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Cdc42 and PAK-mediated Signaling Leads to Jun Kinase and p38 Mitogen-activated Protein Kinase Activation*

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The PAK family of protein kinases has been suggested as a potential target of the Cdc42 and Rac GTPases based on studies *in vitro*. We show that PAK-3 is activated by Cdc42 *in vivo*. Both, activated (GTPase-defective) Cdc42 and a constitutively active PAK-3 mutant stimulated the activity of Jun kinase 1 (JNK1) in transfected cells. Activated Cdc42 also stimulated the activity of the related p38 mitogen-activated protein kinase but was a less effective activator of ERK2. The effect of Cdc42 on JNK activity was similar to that of the potent inflammatory cytokine interleukin-1 (IL-1). The observation that a dominant-negative Cdc42 mutant inhibited IL-1 activation of JNK1 indicates a role for Cdc42 in IL-1 signaling. These results suggest that Cdc42 and PAK may mediate the effects of cytokines on transcriptional regulation.

The Ras-like GTP-binding protein Cdc42, a member of the Rho family, plays an important role in the initiation of cytoskeletal alterations and in the establishment of cell polarity (1). In *Saccharomyces cerevisiae*, this is manifested by the assembly of the bud site (2), while in *Schizosaccharomyces pombe* Cdc42 is essential for both unidirectional and bidirectional cell growth (3). Moreover, Cdc42 has recently been implicated in cell polarity-dependent processes that are critical to T cell activation (4) and the formation of filopodia in fibroblasts (5, 6). Other members of the Rho family, Rac and Rho, promote formation of lamellipodia and actin stress fiber assembly, respectively (7, 8). Indeed, Cdc42 may lie upstream from Rac and RhoA in growth factor-stimulated rearrangements of the cytoskeleton (5, 6, 8). A key question concerns the identity of the cellular targets for Cdc42 and how the stimulation of these targets coordinate cytoskeletal changes and cell polarity-dependent processes with cell cycle events occurring in the nucleus.

A potential target of Cdc42 was identified as a serine/threonine protein kinase, termed p65 PAK (p21 activated kinase) (9). Recently, we and others have cloned other closely related kinases pointing to the existence of a family of PAKs, which are activated by the GTPases Cdc42 and Rac *in vitro* (10, 11).[1] The PAK family of protein kinases is homologous to the yeast Ste20 protein kinase. Ste20 acts at an early step in a signaling pathway leading from a receptor-activated heterotrimeric G protein to the MAP[2] kinases KSS1 and FUS3 in the response to mating pheromone (12). Indeed, we have shown that mPAK-3 can partially restore the Ste20 null defect in *S. cerevisiae* (11). It is therefore possible that PAK may function upstream of MAP kinases in mammalian signaling pathways. A mammalian protein kinase cascade initiated by activated Ras, involving Raf and MAP kinase kinase (MEK) and leading to activation of the MAP kinases ERK1 and ERK2 in response to growth factors, is well established (13, 14). However, the Ras-dependent and -independent pathways leading to activation of the JNK group of MAP kinases (15, 16), which phosphorylate and activate the c-Jun transcription factor, have not been fully elucidated (17). Components of the JNK protein kinase cascade that have been identified include the MAP kinase kinase MKK4 (also designated SEK1/JNKK) (18–20) and the MAP kinase kinase kinase MEKK1 (21, 22). The components of the JNK pathway upstream of MEKK1 may include Ras (23) and/or additional protein kinases. The purpose of this study was to evaluate the possible role of Cdc42 and PAK as regulators of mammalian MAP signal transduction pathways.

