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The Small GTP-Binding Proteins Rac1 and Cdc42 Regulate the Activity of the JNK/SAPK Signaling Pathway

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Summary

c-Jun amino-terminal kinases (JNKs) and mitogen-activated protein kinases (MAPKs) are closely related; however, they are independently regulated by a variety of environmental stimuli. Although molecules linking growth factor receptors to MAPKs have been recently identified, little is known about pathways controlling JNK activation. Here, we show that in COS-7 cells, activated Ras effectively stimulates MAPK but poorly induces JNK activity. In contrast, mutationally activated Rac1 and Cdc42 GTPases potently activate JNK without affecting MAPK, and oncogenic guanine nucleotide exchange factors for these Rho-like proteins selectively stimulate JNK activity. Furthermore, expression of inhibitory molecules for Rho-related GTPases and dominant negative mutants of Rac1 and Cdc42 block JNK activation by oncogenic exchange factors or after induction by inflammatory cytokines and growth factors. Taken together, these findings strongly support a critical role for Rac1 and Cdc42 in controlling the JNK signaling pathway.

Introduction

Mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases, are protein-serine/threonine kinases that are rapidly activated upon stimulation of a variety of cell surface receptors (Ray and Sturgill, 1987). Their function is to convert extracellular stimuli to intracellular signals controlling the expression of genes essential for many cellular processes, including cell growth and differentiation (Marshall, 1995). These kinases play a central role in mitogenic signaling, as impeding their function prevents cell proliferation in response to a number of growth-stimulating agents (Pages et al., 1993). Furthermore, aberrant functioning of proteins known to be upstream of MAPK can induce cells to acquire the transformed phenotype, and constitutive activation of the MAPK pathway is itself sufficient for tumorigenesis (Cowley et al., 1994; Mansour et al., 1994). Thus, MAPKs appear to be a critical component of growth-promoting pathways.

Recently, two novel classes of mammalian enzymes closely related to MAPKs have been identified. One class presents extended similarity to the Saccharomyces cerevisiae HOG1 kinase (Han et al., 1994), which is involved in protecting S. cerevisiae from hyperosmotic solutions (reviewed by Herskowitz, 1995). The role of this mammalian HOG1 homolog is largely unknown. Although it can also be activated by changes in osmolarity, it appears to participate in the inflammatory response to lipopolysaccharides or to inflammatory mediators such as interleukin-1 (IL-1) (Han et al., 1994; Freshney et al., 1994). The other class of MAPKs represents a family of closely related enzymes activated by cellular stress, which have been named stress-activated protein kinases (SAPKs) (Kyriakis et al., 1994). SAPKs were independently identified by virtue of their ability to phosphorylate the amino terminus of the c-Jun transcription factor; hence, they have been also termed c-Jun amino-terminal kinases (JNKs) (Dérijard et al., 1994). JNKs can be potently activated by inhibitors of protein synthesis such as cycloheximide and anisomycin, inflammatory cytokines such as tumor necrosis factor α (TNFα) and IL-1, changes in osmolarity, heat shock, and ultraviolet irradiation (Dérijard et al., 1994; Kyriakis et al., 1994). JNKs are thought to be responsible for phosphorylating the transactivating domain of c-Jun protein in vivo (Dérijard et al., 1994; Kyriakis et al., 1994), and, in turn, phosphorylated c-Jun homodimers have potent AP-1 activity and can control the expression of a number of genes, including c-*jun* itself (Angel et al., 1988).

The mechanism of activation of JNKs has just begun to be explored. Although initial observations suggested that these enzymes were located downstream of Ras (Dérijard et al., 1994), this hypothesis is in conflict with recently available data (Sun et al., 1994), including the lack of activation of JNK in certain cellular systems by agonists acting on receptors that are known to couple to the Ras–MAPK pathway (Kyriakis et al., 1994; Coso et al., 1995). Furthermore, agonists such as TNFα and IL-1 potently induce JNK, but they activate MAPK poorly (Kyriakis et al., 1994). Taken together, these observations suggest the existence of distinct pathways leading to the activation of either MAPK or JNK. In this study, we have used the expression of hemagglutinin (HA) epitope-tagged JNK1 (HA-JNK) in COS-7 cells to explore the mechanism of activation of JNK. We present evidence that the small GTP-binding proteins Rac1 and Cdc42 are an integral part of a novel signaling pathway linking cell surface receptors to JNK activation.

Results

Functional Expression of an Epitope-Tagged JNK

The *JNK1* cDNA was obtained by the polymerase chain reaction (PCR) technique, its coding sequence was altered by the insertion of an amino-terminal tag of 9 amino acids representing influenza HA1 protein (Wilson et al., 1984), and the cDNA was cloned in the expression vector pcDNA3. As shown in Figure 1 (top), this epitope-tagged *JNK1* cDNA

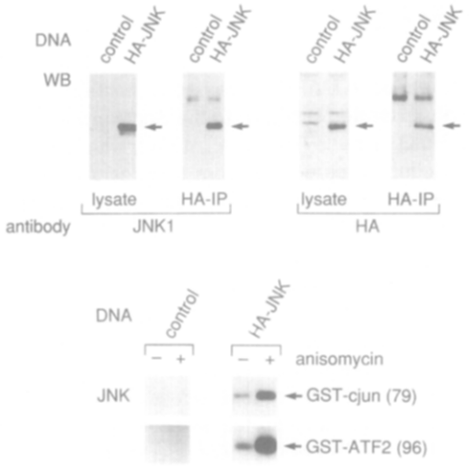

Figure 1. Expression of an Epitope-Tagged JNK1 in COS-7 Cells

Lysates containing 40 μg of protein from COS-7 cells transfected with pcDNA3 vector (control) or with an expression plasmid carrying an epitope-tagged human *JNK1* cDNA (HA-JNK) (1 μg per plate) were subjected to Western blot (WB) analysis with antibodies against HA or JNK1, as indicated. Additional lysates containing 800 μg of cellular protein were first immunoprecipitated with the anti-HA antibody (HA-IP) and then subjected to the same analysis. Bands were visualized by the enhanced chemiluminescence technique using the appropriate horseradish peroxidase–conjugated goat antiserum. Arrows indicate the position of the epitope-tagged JNK1 protein. Parallel immunoprecipitates from serum-starved cells untreated (minus) or exposed to anisomycin (10 μg/ml; 20 min) (plus) were assayed for JNK activity (JNK) as described in Experimental Procedures, using GST–c-Jun(79) or GST–ATF2(96) as substrates. Bands corresponding to the radiolabeled substrate are indicated.

