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The PRK2 Kinase Is a Potential Effector Target of both Rho and Rac GTPases and Regulates Actin Cytoskeletal Organization

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The Ras-related Rho family GTPases mediate signal transduction pathways that regulate a variety of cellular processes. Like Ras, the Rho proteins (which include Rho, Rac, and CDC42) interact directly with protein kinases, which are likely to serve as downstream effector targets of the activated GTPase. Activated RhoA has recently been reported to interact directly with several protein kinases, p120 PKN, p150 ROKα and -β, p160 ROCK, and p164 Rho kinase. Here, we describe the purification of a novel Rho-associated kinase, p140, which appears to be the major Rho-associated kinase activity in most tissues. Peptide microsequencing revealed that p140 is probably identical to the previously reported PRK2 kinase, a close relative of PKN. However, unlike the previously described Rho-binding kinases, which are Rho specific, p140 associates with Rac as well as Rho. Moreover, the interaction of p140 with Rho in vitro is nucleotide independent, whereas the interaction with Rac is completely GTP dependent. The association of p140 with either GTPase promotes kinase activity substantially, and expression of a kinase-deficient form of p140 in microinjected fibroblasts disrupts actin stress fibers. These results indicate that p140 may be a shared kinase target of both Rho and Rac GTPases that mediates their effects on rearrangements of the actin cytoskeleton.

An emerging theme in signal transduction via the small GTPases is that these proteins often utilize protein kinases to elicit their downstream effector responses (1, 23, 44, 47). In many cases, it appears that the activated GTP-bound form of the GTPase can interact directly with such kinases. Such an interaction is likely to result in the direct activation of kinase activity and/or the redistribution of the GTPase-kinase complex within the cell. For example, the well-documented association of the activated Ras GTPase with the Raf kinase results in the translocation of Raf to the plasma membrane (24) and initiation of a mitogen-activated protein kinase (MAPK) cascade downstream of Raf (13, 25). The events that follow the Ras-Raf interaction appear to be responsible for many of the phenotypic effects of Ras activation that have been reported for a variety of experimental systems.

The Ras-related Rho family of proteins, which includes the Rho, Rac, and CDC42 GTPases, mediates signaling pathways that affect the organization of the actin cytoskeleton (16, 30, 31, 40, 41) and may affect changes in nuclear gene expression (3, 8, 11, 28). These GTPases have been implicated in a variety of cellular functions, including motility, adhesion, cell cycle progression, and endocytosis (10). However, considerably less is known about the signaling cascades downstream of activated Rho family proteins and their relationship to the biological activities of these GTPases. Recently, the downstream targets of activated Rho family proteins have begun to be identified. Activated, GTP-bound forms of Rac and CDC42 interact directly with a family of serine/threonine kinases referred to as PAKs (p21-activated kinase) (23, 26), which are related to the *Saccharomyces cerevisiae STE20* gene, a MAPK pathway component of the yeast pheromone mating response (19, 37). Activation of the PAKs is believed to lead in turn to activation of a group of MAPK-related proteins known as the JunK/SAPK/p38 family (2, 46). Activation of this kinase cascade by Rac and CDC42 has recently been shown to be unrelated to the effects of these proteins on cytoskeletal rearrangements (17), suggesting that the Rho family GTPases utilize multiple effector pathways to elicit distinct cellular responses. Several other candidate targets of Rac and CDC42 have also now been reported, although the biological role of these interactions has not been established (6).

Several proteins that bind the activated Rho GTPase have also now been identified. Five protein kinases, referred to as p120 PKN, p150 ROKα (RhoA-binding kinase) and ROKβ, p160 ROCK (Rho-associated coiled-coil containing protein kinase), and p164 Rho kinase, have recently been reported to associate specifically with the activated GTP-bound form of Rho, but not with Rac or CDC42 (1, 14, 20, 21, 27, 44). These kinases, which share limited structural similarity to PKC and myotonic dystrophy kinase proteins, appear to be effector targets of the activated Rho GTPase. Thus, analogous to the Ras-Raf interaction, ROK is translocated to the plasma membranes of cells in which an activated mutant form of RhoA is expressed (21). In the yeast *S. cerevisiae*, a screen to identify downstream components of a Rho-mediated signaling pathway revealed that a PKC-related protein is required to mediate the phenotypic effects of Rho overexpression, demonstrating a biological role for such Rho-kinase interactions (32). Such a connection was also recently made for the p150 ROKα kinase, which was found to mediate Rho-induced stress fiber formation in cultured HeLa cells (20). It is likely that the substrates of these kinases then serve as the next link in the signaling cascade that mediates the biological effects of the activated Rho protein.

While Rho, Rac, and CDC42 appear to regulate distinct aspects of cytoskeletal organization in microinjection studies (31), there is some overlap in their spectra of phenotypic effects. For example, both Rac and CDC42 can stimulate the SAPK/JunK cascade, an effect that may be due to the ability of each of these proteins to stimulate PAK activity (8, 28). In addition, Rho, Rac, and CDC42 proteins can each promote

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cell cycle progression through an unknown mechanism (33). Finally, Rho and Rac have both been found to be capable of promoting actin stress fibers, focal adhesions, and membrane ruffles (29, 41). The results of epistasis experiments with dominant-negative mutants of the various Rho family GTPases have led to the conclusion that some of these overlapping effects are due to sequential activation of the GTPases in a somewhat linear cascade. For example, Rac-induced stress fiber formation appears to require Rho activity (41). However, those studies do not rule out the possibility that some of the overlapping effects of these GTPases are due to their ability to signal through common effector targets. Indeed, as described above, Rac and CDC42 are each able to activate the PAKs. Thus far, none of the identified Rho targets, including the four kinases described above, or rhophillin, a Rho-binding protein of unknown function (44), has been found to interact with other Rho family members. Here, we describe the purification and identification of a Rho target kinase that also interacts specifically with the activated form of Rac. Thus, this kinase appears to be a shared effector target of both Rho and Rac proteins, and might therefore be utilized by both GTPases to elicit similar biological responses.

MATERIALS AND METHODS

Preparation of recombinant GST fusion proteins. The coding sequences of human RhoA, Rac1, CDC42, and H-Ras cDNAs were expressed as glutathione-*S*-transferase (GST) fusions under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter. Fifty milliliters of an overnight culture of XA90 bacteria transformed with these constructs was diluted with 500 ml of Luria-Bertani medium containing 50 μg of ampicillin per ml, and the bacteria were grown at 37°C with shaking until they were in log phase. Protein expression was then induced for 4 h with 0.1 mM IPTG. The pelleted cells were resuspended in ice-cold bacterial lysis buffer (50 mM Tris [pH 7.5], 5 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol [DTT], 10 μg of phenylmethylsulfonyl fluoride) and sonicated four times for 20 s each. Lysates were spun at 12,000 × *g* for 10 min at 4°C. Supernatants were incubated for 30 min at 4°C with 1 ml (50% slurry) of glutathione agarose beads (Sigma) equilibrated in the same buffer. Beads were washed three times in 10 ml of bacterial lysis buffer and maintained in 0.5 ml of the same buffer containing sodium azide (0.05%).

