Involvement of the Small GTPase Rho in Integrin-mediated Activation of Mitogen-activated Protein Kinase*

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Engagement and clustering of integrins triggers a number of intracellular signaling events, including activation of the mitogen-activated protein (MAP) kinases Erk1 and Erk2. To investigate the mechanism by which integrins mediate the activation of MAP kinases upon binding of NIH 3T3 cells to fibronectin, we assessed the effects of both inhibiting and activating the small GTPase Rho. We observed that inhibition of Rho by the Rho-specific inhibitor C3 exoenzyme or by a dominant negative Rho A (RhoN19) inhibited MAP kinase activation. Conversely, activation of Rho by expression of an activated Rho A mutant (RhoQ63L), or the Rho-specific guanine nucleotide exchange factor Lbc, enhanced and partially mimicked activation of Erk2 by plating on fibronectin. These results therefore show that Rho is involved in the integrin-dependent activation of MAP kinase.

Cell surface receptors allow eukaryotic cells to receive and respond to extracellular stimuli by activating signal transduction pathways leading to changes in gene expression and cell cycle progression. Integrins are a family of transmembrane receptors that bind to proteins of the extracellular matrix, such as fibronectin, collagen, and vitronectin, and mediate a variety of signaling events (1–3). Integrins are heterodimeric proteins composed of noncovalently associated α and β subunits (1). Binding and clustering of integrins leads to the formation of focal adhesions, in which integrins connect to actin stress fibers. This scaffolding structure also contains a number of signaling molecules involved in signal transduction (4).

Recently integrin ligation has been found to induce the activation of the MAP kinases p42 and p44 (4–7) and their translocation to the nucleus (5). MAP kinases, also known as Erks (for extracellular-regulated kinases), become activated when cells adhere to substrata coated with integrin ligands, such as fibronectin, laminin type IV collagen, or a synthetic peptide containing the RGD sequence (Arg-Gly-Asp), found in many of these proteins. MAP kinases are considered to be key molecules for the transmission of extracellular signals to the nucleus. Substrates for MAP kinases include many transcription factors, such as TCF, Jun, Fos, Myc, NF-IL6, TAL1, and ATF2 (8, 9). MAP kinases are also involved in activation of cytoplasmic pathways, for example, phospholipase A2 (10). Cell adhesion has been found to be required for mid-late G1 cell cycle progression (11, 12), suggesting a possible role for integrin-mediated MAP kinase activation in G1 cell cycle progression.

Recently, Rho has also been shown to play an important role in G1 to S phase of cell cycle progression (13). Rho is member of the Ras superfamily subgroup consisting of Rho, Rac, and CDC42 (14–16). Rho regulates the formation of focal adhesions and actin stress fibers. There is also strong evidence indicating that Rho plays a role in integrin-mediated signaling events. First, activated Rho is known to stimulate stress fiber formation (17), and plating of cells onto fibronectin-coated dishes in serum-free medium results in the rapid formation of stress fibers. Second, both Rho and adhesion to fibronectin activate phosphatidylinositol-4-phosphate 5-kinase (18). Third, specific inhibition of Rho by using botulinum C3 exoenzyme blocks cellular responses, similar to the loss of integrin-mediated cell adhesion (18, 19). Last, injection of activated Rho restores the ability of suspended cells to respond to growth factors similar to adhesion (18).

