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Rho Family GTPases Regulate p38 Mitogen-activated Protein Kinase through the Downstream Mediator Pak1*

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Shengjia Zhang‡§, Jiahuai Han‡¶, Mary Ann Sells‖, Jonathan Chernoff‖, Ulla G. Knaus‡§, Richard J. Ulevitch‡, and Gary M. Bokoch‡§¶

From the Departments of ‡Immunology and §Cell Biology, Scripps Research Institute, La Jolla, California 92037 and the ‖Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

The stress-activated p38 mitogen-activated protein (MAP) kinase defines a subgroup of the mammalian MAP kinases that appear to play a key role in regulating inflammatory responses. Co-expression of constitutively active forms of Rac and Cdc42 leads to activation of p38 while dominant negative Rac and Cdc42 inhibit the ability of interleukin-1 to increase p38 activity. p21-activated kinase 1 (Pak1) is a potential mediator of Rac/Cdc42 signaling, and we observe that Pak1 stimulates p38 activity. A dominant negative Pak1 suppresses both interleukin-1- and Rac/Cdc42-induced p38 activity. Rac and Cdc42 appear to regulate a protein kinase cascade initiated at the level of Pak and leading to activation of p38 and JNK.

Rac and Cdc42 are members of the Rho family of small guanosine 5′-triphosphate (GTP)-binding proteins. These GTPases regulate assembly of actin cytoskeletal structures associated with cell motility and metastasis, as well as the generation of bactericidal oxygen metabolites by the phagocyte NADPH oxidase (1, 2). Rac was also shown to be an important component of cellular transformation by Ras oncogenes, although the mechanisms by which Rac contributes to the transformation process are unknown (3). Regulation of nuclear signaling by Rho family GTPases has recently been described (4), possibly through their stimulatory effects on c-Jun amino-terminal kinase (JNK)[1] (5, 6).

JNKs or stress-activated protein kinases represent a second class of the mammalian mitogen-activated protein (MAP) kinases, which includes the "classical" extracellular signal-regulated kinases (ERK) (7, 8). An additional class, which presents substantial similarity to the *Saccharomyces cerevisiae* HOG1 kinase involved in responses to increased extracellular osmolarity (reviewed by Herskowitz (9)), is p38 MAP kinase. Like HOG1, p38 can be activated by changes in osmolarity but also appears to participate in the inflammatory response to lipopolysaccharides or to inflammatory mediators such as interleukin-1 (IL-1) or tumor necrosis factor (10–12). The mechanisms by which p38 activation occurs in response to external stimuli remain to be determined. Induction of p38 activity by IL-1 or tumor necrosis factor α has little effect on ERK activity, suggesting upstream signaling via Ras does not play an important role in p38 activation.

In their active GTP-bound forms, both Rac and Cdc42 bind to and stimulate the activity of a group of 65–68-kDa Ser/Thr kinases in mammalian cells (13–15). These p21-activated kinases (Paks) are homologous to the yeast Ste20 kinase involved in regulating yeast MAP kinase cascades controlling the mating pheromone response pathway, invasive growth of haploid yeast, and pseudohyphal differentiation in diploid yeast (9). As in the yeast mating factor pathway, we have recently established that Pak activity can be regulated by mammalian G protein-coupled receptors through a pertussis toxin-sensitive G protein (15). In the present communication, we show that Pak and its upstream regulators, Rac and Cdc42, couple to and regulate the activity of p38 MAP kinase and are an integral part of the signaling pathway linking cell surface proinflammatory receptors to p38 activation.

EXPERIMENTAL PROCEDURES

Expression Plasmids—The Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; Immunex, Seattle, WA) was inserted between codons 1 and 2 of the p38 and JNK cDNAs by insertional overlapping polymerase chain reaction (16) and placed into the pcDNA3 expression vector (Invitrogen) to generate the plasmids pcDNA3-Flag-JNK1 and pcDNA3-Flag-p38 MAP kinase. Hemagglutinin (HA)-tagged Rac1(T17N), HA-Rac1(Q61L), HA-Cdc42Hs(Q61L), and HA-Cdc42Hs(T17N), were all inserted into the polylinker region of the pcDNA3 vector. HA-RhoA(Q63L) and HA-RhoA(T19N) were inserted into the pCMV5 vector (17), HA-Pak1 into the pJ3H expression vector (18), and c-Myc-tagged ERK1 into the pJ3M vector (18). HRas(Q61L) and Raf 22W (an amino-terminal-truncated active form) (19) were in the pZip-neo-svx(1) vector and were provided by J. Jackson (Scripps Research Institute).