Autokinase assays. Typically, 100 μl of tissue extract or column fraction, 400 μl of incubation buffer (50 mM HEPES [pH 7.5], 25 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EGTA, 10% glycerol, 1% Triton X-100), and 20 μl of GST beads were mixed and incubated for 30 min at 4°C with rocking as a preclearing step. To load the GTPases with guanine nucleotide, 20 μl of GST-GTPase fusion protein beads (approximately 5 μg of protein) was centrifuged and the buffer was removed and replaced with 50 μl of nucleotide exchange buffer (50 mM HEPES [pH 7.0], 5 mM EDTA [pH 8.0], 0.1 mM EGTA, 50 mM NaCl, 0.1 mM DTT) containing a 0.5 mM concentration of the appropriate nucleotide. Beads were then incubated at 37°C with periodic shaking for 15 min. The exchange reaction was stopped on ice by adding 1 M $MgCl_2$ to a final concentration of 20 mM. The precleared extract was then added to beads of nucleotide-loaded GST fusion protein and incubated for 1 h at 4°C with rocking. The beads were then washed three times with 1 ml of cold wash buffer (20 mM HEPES [pH 7.5], 25 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.1% Triton X-100) and once with 0.1 ml of cold kinase buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$). The kinase reaction was performed at room temperature in 25 μl of kinase buffer containing 1.5 μCi of [γ-P^{32}]ATP (3000 Ci/mmol; NEN). After 20 min, the reaction was stopped on ice and the beads were washed with 1 ml of wash buffer, boiled in 50 μl of sodium dodecyl sulfate (SDS)-containing sample buffer (2% SDS, 20% glycerol, 0.7 M beta-mercaptoethanol, 1 mM Tris, pH 6.8) and analyzed by SDS–10% polyacrylamide gel electrophoresis (PAGE). The gel was then dried and autoradiographed.

C3 treatment of RhoA. GST-RhoA protein (or Rac) on beads loaded with GTPγS was incubated for 30 min at 30°C in 20 μl of C3 buffer (20 mM HEPES [pH 8.0], 2 mM $MgCl_2$) containing 200 μM GTP and 0.5 μg of C3 in the presence or absence of 100 μM NAD^+ (Sigma). The reaction was stopped on ice, and the supernatant was aspirated after centrifugation. The beads were then incubated with the precleared extract as described above. ADP ribosylation of RhoA was confirmed by the mobility shift of the modified protein relative to untreated protein as determined by PAGE. Recombinant C3 toxin (kindly provided by L. Feig and S. Dillon) was prepared in bacteria as a GST fusion protein and then cleaved from GST with thrombin.

Phosphoamino acid analysis. Rho-associated proteins were phosphorylated by autokinase as described above in the presence of radiolabeled ATP. Proteins were then separated by SDS–10% PAGE and transferred onto an Immobilon-P membrane (Pharmacia) in 10% methanol–25 mM Tris–0.192 M glycine with a semidry apparatus for 1 h at 10 V. The membrane was autoradiographed, and the radioactive bands were then excised and separately hydrolyzed for 1 h at 110°C in 100 μl of boiling 6 M HCl. The solutions of degraded proteins in HCl were dessicated and redissolved in 15 parts buffer (50 ml of formic acid [88%], 156 ml of acetic acid, and 794 ml of deionized water; pH 1.9) and 1 part cold amino acid solution (1 mg each of phosphoserine, phosphotyrosine, and phosphothreonine per ml in deionized water; 5 μl/500 Cerenkov cpm). Five microliters of each sample was spotted on a thin-layer cellulose plate. Phosphoamino acids were separated by bidimensional electrophoresis with the HTLE 7000 system. The first and second runs were performed, respectively, in the pH 1.9 buffer for 20 min at a 1.5-kV constant voltage and in pH 3.5 buffer (100 ml of acetic acid, 10 ml of pyridine in a final volume of 1 liter of water) for 16 min at a 1.3-kV constant voltage. The air-dried plate was sprayed with ninhydrin (0.25%, in acetone), and staining of cold amino acids was performed by baking the plate for 15 min at 60°C. Radiolabeled phosphoamino acids were detected by autoradiography.

Preparation of tissue extracts. Tissues derived from sacrificed rats or mice were homogenized in a blender for 30 s from low to high speed in 5 volumes (5 vol/wt of tissue) of lysis buffer (25 mM HEPES [pH 7.5], 20 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg of phenylsulfonylfluoride per ml, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml). The crude homogenate was centrifuged at 90,000 × *g* for 30 min at 4°C. The supernatant was centrifuged again under the same conditions, and the clarified supernatant was then used as a protein source for kinase assays or for p140 purification. The protein concentration was estimated by the Bradford method.

Purification of the Rho-associated p140 kinase. Whole-tissue extract was prepared from five adult rat livers as described above. Pure ammonium sulfate salt was added progressively to the tissue protein extract at 4°C to reach 20% saturation. Proteins were precipitated for 10 min on ice and then centrifuged for 10 min at 17,000 × *g*. Supernatants were collected, and pellets were resuspended in the same volume of lysis buffer. An equivalent volume of each fraction was assayed for RhoA-associated kinase activity. Soluble proteins from the 20% saturated ammonium sulfate precipitation were loaded onto a phenyl Superose column equilibrated with 20% ammonium sulfate-saturated buffer A (20 mM HEPES [pH 7.5], 0.1 mM EGTA, 20 mM NaCl, 0.5 mM DTT). The column was washed with several volumes of this buffer, and proteins were eluted by a linear gradient of buffer A containing from 20 to 0% ammonium sulfate. Collected fractions were assayed for kinase activity after binding to GST-RhoA beads. Positive fractions were pooled and dialyzed for 12 h against buffer A with three changes. The phenyl Superose pool was loaded onto a Q Sepharose column preequilibrated with several volumes of buffer A. The column was then washed with several column volumes of buffer A and then with buffer A containing 150 mM NaCl. Proteins were eluted by a linear gradient of NaCl from 150 to 300 mM on a Pharmacia fast protein liquid chromatography (FPLC) system. Positive fractions were pooled and loaded on a Mono S Sepharose column equilibrated with buffer B (same as buffer A except HEPES was replaced by MES [morpholineethanesulfonic acid; pH 6.5]) containing 200 mM NaCl. The column was washed with buffer B containing 350 mM NaCl. The elution of proteins was performed by either a linear gradient from 350 to 650 mM NaCl or a step to 650 mM NaCl in buffer B. Kinase-active fractions from the Mono S Sepharose column were pooled (S pool) and dialyzed twice for 1 h and once for 16 h in 1 liter of buffer A. The protein was then loaded onto a Mono Q Sepharose column preequilibrated with buffer A. The column was washed with buffer A and then with buffer A containing 150 mM NaCl. Proteins were eluted with a 150 to 300 mM NaCl gradient. Collected column fractions were assayed for kinase activity after binding on GST-RhoA beads.

Protein microsequencing. To prepare the p140 protein for microsequencing, pooled kinase-positive fractions obtained from the Mono Q column were precipitated with methanol-chloroform as previously described (45) and then subjected to SDS–6% PAGE and transferred to a polyvinylidene difluoride filter membrane (Bio-Rad). The filter was stained briefly with Ponceau S to identify the p140 protein band. The p140 band was excised and washed extensively with water, and the protein was cleaved on the filter with LysC protease. Peptides were separated by C_{18} reverse-phase liquid chromatography, and peak fractions were subjected to automated Edman degradation and subsequent analysis of phenylthiohydantoin-derived amino acids. Similarity to PRK2 was determined by a BLAST search of the GenBank database.

Plasmid construction and mutagenesis. A complete PRK2 human cDNA (kindly provided by H. Mellor and P. Parker) was first subcloned as an *Xba*I fragment in the *Spe*I cloning site of pBSK2+ (Stratagene) and then recloned by using *Eco*RI and *Xba*I sites of a FLAG tag vector derived from pCDNA3 (Invitrogen; kindly provided by M. Melegari) which places the FLAG epitope at the amino terminus of the encoded protein. The isolated amino-terminal region of PRK2 (amino acids 1 to 405) was similarly cloned into the FLAG vector by using a unique *Bal*I restriction site in the PRK2 coding sequence. The kinase-deficient mutant of PRK2 was generated by a PCR strategy in which the highly conserved lysine at amino acid position 686 was substituted with glutamic acid. This mutant was expressed and found to be defective for autokinase activity (data not shown). The p190 expression construct (30-1) contains a 49-amino-acid deletion within the RhoGAP domain that completely disrupts GTPase-activating protein (GAP) activity (42a).

