak may be regulated by different mechanisms in different cell types.

While Pak1 activation is independent of Ras, our experiments suggest that it occurs downstream of Vav and Cdc42 or Rac. This conclusion is consistent with previous results by many investigators demonstrating that the activation of Pak isoforms is dependent on GTP-bound Rac1 or Cdc42 (Sells and Chernoff, 1997). Pak1 seems to function downstream of Vav, since augmentation of NFAT activity induced by overexpression of Vav was blocked by Pak1DN. Furthermore, we have demonstrated that dominant negative Cdc42 can block TCR-mediated Pak1 activation, indicating that Pak1 activation is dependent on Cdc42. These two results may be connected by the recent finding that tyrosine phosphorylation of Vav by Lck activates its GEF activity towards Rac and Cdc42 (Crespo *et al.*, 1997; Han *et al.*, 1997). We suggest therefore a model in which TCR-stimulated tyrosine phosphorylation of Vav activates its exchange activity towards Rac1 or Cdc42, which then mediate Pak1 activation. Since Rho-family G proteins have been demonstrated to act in cascades (Hall, 1998), we cannot discern which Rho-family G protein is the direct activator of Pak1. However, both Rac1 and Cdc42 may be involved, based on a recently published report that dominant negative Rac1 inhibits TCR-mediated NFAT activation (Genot *et al.*, 1996; our data using Cdc42N17).

The mechanism by which Pak1 facilitates TCR signaling leading to regulation of NFAT is still under investigation. Recent reports demonstrate dramatic effects of activated alleles of Pak1 on the actin cytoskeleton (Manser *et al.*, 1997; Sells *et al.*, 1997). If activated Pak1 also modulates the cytoskeletal organization of T cells, it is possible that Pak1 may act on the TCR complex itself, or on molecules involved in early signaling events, changing their spatial arrangement and facilitating their ability to couple with downstream effector pathways. This model is consistent with the finding that Vav requires a functional TCR to augment basal NFAT activity (Wu *et al.*, 1995). It is also consistent with recent reports demonstrating that Vav is required to mediate TCR capping and actin polymerization in response to TCR stimulation (Fischer *et al.*, 1998; Holsinger *et al.*, 1998). It is therefore possible that Vav and Pak1 both act at a very proximal step, essentially regulating the TCR complex itself.

An alternative hypothesis is that Pak1 activity regulates the function of downstream elements of the TCR pathway. Two potential downstream elements were considered in this work, JNK and Erk.

JNK is a MAP kinase family member which may be regulated by Pak in fibroblasts. Previous studies have demonstrated activation of JNK by constitutively active alleles of Rac and Cdc42 as well as by some activated alleles of Pak (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Brown *et al.*, 1996). Furthermore,

activation of JNK in fibroblasts can be blocked by dominant negative Pak (Minden *et al.*, 1995). However, it has been suggested recently that JNK may be regulated independently of Pak (Westwick *et al.*, 1997). Strikingly, the function of Pak1 within the TCR pathway appears to be unrelated to JNK activation. While Pak1 was activated by TCR stimulation alone and was not affected by CD28 costimulation, JNK activation requires costimulation of both receptors. Furthermore, we have shown that Pak1DN, which completely blocked TCR induction of Pak1 activity, did not affect JNK activation by TCR and CD28 costimulation. These results suggest that JNK activation in T cells occurs by a pathway which is independent of Pak1.

In contrast to JNK, we observed inhibition of TCR-mediated Erk2 activation by Pak1DN. Erk2, another member of the Map kinase family, is activated in a Ras-dependent manner via the Ras–Raf–Mek–Erk cascade (Izquierdo *et al.*, 1993). Pak1DN inhibited activation of Erk2 in response to TCR stimulation, but not in response to PMA. Similarly, we found that Pak1DN inhibited TCR-mediated, but not PMA-mediated, activation of an AP-1-luciferase reporter construct, which is dependent on the Ras pathway for its activation (data not shown). These results suggest that Pak1DN inhibits a receptor proximal step required for TCR-mediated activation of Ras. Alternatively, Pak1 could act in a Ras-independent pathway which facilitates Erk2 activation, but which is bypassed by PMA.