(HA-JNK) was efficiently expressed when transfected into COS-7 cells, as judged by its immunodetection with the anti-HA murine monoclonal antibody 12CA5 (Wilson et al., 1984) or with a JNK1-specific antiserum. Furthermore, only HA-JNK-transfected cells exhibited in vitro phosphorylating activity in an immunocomplex kinase assay, using purified glutathione S-transferase (GST)–c-Jun(79) or GST–ATF2(96) as substrates (Figure 1, bottom). Exposure of serum-starved cells to anisomycin, a potent JNK/SAPK activator (Kyriakis et al., 1994), induced a striking increase in JNK activity (Figure 1, bottom). Thus, this epitope-tagged JNK1 encoded by the HA-JNK cDNA construct functions as a JNK.

Activation of JNK Involves a Signaling Route Different from That of the Ras–MAPK Pathway

Although a number of studies have demonstrated that the activity of c-Jun and JNK is elevated in cells transformed by oncogenic *ras* genes (Angel and Karin, 1991; Dérijard et al., 1994; Westwick et al., 1994), many growth factors known to activate the Ras–MAPK pathway fail to elevate the enzymatic activity of JNK in several cellular systems (Kyriakis et al., 1994; Coso et al., 1995). To examine whether JNK lies in the same biochemical route as MAPK in COS-7 cells, we coexpressed the tagged JNK or MAPK alone or together with activated forms of Ras, Raf, and MEK. Transfection with the wild-type form of MEK and treatment with anisomycin served as controls. As shown in Figure 2, activated forms of Ras, Raf, and MEK potently induced MAPK activity, while anisomycin induced this activity to a lesser extent and the wild-type MEK lacked a demonstrable effect. In contrast, anisomycin potently induced JNK activity, v-*ras* induced a modest increase, and Raf and both MEK constructs failed to stimulate JNK. Thus, in COS-7 cells, v-*ras* can weakly signal JNK activation, however, through a biochemical route different from that of the Raf–MEK–MAPK pathway.

The Small GTP-Binding Proteins Rac1 and Cdc42 Can Potently Activate JNK

The recent observation that two members of the Rho family of small GTP-binding proteins, Rac1 and Cdc42, can bind and activate certain intracellular kinases (Manser et al., 1994), a situation analogous to that of Ras acting on Raf (see McCormick, 1994), prompted us to explore whether this family of GTPases can initiate a pathway leading to JNK activation. We began by expressing wild-type and activated forms of members of the Rho family of GTP-binding proteins in COS-7 cells. This family consists of

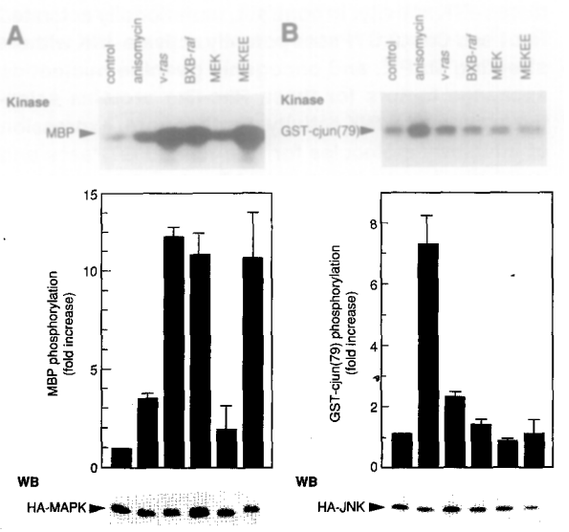

Figure 2. Effect of Activated Ras, Raf, and MEK on MAPK and JNK Activity

COS-7 cells were transfected with pcDNA3-HA-JNK1 (1 μg per plate) or pcDNA3-HA-MAPK (1 μg per plate) for the MAPK (A) or JNK (B) assay, respectively, together with pcDNA3 vector (control) or expression vectors carrying cDNAs for v-*ras*, a constitutively active form of Raf (BXB-*raf*) (previously described by Crespo et al., 1994), MEK, or the mutationally activated MEK (MEKEE), as indicated (2 μg per plate in each case). Treatment of cells with 10 μg/ml anisomycin for 20 min was used as a control. Kinase reactions or Western blot (WB) analysis were performed in anti-HA immunoprecipitates from the corresponding cellular lysates as described in Experimental Procedures. ^{32}P-labeled products are indicated. Slower mobility forms of MAPK are likely to represent phosphorylated species. Autoradiograms correspond to representative experiments for MAPK and JNK assays. Data represent the mean ± SEM of three to five independent experiments, expressed as fold increase with respect to vector-transfected cells (control).

three subfamilies: Rho (RhoA, RhoB, RhoC, and RhoG), Rac (Rac1 and Rac2), and Cdc42 (Cdc42Hs or G25K) (reviewed by Hall, 1994). Thus, we engineered expression vectors for representative members of these subfamilies, including RhoA, Rac1, and Cdc42, and their corresponding activated mutants. The latter were generated by replacing glutamine for leucine in RhoA, Rac1, and Cdc42 (QL mutants) in a position analogous to that of codon 61 of Ras. Such a mutation has been shown to inhibit the GTPase activity of most of these proteins (Bourne et al., 1991). When transfected into COS-7 cells, RhoA, Rac1, and Cdc42 were detectably expressed, as judged by GTPase-specific antibodies (Figure 3C). However, these GTP-binding proteins failed to activate MAPK, although v-*ras* had a very potent effect under the same experimental conditions (Figure 3A). In contrast, Rho-related GTPases effectively activated JNK, although to a variable extent (Figure 3B). Whereas wild-type RhoA and Rac1 did not enhance JNK activity and activated RhoA caused only a very modest increase, expression of wild-type Cdc42 or the activated forms of Rac1 and Cdc42 consistently induced a 5- to 10-fold increase in the in vitro phosphorylating activity of JNK. Thus, expression of activated forms of Rac1 and Cdc42 can potently stimulate JNK, but not MAPK, activity. In the case of wild-type Cdc42, we observed that it can elevate JNK activity only if expressed at very high levels, thus suggesting that a certain proportion of wild-type Cdc42 is in an activated state and, if highly expressed, that active fraction might be sufficient to trigger downstream effectors.