Cell culture, transfection, and microinjection. Cos-7 and NIH 3T3 cells were maintained under standard culture conditions in Dulbecco's modified Eagle's

medium supplemented with 10% fetal calf serum and antibiotics. Subconfluent Cos cells were transfected by the dextran sulfate method with 5 μg of purified plasmid. Three days after transfection, cells were lysed in lysis buffer (described above) and the lysate was centrifuged for 5 min at 4,000 rpm in an Eppendorf microcentrifuge. Supernatant was used for binding assays on GST fusion proteins and for kinase assays following immunopurification of FLAG-tagged PRK2 with M2 antibodies. For M2 immunoprecipitation, lysates were incubated together with 5 μg of M2 antibody and protein G-Sepharose for 2 h at 4°C. Beads were washed as for autokinase assays. For microinjection experiments, NIH 3T3 cells were grown on glass coverslips at 40% confluence. Injections of plasmid DNAs (50 to 100 μg/ml) were performed with an Eppendorf microinjector (model 5242) connected to a Zeiss microscope (Axiovert 35). Twelve to sixteen hours after injection, cells were fixed and analyzed by immunofluorescence as described below.

Immunofluorescence. Cells washed in phosphate-buffered saline (PBS) were fixed on coverslips by 20 min of incubation in 4% formaldehyde in PBS, followed by 5 min of permeabilization in 0.1% Triton X-100 in PBS, followed by a 15-min blocking step in 0.1% bovine serum albumin. Detection of FLAG-tagged PRK2 proteins was achieved by a 45-min incubation with anti-FLAG antibody M2 (Sigma; 15 μg/ml). For detection of the p190 RhoGAP mutant, a p190 monoclonal antibody (D2D6) was used. Coverslips were washed with three changes of PBS (5 min each) and then were incubated for 45 min with a mixture of antimouse antibody conjugated to fluorescein isothiocyanate (3 μg/ml; Jackson Immun. Research Laboratory) and tetramethyl rhodamine isocyanate-coupled phalloidin (Sigma) in PBS. Coverslips were mounted on glass slides and viewed with a Nikon Microphot fluorescence microscope.

Immunoblotting. Following SDS-PAGE, proteins were transferred directly to nitrocellulose by semidry transfer. Filters were blocked for 1 h with 5% milk in Tris-buffered saline with Triton X-100 (TBST) and then were incubated with primary antibodies (either anti-PKN or anti-ROKα; kindly provided by Yoshitaka Ono and Thomas Leung, respectively) in TBST for 1 h. Anti-FLAG antibody M2 (Sigma) was used to detect transfected PRK2. After being washed filters were incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 1 h, and following washing were analyzed by enhanced chemiluminescence (Amersham) and autoradiography.

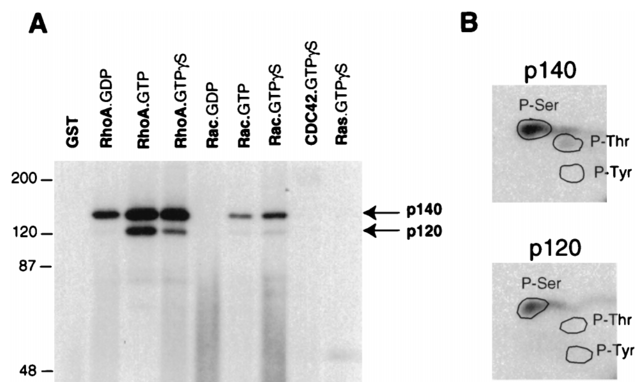

FIG. 1. Detection of RhoA-associated serine/threonine kinases. (A) Liver extracts precleared on GST beads were incubated with beads bound to either GST-GTPase fusion proteins which were preloaded with the indicated nucleotides or GST beads as a control. After several washes, autophosphorylation of bound proteins was assayed in the presence of [γ-^{32}P]ATP and phosphoproteins were analyzed by SDS-PAGE and autoradiography. The p120 and p140 proteins are indicated. (B) RhoA-associated ^{32}P-labeled proteins were separated on an SDS-polyacrylamide gel and transferred to an Immobilon-P membrane. Radioactive bands corresponding to the 140- and 120-kDa proteins were excised, and proteins were hydrolyzed in boiling HCl. Radiolabeled phosphoamino acids were analyzed by two-dimensional thin-layer chromatography. Phosphoserine, phosphotyrosine, and phosphothreonine (indicated by P-Ser, P-Tyr, and P-Thr, respectively) were detected by ninhydrin staining, and radiolabeled phosphoamino acids were revealed by autoradiography.

RESULTS

Detection of protein kinases that associate with RhoA. To identify protein kinases that associate with the human RhoA GTPase, we used a GST-RhoA fusion protein bound to glutathione beads as a solid-phase support on which to isolate potential Rho-interacting kinases from crude tissue extracts. Whole-tissue extracts prepared from rat liver were incubated with immobilized GST-RhoA fusion protein that had been preloaded with guanine nucleotide. After extensive washing of the beads, the autokinase activity of any associated proteins was examined by incubation with radiolabeled ATP followed by SDS-PAGE and autoradiography. As shown in Fig. 1A, two major protein bands of approximately 140 and 120 kDa are seen in association with GST-RhoA but not with GST alone or with CDC42 or Ras GTPases. The p140 protein also associates efficiently with GST-Rac, whereas the p120 protein could only be detected weakly and not reproducibly with Rac. Although these results do not distinguish between the autokinase activities of p120 and p140 and their abilities to serve as substrates of some other Rho-associated kinase (this issue is resolved later), p120 and p140 are hereafter referred to as p120 kinase and p140 kinase, respectively.

GTPases are generally considered to be active when associated with GTP (4), and previously described interactions between small GTPases and protein kinases are promoted by the GTP-bound form of the protein. Therefore, we examined the nucleotide requirement for the observed kinase interaction with RhoA. As shown in Fig. 1A, association of the p140 kinase activity with RhoA is nucleotide independent. RhoA proteins loaded with GDP, GTP, or GTPγS, a nonhydrolyzable GTP analog, are each able to capture the p140 kinase efficiently. On the other hand, the p120 kinase appears to be completely dependent on GTP activation of RhoA and does not bind detectably to GDP-loaded Rho.

To determine whether the Rho-associated kinases are serine/threonine or tyrosine kinases, a phosphoamino acid analysis was performed on the autokinase-labeled p140 and p120 RhoA-associated proteins following excision from a polyacrylamide gel. This analysis revealed that phosphorylation of both proteins is on serine and threonine, with the majority of the phosphate being on serine (Fig. 1B). No detectable phosphotyrosine was observed on either protein, suggesting that the Rho-associated proteins are serine/threonine kinases. The Rho-associated kinases are also able to phosphorylate exogenous substrates including recombinant myelin basic protein and histone H1 (data not shown).

Tissue and subcellular distributions of the Rho-associated kinases. We next examined the tissue and subcellular distributions of the Rho-associated kinases. Various tissue lysates were prepared from a sacrificed mouse and were incubated with GTP-loaded GST-RhoA beads to detect associated kinase activity. As shown in Fig. 2A, the p140 kinase appears to be widely expressed in adult tissues, with the highest levels being in liver and lung. In some tissues, less-prominent bands were observed, whose specificity for RhoA was not examined. This observation raises the possibility that additional tissue-restricted Rho-binding kinases or kinase substrates may exist. There is little correlation between the p140 distribution and that of p120, which is always less prominent in the tissues that have been examined. The prominence of the p140 kinase, together with the observation that p140 associates with Rho as well as Rac (suggesting that it is distinct from previously reported Rho-associated kinases) prompted us to pursue the investigation of this protein.

