lapse of the unfolded state to the folding intermediate was slowed by a factor of $>10^3$ by the chaperones (*26*).

The amide proton exchange data do not require that the major chaperone-bound state of barnase is the unfolded conformation; the data merely require that barnase becomes transiently fully unfolded in order for exchange to occur. Indeed, the kinetics of refolding of barnase in the presence of GroEL or SecB are consistent with the intermediate state being the major bound species (*27–29*). A small amount of transient unfolding, detectable by amide proton exchange, would not be detected in attempts to measure the gross unfolding of the protein by direct spectroscopic techniques (*7*, *9*, *12*), and transient unfolding is all that is required to correct misfolding. Such a transient unfolding is an annealing process. Our data are consistent with both chaperones having a dual role with regard to protein folding: (i) They recognize and bind to partially folded proteins. By this mechanism, GroEL prevents formation of irreversible aggregates, which would lead to off-pathway reactions (*7*), and SecB holds the substrate protein in a state competent for membrane transport (*3*). (ii) GroEL and SecB use protein-protein binding energy to denature the bound polypeptide chain to its fully unfolded state, allowing misfolded protein states to be corrected and guided back to the productive folding or transport pathway.

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18. The concentrations of GroEL and SecB always refer to the oligomeric proteins.
19. It may seem strange that a small amount of GroEL or SecB can appreciably broaden the line widths in barnase. However, if the line width of a signal in free barnase is W_F and that when the enzyme is bound to chaperone is W_B, and the fractions of free and complexed barnase are f_F and f_B, respectively, then the line width that is observed if barnase associates and dissociates rapidly (W_{obs}) is given by $W_F f_F + W_B f_B$. Because W_B is very large, it makes a substantial contribution to W_{obs} even when f_B is low.
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23. In the presence of chaperone, the observed exchange rate constant is given by the sum of the overall exchange rate constants for each of the various species present—namely, free native barnase $k^{obs}_{ex\,(N)}$, free folding intermediate $k^{obs}_{ex\,(I)}$, free unfolded barnase $k^{obs}_{ex\,(U)}$, and the respective chaperone-bound states $k^{obs}_{ex\,(G.N)}$, $k^{obs}_{ex\,(G.I)}$, and $k^{obs}_{ex\,(G.U)}$. The observed exchange rate constants of the locally exchanging amide protons are approximately equal to $k^{obs}_{ex\,(N)}$ in both the presence and absence of chaperones because it is by far the largest term at the low concentrations of chaperone used in the exchange experiments. In the presence of chaperone, the slowly exchanging amide protons tend to exchange with the single limiting rate constant $k^{obs}_{ex\,(G.U)}$.
24. The rate laws in Fig. 1B fit all the known data. If, however, the scheme is less complicated and G.N is converted to G.U directly, or if there is a more complicated scheme in which there is another state (G.X) from which exchange occurs, the same types of rate laws still apply.
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26. If, in order for an EX1 mechanism to occur, the lowest intrinsic rate constant for exchange (*22*) must be greater than five times the rate constant for the reversion of the unfolded state to the folding intermediate, then the rate constant of G.U → G.I must be $<20\ min^{-1}$. At the other extreme, and by a similar argument, the rate constant for the reversion of free barnase, exchanging under EX2 conditions, must be $>2 \times 10^4\ min^{-1}$.
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Protein Kinase N (PKN) and PKN-Related Protein Rhophilin as Targets of Small GTPase Rho

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The Rho guanosine 5′-triphosphatase (GTPase) cycles between the active guanosine triphosphate (GTP)–bound form and the inactive guanosine diphosphate–bound form and regulates cell adhesion and cytokinesis, but how it exerts these actions is unknown. The yeast two-hybrid system was used to clone a complementary DNA for a protein (designated Rhophilin) that specifically bound to GTP-Rho. The Rho-binding domain of this protein has 40 percent identity with a putative regulatory domain of a protein kinase, PKN. PKN itself bound to GTP-Rho and was activated by this binding both in vitro and in vivo. This study indicates that a serine-threonine protein kinase is a Rho effector and presents an amino acid sequence motif for binding to GTP-Rho that may be shared by a family of Rho target proteins.