In this study we find that Rho is required for the activation of MAP kinase in cells plated on fibronectin. We demonstrate that this activation can be blocked by inhibiting Rho using either a dominant negative Rho mutant or C3 exoenzyme. Furthermore, we find that activation of Rho either by expression of a constitutively active mutant (RhoQ63L) or by the guanine nucleotide exchange protein Lbc (20, 21) enhances the activation of MAP kinase observed upon binding to fibronectin and partially mimics this binding in suspended cells. These results demonstrate that Rho is a component of the pathway leading to the activation of MAP kinases by integrins.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfection Procedures—NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum. CMV5 RhoN19 and CMV5 RhoQ63L clones were a gift obtained from Dr. Gary Bokoch (18). CMV5 Erk2 and CMV5 p70 clones have been described previously (22). For transfections, cells were plated at a density of 4 10^6 cells/6-cm dish 24 h before transfection. Cells were transfected with LipofectAMINE (Life Technologies, Inc.) as described previously (22), using 0.2 μg of pCMV5 Erk2, 0.2 μg of pCMV5 p70, and 1.6 μg of each of the empty control plasmid (pCMV5), pCMV5 RhoN19 or pCMV5 RhoQ63L, per plate. 24 h after transfection, cells were transfected to medium containing 0.5% serum for an additional 24 h. Cells from indicated plates were then trypsinized and suspended in serum-free DMEM containing 0.1% BSA (Calbiochem, nuclease- and protease-free), and 0.25 μg/ml soybean trypsin inhibitor (Sigma). They were incubated in suspension for 2 h in dishes which had been coated with 1% heat-denatured BSA (Sigma fraction V). Cells were then transferred to dishes that had been coated with 25 μg/ml fibronectin and blocked with 1% BSA. As a positive control, cells were stimulated with 100 ng/ml TPA for 10 min prior to harvest. For C3 treatment, C3 was introduced into cells using the LipofectAMINE procedure. Cells were transfected with 11.25 μg of C3 per plate, and after 5 h, the medium was replaced with medium containing 0.1% serum for an additional 3 h. Cells were then trypsinized and suspended in serum-free DMEM and

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‡ The abbreviations used are: MAP, mitogen-activated protein; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; IP, immunoprecipitation; TPA, 12-O-tetradecanoylphorbol-13-acetate.
RESULTS

To investigate the role of the small GTPase Rho in the integrin-mediated activation of MAP kinase, we developed an assay using transient expression of hemagglutinin epitope-tagged Erk2 (Fig. 1). Using this assay, we observed activation of MAP kinase upon cell adhesion to fibronectin, similar to the level and time courses previously reported for endogenous Erks (4–7). In these experiments, we found that adherent cells had a low basal level of Erk2 activity (Fig. 1A, lane 1), which after trypsinization and placement of the cells in suspension was reduced to nearly undetectable levels (lane 3). Upon replating onto fibronectin, MAP kinase was substantially activated, consistent with previous work analyzing endogenous Erks (4–7). The peak of activity varied somewhat between experiments, occurring between 10 and 30 min after plating, then declined at later times. As a positive control, cells were treated with 100 ng/ml TPA for 10 min (lane 2). Integrin-stimulated Erk activation was consistently found to be 30–50% of that obtained with TPA.

When dominant negative Rho (RhoN19) (Fig. 1A) was cotransfected with the Erk2, integrin-stimulated activation of the Erk2 kinase was consistently inhibited by 58–65% (Fig. 2). Examination at shorter or longer times showed similar inhibition (data not shown). A 45% reduction in the low basal level was also consistently observed in stably adherent cells. While complete inhibition was never observed, the level of Rho N19 expressed relative to the endogenous Rho protein may not have been sufficient to completely block Rho activity. By contrast, expression of RhoN19 had no effect on the activation of MAP kinase by TPA.

We also analyzed the effects of a constitutively activated Rho (RhoQ63L) (Figs. 1B and 2). Cotransfection with RhoQ63L enhanced Erk2 activation upon plating onto fibronectin by 50 ± 12%. Furthermore, the decline in Erk activity observed when cells were detached and held in suspension was substantially inhibited. When RhoQ63L was cotransfected, RIK activity decreased by only 36% (14.0 ± 5.0 in adherent cells, 9.1 ±

Fig. 1. Integrin-mediated activation of MAP kinase is blocked by dominant negative Rho. NIH 3T3 cells were transiently cotransfected with HA-Erk2 and either a control plasmid or dominant negative RhoN19 (A) or dominant positive RhoQ63L (B). Transfected HA-Erk2 activity was measured by the in-gel kinase assay method, while the amount of immunoprecipitated HA-Erk2 protein was measured using Western blots. HA-Erk2 was measured from lysates of unstimulated adherent cells in 0.5% serum (Min), after stimulation with TPA for 10 min (TPA 10), after suspending the cells for 2 h (Susp. BSA), or after suspending the cells and then replating onto fibronectin-coated dishes for 20 and 30 min (Fibro 20' and Fibro 30', respectively).

Fig. 2. Quantitative effects of dominant activating or inhibiting mutants of Rho on Erk2 activation by integrins. Transiently expressed HA-Erk2 activity was measured in cells cotransfected with either RhoN19, RhoQ63L, or the empty control plasmid. A value of 100% was set for the level of Erk2 activity in TPA-stimulated control cells to give a reference point from which to compare the relative activities of the remaining samples. Values depicted in the bar graph represent the mean values from three independent experiments.