To determine whether the Rho-associated p140 kinase is cytoplasmic or associated with a membranous subcellular fraction, we prepared soluble and detergent-extractable subcellular fractions from mouse liver (where p140 is abundant relative to p120) and examined the distribution of Rho-associated kinase activity as described above. As shown in Fig. 2B, the vast majority of Rho-associated kinase activity is in the soluble, presumably cytoplasmic fraction. The absence of significant membrane-associated kinase is not due simply to an inhibition

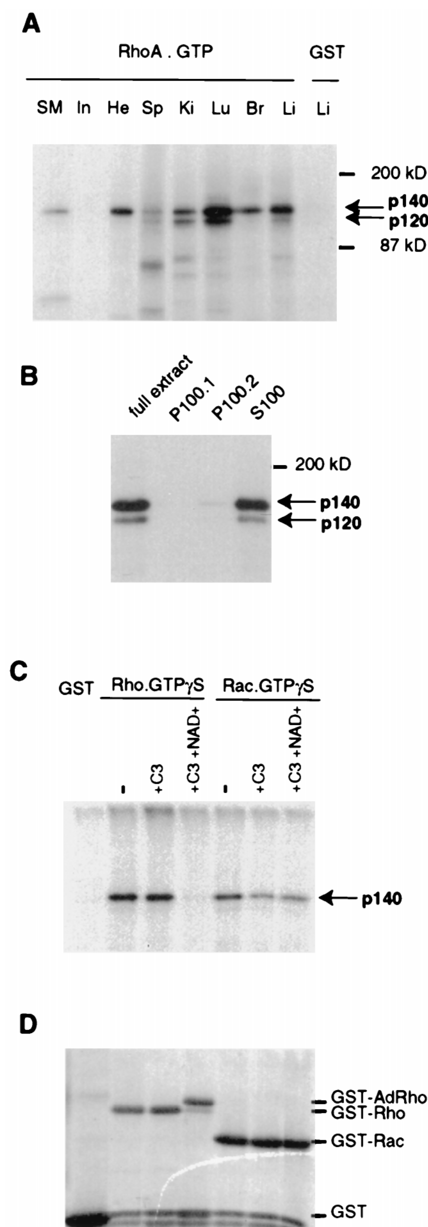

FIG. 2. Tissue and subcellular distributions of RhoA-associated kinase activity and requirement for an intact Rho effector site. (A) Protein extracts were prepared from the following mouse tissues: skeletal muscle (SM), intestine (In), heart (He), spleen (Sp), kidney (Ki), lung (Lu), brain (Br), and liver (Li). Equivalent protein amounts of each tissue extract were precleared on GST beads, incubated with GTP-loaded GST-RhoA beads, and analyzed for autokinase activity as previously described. Liver extract was also incubated on GST beads to detect any GST-associated kinase activity. This experiment was repeated with two independent preparations of tissue extracts. (B) Liver extract, prepared in the absence of detergent, was centrifuged twice at 100,000 $\times$ *g*. The two pellets resuspended in the presence of detergent constitute the membrane protein extract (P100.1 and P100.2). The supernatant represents the cytosolic protein (S100). Each fraction was assayed for autokinase activity on GST-RhoA beads. Full extract corresponds to liver lysate prepared in the presence of 1% Triton X-100. (C) GTPγS-loaded GST-RhoA and GST-Rac beads were incubated with or without (−) the recombinant C3 toxin from *C. botulinum* in the presence or absence of its substrate (NAD$^+$). Beads were then incubated with precleared liver extract and analyzed for autokinase activity. (D) Coomassie blue staining of the GST fusion proteins used in the experiment shown in panel C. As expected, ADP ribosylation of RhoA by the C3 toxin results in a shift in RhoA migration in the gel (GST-AdRho), whereas Rac is unaffected by C3.

of GTPase-binding ability or kinase activity by the presence of nonionic detergent, since whole-tissue lysates prepared in the same detergent-containing solution contain readily precipitable Rho-associated kinase activity (all previous experiments). In cultured cells, however, we have found that there is a significant amount of membrane-associated (P100 fraction) p140, raising the possibility that localization may be regulated during cell growth (data not shown).

Association of the p140 kinase with RhoA requires a Rho effector domain. In addition to the nucleotide requirement, small GTPases require an intact effector domain to associate with their downstream targets. The effector domain of RhoA has previously been identified (42) and can be specifically inactivated by incubation with the *Clostridium botulinum* C3 toxin, an enzyme that ADP-ribosylates a single critical asparagine (Asn-41) in the effector domain (7). The resulting Rho modification renders the protein defective in its ability to promote actin stress fiber formation in serum-starved cells upon microinjection, presumably by blocking the ability of Rho to transmit a signal to its effector targets (7, 40). To determine whether the interaction of RhoA with the 140-kDa kinase requires the Rho effector domain, we examined the effect of pretreating the GST-RhoA protein with C3 toxin on its ability to bind the kinase. As a control to eliminate potential nonspecific inhibitory effects of the recombinant bacterial C3 preparation, samples were incubated with C3 in the presence or absence of the C3 enzyme substrate, NAD$^+$. As shown in Fig. 2C, C3 treatment of RhoA (with NAD$^+$) blocks the majority of the RhoA-kinase interaction, indicating that the interaction requires an intact RhoA effector domain. As expected, similar treatment of Rac with C3 had no effect on p140 association since C3 does not modify Rac (Fig. 2C and D).

Purification and identification of the Rho-associated p140 kinase. In an effort to identify the Rho-associated p140 kinase, we utilized a protein purification strategy that relies on the ability to detect autokinase activity associated with GST-RhoA following incubation with rat liver extract. Using this strategy, we identified several effective purification steps. First, crude, clarified liver lysate from five rats was subjected to a 20% ammonium sulfate precipitation. The supernatant was then fractionated by phenyl Superose chromatography, followed by Q-Sepharose, then Mono S, and finally Mono Q chromatography. At each step, FPLC column fractions were analyzed by incubation with GST-RhoA beads, followed by a kinase assay in the presence of [^{32}P]ATP, after which the GTPase-associated phosphorylated proteins were resolved by electrophoresis and detected by autoradiography (Fig. 3). Efficient separation of the p140 and p120 proteins could be achieved on the Mono Q column (Fig. 3B). At the time that these studies were undertaken, Rho-associated proteins of 120 kDa (PKN) and 150 kDa (ROKα) had been reported (1, 21, 44). Using PKN-specific antibodies, we were able to identify the 120-kDa protein as the previously reported PKN, and with ROK-specific antibodies we were able to demonstrate that the 140-kDa protein is distinct from ROK (data not shown). For both PKN and ROK, it was possible to detect these proteins in association with GST-RhoA beads from whole-liver extracts, but not in Mono S-enriched p140-containing fractions.