The Ras-related small GTPase Rho regulates certain types of actin-based cytoskeletal structures, including focal adhesions, stress fibers, and the contractile ring, and works as a switch in stimulus-evoked cell adhesion (*1*) and cytokinesis (*2*). Rho also functions to regulate smooth muscle contraction (*3*), transcription (*4*), and cell proliferation (*5*). However, the biochemical mechanism of these actions is unknown. Although protein kinases (*6*), lipid kinases, and a phospholipase (*7*) have been implicated in the Rho signaling pathway, the direct binding and activation of these molecules by activated GTP-bound Rho has not been demonstrated.

To isolate complementary DNAs (cDNAs) encoding proteins that bound to GTP-Rho, human RhoA truncated at

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Ala[181] was fused to a LexA DNA binding protein and was used to screen a mouse embryo cDNA library in a yeast two-hybrid system (*8*). One clone obtained by this procedure, clone 79, contained a 435–base pair (bp) cDNA. In the two-hybrid system, this clone interacted strongly with RhoA but little with RhoC and less with RhoB (Fig. 1A). Among the RhoA mutants, Val[14]-RhoA yielded a strong signal, whereas only a weak signal was found with Asn[19]-RhoA. Because Val[14]-RhoA is a constitutively active, GTP-bound form, and Asn[19]-RhoA, a dominant-negative mutant, is preferentially in the guanosine diphosphate (GDP)–bound form (9), these results suggested that a peptide encoded by clone 79 interacted preferentially with GTP-Rho. Clone 79 was expressed as a glutathione-S-transferase (GST) fusion protein and its binding to Rho was examined in a ligand overlay assay (*10*). The recombinant protein bound to [^{35}S]guanosine 5′-O-(δ-thiotriphosphate)–Rho (GTP-γ-S–Rho) but did not bind to [^{35}S]guanosine 5′-O-(β′-thiodiphosphate)–Rho (GDP-β-S–Rho) (Fig. 1B). Furthermore, little binding was observed to either Rac or Cdc42 (Fig. 1C).

A full-length cDNA of clone 79 was obtained by screening of mouse embryo and

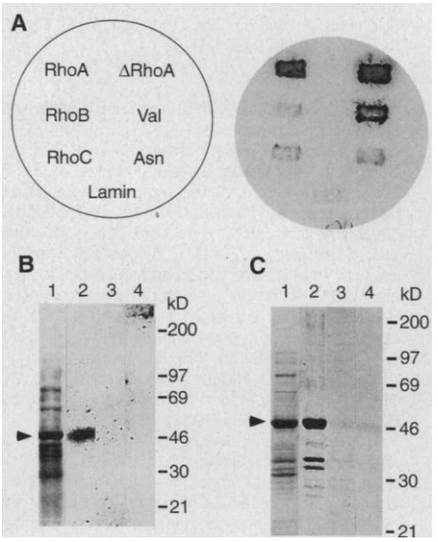


Fig. 1. Interaction of clone 79 peptide with Rho and Rho-related proteins. (**A**) Analysis by the yeast two-hybrid system. Clone 79 was expressed as a fusion protein with the VP-16 activating domain, and its interaction with various Rho or RhoA mutants expressed as fusion proteins with the LexA-binding domain was examined by a filter assay for β-galactosidase activity. ΔRhoA, Val, and Asn indicate RhoA truncated at Ala[181], Val[14]-RhoA, and Asn[19]-RhoA, respectively. Lamin was used as a negative control. (**B**) Analysis by overlay assay. Clone 79 was expressed as a GST fusion protein (indicated by an arrowhead), and its binding to [^{35}S]GTP-γ-S–RhoA (lane 2) or to [^{35}S]GDP-β-S–RhoA (lane 3) or to [^{35}S]GTP-γ-S alone (lane 4) was examined. An autoradiogram is shown. Lane 1, protein staining. (**C**) Binding specificity to the Rho-related proteins. An overlay assay was used to evaluate binding to [^{35}S]GTP-γ-S–bound RhoA (lane 2), to Rac1 (lane 3), and to Cdc42 (lane 4). Lane 1, protein staining. An arrowhead indicates the position of a GST-fused clone 79 protein.