EXPERIMENTAL PROCEDURES

Plasmids—The HA-tagged ERK2 expression vector was provided by M. Weber (University of Virginia). Plasmids J3HmPAK-3, pCMV-p38, pCDNA3-JNK1 expressing HA-tagged mouse mPAK-3, Flag-tagged mouse p38 MAP kinase, and Flag-tagged human JNK1 have been described (11, 19, 27). The mutations F91S, G93A, and P95A in the Cdc42/Rac binding domain (or p21 binding domain, designated PBD) of mPAK-3 were made by the overlap extension method using the polymerase chain reaction to generate plasmid J3H91,93,95 mPAK-3. The sequence was confirmed with a model 373A DNA sequencer (Applied Biosystems). Plasmids J3HCdc42Hs, J3HCdc42L61, and J3HCdc42N17 expressing HA-tagged wild type Cdc42, GTPase-defective Cdc42L61, and dominant-negative Cdc42N17 under the control of the SV40 promoter were constructed by inserting the *Bam*HI-*Eco*RI fragment of wild type and mutant Cdc42 from a PGEX-KG series of Cdc42 constructs (35) into the HA-tagged plasmid J3H (provided by J. Chernoff, Fox Chase Center). The *Sal*I-*Eco*RI fragment from the J3H series of Cdc42 constructs was subcloned into plasmid pCMV6 to express HA-Cdc42 under the cytomegalovirus promoter. Plasmids pcDNA3Cdc42 and pcDNA3Cdc42L61 expressing untagged Cdc42 were constructed by inserting the *Bam*HI-*Eco*RI Cdc42 fragment into the pCDNA3 vector (Invitrogen).

Cell Culture and Transfections—COS1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) or 5% bovine serum albumin (Life Technologies, Inc.). Plasmid DNA (1 μg) was transfected into COS1 cells using the lipofectamine method (Life Technologies, Inc.). The cells were harvested after 48–72 h, and where indicated the cells were treated with human IL-1 (Genzyme Corp.) for 1 h.

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[1] J. Chernoff, personal communication.

[2] The abbreviations used are: MAP, mitogen-activated protein; JNK, Jun amino-terminal kinase; MEKK, MAP kinase kinase kinase; HA, hemagglutinin; PBD, p21 binding domain; IL, interleukin; mAb, monoclonal antibody; MBP, myelin basic protein; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; WT, wild type.

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Fusion Protein Purification—His-Cdc42, GST-Jun, and GST-ATF2 proteins were expressed in *Escherichia coli* and purified by glutathione-Sepharose affinity chromatography as described (19, 27, 30). The His-tag was removed from His-Cdc42 with thrombin (27).

Immunoprecipitation and Western Blot Analysis—COS1 cells were lysed in 40 mM Hepes (pH 7.4), 1% Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin centrifuged at 12,000 × *g* for 25 min at 4 °C. Flag-tagged JNK1 was immunoprecipitated with monoclonal antibody (mAb) M2 (Kodak Scientific Imaging Systems) prebound to protein G-Sepharose. mAb M2 recognizes the Flag epitope Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys. HA-tagged mPAK-3 was immunoprecipitated with mAb 12CA5 (Berkeley Antibody Co.) primary antibody for 1 h followed by incubation with protein A-Sepharose prebound to rabbit anti-mouse IgG for 1 h. mAb 12CA5 recognizes the hemagglutinin epitope Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala. Immunoprecipitates were washed three times with lysis buffer. The proteins were eluted with Laemmli sample buffer, boiled for 3–5 min, and resolved by 10 or 12.5% SDS-polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P membranes (Millipore). The membranes were probed with primary antibody mAb M2 or mAb 12CA5. The primary antibody was detected with horseradish peroxidase-coupled sheep anti-mouse antibody using enhanced chemiluminescence detection reagent ECL (Amersham Corp).

Protein Kinase Assays—JNK, ERK2, p38 MAP kinase, and PAK protein kinase activities were measured in an immune complex kinase assay.

mPAK-3 immunoprecipitates were washed in 2 × phosphorylation buffer (10 mM $MgCl_2$ and 40 mM Hepes (pH 7.4)) and divided into three equal aliquots. One aliquot was subjected to Western blot analysis. The remaining aliquots were mixed with or without Cdc42L61 (~5 μg) and 5 μg of the substrate myelin basic protein (MBP) (Sigma). Kinase assays were initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 20 μM ATP in 30 μl (final volume) for 10 min at 22 °C.

JNK, ERK2, and p38 MAP kinase immunoprecipitates were incubated with 20 μM ATP, 5 μCi of [γ-^{32}P]ATP in 30 μl of kinase buffer (20 mM $MgCl_2$, 25 mM Hepes (pH 7.6), 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM Na_3VO_4, and 2 mM dithiothreitol). JNK assays were performed with 3 μg of substrate GST-Jun (19), ERK2 assays were performed using 3 μg of GST-Myc (36), and p38 MAP kinase assays were performed using 3 μg of GST-ATF2 (27) as substrates. The kinase reactions were terminated after 20 min at 22 °C with EDTA containing Laemmli sample buffer, and the products were resolved by SDS-PAGE (12.5% gel). In control experiments, the phosphorylation reaction was shown to be linear up to 30 min. The incorporation of ^{32}P was visualized by autoradiography and by PhosphorImager analysis (Molecular Dynamics). The protein kinase activities of ERK2 and p38 MAP kinase were measured using the same procedure.