Oncogenic Guanine Nucleotide Exchange Factors for Rho-like Proteins Potently Activate JNK

Recent observations that two oncogenes, *ost* and *dbl* (Hart et al., 1991, 1994; Horii et al., 1994), induce in vitro the exchange of GDP for GTP on RhoA and Cdc42 have suggested that these oncogenes can activate in vivo both small GTP-binding proteins. In view of our results, we next examined whether expression of *dbl* and *ost* can induce JNK activity in COS-7 cells. We constructed expression plasmids for the *ost* (Horii et al., 1994) and the *dbl* oncogene (onc-*dbl*) (Eva et al., 1988) and for the *dbl* proto-oncogene (proto-*dbl*) (Ron et al., 1988). As a control, we also expressed the catalytic domain (Cdc25 domain) of SOS, a guanine nucleotide exchange factor (GEF) for Ras (Chardin et al., 1993), upon targeting to the plasma membrane by fusing its coding sequence to that of the amino-terminal myristoylation signal from c-Src (Takeya and Hanafusa, 1983). As expected, when expressed in COS-7 cells, the myristoylated Cdc25 domain of SOS was localized to the plasma membrane fraction (data not shown) and potently induced MAPK (Figure 4), but it had little effect on JNK. In contrast, transfection of COS-7 cells with expression vectors carrying the cDNA for the *ost*, onc-*dbl*, or proto-*dbl* oncogene did not affect MAPK functioning, but induced a remarkable increase in JNK enzymatic activity, which was nearly as great as that caused by exposing cells to anisomycin (Figure 4). Therefore, we conclude that Rac1 and Cdc42 Rho-like GTP-binding proteins and their exchange factors can effectively induce pathways leading to the activation of JNK.

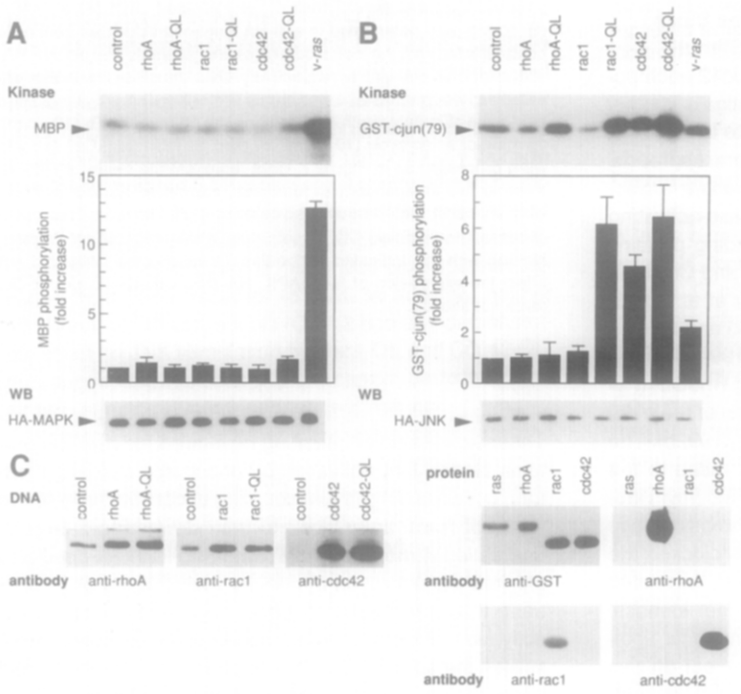

Figure 3. Rac1 and Cdc42 Can Induce JNK Activity but Fail to Activate MAPK

(A and B) COS-7 cells were transfected with pcDNA3 vector (control) or with the same expression vector carrying cDNAs for the wild-type or activated (QL) forms of RhoA, Rac1, or Cdc42 (2 μg per plate in each case). Cells were transfected with the indicated expression plasmids together with pcDNA3-HA-MAPK or pcDNA3-HA-JNK (1 μg per plate) for MAPK (A) or JNK (B) assay, respectively. Cell extracts were processed as in Figure 2. Data represent the mean ± SEM of four independent experiments, expressed as fold increase with respect to vector-transfected cells (control).

(C) Lysates from transfected cells containing 40 μg of total cellular protein (left) or 1 μg of purified bacterially expressed GST–HA-Ras (Ras), GST–RhoA (RhoA), GST–Rac1 (Rac1), and GST–Cdc42 (Cdc42) fusion proteins (right) were subjected to Western blot analysis with the corresponding antibodies.

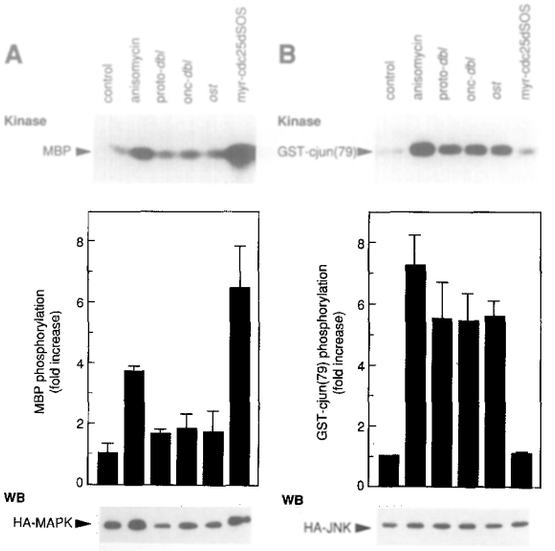

Figure 4. Activation of JNK by Expression of GEFs for the Rho Family of Small GTP-Binding Proteins

COS-7 cells were transfected with the indicated expression plasmids for *ost*, onc-*dbl*, and proto-*dbl* and for the Cdc25 domain of SOS fused to the c-Src amino-terminal myristoylation signal (see Experimental Procedures) (2 μg per plate in each case), together with plasmids carrying HA-MAPK or HA-JNK1 for MAPK (A) or JNK (B) immunocomplex kinase assay, respectively. Cells cotransfected with the pcDNA3 vector (control) untreated or stimulated with anisomycin (10 μg/ml; 20 min) served as controls. Cell extracts were processed as in Figure 2. Data represent the mean ± SEM of three independent experiments, expressed as fold increase with respect to vector-transfected cells (control). Expression of each exchange factor was confirmed by Western blotting with the corresponding antibodies (data not shown).