A

MILEERPDGQGTGEESSRPQDDGSIRKGYGSFVQNQPGQLQSHRARLHQQISKELRMRTGAENLYRATSNTWVRETVALELSYVNSNLQLLKEELAELST 100

SVDVDQPEGEGITIPMIPLGLKETKELDWATPLKELISEHFGEDGTSFETEIQELEDLRQATRTPSRDEAGLDLLAAYYSQLCFLDARFFSPSRSPGLLF 200

HWYDSLTGVPAQQRALAFEKGSVLFNIGALHTQIGARQDCSCTEGTNHAAEAFQRAAGAFRLLRENFSHAPSPDMSAASLSMLEQLMIAQAQECIFKGLL 300

LPASATPDICPDQLQLAQEAAQVATEYGLVHRAMAQPPVRDYLPASWTNLAHVKAEHFCALAHYHAAMALCESHPAKGELARQEHVFQPSTPHEPLGPTL 400

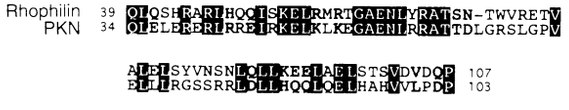
PQHPEDRRKLAKAHLKRAILGQEEALRLHTLCRVLRKVDLLQVVVTQALRRSLAKYSQLEREDDFFEATEAPDIQPKTHQTPEGPLSVFSTKNRWQLVGP 500

VHMTRGEGGFGFTLRGDSPVLIAAVVPGGQAESAGLKEGDYIVSVNGQPCKWWKHLEVVTQLRSMGEEGVSLQVVSLLPSPEPRGTGPRRAALLWNQREC 600

GFETPMPTRTRPWPILGWSRKNKQGKTGSHPDPCTNRNCVTCP 643

Fig. 2. (**A**) Deduced amino acid sequence of Rhophilin (*24*). The sequence encoded by clone 79 is underlined. (**B**) A sequence comparison of Rhophilin and human PKN. Identical amino acids are shown in white against a black background. Conservative replacements are grouped as D, E, Q, N; R, K, H; T, S; V, L; and are shaded.