Recently, others have suggested that Pak1 may participate in regulation of Erk activity. Constitutive activation of Pak1 has been achieved by targeting the second SH3 domain of Nck to the membrane, leading to Pak1-dependent activation of Erk (Lu *et al.*, 1997). Another group has demonstrated that constitutively active Pak can synergize with Raf1 to activate Erk2, while a kinase-dead construct can block activation of the Erk cascade by Rho-family G proteins (Frost *et al.*, 1997). However, this work is the first to examine the role of Pak1 in a more physiologically relevant system involving receptor-mediated activation of Erk. Furthermore, for the first time, this work demonstrates that Pak1 activity is required to mediate a physiological response to receptor stimulation, that is, regulation of gene expression.

Materials and methods

Cell culture and transfections

The Jurkat cell lines used were TAg Jurkat, Jurkat T cells stably transfected with SV40 TAg (provided by G.Crabtree, Stanford University; Clipstone and Crabtree, 1992); J.HM1.2.2, Jurkat T cells stably transfected with the human muscarinic receptor 1 (Goldsmith *et al.*, 1989); the Jurkat-derived signaling mutant JCaM1.6; and the Lck-reconstituted JCaM1/Lck (Goldsmith *et al.*, 1988; Straus and Weiss, 1992). All cells were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), penicillin, streptomycin, and glutamine.

Transient transfections were performed by electroporation using the Gene Pulser (Bio-Rad Laboratories, Hercules, CA), at a setting of 250 V and 960 μ F, in cuvettes containing 2×10^7 cells in 0.4 ml serum-free RPMI and the amount and type of DNA as indicated in the figure legends. Following transfection, cells were incubated for 20–30 h in RPMI containing 10% FCS and processed as indicated for each experiment.

Plasmids

HA-tagged human Pak1 cDNA (Brown *et al.*, 1996) was provided by J.Chant, Harvard. The *Kpn1* fragment encoding HA-Pak1 was subcloned

into pEF-BOS, a mammalian expression vector (Mizushima and Nagata, 1990), to create pEFhPak1WT. A Pak1 dominant negative allele was created using PCR to insert a stop codon followed by a *ClaI* site, immediately following codon D265 of Pak1. The resulting *KpnI*–*ClaI* fragment encoding HA-tagged Pak1 residues 1–265 was subcloned into pEF-BOS to create pEFPak1DN. PCR-based mutagenesis was used to mutate the histidines at codons 83 and 86 of Pak1DN, creating pEFPak1DN83,86L. The resultant Pak1DN and Pak1DN83,86L inserts were verified by sequencing. Dominant negative Nck, in which a W143K mutation inactivates the second SH3 domain, was provided by Wange Lu (Lu *et al.*, 1997). Constructs encoding myc-tagged Raf1DN, myc-tagged Erk2, RasN17 and v-H-Ras in pEF-BOS were provided by D.Cantrell (Izquierdo *et al.*, 1993). pCMVneoCdc42N17, encoding myc-tagged dominant negative Cdc42 was provided by Matt Hart, Onyx Pharmaceuticals. The dominant negative ZAP-70 construct, SH2(N+C), has been previously described (Qian *et al.*, 1996). The NFAT-luciferase reporter construct, in which the expression of luciferase is driven by multiple copies of the NFAT DNA-binding element, was provided by G.Crabtree, Stanford University. PEF116N, encoding myc-tagged Vav in pEF-BOS, is identical to the previously described EF115myc (Wu *et al.*, 1995). HA-tagged JNK1 was provided by Michael Karin, UCSD.