RESULTS AND DISCUSSION

To determine whether mPAK-3 is a target of Cdc42 *in vivo*, we examined the effect of the GTPase-defective Cdc42L61 mutant on mPAK-3 activity by co-transfection in COS1 cells (Fig. 1, *A* and *B*). HA-tagged mPAK-3 was immunoprecipitated from COS cells expressing HA-mPAK-3 together with Cdc42L61 (Fig. 1*B*, *lane 1*) or from cells expressing HA-mPAK-3 alone (Fig. 1*B*, *lane 2*). Expression of constitutively activated Cdc42L61 caused a marked stimulation of mPAK-3 protein kinase activity measured using MBP as a substrate (Fig. 1*A*). mPAK-3 autophosphorylation was not detected. This is probably due to phosphorylation of the major autophosphorylation site(s) in COS1 cells. However, *in vitro* studies demonstrate that mPAK-3 autophosphorylation is detectable and stimulated by recombinant Cdc42L61 (11).

It has been established that MAP kinase signal transduction pathways require specific protein-protein interactions for normal function (17). Therefore, in initial studies to investigate the role of Cdc42, we examined the complexes formed between Cdc42 and components of MAP kinase cascades. Immunoblot analysis demonstrated the GTPγS-dependent association of PAK (~66 kDa) and MEKK (~78 and ~200 kDa) with immobilized Cdc42.[3] Given that MEKK1 functions as an upstream kinase in the JNK MAP kinase pathway (21, 22), the observation of direct or indirect GTPγS-dependent association of MEKK with Cdc42 complexes pointed to the involvement of Cdc42 in a signaling pathway that activates the JNK group of MAP kinases.

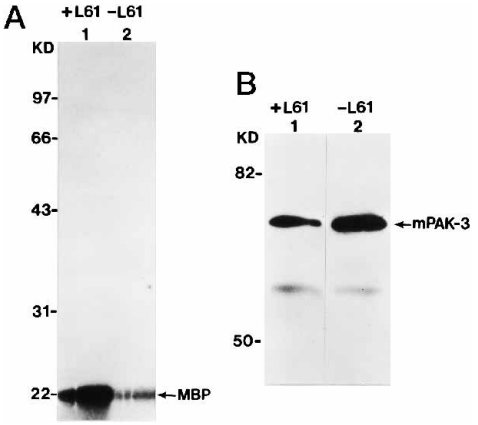

FIG. 1. **Cdc42 stimulates mPAK-3 protein kinase activity *in vivo*.** *A*, COS1 cells were transfected with plasmid J3HmPAK-3 expressing HA-tagged WT mPAK-3 together with plasmid pcDNA3Cdc42L61 (*lane 1*) or with J3HmPAK-3 alone (*lane 2*). The mPAK-3 proteins were isolated by immunoprecipitation using the 12CA5 mAb. mPAK-3 protein kinase activity was measured in the immune complex using [γ-^{32}P]ATP and MBP as substrates. The reaction was terminated after 10 min, and the products of the phosphorylation reaction were visualized after SDS-PAGE by autoradiography. *B*, the mPAK-3 immunoprecipitates of COS cell lysates expressing HA-mPAK-3 and Cdc42L61 (*lane 1*) or HA-mPAK-3 (*lane 2*) were examined by immunoblot analysis and probed with the 12CA5 mAb to detect HA-mPAK-3 followed by chemiluminescence. Molecular mass standards (kilodaltons) are indicated.

