Communication

The Small GTP-binding Protein Rho Activates c-Jun N-terminal Kinases/Stress-activated Protein Kinases in Human Kidney 293T Cells

EVIDENCE FOR A Pak-INDEPENDENT SIGNALING PATHWAY*

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Work from a number of laboratories has established a role for certain small GTP-binding proteins in controlling the enzymatic activity of a family of serine-threonine kinases known as mitogen-activated protein kinases (MAPKs). MAPKs have been classified into three subfamilies: extracellular signal-regulated kinases (ERKs), also known as MAPKs; c-Jun N-terminal kinases (JNKs); and p38 kinase. Whereas Ras controls the activation of MAPKs, we and others have recently observed that in certain cells, the small GTP-binding proteins Rac1 and Cdc42 but not Rho regulate the activity of JNKs. Furthermore, because Rac1 and Cdc42 but not Rho bind and activate a kinase known as Pak1, it has been suggested that Pak1 is the most upstream component of the pathway linking these GTPases to JNK. However, in both yeast and mammalian cells, Rho1p, a Rho homologue, and RhoA, respectively, directly interact with a number of proteins, including kinases related to protein kinase C. In addition, in yeast, Rho1p controls the activity of a MAPK cascade involved in bud formation. Considering this diversity of target molecules for small GTP-binding proteins, their likely tissue specific distribution, and the potential role for Rho in signaling to a kinase cascade, we decided to extend our initial analysis, exploring the ability of Ras and Rho-related GTP-binding proteins to activate MAPK or JNK in a variety of cell lines. We found that in the human kidney epithelial cell line, 293T, Cdc42 and all Rho proteins, RhoA, RhoB, and RhoC, but not Rac or Ras can induce activation of JNK. Furthermore, we provide evidence that signaling from Rho proteins to JNK in 293T cells does not involve Pak1. Taken together these findings demonstrate that Rho signals to JNK in a cell type-specific manner and suggest the existence of a novel, Pak1-independent signaling route communicating the Rho family of small GTP-binding proteins to the JNK pathway.

The Ras superfamily of GTPases comprises more than 50 members, which have been divided into six families based upon sequence similarity: Ras, Rho, Arf, Sar, Ran, and Rab (1). These proteins function as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state (2). One function of Ras is to regulate the activity of mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs). These protein serine/threonine kinases are rapidly activated upon stimulation of a variety of cell surface receptors, and they play a key role converting extracellular stimuli to intracellular signals that in turn control the expression of genes essential for many cellular processes (3, 4). At the molecular level, Ras exchanges GDP for GTP upon activation of Ras-specific guanine-nucleotide exchange factors. In the GTP-bound state, Ras then physically associates with the N-terminal region of Raf (5, 6), thereby recruiting this serine-threonine kinase to the plasma membrane and allowing its activation by a still unknown mechanism (7). In turn, Raf activates a linear cascade of protein kinases, defined sequentially as MAPK kinase, such as MEK (8), which ultimately phosphorylate MAPKs on both threonine and tyrosine residues, thereby increasing their enzymatic activity (9).

Interestingly, in yeast, Rho1p, a homologue of the mammalian RhoA, interacts directly with PKC1, a yeast homologue of protein kinase C (10). This interaction causes the activation of a MAPK cascade involved in bud formation. Similarly, the yeast Cdc42 homologue participates in the mating pheromone response by acting on a serine-threonine kinase, Ste20, initiating activity from a different cascade of kinases including, sequentially, Ste11 and Ste7, which phosphorylate and activate the yeast MAPK homologue Fus3 and Kss1 (11). Thus, available information suggests that in yeast, members of the Rho family of GTPases control the activity of at least two distinct MAPK pathways.

Recently, we and others have observed that in certain mammalian cell types, Rac1 and Cdc42 but not Rho control the activity of a novel member of the MAPK superfamily, known as stress-activated protein kinase (SAPK), also termed c-Jun N-terminal kinase (JNK) (12–14). In addition, Rac1 and Cdc42 have been shown to bind and activate a kinase known as Pak1 (15), thus suggesting that Pak1 is the most upstream component of the biochemical route leading to JNK activation (16). On the other hand, Rho has been shown to regulate the activity of a number of enzymes, such as phosphatidylinositol 3-kinase (17), phosphatidylinositol 4-phosphate 5-kinase (18), and phospholipase D (19). Very recently, two groups have reported that Rho can physically associate with a number of proteins, including protein kinase N, a kinase closely related to but distinct from protein kinase C (20, 21), a situation strikingly similar to that described in yeast (see above). In view of this diversity of target molecules for small GTP-binding proteins, their likely

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† The abbreviations used are: MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase.