Antibodies

Mouse monoclonal antibodies used were C305, (specific for the Jurkat T β chain) (Weiss and Stobo, 1984); mAb 235 (against the CD3 subunit of the TCR) was provided by S.M.Fu, University of Virginia (Hara and Fu, 1985); Leu4 (anti-CD3 ϵ chain); 9.3 (anti-human CD28) was the generous gift of the Bristol-Myers-Squibb Pharmaceutical Research Division; one anti-Nck mAb was obtained from Transduction Laboratories, Lexington, KY; a second anti-Nck mAb was provided by Rusty Williams, UCSF; 9E10 (anti-myc epitope) was provided by J.M.Bishop; 12CA5 (anti-HA epitope) was obtained from Boehringer Mannheim, Indianapolis, IN; and RC20 (anti-phosphotyrosine) was obtained from Transduction Laboratories, Lexington, KY. Rabbit polyclonal anti-Pak1 antibodies sc-882 (anti-Pak1 N-terminus) and sc-881 (anti-Pak1 C-terminus) and their respective blocking peptides were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Polyclonal anti-Nck antiserum was provided by Joseph Schlessinger, NYU.

Cell stimulation, lysis and immunoprecipitations

Cells were washed in phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} (PBS), preheated to 37°C for 15 min, and were either mock stimulated with PBS (no stimulation), or were stimulated for the time indicated in the figure legend with one or more of C305 ascites (1:500) or other antibody as indicated; ionomycin (1 μ M); or phorbol myristate acetate (PMA) (50 ng/ml). Cells were then collected and lysed at 2×10^7 cells/ml in cold lysis buffer containing 1% Nonidet P-40, 10 mM Tris pH 7.6 and 150 mM NaCl (NP-40 lysis buffer) supplemented with protease and phosphatase inhibitors as described (Straus and Weiss, 1992). For Pak kinase assays, the lysis buffer was also supplemented with 50 mM NaF, 50 mM β -glycerol phosphate pH 7.5 and 20 mM sodium pyrophosphate pH 7.5. After 15 min at 4°C, lysates were cleared by centrifugation in a microfuge at 13 000 r.p.m. for 10 min, and were immunoprecipitated with the indicated antibody. For subsequent Western blotting, immunoprecipitates were washed 3 times with lysis buffer and once with 10 mM Tris pH 7.6 and 150 mM NaCl. Whole cell lysates or immune complexes were resolved by SDS–PAGE, transferred to Immobilon-P (Millipore Corporation, Bedford, MA) and probed with primary and secondary antibodies as previously described (Wu *et al.*, 1995). For Pak1 kinase assays, immunoprecipitates were processed as described below.

Pak1 kinase assays

Pak1 was immunoprecipitated from lysates of 4×10^6 cells using sc-882 bound protein A beads for endogenous Pak or 12CA5-bound protein A–Sephacrose beads to immunoprecipitate HA-tagged, transfected Pak. The immune complexes were washed 2 times with lysis buffer and 2 times with kinase buffer containing 50 mM Tris pH 7.5, 100 mM NaCl and 10 mM MgCl_2 . Beads were resuspended in 40 μ l of kinase buffer containing 3 μ g of histone H4 (Boehringer Mannheim), and reactions were initiated by the addition of 10 μ l kinase buffer containing 5 μ M ATP and 10 μ Ci [γ - 32 P]ATP. Reactions proceeded for 20 min at 30°C and were stopped by the addition of sample buffer for subsequent electrophoresis, or by the removal of 40 μ l of the supernatant to a Whatman 3 mm filter for quantitative analysis of histone H4 phosphorylation. Filters were washed extensively in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate until background levels reached

<600 c.p.m., and were then washed sequentially with ethanol and with acetone, dried, and Cherenkov counted in a scintillation counter. Background levels were determined by measuring the nonspecific activity immunoprecipitated in the presence of the sc-882 epitope blocking peptide (for assays of endogenous Pak) or immunoprecipitated from lysates of untransfected cells (for transfected Pak); the background was subtracted from the results presented.

Luciferase assays

Cells were transiently cotransfected with 10–20 µg of an NFAT luciferase reporter plasmid along with the plasmids indicated in the figure legends. Twenty to twenty-four hours later, cells were aliquoted into a 96 well cell culture dish at 10^5 cells/well and stimulated for 6 h at 37°C, with stimuli indicated in the figure legends. Cells were lysed and assayed for luciferase activity as previously described (Wu *et al.*, 1995). To correct for variations in transfection efficiency between experiments, in some cases the NFAT luciferase activity obtained upon receptor stimulation was normalized to the activity obtained upon PMA and ionomycin treatment.