To test whether Cdc42 activates JNK, we expressed wild type (WT) Cdc42 or activated Cdc42L61 in COS1 cells and measured JNK protein kinase activity using GST-Jun as a substrate (Fig. 2). Activated, but not WT, Cdc42 stimulated JNK activity (Fig. 2, compare *lanes 1* and *4*) to an extent that was similar to that caused by the potent inflammatory cytokine IL-1 (Fig. 2, *lane 2*). Interestingly, a dominant-negative mutant of Cdc42 (Cdc42N17) was able to block IL-1 activation of JNK (Fig. 2, *lane 6*), indicating that endogenous Cdc42 may have a role in the IL-1 signaling pathway that causes JNK activation. These data establish Cdc42 as an activator of the JNK signal transduction pathway and are consistent with recent findings from other laboratories (28, 29).

To determine whether mPAK-3 is an intermediate in a Cdc42-initiated signaling pathway leading to JNK1 activation, we created a constitutively activated mPAK-3 protein kinase. The putative Cdc42/Rac binding domain (designated PBD) is highly conserved among the mammalian PAK family members including rat p65PAK, hPAK-1, hPAK-2, and mPAK-3 (9–11).[1] The region of rat p65PAK (amino acids 67–150), which binds to Cdc42 and Rac, is conserved in mPAK-3 (residues 67–137) and mediates the binding of mPAK-3 to Cdc42 and Rac *in vitro*.[4] Since the binding of Cdc42 to mPAK-3 stimulates PAK protein kinase activity (11), it is possible that Cdc42 binding relieves a negative constraint conferred by an intramolecular interaction between the PBD and another subregion of the PAK protein kinase. This hypothesis suggests that mutations in the PBD may cause activation of PAK protein kinase activity by relieving this negative constraint. We made three mutations in the PBD of mPAK-3 (F91S, G93A, and P95A). The protein kinase activity of WT and PBD-mutated mPAK-3 was examined in an

[3] S. Bagrodia, S. Pelech, and R. A. Cerione, unpublished observations.

[4] S. Bagrodia, unpublished observations.

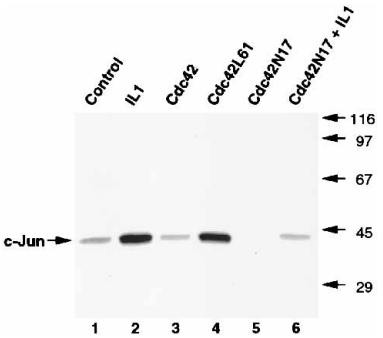

FIG. 2. **Cdc42 stimulates JNK activity *in vivo*.** COS1 cells were transfected with plasmid pcDNA3-Flag-JNK1 together with an empty expression vector (*lanes 1* and *2*) or an expression vector encoding Cdc42 (*lane 3*), Cdc42L61 (*lane 4*), Cdc42N17 (*lanes 5* and *6*). Some cultures were treated with IL-1 (*lanes 2* and *6*). The JNK activity was measured by immune complex kinase assays using [γ-^{32}P]ATP and c-Jun as substrates. The products of the phosphorylation reaction were visualized after SDS-PAGE by PhosphorImager analysis (Molecular Dynamics) and quantitated. The relative incorporation of $^{32}P_i$ in c-Jun was 1, 4.6, 0.6, 4.1, 0.007, and 0.6 for *lanes 1–6*, respectively. Molecular mass standards (kilodaltons) are indicated.