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tissue-specific distribution, and the potential role for Rho in signaling to a kinase cascade, we decided to extend our initial analysis, exploring the ability of Ras and Rho-related GTP-binding proteins to activate MAPK or JNK in a variety of cell lines. We found that in the human kidney epithelial cell line, 293T, Cdc42 and all Rho proteins, RhoA, RhoB, and Rhoc, but not Rac or Ras can induce activation of JNK. Furthermore, we provide evidence that signaling from Rho proteins to JNK in 293T cells does not involve Pak1, raising the possibility of the existence of a novel, Pak-independent pathway communicating small GTP-binding proteins to JNK.

MATERIALS AND METHODS

Cell Lines and Transfection—Human kidney 293T cells and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. 293T cells were transfected by the calcium-phosphate precipitation technique, and COS-7 cells were transfected by the DEAE-dextran method, adjusting the total amount of DNA to 5–10 μg/plate with vector alone. For 293T cells, tissue culture plates were treated with phosphate-buffered saline containing 20 μg/ml poly-n-lysine for 15 min before seeding the cells to prevent detachment from the plates in serum-free conditions.

DNA Constructs—Plasmids expressing epitope-tagged MAPK and JNK, pcDNA3 HA-MAPK and pcDNA3 HA-JNK, respectively, as well as expression plasmids for wild type and constitutively activated forms of Ras, RhoA, Rac1, and Cdc42 were described previously (13). pEBG-SEK and pEBG SEK KR were kindly provided by Dr. Leonard Zon (22). pcDNA3-rat Pak1 was kindly provided by Dr. Toru Miki. An expression plasmid for the N-terminal 150-amino acid noncatalytic domain of Pak1, which contains the Ras/Cdc42 binding region, was obtained upon amplification of the corresponding coding sequence by the polymerase chain reaction technique and subcloning the obtained DNA fragment into the BamHI and EcoRI site of pCEFL-GST, a modified pEBG plasmid (see below). The final construct was designated pCEFL-GST Pak(N). pEBG ras, pEBG rasV12, pEBG rhoA WT, pEBG rhoA QL, pEBG rac1 WT, pEBG rac1 QL, pEBG cdc42 WT, and pEBG cdc42 QL were obtained by subcloning the corresponding cDNAs as BglII-NotI or BamHI-NotI fragments into the BamHI and NotI sites of pEBG, an expression plasmid encoding the N-terminal region of the glutathione S-transferase protein (GST) in frame with the BamHI site (23). RhoB WT and RhoC WT were obtained by polymerase chain reaction, using human cDNA as template. Their respective constitutively activated mutants were obtained by replacing a glutamine residue corresponding to codon 63 of RhoB and RhoC for a leucine residue (QL mutants), using polymerase chain reaction-directed mutagenesis. Sequences of each oligonucleotide will be made available upon request. Final DNA constructs were expected to express either the wild type or the activated forms of RhoB and RhoC.

Kinase Assays—MAPK activity in cells transfected with an epitope-tagged MAPK (HA-ERK2, referred in here as HA-MAPK) was determined, as described previously (13), using myelin basic protein (Sigma) as a substrate. JNK assays in cells transfected with an epitope-tagged JNK (HA-JNK) were carried out as described previously (13), using myelin basic protein (Sigma) as a substrate. Samples were analyzed by SDS gel electrophoresis on 12% polyacrylamide gels. Autoradiography was performed with the aid of an intensifying screen. Parallel lysates of cells transfected with the HA-JNK expression plasmids and cultured for 48 h in incomplete medium. Cells were then washed three times with cold buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 500 mM NaCl, 0.5% Nonidet P-40), and bound nucleotides were released by heating samples at 68 °C for 3 min. Eluted nucleotides were analyzed by polyethyleneimine thin layer chromatography (J. T. Baker), using unlabeled guanine nucleotides as standards.

RESULTS AND DISCUSSION

Using COS-7 cells, we and others have recently observed that whereas Ras initiates activity of a kinase cascade leading to the activation of MAPK, two members of the Rho family of small GTP-binding proteins, Rac1 and Cdc42, can activate JNK, utilizing a biochemical route distinct from that of the Ras-Raf-MEK-MAPK pathway (13). Similar results were obtained in NIH 3T3 cells, Hela cells, Chinese hamster ovary cells, and the human keratinocyte cell line HaCat (data not shown). Consistent with those observations, we found that expression of activated Ras (V12Ras) but not activated mutants of RhoA, Rac1, and Cdc42 (RhoA-QL, Rac1-QL, and Cdc42-QL, respectively) potently elevates the enzymatic activity of a cotransfected epitope-tagged MAPK (HA-MAPK) in
either COS-7 cells or in the human kidney epithelial cell line 293T. Surprisingly, a very different result was obtained for JNK in the latter cells. Although constitutively active mutants of Rac1 and Cdc42 potently enhanced JNK activity in COS-7 cells, as reported (13), parallel experiments in 293T cells revealed that in this cell line, Cdc42-QL and RhoA-QL increased the in vitro phosphorylating activity of JNK without affecting the expression level of the tagged JNK (Fig. 1). These results suggest that in 293T cells, RhoA and Cdc42 but not Rac1 can initiate the activity of a biochemical route leading to JNK stimulation.