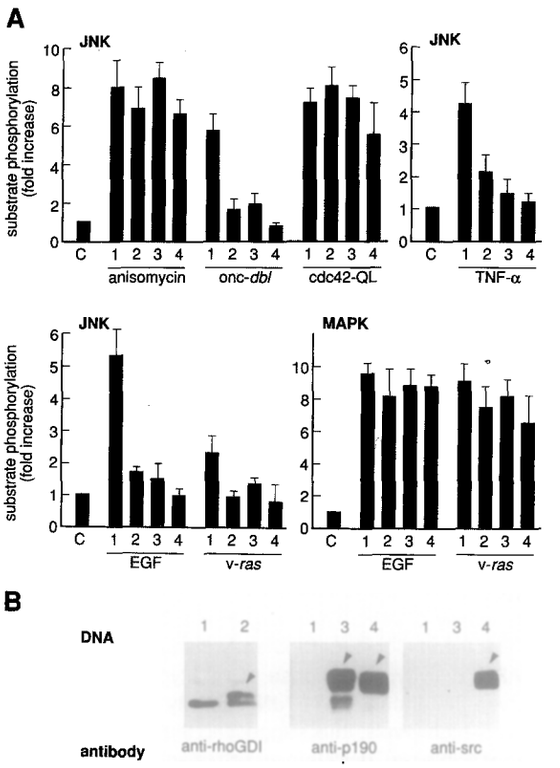

Figure 5. Effect of Coexpressing Inhibitory Molecules for Rho-Related GTPases on JNK Activation

COS-7 cells were transfected with expression plasmids for onc-*dbl*, *cdc42-QL*, or v-*ras* (1 μg per plate in each case) together with pcDNA3 vector without insert (lanes 1) or carrying cDNAs for RhoGDI (1 μg per plate) (lanes 2) or the RhoGAP domain of the p190 RasGAP-associated protein, either alone (RhoGAPd p190) (0.5 μg per plate) (lanes 3) or fused to the c-Src amino-terminal myristoylation signal (myr RhoGAPd p190) (0.5 μg per plate) (lanes 4), as indicated. JNK (A) or MAPK (B) activity was determined in anti-HA immunoprecipitates from cells cotransfected with the indicated expression plasmids and pcDNA-HA-JNK or pcDNA-HA-MAPK, respectively. Cells transfected with the indicated expression plasmids and empty vector were serum starved and then were stimulated with EGF (100 ng/ml; 15 min), TNFα (10 μg/ml; 15 min), or anisomycin (10 μg/ml; 20 min), as indicated. Cell extracts were processed as in Figure 2. Data represent the mean ± SEM of three to five independent experiments, expressed as fold increase with respect to vector-transfected cells (control). The letter C indicates lysates of transfected COS-7 cells that were analyzed by Western blotting with the indicated antibodies. Cotransfected DNAs did not affect the expression of HA-MAPK, HA-JNK, Cdc42-QL, or onc-Dbl (data not shown).

Inhibitory Molecules for Rho-like GTPases Block JNK Activation Induced by a Variety of Stimuli

Because we have shown that Rac1 and Cdc42 each potently induce JNK activity, we set out to investigate whether these small GTP-binding proteins participate in signaling from cell surface receptors to JNK. As a general approach, we took advantage of the observation that guanine nucleotide dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs) for this family of GTP-binding proteins can inhibit the functioning of RhoA, Rac1, and Cdc42 in vitro and in vivo (Ueda et al., 1990; Ridley et al., 1993; Nishiyama et al., 1994; reviewed by Boguski and McCormick, 1993; Hall, 1994; Lamarche and Hall, 1994). Initially, we engineered expression plasmids carrying RhoGDI (Fukumoto et al., 1990) and the catalytic domain of the p190 RhoGAP (RhoGAPd p190) (Settleman et al., 1992b). The latter was expressed either in isolation or fused to the amino-terminal myristoylation signal from c-Src (myr RhoGAPd p190). As shown in Figure 5, coexpression of these inhibitory molecules for all Rho-like proteins blocked the activation of JNK induced by onc-*dbl*; however, they failed to inhibit JNK stimulation by Cdc42-QL. Under identical conditions, RhoGDI and both RhoGAP constructs abolished JNK stimulation by *ost* but had no effect on this response when elicited by Rac1-QL (data not shown).

Thus, expression of inhibiting molecules for Rho-like proteins blocks JNK activation by oncogenic exchange factors, but does not prevent coupling of constitutively active forms of Rac1 and Cdc42 to their downstream targets. Interestingly, molecules inhibiting Rho-like GTPases also blocked the activation of JNK by v-*ras* (Figure 5). In contrast, they had nearly no effect on v-*ras*-induced MAPK activation (Figure 5). This observation further demonstrates the specificity of this experimental approach and raises the possibility of the existence of a pathway commu-

nicating Ras with the Rho family of small GTP-binding proteins.

We next examined the effect of RhoGDI and RhoGAP proteins on JNK activation in response to agonists acting through receptors endogenously expressed in COS-7 cells. We initially found that TNFα (10 μg/ml) and epidermal growth factor (EGF) (100 ng/ml) were both able to activate JNK (Figure 5). As shown in Figure 5, coexpression of inhibiting molecules for Rho-related proteins potently blocked stimulation of JNK by both agonists, but they had little effect on the JNK activity induced by anisomycin when used as a control. In contrast, RhoGDI, RhoGAPd p190, and myr RhoGAPd p190 did not significantly affect the MAPK response to EGF (Figure 5). Thus, inhibitory proteins for Rho-related GTPases selectively prevent coupling of TNFα and EGF receptors to the JNK pathway.