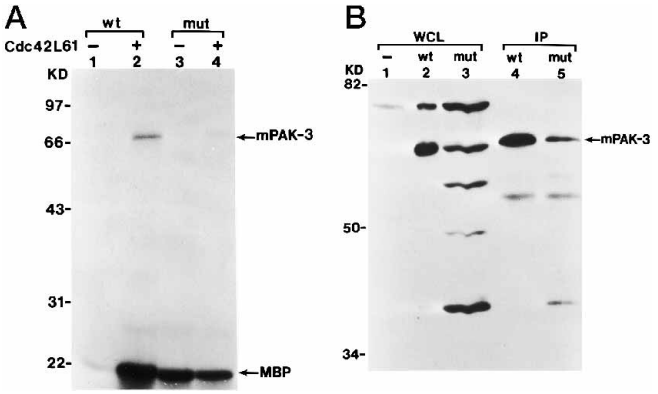

FIG. 3. **PBD-mutated mPAK-3 is constitutively active.** *A*, COS1 cells were transfected with plasmid expressing WT HA-mPAK-3 (*lanes 1* and *2*) or PBD-mutated HA-mPAK-3 (*lanes 3* and *4*). The mPAK-3 proteins were isolated by immunoprecipitation using the 12CA5 mAb. The HA-mPAK-3 immune complexes were incubated with (*lanes 2* and *4*) or without (*lanes 1* and *3*) activated Cdc42L61 (~5 μg). The protein kinase activity was measured in the immune complex using [γ-^{32}P]ATP and MBP as substrates. The reaction was terminated after 10 min, and the products of the phosphorylation reaction were visualized after SDS-PAGE by autoradiography. *B*, whole cell lysates (*WCL*) and 12CA5 mAb immunoprecipitates (*IP*) isolated from untransfected COS1 cells (*lane 1*) or COS cells transfected with WT HA-mPAK-3 (*lanes 2* and *4*), or PBD-mutated HA-mPAK-3 (*lanes 3* and *5*) were examined by immunoblot analysis and probed with the 12CA5 mAb to detect HA-mPAK-3. The highest molecular weight band observed in whole cell lysates (see *lanes 2* and *3*) is also present in untransfected COS cells (although it is not readily observed in *lane 1*). The molecular weight bands below 65 kDa detected in *lane 3* likely represent proteolyzed forms of PBD-mutated HA-mPAK-3. Molecular mass standards (kilodaltons) are indicated.

immune complex kinase assay (Fig. 3*A*); Fig. 3*B* shows the relative expression of wild type and PBD-mutated mPAK-3. The WT mPAK-3 protein kinase was activated by the addition of Cdc42L61 (Fig. 3*A*, compare *lanes 1* and *2*). In contrast, the PBD-mutated mPAK-3 exhibited a high basal protein kinase activity that was not further activated by Cdc42L61 (Fig. 3*A*, compare *lanes 3* and *4*). These data establish that the PBD-mutated mPAK-3 is constitutively activated *in vitro*.

To examine the possible role of mPAK-3 as a regulator of the JNK signal transduction pathway, we investigated the effect of WT and activated (PBD-mutated) mPAK-3 on JNK activity. Expression of WT mPAK-3 caused a small increase in JNK protein kinase activity compared with control cells transfected with the empty expression vector (Fig. 4, compare *lanes 1* and *3*). A larger increase in JNK protein kinase activity was detected in cells transfected with the activated (PDB-mutated) mPAK-3 (*lane 4*). Co-expression of activated mPAK-3 with activated Cdc42L61 (*lane 6*) did not cause JNK activation that was greater than that caused by Cdc42L61 alone (*lane 5*). The absence of additive JNK activation caused by Cdc42L61 and PDB-mutated mPAK-3 is consistent with the hypothesis that Cdc42 and mPAK-3 do not activate JNK through independent pathways. Instead, these data indicate that Cdc42 and mPAK-3 may function as components of the same signal transduction pathway that leads to JNK protein kinase activation.

To address the specificity of MAP kinase activation by Cdc42, we compared the effect of activated Cdc42L61 on JNK and the related MAP kinases p38 (22, 24, 25) and ERK2 (26). Activated Cdc42L61 stimulated the activity of JNK (Fig. 5, *lanes 3* and *4*) and p38 MAP kinase (*lanes 5* and *6*). In contrast, Cdc42L61 caused only a small increase in the activity of ERK2 (*lanes 1* and *2*). Previous studies have demonstrated that JNK and p38 are similarly regulated (27). The results of this study indicate that the Cdc42 signal transduction pathway leads to the selective activation of the JNK and p38 groups of stress-activated MAP kinases (17). The activation of Cdc42 therefore represents one mechanism that may mediate the stimulation of JNK and p38 activity in response to different environmental stimuli.

Cdc42 and Rac have been shown to activate PAK kinases (9–11), indicating that both of these GTP-binding proteins may have a role in mediating signaling to JNK1 and/or p38 MAP kinases. While this paper was in preparation, Coso *et al.* (28) and Minden *et al.* (29) reported that the Cdc42 and Rac