To assess the purification of the p140 protein, fractions from the final Mono Q purification step were analyzed by gel electrophoresis followed by either Coomassie blue staining (Fig. 4A), direct autokinase activity assay followed by electrophoresis (Fig. 4B), or RhoA-associated kinase activity assay as described above (Fig. 4C). As shown, a prominent protein band corresponding to p140 could be detected in several column fractions by Coomassie blue staining. In addition, these same

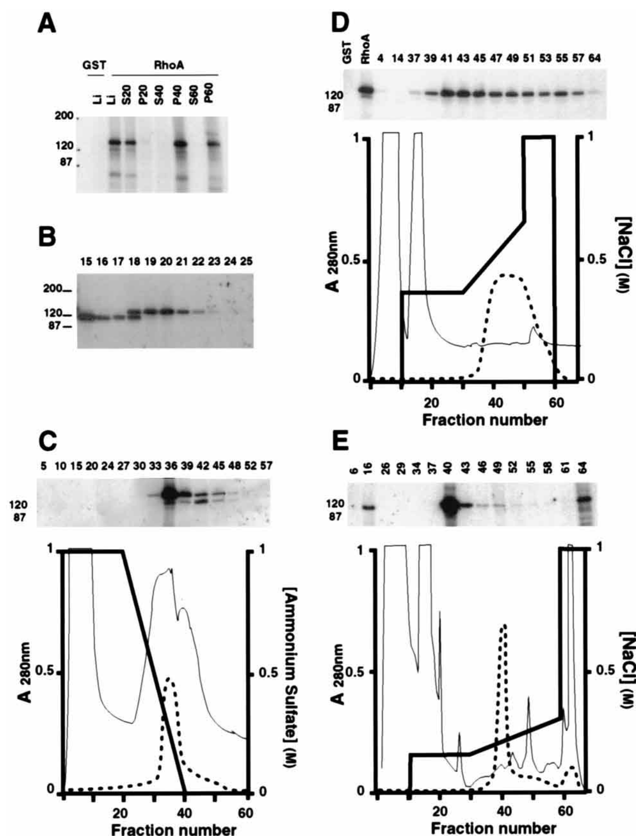

FIG. 3. Purification of the p140 kinase. (A) Liver cytosolic proteins precipitated in the presence of 20, 40, or 60% saturated ammonium sulfate were centrifuged, and the supernatants (S20, S40, and S60) and redissolved pellets (P20, P40, and P60) were assayed for kinase activity on GTP-loaded GST-RhoA (RhoA) beads. The starting material (Li) was also used as a control for kinase activity detected on GST or GST-RhoA-GTP beads. In panels B, C, D, and E, RhoA-associated kinase activity was assayed as described above with column fractions after separation of liver proteins by FPLC. Fraction numbers are indicated on the top of each lane of the autoradiograms. Panel B shows the separation of the two major Rho-associated proteins, p120 and p140, on a Mono Q-Sepharose column with a linear NaCl gradient of 200 to 400 mM. In panel C, a 25% ammonium sulfate (1 M) soluble fraction was subjected to phenyl Superose chromatography with a 1.0 to 0 M ammonium sulfate gradient. Subsequent fractionations were performed by Mono S Sepharose chromatography with a 350 to 650 mM NaCl gradient (D) and by Mono Q Sepharose chromatography with a NaCl gradient of 150 to 300 mM (E). Graphic representations in panels C, D, and E show the profiles of protein concentration (estimated by absorbance at 280 nm; thin line), of the salt gradient (thick line), and of the detected p140 kinase activity (dotted line).

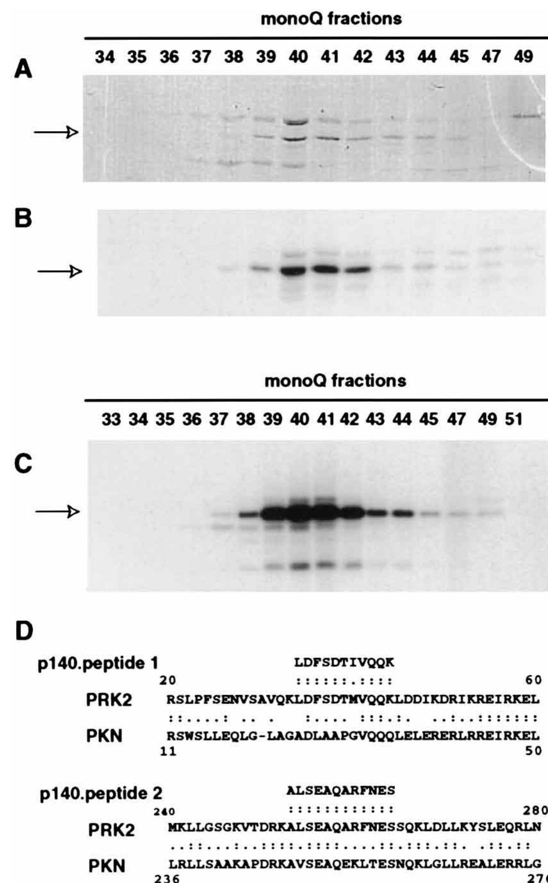

FIG. 4. Kinase activity of partially purified p140 and alignment of p140-derived peptide sequences with PRK2 and PKN. Several fractions of the final Mono Q chromatography step were analyzed by Coomassie blue staining of total protein (panel A; 20 μl/fraction), by assay of total autokinase activity in each fraction (panel B; 2 μl/fraction), or by assay of RhoA-associated kinase activity, (panel C; 2 μl/fraction) as previously described. The arrows indicate the position of p140. (D) p140 prepared from the Mono Q fractions was cleaved by endopeptidase (LysC) and subjected to microsequencing. An analysis of the amino acid sequences of two purified peptides with the BLAST system revealed a highly significant homology with human PRK2. The alignment of p140 peptides 1 and 2 with PRK2 and PKN was performed with the EERIE program. Identical amino acids are indicated by colons; single dots indicate conservative amino acid differences.

fractions were positive both for direct autokinase activity of the p140 protein and Rho-associated autokinase activity. By aligning the autoradiogram of the kinase assays with the Coomassie-stained gel, we were able to demonstrate comigration of a single major protein of 140 kDa. These results suggest that the Coomassie-stained p140 protein is likely to represent the Rho-binding kinase and additionally indicate that the kinase activity of p140 is not completely dependent on the presence of Rho or Rac for activity.

Following the final column step (see Table 1 for a purification summary), p140-positive fractions were pooled and subjected to SDS-PAGE and the p140 protein (approximately 8 μg) was transferred to a polyvinylidene difluoride membrane. A region of the filter containing p140 was excised and subjected to cleavage with the LysC protease. The cleaved peptides were resolved by reverse-phase liquid chromatography, and the sequences of two peptides were determined. One of these peptides, p140 peptide 2, exactly matches a region of the human PRK2 (36) sequence (12 of 12 amino acids) corresponding to amino acids 253 to 264, and a second peptide was identical to PRK2 at 11 of 12 residues, with one conservative

TABLE 1. Summary of p140 purification

Purification step	Protein concn ($mg \cdot ml^{-1}$)	$Q_{protein}$[a] (mg)	Estimated activity (%)	Fold purification
Liver lysate	10	1,700	100	1
S20 (Amm. sulf.)[b]	9	1,530	99	1.1
Phenyl Superose	1.5	300	90	6.1
Q-Sepharose	0.15	22.3	80	92.3
Mono S	2.5×10^{-3}	0.1	72	22,870
Mono Q	24×10^{-3}	0.048	35	78,000

[a] $Q_{protein}$, amount of protein.
[b] Addition of pure ammonium sulfate (Amm. sulf.) salt to reach 20% saturation.

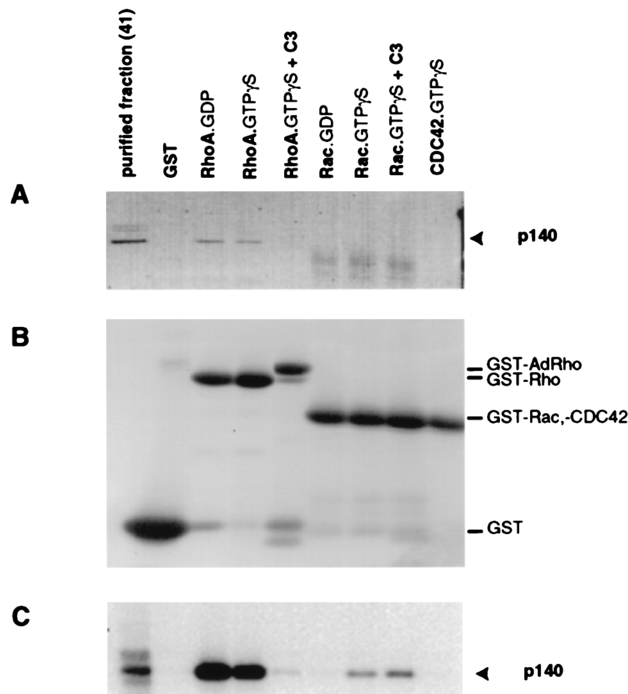

FIG. 5. Binding and kinase activation of purified p140 by Rho and Rac GTPases. Twenty microliters of Mono Q fraction 41 was incubated with several GST fusion beads preloaded with the indicated nucleotides. Ninety percent of the bead-bound material was analyzed by SDS-PAGE and Coomassie blue staining to visualize associated protein (A) and the corresponding GST fusion proteins (B). The rest was assayed for autokinase activity present on the beads and then analyzed by SDS-PAGE and autoradiography (C). Purified fraction 41 (left lane) is the starting material unincubated with beads.