We next asked which member of the Rho family can signal to JNK in 293T cells. Initially, we engineered expression vectors for RhoB and RhoC and their corresponding activated mutants. When transfected into 293T cells, each small GTP-binding protein was found to be detectably expressed (data not shown). Furthermore, as shown in Fig. 2, activated forms of RhoA, RhoB, and RhoC effectively elevated the enzymatic activity of JNK in 293T cells, whereas their corresponding wild type forms enhanced JNK activity only to a very limited extent. Thus, all Rho GTP-binding proteins can signal to JNK activation in 293T cells.

The observations that Pak1 binds in vitro Rac1 and Cdc42 in a GTP-dependent manner and that both Rac1 and Cdc42 activate JNK in several cell lines provided strong evidence supporting a role for Pak1 as the most upstream kinase connecting these GTPases to JNK. However, we showed here that in 293T cells, JNK is activated by Rho proteins but not by Rac1. This observation prompted us to investigate whether Pak1 participates in signaling from Rho to JNK. As an experimental approach, we determined the ability of Pak1 to associate exclusively with activated mutants of Ras, RhoA, Rac1, and Cdc42, fused to GST. As shown in Fig. 3B, each GST fusion protein was expressed at detectable levels in these mammalian cells. Furthermore, analysis of the nucleotide bound to these GTPases revealed that wild type GTP-binding proteins were almost exclusively in the GDP-bound form and that the majority of the nucleotide bound in vivo to the corresponding activated mutants was GTP (Fig. 3A). Using these experimental conditions, we found that Pak1 was associated exclusively with activated Rac1 and Cdc42 in 293T cells (Fig. 3). Similar results were obtained in COS-7 cells (data not shown). In addition, we observed that overexpression of the noncatalytic domain of Pak1 blocks JNK activation by Cdc42 but does not affect JNK stimulation by RhoA (Fig. 3C). These and additional findings, including the observations that Rac1 can bind and activate Pak1 (15) but fails to induce JNK in 293T cells (this study) and that, conversely, Rho proteins can induce JNK in these cells but fail to bind Pak1 in vitro (14) and in vivo (this study), suggest that Pak1 does not participate in signaling from GTP-binding proteins to JNK in 293T cells and lend support to the conclusion that signaling from Rho to JNK utilizes a Pak1-independent pathway. Whether Pak1 mediates JNK activation by Cdc42 and Rac1 in other cell types is not yet
required for cell wall integrity. Recently available data also suggest that Rho regulates the activity of an enzyme responsible for cell wall glucan synthesis (26). Thus, in yeast, Rho functions upstream from a MAPK signaling pathway and controls the activity of enzymes directly involved in cell wall formation. In mammalian cells, Rho has been proposed to participate in a number of cell functions by regulating the reorganization of actin cytoskeleton (27). The recent finding that Rho physically associates with a mammalian kinase, PKN, suggests that Rho might also participate in the control of a kinase cascade in these cells (20, 21), but in this regard, we and others have previously observed that only Ras activates MAPK and that Rac and Cdc42 but not Rho activate a signaling pathway leading to JNK stimulation (19). However, a more extensive study using a variety of cell lines revealed that all members of the Rho subfamily, RhoA, RhoB, and RhoC, can activate JNK but not MAPK in a commonly used cell line, human kidney 293T cells. Furthermore, we obtained evidence to suggest that signaling from Rho to JNK involves a Pak1-independent pathway. Thus, taken together, these observations and those from others (28) strongly suggest the existence of distinctly regulated kinases acting upstream of JNK that based on the present study are expected to function in a tissue-specific manner. The nature of the Rho-dependent kinase(s) regulating JNK in 293T cells remains unknown and is currently being investigated.

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**Fig. 4. Effect of Dominant Negative Mutant of SEK on JNK/ SAPK Activation.** 293T cells were transfected with empty vector (pEBO) or expression plasmids for SEK and dominant negative SEK (SEK KR) (3 µg/plate in each case) together with pcDNA3 vector without insert (control) or carrying cDNAs for RhoA QL, RhoB QL, RhoC QL, Cdc42 QL, and MEKK (3 µg/plate), as indicated, and pcDNA3-HA-JNK (1 µg/plate in each case) for JNK assays. Data represent the means ± S.E. of three independent experiments, expressed as fold increase with respect to vector-transfected cells (control). Western blot (WB) analysis was performed with SEK antiserum upon affinity purification of GST SEK and GST-SEKKR fusion proteins from the corresponding cellular lysates, as described under “Experimental Procedures.” Autoradiograms correspond to representative experiments.
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