Dominant Negative Mutants of Rac1 and Cdc42 Inhibit Signaling from Cell Surface Receptors to JNK

To determine which Rho-related GTPase participates in signaling to JNK, we have made use of dominant negative mutants for RhoA, Rac1, and Cdc42. In each case, we replaced threonine for asparagine in a position analogous to codon 17 of Ras, thus generating N19RhoA, N17Rac1, and N17Cdc42, respectively. By analogy to the activity of N17Ras, these inhibitory mutants are thought to act by competitively inhibiting the interaction of endogenous GTP-binding proteins with their respective GEFs (Feig and Cooper, 1988). When transfected into COS-7 cells, each mutated GTPase was found to be detectably expressed (data not shown). We next examined the ability of N19RhoA, N17Rac1, and N17Cdc42 to affect JNK activation by stress-inducing agents or by agonists acting on cell surface receptors. As shown in Figure 6, none of these inhibitory mutants affected the JNK response to anisomycin. In contrast, expression of N17Rac1 and N17Cdc42 potently blocked the JNK activation elicited by EGF (Figure 6), but did not affect the MAPK response to this growth factor (data not shown). N17Rac1 and N17Cdc42 also diminished JNK stimulation produced by TNFα (Figure 6). However these mutated GTPases were less effective as blockers than the inhibitory proteins utilized in the experiments shown in Figure 5, thus raising the possibility that additional GTP-binding proteins might also participate in signaling from TNFα receptor to JNK. In addition, neither N19RhoA, N17Rac1, nor N17Cdc42 had any demonstrable effect on JNK stimulation by Rac1-QL and Cdc42-QL, thus suggesting that each GTPase can couple to the downstream effector(s) in an independent manner.

We also examined whether Ras plays a role in signaling JNK activation by extracellular stimuli, using a dominant negative mutant of Ras (N17Ras) (Feig and Cooper, 1988). We found that the JNK response to TNFα, anisomycin, Rac1-QL, and Cdc42-QL was not affected by N17Ras, but that this molecule abolishes signaling from EGF receptors to both JNK and MAPK (Figure 6; Crespo et al., 1994). Thus, taken together these findings demonstrate that tyrosine kinase receptors require functioning of Ras and Rac1, Cdc42, or both to activate JNK.

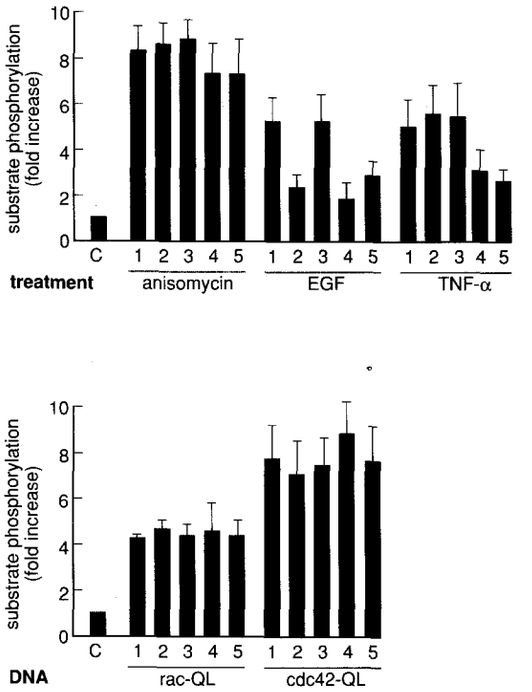

Figure 6. Effect of Dominant Negative Mutants of Ras, RhoA, Rac1, and Cdc42 on JNK Activation

COS-7 cells were transfected with empty vector (top) or expression plasmids for Rac1-QL and Cdc42-QL (1 μg per plate in each case, where indicated) together with pcDNA3 vector without insert (bars 1) or carrying cDNAs for N17Ras (0.5 μg per plate) (bars 2), N19RhoA (1 μg per plate) (bars 3), N17Rac1 (1 μg per plate) (bars 4), or N17Cdc42 (1 μg per plate) (bars 5) together with pcDNA3-HA-JNK. Serum-starved cells were stimulated with EGF (100 ng/ml; 15 min), TNFα (10 μg/ml; 15 min), or anisomycin (10 μg/ml; 20 min), as indicated, and processed for immunocomplex JNK assays as described in Figure 2. Data represent the mean ± SEM of three to five independent experiments, expressed as fold increase with respect to vector-transfected cells (control).

Discussion

A large number of extracellular stimuli can potently activate JNK, including inflammatory cytokines, certain growth factors, G protein–coupled receptors, protein synthesis inhibitors, changes in osmolarity, ultraviolet light, et cetera (Dérijard et al., 1994; Kyriakis et al., 1994; Coso et al., 1995). Many of these agents also weakly activate MAPKs. Conversely, potent MAPK stimulants such as phorbol esters and growth-promoting agents acting on tyrosine kinase receptors fail to activate JNK in a number of cell types (Kyriakis et al., 1994; Coso et al., 1995). Thus, the emerging picture is that JNK is regulated independently of MAPKs. In this regard, expression of constitutively active oncogenic forms of *ras* has been shown to induce MAPK potently but to raise JNK phosphorylating activity to a much more limited extent (Dérijard et al., 1994; Sun et al., 1994). In line with these observations, transient expression of v-*ras* in COS-7 cells potently activated an epitope-

tagged MAPK (Crespo et al., 1994; this study), but had little effect on an epitope-tagged JNK. Furthermore, constitutively activated forms of molecules connecting Ras to MAPK dramatically activated MAPK, but did not have any demonstrable effect on JNK. Under the same experimental conditions, exposure of cells to a protein synthesis blocker, anisomycin, elicited a marked increase of JNK activity, but weakly stimulated the epitope-tagged MAPK. Thus, our results in COS-7 cells are consistent with those recently reported in NIH 3T3 (Yan et al., 1994), HeLa, and PC12 cells (Minden et al., 1994) and provide compelling evidence that the pathway communicating Ras to JNK is different from that controlling MAPKs.