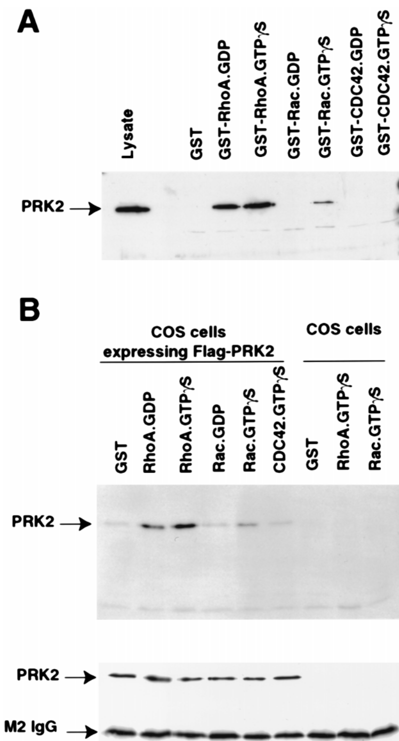

FIG. 6. Recombinant PRK2 binds to and is activated by both Rho and Rac. (A) Lysates of transfected Cos cells expressing a FLAG-tagged PRK2 protein were incubated with GST or the indicated GST-GTPase fusion proteins (loaded with the indicated nucleotides) on beads. The binding of PRK2 to the various GTPases was analyzed by SDS-PAGE and immunoblotting with M2 anti-FLAG antibody. (B) FLAG-tagged PRK2 expressed in transfected Cos cells was immunoprecipitated with M2 antibodies and assayed for kinase activity in the presence of the indicated GST fusion proteins (upper autoradiogram). The relative quantity of immunoprecipitated PRK2 was then determined by immunoblotting with M2 antibody, which revealed both the FLAG-tagged PRK2 protein (PRK2) and the M2 antibody (M2 IgG) used for immunoprecipitation (lower autoradiogram). Mock-transfected Cos cells (no DNA) were included as a control (right three lanes).

difference (Met to Ile) (Fig. 4D). Therefore, it was concluded that the purified p140 Rho-associated kinase very likely represents the rat homolog of the previously reported human PKN-related protein, PRK2. The predicted size difference between PRK2 and PKN is also consistent with our observed size difference between PKN and p140 (36).

p140 kinase activity is stimulated by both Rho and Rac proteins. By comparing levels of Coomassie-stained p140 protein to kinase activity with or without Rho or Rac association, we were able to determine whether kinase activity is promoted by the presence of these GTPases. For these experiments, a p140-positive fraction from the final purification step was used. The kinase activities of equivalent amounts of the p140 fraction were compared in the absence of GTPase or in association with GST-Rho, Rac, or CDC42 beads. In addition, GDP- or GTPγS-loaded and C3-treated forms of RhoA and Rac were examined. As shown (Fig. 5), p140 kinase activity is increased severalfold in association with GDP and GTPγS forms of RhoA relative to unassociated p140 activity (compare lane 1 with lanes 3 and 4 in Fig. 5C). Moreover, C3 treatment eliminated most of the association. The fact that C3 treatment blocks binding (Fig. 5A) as well as kinase association (Fig. 5C) of RhoA with p140 also indicates that the interaction of RhoA with the p140 protein requires an intact Rho effector domain but, notably, does not require the GTP-activated form of Rho. Although it was not possible to detect Coomassie blue-stained p140 in association with Rac (Fig. 5A), the fact that comparable levels of p140 kinase activity are seen in association with Rac-GTPγS and the purified p140 fraction, despite substantially lower levels of detectable p140 protein bound to Rac, suggests that Rac is also able to stimulate the kinase activity of p140. Unlike activation by Rho, however, activation of p140 by Rac is completely dependent on the GTP-bound form of Rac. These results indicate that p140 kinase activation, as well as binding, is nucleotide dependent for Rac and nucleotide independent for Rho.

Recombinant PRK2 binds Rho and Rac proteins. In order to verify that the p140 kinase that we detected is indeed identical to the PKN-related PRK2 protein, we expressed an epitope-tagged (FLAG) form of full-length human PRK2 from a mammalian expression construct in transfected Cos cells and examined its ability to interact with Rho and Rac GTPases. In the first experiment, lysates from Cos cells transfected with FLAG-tagged PRK2 were incubated with GDP- and GTP-loaded forms of either Rho, Rac, or CDC42 as described above. Bound proteins were assayed by immunoblotting with anti-FLAG antibodies. As shown (Fig. 6A), PRK2 associates with both GDP- and GTP-bound Rho, as well as with GTP-bound Rac, but not detectably with CDC42 or GST alone. Therefore, it appears that PRK2 exhibits GTPase-binding properties identical to those seen with purified p140. In the next experiment, we addressed the ability of Rho and Rac proteins to stimulate PRK2 kinase activity. For this analysis, tagged PRK2 was immunoprecipitated from lysates of transfected Cos cells and autokinase activity of precipitated PRK2 was assayed in the presence or absence of Rho and Rac GST

fusion proteins in solution. A detectable autokinase activity, which was observed in the absence of GTPase addition, is stimulated by both the GDP- and GTP-bound forms of Rho and weakly by the GTP-bound form of Rac (Fig. 6B). The expression of equivalent levels of transfected PRK2 in each sample was confirmed by immunoblotting of the same immunoprecipitations with anti-FLAG antibodies (Fig. 6B). Together, these results strongly suggest that the purified Rho- and Rac-binding p140 kinase is identical to the PKN-related PRK2 protein.

Expression of a kinase-deficient form of PRK2 disrupts actin stress fibers. Although the Rho family GTPases have been identified as important regulators of the actin cytoskeleton, the effector pathway(s) that links these GTPases to their effects on the cytoskeleton has yet to be clearly defined. To investigate the possibility that PRK2 plays a role in the regulation of actin assembly, we used a microinjection strategy to determine whether expression of a kinase-deficient form of PRK2 could affect the organization of the actin cytoskeleton in NIH 3T3 cells. As a control for nonspecific effects of microinjection on the cytoskeleton, we expressed a deletion mutant of p190 RhoGAP (43) that is defective for Rho-binding and RhoGAP activity. The appearance of the actin cytoskeleton in cells expressing this protein (as detected by immunostaining and phalloidin staining) is indistinguishable from that seen in surrounding uninjected cells (Fig. 7A and E).

In cells expressing the kinase-deficient PRK2 protein, there is a dramatic and nearly complete loss of actin stress fibers, as revealed by phalloidin staining (Fig. 7B and F). In addition, many of the cells expressing the PRK2 mutant also exhibit an increase in the level of detectable subcortical actin, raising the possibility that PRK2 may regulate the distribution of filamentous actin within the cell. To determine whether the observed effect is due simply to a competition effect for binding to the Rho GTPases, we also expressed an amino-terminal domain of PRK2 which contains the GTPase-binding region (amino acids 1 to 405) and which is sufficient for binding Rho proteins in vitro (data not shown). This form of PRK2 was highly expressed in injected cells but did not significantly affect the appearance of the actin cytoskeleton (Fig. 7C and G). Expression of wild-type PRK2 leads to a modest reduction in the number of stress fibers; however, in these cells, the actin appears to be recruited into somewhat larger cables that are qualitatively distinct from those seen in uninjected cells (Fig. 7D and H).