JNK assay

Tag Jurkat cells were cotransfected with 15 µg of pSR-JNK (HA-tagged) and 40 µg of other indicated plasmids. Twenty hours after transfection, 2×10^7 cells per point were resuspended in fresh RPMI + 5% FCS at a final concentration of 5×10^6 cells/ml. Cells were preincubated for 45 min at 37°C, and were then stimulated for 15 min with anti-TCR and anti-CD28 antibodies (C305 + 9.3), at 1:500 each of concentrated supernatant. Cells were pelleted at room temperature (rt) for 3 min at 500 g, and washed once with 1 ml of rt PBS. Cells were lysed with 0.5 ml hypotonic buffer (10 mM HEPES pH 7.6, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM each EDTA and EGTA, 2 mM DTT, 10 mM KCl, and protease inhibitors). After 10 min on ice, NP-40 was added to 0.1%, and lysates were layered onto 0.5 ml of 50% sucrose in hypotonic buffer. Samples were centrifuged for 5 min at 5000 r.p.m., after which 450 µl was removed from the top layer and added to 50 µl of restore buffer (500 mM HEPES pH 7.6, 7.5 mM spermidine, 4.5 mM spermine, 100 mM KCl, 2 mM EDTA, 1 mM DTT, and protease and phosphatase inhibitors). Anti-HA antibody (12CA5)-coated protein G-Sepharose beads were added to the reconstituted hypotonic lysates, and samples tumbled for 2 h at 4°C. Beads were washed and kinase assays were performed as described by Su *et al.* (1994). Reaction products were resolved on 10% SDS-PAGE and blotted to PVDF. Phosphorylation of c-jun substrate was monitored by autoradiography or by a Fuji Multimager. Expression of Pak1DN and JNK was confirmed by probing the blot with 12CA5, followed by enhanced chemiluminescence (ECL) detection (Amersham).