Fig. 3. Activation of endogenous Rho enhances and partially mimics adhesion to fibronectin. Endogenous Erk2 activities were measured in normal NIH 3T3 cells (Control) and a polyclonal population of cells stably expressing the Rho-specific guanine nucleotide exchange protein Lbc (A) and in cells treated with LipofectAMINE alone or transfected with C3 exoenzyme (B). Cells were placed in suspension for 2 h and then transferred to fibronectin-coated dishes for 10, 20, 30, 45, and 60 min. Erk2 activity and protein levels were measured as described previously.

For all immunoprecipitations, one-fifth of the samples were saved and run on a 10% SDS-polyacrylamide gels, transferred to Hybond C (Amersham Corp.), and immunoblotted using the anti-Erk2 antibody, to measure the amount of Erk2 immunoprecipitated. Erk2 activity was measured using the in-gel kinase assay method as described previously (23). Samples were run on 12.5% SDS-polyacrylamide gels containing 0.5 mg/ml myelin basic protein (23). Kinase reactions were performed soaking gels in kinase buffer (26) containing 25 μCi/ml [γ-32P]ATP and 10 μM cold ATP. Gels were washed exhaustively and analyzed by autoradiography and scanning densitometry using a model I.S. 1000 digital imaging system from Alpha-Innotech Corp.
with a constitutively activated Rho mutant or by expression of the nucleotide exchange factor lbc specifically enhanced the activation of MAP kinase by fibronectin. Constitutive activation of Rho also partially prevented the decline in basal MAP kinase activity that occurred after cell detachment, indicating that maintaining Rho function in suspended cells can partially substitute for cell adhesion. Essentially identical results were obtained with transiently transfected proteins and endogenous proteins. These data therefore demonstrate that Rho is involved in the activation of MAP kinase by fibronectin.

It was recently reported that plating serum-starved Swiss 3T3 cells onto fibronectin did not induce MAP kinase activation (29). In that system, stress fibers and focal adhesions do not form due to inactivation of the Rho pathway by serum starvation. Although serum starvation is likely to alter many cellular pathways, these results are consistent with our findings that Rho activation must occur in order to observe MAP kinase activation by integrins.

There are now several examples of signaling pathways in which inhibition of Rho in adherent cells results in behavior similar to suspended cells, while activation of Rho in suspended cells gives rise to behavior that mimics adherent cells (18, 19). Our data join this body of evidence demonstrating that Rho can mediate integrin signaling events and that constitutive activation of Rho prevents inactivation of integrin pathways upon cell detachment. Taken together, these data tend to support a model in which integrins activate Rho, which then mediates downstream integrin-dependent events. This model does not exclude modulation of Rho function by soluble factors such as lysophosphatidic acid or platelet-derived growth factor; indeed, there is ample evidence that Rho is also an important mediator of growth factor-dependent pathways (30, 31).

How Rho contributes to MAP kinase activation is unknown. However, Ras has been indicated via a connection to focal adhesion kinase through a Grb2/SOS interaction (32), and Rho has been shown to modulate focal adhesion kinase activation (19), providing a possible link. These effects could be mediated by effects of Rho on the actin cytoskeleton, as cytochalasin D, which disrupts actin filaments can also block integrin-mediated activation of both focal adhesion kinase and MAP kinase. Thus, one possible pathway involves activation of Rho which leads to assembly of an actin scaffold required for the proper formation of focal adhesions. These structures may promote the association of signaling molecules such as focal adhesion kinase, which would then interact to induce activation of MAP kinase.

DISCUSSION

Our results show that inhibiting Rho, either by cotransfection of HA-Erk2 with a dominant negative Rho construct or by treating cells with C3 exoenzyme, had little effect on TPA-stimulated MAP kinase activity, but substantially inhibited the activation by fibronectin. Conversely, activating Rho either with a constitutively activated Rho mutant or by expression of the nucleotide exchange factor lbc specifically enhanced the activation of MAP kinase by fibronectin. Constitutive activation of Rho also partially prevented the decline in basal MAP kinase activity that occurred after cell detachment, indicating that maintaining Rho function in suspended cells can partially substitute for cell adhesion. Essentially identical results were obtained with transiently transfected proteins and endogenous proteins. These data therefore demonstrate that Rho is involved in the activation of MAP kinase by fibronectin.
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