DISCUSSION

In a search for protein kinases that serve as effector targets of the RhoA GTPase, we detected and purified a serine/threonine kinase referred to as p140 which is activated upon binding to RhoA or Rac but not to CDC42 or Ras. These biochemical properties clearly distinguish p140 from several recently identified protein kinases which interact specifically with Rho but not with Rac or CDC42. Moreover, with the exception of PKN, the other identified Rho-binding kinases (p160 ROCK, p150 ROKα, and p164 Rho kinase) do not appear to be activated substantially upon binding to Rho (14, 21, 27). In addition, p140 kinase activity is detected in a variety of tissues and cell lines, while expression of the other candidate Rho targets seems more restricted. The Rho-activated kinase PKN is also detected in most tissues; however, in the autokinase assay employed in these studies, p140 is always the predominant Rho-associated kinase and is detected at substantially greater levels than PKN. These results suggest that p140 may be a ubiquitous and relatively abundant effector target of the Rho GTPase.

Several lines of evidence suggest that p140 is identical to the recently described PKC-related kinase 2 (PRK2), which is closely related to Rho-binding kinase PKN (58% sequence identity) (36). First, the near identity of the microsequenced p140 peptides with sequences within the predicted human PRK2 sequence suggests that p140 is the rat homolog of human PRK2. Furthermore, the reported electrophoretic mobility of PRK2 relative to PKN is consistent with our observed size difference between p140 and the 120-kDa PKN. Finally, we have found that a PRK2 cDNA clone, when expressed in transfected Cos cells, exhibits GTPase-binding properties that are indistinguishable from those observed with purified p140.

PRK2 shares several structural properties with PKN. Its amino-terminal domain (HR1) includes three repeated motifs containing the peptide sequence GAEN followed by a leucine zipper structure. As shown in Fig. 8, the sequence surrounding the first repeat is very similar to the REM-1 sequence which was found in several RhoA-interacting proteins, including rhotekin, PKN, and rhophilin, and was therefore defined as the consensus binding site for the RhoA protein (38). Thus, it is likely that this sequence constitutes the Rho-binding site of p140. A central domain (HR2) which is conserved in PRK1/PKN and PRK2 includes sequences found not only in these proteins but also within the amino-terminal part of the atypical PKC members, PKCε, PKCη, and DBK (34). The function of this domain has yet to be defined. The carboxy-terminal kinase domains of PRK1 and PRK2 are closely related to those of all the known members of the PKC family. However, PKN/PRK1 and p140/PRK2 clearly constitute a distinct family of kinases which, unlike the PKCs, are not regulated by phorbol esters and calcium (15, 34, 35).

Surprisingly, the structural similarity between p140/PRK2 and PRK1/PKN does not correlate exactly with their GTPase-binding properties. While PKN specifically binds to and is activated by the GTP-bound form of RhoA (1, 44), p140 kinase activity can be significantly stimulated by the binding of RhoA protein associated with either GDP or GTP. In addition, the binding of p140/PRK2 to RhoA in vitro is essentially equally efficient regardless of whether RhoA is bound to GDP or GTP. The fact that PKN and PRK2 exhibit different nucleotide requirements for Rho binding, even when assayed in the same extract, suggests distinct binding mechanisms, and possibly distinct biological roles for these two related kinases in Rho signaling in vivo. Notably, it has previously been demonstrated that both the GTP- and the GDP-bound forms of RhoA are able to bind the 68-kDa human erythrocyte phosphatidylinositol-5 kinase type 1 (PI 5-K) (39). Furthermore, the recently described IQGAP protein has been reported to interact equally well with GDP- and GTP-bound forms of CDC42 (5). These observations, of course, counter the well-established paradigm in which GTPases are viewed as switches whose GTP-bound forms are active and are uniquely able to interact with downstream targets (4). However, the fact that ADP ribosylation of the effector domain of RhoA by C3 toxin abolishes the association of RhoA with p140 and the activation of p140 kinase activity implicates p140 as a Rho effector target. Moreover, we have found that GDP-bound C3-treated RhoA also fails to activate p140 kinase activity, indicating that the interaction of p140 with Rho-GDP is also mediated by the Rho effector domain (unpublished observation). It is not possible, however, to formally exclude the possibility that p140 and PRK2 only interact with the GTP-bound form of Rho in vivo.

The association of the p140 kinase with the GTP-bound form of Rac suggests that p140 may function as a downstream