Transient Cell Expression—COS-7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% bovine serum. Cells on 35-mm plates were transiently transfected with 1 μg of each plasmid DNA (see below) using Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer's recommendations. Transfection efficiency was evaluated using a luciferase co-transfection assay (Promega). After 48 h, the cells were treated with or without UV radiation or IL-1 as described (12, 20). Cells were solubilized with lysis buffer (25 mM Hepes, pH 7.6, 3 mM β-glycerophosphate, 3 mM EDTA, 3 mM EGTA, 250 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.078 trypsin inhibitory units/ml aprotinin) for 30 min with shaking at 4 °C and then cleared by centrifugation at 100,000 × *g* for 30 min at 4 °C prior to immunoprecipitation and kinase assay. Expression levels of cDNA constructs after transient transfection were verified using the respective epitope tag antibodies.

Kinase Assays—Mouse monoclonal antibodies against the Flag epitope, M2 (Kodak Scientific Imaging Systems), the c-*myc* epitope, 9E10 (Santa Cruz Biotechnology), HA epitope, and 12CA5 (kindly provided by I. Wilson, Scripps Research Institute) or rabbit polyclonal Pak1 antibody (15) were prebound to protein G-Sepharose or protein A-Sepharose beads, respectively. 20 μl of a 1:1 suspension of beads was added to 300-μl cell lysates and gently shaken for 3 h at 4 °C. The precipitates were washed 6 times with 1 ml of wash buffer containing 25

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¶ To whom correspondence should be addressed: Depts. of Immunology and Cell Biology, IMM14, Scripps Research Inst., 10666 N. Torrey Pines Rd., La Jolla, CA 92037.

[1] The abbreviations used are: JNK, c-Jun amino-terminal kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; IL, interleukin; Pak, p21-activated kinase; HA, hemagglutinin.

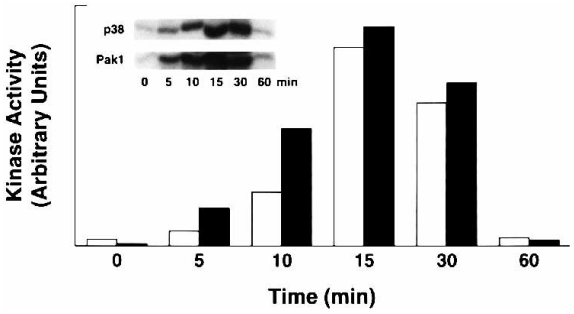

FIG. 1. **Time course of p38 MAP kinase and Pak1 activation by IL-1.** Epitope-tagged p38 MAP kinase or Pak1 was expressed in HeLa cells as described under "Experimental Procedures." After 48 h, the cells were treated with 10 ng/ml IL-1α (Genzyme Corp.) for the indicated time periods at 37 °C prior to immunoprecipitation with Flag or Pak1 antibody, respectively, and kinase assay. Phosphorylated myelin basic protein and activating transcription factor-2 were detected after 12% SDS-polyacrylamide gel electrophoresis by autoradiography and quantitated using PhosphorImager and ImageQuant software (Molecular Dynamics). *Open bars* represent p38 activity and *solid bars* Pak1 activity. The data presented represent the relative kinase activity quantified from a single experiment representative of two; the autoradiograph from this experiment is shown in the *inset*.

mM Hepes, pH 7.6, 50 mM NaCl, 0.1 mM EDTA, 0.05% Triton X-100, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.078 trypsin inhibitory units/ml aprotinin prior to kinase assay. Immunocomplex kinase assays were performed at 30 °C for 20 min using 30 μl of kinase buffer (20 mM Hepes, pH 7.6, 20 mM $MgCl_2$, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol), 3–5 μg of substrate, 20 μM ATP, and 10 μCi of [γ-^{32}P]ATP per assay. Substrates utilized were: a glutathione *S*-transferase amino-terminal truncated (amino acids 1–109) activating transcription factor-2 (20) for p38, glutathione *S*-transferase-c-Jun for JNK (21–23), and myelin basic protein for ERK1 and Pak1. The incubation was terminated with 20 μl of 4 × Laemmli sample buffer, and then the products were resolved by SDS-polyacrylamide gel electrophoresis on 12% gels and visualized by autoradiography.

RESULTS AND DISCUSSION

The proinflammatory cytokine IL-1 is a physiological regulator of p38 (12), causing a marked and rapid stimulation of p38 activity in HeLa cells (Fig. 1). We observed that IL-1 also stimulated Pak1, with Pak1 activation slightly preceding that of p38 (Fig. 1). Since Rac and Cdc42 are known regulators of Pak1 (13–15), we speculated that IL-1 might be linked to Pak1 activation through these GTPases and that this pathway might be involved in regulation of p38. In support of this hypothesis, we observed that expression of dominant negative forms of both Rac and Cdc42 effectively inhibited the ability of IL-1 to stimulate p38 activity (Fig. 2). Inhibition was directly dependent upon the amount of the dominant negative plasmid used.