These observations raise the possibility that proteins other than Ras may directly regulate biochemical pathways leading to the activation of JNK. In this regard, the Ras superfamily of GTPases comprises more than 50 members, which have been divided into six families based upon sequence similarity: Rab, Arf, Sar, Ran, Rho, and Ras (see Lamarche and Hall, 1994). Whereas the Rab, Arf, and Sar groups have been shown to participate in the transport of proteins and vesicles among different intracellular compartments (Boguski and McCormick, 1993; Hall, 1994), the Ras family is involved in growth control (see below). The Rho family of GTP-binding proteins consists of the Rho, Rac, and Cdc42 subfamilies and has been shown to regulate several aspects of cytoskeleton functioning (reviewed by Hall, 1994). The Rho subfamily has at least four members, RhoA, RhoB, RhoC, and RhoG. RhoA has been shown to participate in the formation of actin stress fibers, as well as in mediating redistribution of cytoskeletal components (Ridley and Hall, 1992; Hall, 1994). The Rac subfamily includes Rac1 and Rac2, and Rac1 has been shown to be involved in membrane ruffling (Ridley et al., 1992). The Cdc42 group consists of Cdc42Hs (referred to here as Cdc42) and G25K (see Hall, 1994), and its function in mammalian cells is still largely unknown.

Ras has been shown to play a central role in cell proliferation and differentiation. At the molecular level, Ras exchanges GDP for GTP upon activation of Ras-specific GEFs, and in the GTP-bound state, Ras physically associates with the amino-terminal domain of Raf (Vojtek et al., 1993). This process recruits Raf to the plasma membrane, allows the activation of Raf by a still unknown mechanism (see McCormick, 1994), and initiates a sequential cascade of kinases leading to MAPK activation. Interestingly, it has been recently reported that GTP-bound forms of the Rho-related proteins Rac1 and Cdc42 can specifically associate and activate a novel serine/threonine kinase, the p21-activated kinase (PAK) (Manser et al., 1994). This situation is highly analogous to that of the Ras–Raf interaction, thus suggesting that the Rho family of GTP-binding proteins might also initiate activity of a kinase cascade (Manser et al., 1994). This observation and the failure of Ras fully to activate JNK prompted us to ask whether the Rho family of GTPases participates in signaling to the JNK pathway.

Our findings demonstrate that expression of activated forms of Rac1 and Cdc42 can efficiently stimulate JNK without affecting MAPK activity. Interestingly, although activated RhoA is known to induce the most dramatic changes in the cytoskeletal structure (Ridley and Hall, 1992; data not shown), it stimulates JNK very poorly. Furthermore, we have found that treatment of cells with cytoskeletal-disrupting agents such as cytochalasin D fails to block JNK activation induced by Rac1 and Cdc42 (our unpublished data). We conclude that Rac1 and Cdc42 can effectively stimulate signaling pathways leading to JNK activation, probably through a mechanism distinct from that involved in cytoskeletal reorganization.

The functioning of Rho-like GTP-binding proteins is tightly regulated in vivo by proteins that control their GDP/GTP state. GAPs (reviewed by Lamarche and Hall, 1994) and GDIs (Ueda et al., 1990) are negative modulators, whereas GEFs (Boguski and McCormick, 1993) promote the exchange of GDP for GTP, thus activating Rho-like proteins. Based upon our results, we asked whether GEFs for Rho-related GTPases were also capable of inducing JNK activity. Two GEFs for this family have been described: the protein products of the *ost* and onc-*dbl* oncogenes (Hart et al., 1991; Horii et al., 1994). Our data demonstrate that both exchange factors for Rho-like proteins can potently induce JNK activity. In addition, it was noticeable that both oncogenic exchange factors poorly induced MAPK in COS-7 cells, similar to that previously reported for *dbl* and *vav* in NIH 3T3 cells (Khosravi-Far et al., 1994). Conversely, we have observed that a plasma membrane–targeted catalytic (Cdc25-like) domain of SOS greatly induces MAPK, but has a minimal effect on JNK. Taken together, these data provide additional evidence that molecules activating the Ras pathway preferentially stimulate MAPKs and establish that proteins acting on Rho-related GTPases can efficiently signal to JNK.

A number of RhoGAPs have been identified and have been shown to display variable specificity with respect to their activity on each member of the Rho family (Lamarche and Hall, 1994). For example, RhoGAPd p190, a RasGAP-associated protein (Settleman et al., 1992a), can stimulate in vitro the GTPase activity of RhoA, Rac1, and Cdc42 to a comparable extent (Lamarche and Hall, 1994). RhoGDI forms a cytosolic complex preferentially with GDP-bound forms of RhoA, Rac1, and Cdc42 (Ueda et al., 1990; Sasaki et al., 1993; Hall, 1994), thus preventing their activation. Both GAPs and GDIs have proven to be useful tools for blocking the function of Rho-related GTPases in a variety of biological systems (Ridley et al., 1993; Nishiyama et al., 1994; Hall, 1994); thus, we utilized their expression as a simple approach to investigate a role for Rho-related proteins in the JNK signal transduction pathway. Here, we show that expression of RhoGDI and RhoGAPd p190 as well as a membrane-targeted RhoGAPd p190 (myr RhoGAPd p190) effectively block JNK activation by *ost* and *dbl*. In contrast, these inhibitory molecules for Rho-related GTPases failed to block JNK stimulation by the mutationally activated Cdc42. This observation is consistent with the finding that RhoGDI poorly associates with GTP-bound proteins of the Rho family (Sasaki et al., 1993) and with the lack of effect of GAPs on mutationally activated proteins of the Ras superfamily (McCormick et al., 1991).

It also demonstrates that neither RhoGDI nor RhoGAPd p190 exerts a direct inhibitory effect on JNK. In addition, Rho-blocking proteins prevented JNK activation by v-*ras*, EGF, and TNFα, but did not affect signaling from EGF receptors or v-*ras* to MAPK. These findings further support the specificity of this approach and provide strong evidence that Ras, as well as EGF and TNFα receptors, signals to JNK through Rho-related GTPases.