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References

- Bagrodia, S., Derijard, B., Davis, R.J. and Cerione, R.A. (1995) Cdc42 and PAK-mediated signaling leads to jun kinase and p38 mitogen-activated protein kinase activation. *J. Biol. Chem.*, **270**, 27995–27998.
- Bokoch, G.M., Wang, Y., Bohl, B.P., Sells, M.A., Quilliam, L.A. and Knaus, U.G. (1996) Interaction of the Nck adapter protein with p21-activated kinase (PAK1). *J. Biol. Chem.*, **271**, 25746–25749.
- Brown, J.L., Stowers, L., Baer, M., Trejo, J., Coughlin, S. and Chant, J. (1996) Human ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. *Curr. Biol.*, **6**, 598–605.
- Burbelo, P.D., Drechsel, D. and Hall, A. (1995) A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J. Biol. Chem.*, **270**, 29071–29074.
- Bustelo, X.R. (1996) The Vav family of signal transduction molecules. *Crit. Rev. Oncog.*, **7**, 65–88.
- Bustelo, X.R., Ledbetter, J.A. and Barbacid, M. (1992) Product of vav proto-oncogene defines a new class of tyrosine protein kinase substrates. *Nature*, **356**, 68–71.
- Cerione, R.A. and Zheng, Y. (1996) The Dbl family of oncogenes. *Curr. Opin. Cell Biol.*, **8**, 216–222.
- Clipstone, N.A. and Crabtree, G.R. (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature*, **357**, 695–697.
- Coso, O.A., Chiariello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J.S. (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell*, **81**, 1137–1146.
- Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S. and Bustelo, X.R. (1997) Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature*, **385**, 169–172.
- Desai, D.M., Newton, M.E., Kadlecsek, T. and Weiss, A. (1990) Stimulation of the phosphatidylinositol pathway can induce T-cell activation. *Nature*, **348**, 66–69.
- Downward, J., Graves, J.D., Warne, P.H., Rayter, S. and Cantrell, D.A. (1990) Stimulation of p21ras upon T-cell activation. *Nature*, **346**, 719–723.
- Fischer, K.D. *et al.* (1998) Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. *Curr. Biol.*, **8**, 554–562.
- Fischer, K.-D., Zmuidzinas, A., Gardner, S., Barbacid, M., Bernstein, A. and Guidos, C. (1995) Defective T-cell receptor signalling and positive selection of Vav-deficient CD4+ and CD8+ thymocytes. *Nature*, **374**, 474–477.
- Frost, J.A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P.E. and Cobb, M.H. (1997) Cross-cascade activation of Erks and ternary complex factors by Rho family proteins. *EMBO J.*, **16**, 6426–6438.
- Galisteo, M.L., Chernoff, J., Su, Y.-C., Skolnik, E.Y. and Schlessinger, J. (1996) The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. *J. Biol. Chem.*, **271**, 20997–21000.
- Genot, E., Cleverley, S., Henning, S. and Cantrell, D. (1996) Multiple p21ras effector pathways regulate nuclear factor of activated T cells. *EMBO J.*, **15**, 3923–3933.
- Goldsmith, M.A., Dazin, P.F. and Weiss, A. (1988) At least two non-antigen-binding molecules are required for signal transduction by the T-cell antigen receptor. *Proc. Natl Acad. Sci. USA*, **85**, 8613–8617.
- Goldsmith, M.A., Desai, D.M., Schultz, T. and Weiss, A. (1989) Function of a heterologous muscarinic receptor in T Cell antigen receptor signal transduction mutants. *J. Biol. Chem.*, **15**, 4337–4346.
- Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279**, 509–514.
- Han, J., Das, B., Wei, W., Van Aelst, L., Mosteller, R.D., Khosravi-Far, R., Westwick, J.K., Der, C.J. and Broek, D. (1997) Lck regulates Vav activation of members of the Rho family of GTPases. *Mol. Cell Biol.*, **17**, 1346–1353.
- Hara, T. and Fu, S.M. (1985) Human cell activation. I. Monocyte-independent activation and proliferation induced by anti-T3 monoclonal antibodies in the presence of tumor promoter 2-o-tetradecanoyl phorbol-3-acetate. *J. Exp. Med.*, **161**, 641–656.
- Holsinger, L.J., Graef, I.A., Swat, W., Chi, T., Bautista, D.M., Davidson, L., Lewis, R.S., Alt, F.W. and Crabtree, G.R. (1998) Defects in actin cap formation in Vav-deficient mice implicate an actin requirement for lymphocyte signal transduction. *Curr. Biol.*, **8**, 563–572.
- Izquierdo, M., Leever, S.J., Marshall, C.J. and Cantrell, D. (1993) p21ras couples the T cell antigen receptor to extracellular signal-regulated kinase 2 in T lymphocytes. *J. Exp. Med.*, **178**, 1199–1208.
- Knaus, U.G., Morris, S., Dong, H.-J., Chernoff, J. and Bokoch, G.M. (1995) Regulation of human leukocyte p21-activated kinases through G protein-coupled receptors. *Science*, **269**, 221–223.
- Lamarche, N., Tapon, N., Stowers, L., Burbelo, P.D., Aspenstrom, P., Bridges, T., Chant, J. and Hall, A. (1996) Rac and Cdc42 induce actin polymerization and G₁ cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. *Cell*, **87**, 519–529.
- Lu, W., Katz, S., Gupta, R. and Mayer, B.J. (1997) Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. *Curr. Biol.*, **7**, 85–94.
- Manser, E., Huang, H.-Y., Loo, T.-H., Chen, X.-Q., Dong, J.-M., Leung, T. and Lim, L. (1997) Expression of constitutively active α-PAK reveals effects of the kinase on actin and focal complexes. *Mol. Cell Biol.*, **17**, 1129–1143.
- Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-s. and Lim, L. (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature*, **367**, 40–46.
- Margolis, B., Hu, P., Katzav, S., Oliver, J.M., Ullrich, A., Weiss, A. and Schlessinger, J. (1992) Tyrosine phosphorylation of vav: a proto-oncogene combining SH2 and SH3 domains with motifs found in transcriptional factors. *Nature*, **356**, 71–74.