While dominant negative forms of Rac and Cdc42 inhibited p38 activation by IL-1, we wanted to determine whether Rac and Cdc42 were sufficient to stimulate p38 activity. Co-expression of active Rac or Cdc42 with p38 in COS cells caused a large enhancement of p38 activity, comparable with that seen with stimulation by UV radiation, which maximally activates the enzyme and which serves an indicator of the total levels of p38 expressed and present in the immune precipitates (Fig. 3, *A* and *B*). Expression of wild type Rac had only a slight effect on p38 activity (data not shown). This effect was specific for the GTPases Rac and Cdc42, as we failed to observe stimulation when activated forms of H-Ras, Raf, or RhoA were co-transfected with p38 (Fig. 3*C*).

The role of Pak in the p38 activation process was also assessed. Co-expression of wild type Pak1 itself with p38 caused a marked increase in p38 activity (Fig. 3, *A* and *B*). Pak1 appears to become activated when expressed in a COS cell environment, possibly due to the presence of low levels of active

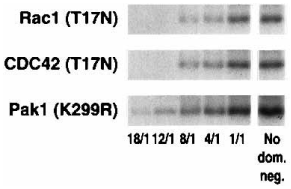

FIG. 2. **Inhibition of IL-1-stimulated p38 MAP kinase activity by dominant negative forms of Rac1, Cdc42, and Pak1.** Epitope-tagged p38 MAP kinase in pcDNA3 vector (0.2 μg/plate) was transiently transfected into HeLa cells together with either the dominant negative form of Rac1 (*top panel*), Cdc42 (*middle panel*), or Pak1 (*bottom panel*). The molar ratio of the dominant negative plasmids to p38 cDNA, respectively, is indicated at the *bottom* of the figure, with the total DNA concentration kept constant by supplementation with pcDNA3 vector. *No dom. neg.* indicates the IL-1-activated control in the absence of any dominant negative DNA. Expression of p38 was similar under each condition as determined by Western blotting (not shown). The cells were stimulated 48 h after transfection with 10 ng/ml IL-1 for 30 min at 37 °C, and then p38 MAP kinase activity was measured. Results are representative of two similar experiments.

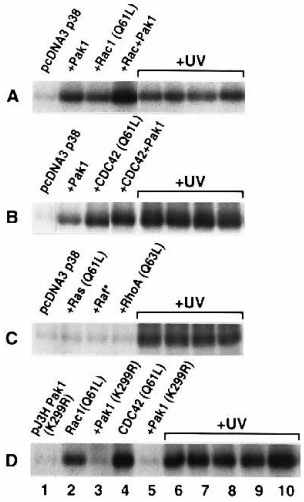

FIG. 3. **Stimulation of p38 MAP kinase by Rho family GTPases acting through Pak.** Epitope-tagged p38 MAP kinase was co-expressed in COS-7 cells with the following cDNAs and then immunopurified after 48 h and assayed for kinase activity. The total DNA concentration in each condition was maintained constant by supplementation with pcDNA3 vector. Results are representative of two or more experiments. *A*, pcDNA3-p38 and: *lanes 1* and *5*, + empty pcDNA3 vector; *lanes 2* and *6*, + pJ3H-Pak1; *lanes 3* and *7*, + pcDNA3-Rac1(Q61L); *lanes 4* and *8*, + pcDNA3-Rac1(Q61L) and pJ3H-Pak1. *B*, pcDNA3-p38 and: *lanes 1* and *5*, + empty pcDNA3 vector; *lanes 2* and *6*, + pJ3H-Pak1; *lanes 3* and *7*, + pcDNA3-Cdc42(Q61L); *lanes 4* and *8*, + pcDNA3-Cdc42(Q61L) and pJ3H-Pak1. *C*, pcDNA3-p38 and: *lanes 1* and *5*, + empty pcDNA3 vector; *lanes 2* and *6*, + pZip-neo-HRas(Q61L); *lanes 3* and *7*, + pZip-neo-Raf*(22W); *lanes 4* and *8*, + pCMV5-RhoA(Q63L). *D*, pcDNA3-p38 and: *lanes 1* and *6*, + pJ3H-Pak1(K299R) alone; *lanes 2* and *7*, + pcDNA3-Rac1(Q61L); *lanes 3* and *8*, + pcDNA3-Rac1(Q61L) and Pak1(K299R) at a 1:10 DNA ratio); *lanes 4* and *9*, + pcDNA3-Cdc42(Q61L); *lanes 5* and *10*, + pcDNA3-Cdc42(Q61L) and Pak1(K299R) at a 1:10 DNA ratio. Each condition is also shown after stimulation with UV light (*lanes 5–8* of *panels A–C* and *lanes 6–10* of *panel D*) to assess maximal stimulation of p38 activity and p38 expression under each condition.