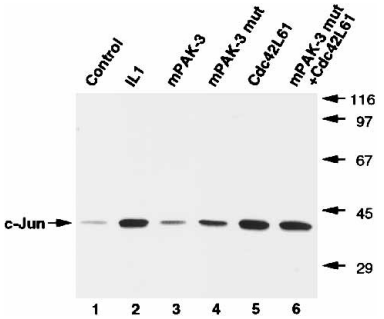

FIG. 4. **Activated PAK stimulates JNK protein kinase activity *in vivo*.** COS1 cells were transfected with pcDNA3-Flag-JNK1 together with an empty expression vector (*lanes 1* and *2*) or an expression vector encoding mPAK-3 (*lane 3*), PBD mutant mPAK-3 (*lane 4*), Cdc42L61 (*lane 5*), Cdc42L61, and PBD-mutated mPAK-3 (*lane 6*). The JNK activity was measured by immune complex kinase assays using [γ-^{32}P]ATP and c-Jun as substrates. The products of the phosphorylation reaction were visualized after SDS-PAGE by PhosphorImager analysis (Molecular Dynamics) and quantitated. The relative incorporation of $^{32}P_i$ in c-Jun was 1, 4.1, 1.3, 2.2, 4.8, and 4.5 for *lanes 1–6*, respectively. Molecular mass standards (kilodaltons) are indicated.

GTPases can cause JNK activation. The present study extends these findings by demonstrating that: 1) dominant-negative Cdc42 blocks the activation of JNK and p38 MAP kinases caused by the inflammatory cytokine IL-1; 2) Cdc42 activates mPAK-3 *in vivo*; and 3) constitutively active mPAK-3 causes JNK activation. These data indicate a role for Cdc42 and PAK in the IL-1 signaling pathway that leads to JNK and p38 activation. At present, the intermediate steps between Cdc42/PAK and JNK1 or p38 are unknown, although recent results suggest the possibility that MEKK (21, 22) and/or MAP kinase kinase (18–20) family members are downstream from Cdc42 and PAK. Taken together, these results (*cf.* Refs. 28 and 29)

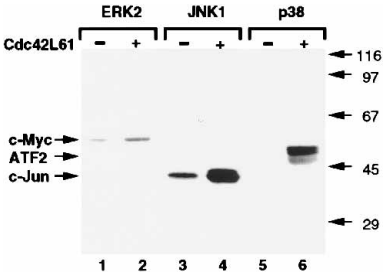

FIG. 5. **Cdc42 activates the JNK and p38 groups of MAP kinases.** COS1 cells were transfected with pCMV-HA-ERK2 (*lanes 1* and *2*) (32), pcDNA3-Flag-JNK1 (*lanes 3* and *4*), and pCMV-Flag-p38 (*lanes 5* and *6*) together with an empty expression vector (*lanes 1*, *3*, and *5*) or an expression vector encoding Cdc42L61 (*lanes 2*, *4*, and *6*). The MAP kinase activity was measured by immune complex kinase assays using [γ-^{32}P]ATP and the substrate c-Myc for ERK2, c-Jun for JNK1, and ATF2 for p38 MAP kinase. The products of the phosphorylation reaction were visualized after SDS-PAGE by PhosphorImager analysis (Molecular Dynamics). Molecular mass standards (kilodaltons) are indicated.

also support a possible connection between the actions of Dbl and related oncoproteins and nuclear MAP kinases. The Dbl (30) and Ost (31) oncoproteins serve as guanine nucleotide exchange factors for the Cdc42 and RhoA proteins while the product of the cell invasion gene *tiam-1* is an exchange factor for Cdc42 and Rac (30). An intriguing possibility is that the proliferative or invasive response of cells to these oncoproteins may be mediated by common signal transduction pathways leading to JNK and/or p38 MAP kinase activation.

Interestingly, Rac has recently been shown to be involved downstream of Ras in cellular transformation and to possess transforming activity itself (33). It is therefore possible that Rac, and perhaps Cdc42, act downstream of Ras in the Ras-dependent pathway leading to JNK1/p38 activation. While it is well established that growth factor stimulation of Ras leads to the activation of the Raf/Mek/Erk pathway, it has become clear that Ras is involved in signaling to the cytoskeleton. A possible point of convergence between the Ras and Cdc42 signaling pathways is through the phosphatidylinositol 3-kinase, which has been implicated in cytoskeleton signaling and shown to bind to both of these GTPases (34). Therefore, these findings suggest that signaling pathways through the Ras and Cdc42 GTPases are orchestrated to yield a coordinated response of cytoskeletal and nuclear events to stress, cytokines, and growth factors.

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