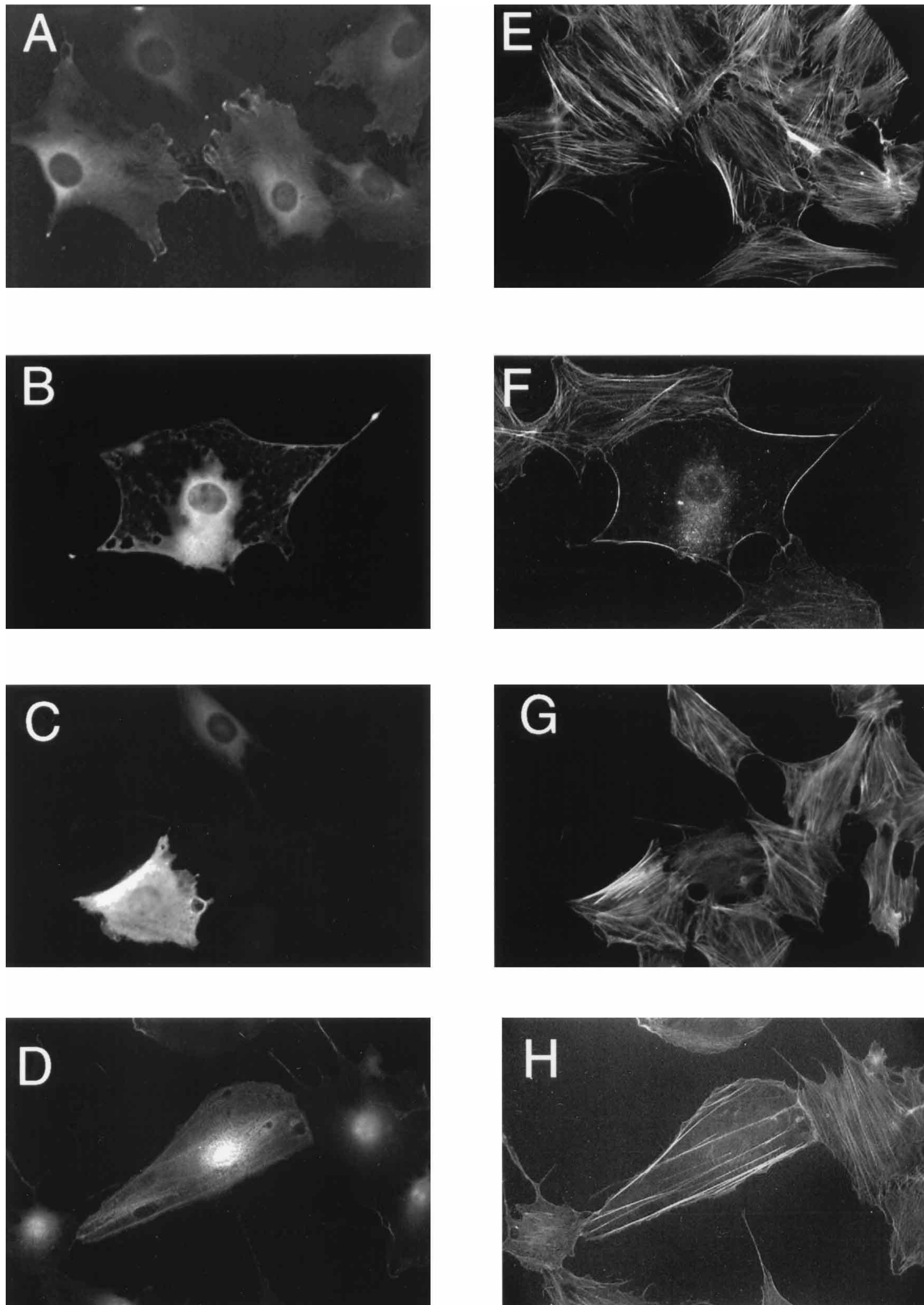

FIG. 7. Expression of kinase-deficient PRK2 disrupts actin stress fiber formation. NIH 3T3 fibroblasts on glass coverslips were microinjected with plasmid expression vectors encoding either a p190 RhoGAP mutant (A and E), a kinase-deficient PRK2 mutant (B and F), the PRK2 amino terminus (amino acids 1 to 405) (C and G), or wild-type PRK2 (D and H). After 12 to 16 h, cells were fixed and subjected to immunostaining with either p190-specific antibody D2D6 (A) or the M2 anti-FLAG antibody (B, C, and D) to detect PRK2. Filamentous actin was visualized in the same fields with fluorescent phalloidin (E, F, G, and H). Expression-positive cells can be seen among surrounding uninjected cells in panels A, B, C, and D.

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PRK2      KLDDIKDR IKREIRKELK IKEGAENLRK VTTD.KKSLA YVDNILKKSN KKLE
PRK1/PKN  QLELERER LRREIRKELK LKEGAENLRR ATTDLGRSLG PVELLLRGSS RRLD
Rhophilin QLQSHRAR LHQQISKELR MRTGAENLYR ATSN.TWVRE TVALELSYVN SNLQ
Rhotekin  ALSLEDTE LQRKLDHEIR MRDGACKLLA ACSQREQALE ATKSLLVCNS RILS

consensus  *   * * *   * **** ** ***** * **      *   *  **     * *
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FIG. 8. Identification of the consensus sequence for Rho binding in PRK2. A comparison of the Rho-binding region of PRK1/PKN, rhophillin, and rhotekin with an amino-terminal region of the predicted human PRK2 sequence. Asterisks indicate conserved amino acids, and boldface type indicates nearly invariant amino acids.

effector of the Rac protein as well as of the Rho protein. Although several targets of Rho and Rac proteins have now been identified, only p140 and the recently described protein Citron (22) are able to interact with both GTPases. In the original report describing p160 ROCK as a Rho target (14), binding was shown to be Rho specific; however, in a subsequent study using a filter-binding assay, ROCK was found to bind to both Rho and Rac (17). It has previously been reported that the interactions of proteins with activated forms of both Rac and CDC42 are mediated by a conserved consensus sequence, referred to as CRIB (CDC42/Rac interactive binding) (6). This sequence was first observed in the p65PAK protein and has now been seen in at least two other related PAK family proteins as well as in several other putative targets of Rac and CDC42, including MLK-3 and WASP. None of these proteins interacts detectably with Rho. An analysis of the PRK2 sequence did not reveal the presence of the conserved binding site, suggesting that there may be more than one structural mechanism for establishing interactions between Rac and its targets. Indeed, Citron also lacks the CRIB sequence and, like p140, binds to Rac and Rho but not to CDC42 (22). However, Citron does not share any obvious sequence similarity with PRK2 and p140. It is also notable that several proteins that have been found to interact specifically with the GTP-bound form of Ras, including Raf, RasGAP, and RalGDS, do not share a domain of significant sequence similarity.

Because both Rho and Rac proteins were found to interact with p140, we have begun to investigate the possibility that these two proteins interact with distinct regions of p140. However, thus far we have not observed either cobinding or synergistic kinase activation by the Rac and Rho GTPases when they are coincubated with p140 (unpublished observation). Moreover, we have found that both Rho and Rac GST fusion proteins can bind to an isolated region of PRK2 that comprises only the amino-terminal repeated leucine zipper motifs (epitope-tagged) when expressed in transfected Cos cells (unpublished observation). Despite the binding of p140 to both Rho and Rac proteins, it is possible that the effector response promoted by each complex is distinct. For example, Rho and Rac proteins might recruit p140 to different subcellular locations. Additionally, it is conceivable that the substrate specificity of the p140 kinase is directed by the interaction with either Rac or Rho. In such scenarios, it is easy to envision a mechanism by which an effector target shared by at least two GTPases can mediate distinct downstream events.

Although Rho and Rac proteins have been reported to regulate distinct biological processes, there is increasing evidence of functional overlap between these two GTPases. Specifically, such processes as membrane ruffling, receptor-mediated endocytosis, and cell cycle progression can be affected by both Rho and Rac proteins (18, 29, 33). Thus, it is not surprising to find that these GTPases share some downstream effector targets. Upstream regulation of these two proteins also appears to be coordinated. Two different nucleotide exchange proteins have now been described (Trio and Ost) (9, 12) which exhibit binding to both Rho and Rac proteins through two separate binding domains. Hence, it is likely that the concerted regulation of the Rho and Rac GTPases is essential for at least some of their cellular functions and that shared effector targets, such as PRK2, may explain the overlap in their spectra of biological activities.

The results of the microinjection studies described here indicate that PRK2 is likely to play a role in regulating the organization of filamentous actin within the cell. The complete inhibition of actin stress fibers seen in cells injected with kinase-deficient PRK2 is similar to that seen in cells injected with the Rho-specific C3 toxin. Moreover, we have found that injection of serum-starved Swiss 3T3 cells with PRK2 blocks the ability of the activated Rho V14 mutant to induce stress fibers (unpublished observation). The kinase-deficient form of PRK2, by analogy to other similarly mutated kinases, is expected to function as a dominant-inhibitory protein. However, the observation that the isolated N-terminal region of PRK2 (which binds Rho and Rac in vitro) does not inhibit stress fibers suggests that kinase-deficient PRK2 is not simply competing for available Rho or Rac proteins but may be inhibiting signaling by a more subtle mechanism. For example, it is possible that the mutant binds to, but fails to phosphorylate, PRK2 substrates, thereby preventing their phosphorylation by the endogenous kinase. Similarly, a kinase-deficient PAK1 protein appears to block downstream signaling by a mechanism that does not involve simply titrating Rac and CDC42 (46). Consistent with this notion, we have observed a substantial difference in the effects of wild-type and kinase-deficient PRK2 on actin stress fibers when expressed at comparable levels.

Since it is well documented that the regulation of the actin cytoskeleton in cultured fibroblasts is mediated by the Rho family GTPases, the most straightforward interpretation of our results is that PRK2 participates in a Rho-mediated signaling pathway that affects the organization of filamentous actin. The fact that the amino-terminal GTPase-binding domain of PRK2 does not induce a similar effect on the cytoskeleton raises the possibility that some other region of PRK2, outside of the kinase domain, may also be necessary to mediate its effects on actin. Notably, PRK2 contains a proline-rich region that could potentially function as an SH3-binding domain, thereby linking PRK2 function to additional proteins.

Recently, the p150 ROKα kinase has been shown to affect Rho-dependent actin stress fiber formation, suggesting that this protein mediates a signaling pathway from Rho to the cytoskeleton (20). Our results indicate that PRK2 probably also participates in a Rho-mediated signaling pathway that regulates the actin cytoskeleton. Thus, it is clear that there are likely to be multiple effector pathways that mediate the effects of Rho GTPases on actin rearrangement. Therefore, future studies will undoubtedly need to address the biochemical and biological distinctions between the various effector signals that emanate from the Rho proteins in order to understand the precise roles of these diverse Rho targets.

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ADDENDUM

Following submission of the manuscript, Quilliam et al. reported the identification of PRK2 as a potential Rho target (36a).

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