GTP-bound Cdc42.[2] However, when we co-expressed Pak1 with constitutively GTP-bound Rac or Cdc42, we observed a greater increase in p38 activity, indicating that the action of Pak could be enhanced by these known activators of the enzyme's catalytic function.

We utilized a Pak1 containing a single point mutation (K299R) in the kinase domain, which renders the enzyme catalytically inactive (24), to investigate the role of Pak1 in p38

[2] S. Zhang and G. M. Bokoch, unpublished observations.

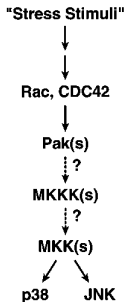

FIG. 4. **Proposed signal transduction pathway for activation of the stress-regulated p38 and JNK MAP kinases.** *MKK*, MAP kinase kinase; *MKKK*, MAP kinase kinase kinase.

activation by Rac and Cdc42. This construct behaves as a dominant negative inhibitor of Pak activity *in vivo*,[3] but does not appear to act merely by titrating out Rac and/or Cdc42, as it does not inhibit Rac-potentiated cell transformation.[4] Dominant negative Pak inhibited nearly all of the p38 stimulatory capability of Rac and Cdc42, suggesting that the effects of both of these GTPases on p38 were mediated via Pak activation (Fig. 3*D*). In studies not shown we observed that UV-induced p38 activation can be blocked by increasing the amount of plasmid DNAs encoding Pak1 (K299R) used in the co-transfection assay using ratios similar to those shown in Fig. 2. Additionally, Pak1 (K299R) was able to effectively block activation of p38 by IL-1. Taking into account the ability of IL-1 to stimulate Pak1 activity with a similar time course as that for p38 activation, the ability of a dominant negative Pak1 to block p38 activation by IL-1, and the ability of Pak1 itself to stimulate p38 activity, we conclude that the activity of Pak1, regulated by the upstream GTPases Rac and/or Cdc42, is an integral component of the signaling process linking cytokine receptors to p38 activation.

The regulatory effects of Pak1 are not limited to the p38 pathway. The JNKs form an additional branch of the mammalian MAP kinase family, which are regulated by many of the same upstream stimuli as p38 (7–9). In addition to the recently reported ability of Rac and Cdc42 to stimulate JNK activity (5, 6), we observed that Pak1 could activate JNK activity as well (data not shown). In contrast, we could detect no stimulatory effect of activated Rac, Cdc42, or Pak1 on the ERK branch of the MAP kinase family; the latter are responsive to upstream regulators quite distinct from the "stress-activated" MAP kinases (7, 8). Since we have shown that Pak(s) can be activated by mammalian G-protein-coupled receptors (15) and growth factor receptors,[5] it is likely that signaling through Pak contributes to the activation of stress-activated MAP kinases by such stimuli as well (25). Based on these data, we suggest a pathway, depicted in Fig. 4, through which a variety of upstream signaling molecules can stimulate activity of the p38 and JNK kinases. Activation of Rac and/or Cdc42 by upstream signals leads to increased activity of Pak kinase(s). Pak does not directly phosphorylate p38 or JNK1, and both p38 and JNK are known to require phosphorylation of both Thr and Tyr residues for activation to occur (12). This dual phosphorylation is mediated by the action of upstream MAP kinase kinases, which are in turn controlled by MAP kinase kinase kinases in a typical MAP kinase regulatory cascade (21–23). We therefore suggest it is likely that, by analogy with the Ste20 kinase cascade in *S. cerevisiae*, Paks regulate the activity of MAP kinase kinase kinases, which act in turn on MAP kinase kinases to directly phosphorylate and regulate p38 and JNK. Potentially, Paks may serve to coordinate stress responses at the transcriptional level with morphological and cytoskeletal changes that occur concomitantly. Thus, regulation of Rac and Cdc42 function may be an important component of the mammalian response to shock and other inflammatory disorders.

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[3] M. A. Sells, U. G. Knaus, D. Ambrose, S. Bagrodia, G. M. Bokoch, and J. Chernoff, submitted for publication.
[4] G. M. Bokoch and C. J. Der, unpublished observations.
[5] G. M. Bokoch, unpublished observations.

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