To establish the identity of the endogenously expressed GTP-binding protein(s) that participates in linking cell surface receptors to JNK, we made use of dominant negative mutants for each class of GTPases, Ras, RhoA, Rac1, and Cdc42. We found that the dominant negative mutant of Ras effectively prevents MAPK (Crespo et al., 1994) and JNK activation by EGF, but not JNK stimulation induced by either TNFα or anisomycin. Thus, functioning of Ras appears to be strictly required for linking EGF receptors to JNK, whereas other stimuli utilize Ras-independent pathways. In this regard, we have found that the dominant negative mutants of Rac1 and Cdc42 effectively reduce JNK stimulation elicited by both EGF and TNFα, suggesting that the pathway connecting TNFα and EGF receptors to JNK involves Rac1 and Cdc42 or, alternatively, a common GEF acting on either GTPase. Thus, taking the inhibitory action of N17Rac1 and N17Cdc42, the blockade of JNK activation upon expression of negative regulators of Rho-related proteins, and the pattern of JNK stimulation by mutationally activated GTPases, we conclude that the small GTP-binding proteins Rac1 and Cdc42 are an integral part of the biochemical route linking cell surface receptors to JNK.

The JNK response to anisomycin was not affected by inhibitors of Rho-related proteins, nor by N17Rac1 or N17Cdc42, thus suggesting that cell-stressing stimuli might induce JNK activity independently of these GTP-binding proteins. On the other hand, expression of the inhibitory mutants of RhoA, Rac1, or Cdc42 did not affect JNK stimulation by constitutively activated Rac1 and Cdc42, which is not consistent with the existence of a hierarchically arranged cascade of GTPases, as recently reported for cytoskeletal regulation (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). Instead, these results strongly suggest that each GTPase, Rac1 and Cdc42, can activate effector pathways independently. Whether PAK or a PAK-related kinase, which can bind either Rac1 or Cdc42, is immediately downstream of these GTPases in the biochemical route to JNK warrants further investigation.

The emerging picture from our study and from recent reports (Minden et al., 1994; Sanchez et al., 1994; Yan et al., 1994; Dérijard et al., 1995) is that mammalian cells have independent signaling pathways connecting cell surface receptors with each member of the MAPK superfamily. This situation is remarkably similar to that of the budding yeast S. cerevisiae, in which genetic studies have revealed the existence of parallel cascades of kinases leading to the activation of at least three distinct MAPK-related enzymes (reviewed by Herskowitz, 1995). In the case of the mating pheromone response, extracellular ligands (α or **a** factors) activate cell surface receptors that in turn induce the dissociation of a heterotrimeric G protein into α (GPA1) and βγ (Ste4 and Ste18) subunits (reviewed by Bardwell et al., 1994; Herskowitz, 1995; see Figure 7). Free βγ dimers then activate a serine/threonine kinase, Ste20 initiating activity from a linear cascade of kinases, including, sequentially, Ste11 and Ste7, which phosphorylate and activate the yeast MAPK homologs Fus3 and Kss1 (Figure 7). As discussed above, JNK and MAPK are highly related to each other and to the budding yeast MAPKs. Four mammalian homologs of Ste7 have been identified, two of them (MEK1 and MEK2) specifically activate MAPK (Crews et al., 1992), and one (designated SEK [Sanchez et al., 1994] or MKK4 [Dérijard et al., 1995]) directly phosphorylates and activates JNK. The known mammalian

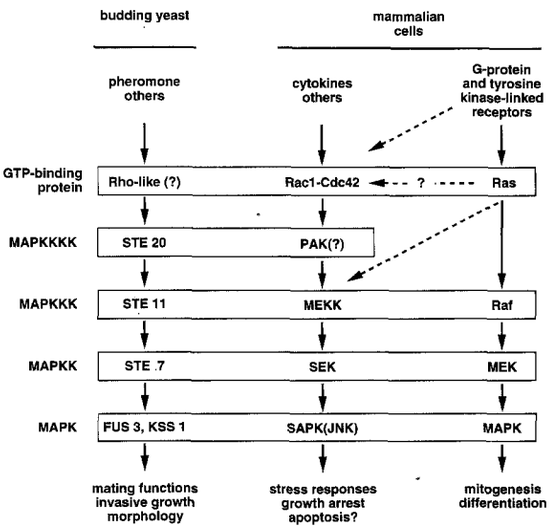

Figure 7. Schematic Representation of Protein Kinase Cascades Leading to the Activation of Distinct Members of the MAPK Superfamily

Accumulating evidence suggests that parallel kinase cascades control the activity of MAPK and JNK. Following the mating pheromone response pathway in S. cerevisiae as an example of such a multicomponent signaling route, each level of the cascade can be functionally defined as MAPK, MAPKK, MAPKKK, and MAPKKKK, represented in this case by Fus3/Kss1, Ste7, Ste11, and Ste20, respectively, as depicted (see Herskowitz, 1995). In the mammalian MAPK pathway, only three levels have been identified, represented by MAPK, MEK, and Raf (see Marshall, 1995). In the mammalian JNK signaling route, JNK, SEK, and MEKK represent the first three hierarchical levels (see text for details), and we postulate that PAK (Manser et al., 1994) or related enzymes represent the most upstream kinase in this cascade. Whether that is the case remains to be established. Activity of these kinase cascades is tightly regulated. Ras controls mammalian MAPK by acting directly on Raf. Our study has shown that the small GTP-binding proteins Rac1 and Cdc42 regulate the JNK pathway. Rho-related GTPases have been previously suggested to regulate the activity of budding yeast Ste20 or of its newly identified homologous proteins (see Bardwell et al., 1994), although biochemical evidence is still missing. Broken lines represent potential cross-talks among signaling systems. The pathway connecting cell surface receptors to low molecular weight GTP-binding proteins and the identity of biologically relevant targets for these kinase cascades are yet to be fully elucidated.

functional homologs of Ste11 are Raf and MEK kinase (MEKK) (Lange-Carter et al., 1993). Raf is located upstream of MEK, thus defining a linear kinase cascade represented by the Raf–MEK–MAPK signaling module, whose activity is dependent on Ras (see above). MEKK is closely related to Ste11 by primary sequence similarity, and two different groups have recently observed that MEKK functions preferentially in the JNK pathway, specifically controlling SEK activity (Yan et al., 1994; Minden et al., 1994). The identity of molecules that directly regulate MEKK is still unknown. In this regard, a mammalian homolog of Ste20 has been recently isolated, $p65^{PAK}$ (Manser et al., 1994), and it has been shown that Ste20 and PAKs are activated upon interaction with GTP-bound forms of Rac1 and Cdc42. Taken together, these observations and our present results demonstrating that Rac1 and Cdc42 can regulate the activity of JNK enable us to postulate that either PAK or related kinases link Rac1 and Cdc42 to the MEKK–SEK–JNK module (Figure 7). Such a hypothesis linking small GTP-binding proteins of the Rho family to the JNK signaling pathway is testable and will be the target for future investigation.