- Martin,G.A., Bollag,G., McCormick,F. and Abo,A. (1995) A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20. *EMBO J.*, **14**, 1970–1978.
- Minden,A., Lin,A., Claret,F.-X., Abo,A. and Karin,M. (1995) Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell*, **81**, 1147–1157.
- Mizushima,S. and Nagata,S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.*, **18**, 5322.
- Qian,D., Mollenauer,M.N. and Weiss,A. (1996) Dominant-negative zeta-associated protein 70 inhibits T cell antigen receptor signaling. *J. Exp. Med.*, **183**, 611–620.
- Qian,D. and Weiss,A. (1997) T cell antigen receptor signal transduction. *Curr. Opin. Cell Biol.*, **9**, 205–212.
- Ridley,A.J. (1996) Rho: theme and variations. *Curr. Biol.*, **6**, 1256–1264.
- Rodriguez-Viciana,P., Warne,P.H., Khwaja,A., Marte,B.M., Pappin,D., Das,P., Waterfield,M.D., Ridley,A. and Downward,J. (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by ras. *Cell*, **89**, 457–467.
- Sells,M.A. and Chernoff,J. (1997) Emerging from the Pak: the p21-activated protein kinase family. *Trends Cell Biol.*, **7**, 162–167.
- Sells,M.A., Knaus,U.G., Bagrodia,S., Ambrose,D.M., Bokoch,G.M. and Chernoff,J. (1997) Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr. Biol.*, **7**, 202–210.
- Stowers,L., Yelon,D., Berg,L. and Chant,J. (1995) Regulation of the polarization of T cells toward antigen presenting cells by Ras-related GTPase CDC42. *Proc. Natl Acad. Sci. USA*, **92**, 5027–5031.
- Straus,D.B. and Weiss,A. (1992) Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell*, **70**, 585–593.
- Su,B., Jacinto,E., Hibi,M., Kallunki,T., Karin,M. and Ben-Neriah,Y. (1994) JNK is involved in signal integration during costimulation of T Lymphocytes. *Cell*, **77**, 727–736.
- Tarakhovskiy,A., Turner,M., Schaal,S., Mee,P.J., Duddy,L.P., Rajewsky,K. and Tybulewicz,V.L.J. (1995) Defective antigen receptor mediated proliferation of B and T cells in the absence of Vav. *Nature*, **374**, 467–470.
- Teo,M., Manser,E. and Lim,L. (1995) Identification and molecular cloning of a p21cdc42/rac1-activated serine/threonine kinase that is rapidly activated by thrombin in platelets. *J. Biol. Chem.*, **270**, 26690–26697.
- Tsakiridis,T., Taha,C., Grinstein,S. and Klip,A. (1996) Insulin activates a p21-activated kinase in muscle cells via phosphatidylinositol 3-kinase. *J. Biol. Chem.*, **271**, 19664–19667.
- Wange,R.L. and Samelson,L.E. (1996) Complex complexes: signaling at the TCR. *Immunity*, **5**, 197–205.
- Weiss,A. and Stobo,J.D. (1984) Requirement for the coexpression of t3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.*, **160**, 1284–1299.
- Westwick,J.K., Lambert,Q.T., Clark,G.J., Symons,M., Van Aelst,L., Pestell,R.G. and Der,C.J. (1997) Rac regulation of transformation, gene expression and actin organization by multiple, PAK-independent pathways. *Mol. Cell Biol.*, **17**, 1324–1335.
- Woodrow,M., Clipstone,N.A. and Cantrell,D. (1993) p21ras and calcineurin synergize to regulate the nuclear factor of activated T Cells. *J. Exp. Med.*, **178**, 1517–1522.
- Wu,J., Katzav,S. and Weiss,A. (1995) A functional T-cell receptor signaling pathway is required for p95Vav activity. *Mol. Cell Biol.*, **15**, 4337–4346.
- Zhang,R., Alt,F.W., Davidson,L., Orkin,S.H. and Swat,W. (1995a) Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. *Nature*, **374**, 471–473.
- Zhang,S., Han,J., Sells,M.A., Chernoff,J., Knaus,U.G., Ulevitch,R.J. and Bokoch,G.M. (1995b) Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.*, **270**, 23934–23936.

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