Experimental Procedures

Expression Plasmids

JNK1 cDNA (Dérijard et al., 1994) was obtained by the PCR technique using as a template human skeletal muscle cDNA (Invitrogen) and was cloned into the BglII and SalI sites of pcDNA3-HA, a modified pcDNA3 expression plasmid (Invitrogen) encoding the HA nonapeptide epitope (Wilson et al., 1984; J. S. G., unpublished data).

The previously described cDNAs for human RhoA, Rac1, and Cdc42Hs (referred to in this paper as Cdc42) GTP-binding proteins (Horii et al., 1994) were cloned in pcDNA3 as BamHI–EcoRI fragments. Their respective constitutively activated mutants were obtained by replacing a glutamine residue corresponding to codon 63 of RhoA or codon 61 of Rac1 and Cdc42 for a leucine residue (QL mutants), using PCR-directed mutagenesis. Dominant negative mutants for each small GTP-binding protein were obtained by replacing threonine for asparagine in a position analogous to codon 17 of Ras, generating N19RhoA, N17Rac1, and N17Cdc42.

Wild-type *MEK1* (Crews et al., 1992) (provided by R. Erickson) was cloned into the pcDNA3 vector as a BamHI–NotI fragment. Activated MEK1 was generated by replacing Ser-218 and Ser-222 by glutamic acid; this construct was designated MEKEE (Cowley et al., 1994). A 1.4 kb DNA fragment encoding the Cdc25 domain of the human SOS protein (codons 568–1042) was amplified by PCR using human SOS cDNA (Chardin et al., 1993) as a template and was subcloned into pcDNA3-myr, a modified pcDNA3 expression plasmid encoding the amino-terminal 21 amino acids of chicken c-Src (Takeya and Hanafusa, 1983), which includes the c-Src amino-terminal myristoylation signal (H. T. and J. S. G., unpublished data). Onc-*dbl* and proto-*dbl* cDNAs (Eva et al., 1988; Ron et al., 1988) were subcloned as BamHI fragments in the pcDNA3 expression vector. A 1.1 kb DNA fragment encoding the RhoGAP domain of human p190 protein associated to RasGAP (Settleman et al., 1992b) (codons 1177–1512) was amplified by PCR using rat brain cDNA and appropriate oligonucleotides and was subcloned into pcDNA3-KZ, a modified pcDNA3 vector containing an efficient translational initiation sequence as described by Kozak (1989) upstream of an artificial initiation codon in-frame with the BamHI site (O. A. C. and J. S. G., unpublished data) or pcDNA3-myr (see above). RhoGDI (Fukumoto et al., 1990) (provided by Y. Takai) was subcloned as a BamHI–EcoRI fragment into the pcDNA3-KZ vector. In all cases, nucleotide sequences of wild-type and mutated cDNAs were verified by DNA sequencing. The sequence of oligonucleotides utilized for each DNA construct will be made available upon request.

Kinase Assays

JNK Assay

Subconfluent COS-7 cells were transfected with pcDNA3-HA-JNK and additional DNAs by the DEAE–dextran technique. Total amount of plasmid DNA was adjusted to 5–10 μg per plate with vector DNA (pcDNA3; Invitrogen) when necessary, and 2 days later, transfected COS-7 cells were cultured overnight in serum-free medium. Cells were then left untreated or stimulated with various agents, washed with cold PBS, and lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. The epitope-tagged JNK was immunoprecipitated from the cleared lysates by incubation with the specific antibody 12CA5 (BABCO) for 1 hr at 4°C. Immunocomplexes were recovered with the aid of Gamma-Bind Sepharose beads (Pharmacia) and washed three times with PBS containing 1% NP-40 and 2 mM sodium vanadate, once with 100 mM Tris (pH 7.5), 0.5 M LiCl, and once with kinase reaction buffer (12.5 mM MOPS [pH 7.5], 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate). The JNK activity present in the immunoprecipitates was determined by resuspension in 30 μl of kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP per reaction and 20 μM unlabeled ATP, using 1 μg of GST–c-Jun(79) or GST–ATF2(96) fusion protein as a substrate, as previously described (Coso et al., 1995). After 20 min at 30°C, reactions were terminated by addition of 10 μl of 5× Laemmli's buffer. Samples were heated at 95°C for 5 min and analyzed by SDS electrophoresis on 12% polyacrylamide gels. Autoradiography was performed with the aid of an intensifying screen. Parallel anti-HA immunoprecipitates were processed for Western blot analysis using a JNK-specific antiserum.

MAPK Assay

MAPK activity in COS-7 cells transfected with an epitope-tagged MAPK (HA-ERK2, referred to here as HA-MAPK) was determined as previously described (Crespo et al., 1994), using myelin basic protein as a substrate. Parallel samples were immunoprecipitated with anti-HA antibody and processed for Western blot analysis using a MAPK-specific antiserum.

Western Blots

Lysates containing approximately 40 μg of total cellular protein or anti-HA immunoprecipitates were analyzed by Western blotting after SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with the corresponding rabbit antiserum or mouse monoclonal antibody. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham) using goat anti-rabbit or anti-mouse antiserum coupled to horseradish peroxidase as a secondary antibody (Cappel). Mouse monoclonal antibodies anti-HA epitope clone 12CA5 and anti-p190 were purchased from BABCO and from Transduction Laboratories, respectively. Rabbit polyclonal antisera anti-JNK1, anti-RhoA, anti-Rac1, anti-Cdc42, anti-RhoGDI, and anti-c-Src were purchased from Santa Cruz Laboratories. In all cases, specificity was confirmed using a battery of bacterially produced recombinant proteins (Figure 3; data not shown).

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