B



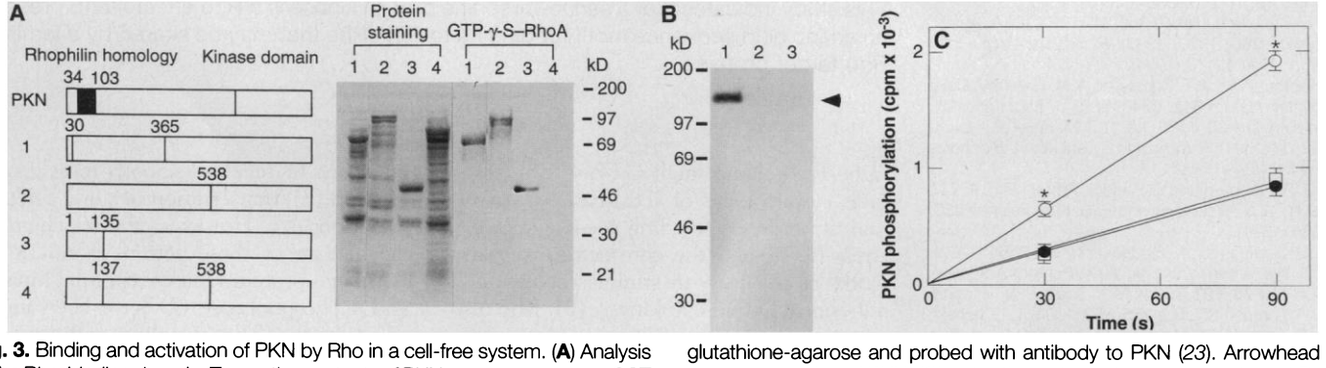


Fig. 3. Binding and activation of PKN by Rho in a cell-free system. (**A**) Analysis of the Rho-binding domain. Truncation mutants of PKN were expressed as GST fusion proteins (*25*), and their binding to [^{35}S]GTP-γ-S–Rho was examined by an overlay assay. Mutants used (1 to 4) are schematically shown on the left, and lysates of *E. coli* expressing each protein were applied to lanes 1 to 4, respectively. (**B**) Association of Rho and PKN. PKN incubated with GST-Rho bound to GTP-γ-S (lane 1), to GDP (lane 2), or to GST alone (lane 3) was precipitated with glutathione-agarose and probed with antibody to PKN (*23*). Arrowhead indicates the position of PKN. (**C**) Activation of the autophosphorylation of PKN by Rho. PKN was incubated with GTP-γ-S–bound Rho (○) or with GDP-bound Rho (●) or with vehicle (□), and autophosphorylation was examined (*23*). Results are shown as mean ± SEM of four experiments. Asterisks denote significant differences ($P < 0.01$) between the results of PKN phosphorylations with GTP-γ-S–bound Rho and with either GNP-bound Rho or vehicle.

brain libraries (*11*). It encoded a protein of 643 amino acid residues with a calculated molecular size of ~71 kD that we named Rhophilin (Fig. 2A). Northern (RNA) blot analysis revealed a 3.2-kb transcript, expressed most highly in the testis (*12*). The original cDNA (clone 79) encoded a 130–amino acid NH_2-terminal fragment, which was thought to be the Rho binding domain. A search of protein sequence databases with FASTA (*13*) and BLAST (*14*) revealed the similarity of this domain to a region found in a 120-kD serine-threonine kinase designated protein kinase N (PKN) or PRK (*15*); 41% of 69 amino acids in this region were identical in the two proteins (Fig. 2B). The remainder of the Rhophilin sequence was similar to that of the NH_2-terminal region in YNK-1, a protein of unknown function from *Caenorhabditis elegans* (*16*) (30% identity in residues 153 to 454) (*12*).

The region of PKN that is similar to Rhophilin is localized to the NH_2-terminal portion (Gln^{34} to Pro^{103}). Because this region has been suspected to be a regulatory domain of PKN (*15*), we examined whether Rho could bind to the region and activate the kinase activity. Several truncation mutants of PKN were expressed as GST fusion proteins, and their binding to Rho was examined in an overlay assay. GTP-γ-S–Rho bound to PKN fragments containing amino acids 30 to 135 (Fig. 3A). This binding was specific for Rho, and little binding was observed with either Rac or Cdc42 (*12*). PKN was precipitated from the lysates by incubation with GST-Rho coupled to glutathione-agarose, and this precipitation was dependent on prior incubation of Rho with GTP-γ-S (Fig. 3B). GTP-γ-S–Rho also stimulated the autophosphorylation of PKN by more than twofold (Fig. 3C). Little precipitation and activation were observed with GDP-Rho.

Rho-dependent activation and phosphorylation of PKN were also tested in cells. Myc-tagged PKN was expressed in COS-7 cells with either wild-type or Val^{14}-Rho or with vector alone. Cells were then lysed, and the kinase activity immunoprecipitated with antibody to Myc was examined. PKN activity in the precipitates was enhanced by co-expression of Rho. Stimulation was more pronounced with the Val^{14} mutant than with the wild-type Rho (Fig. 4A). Involvement of PKN in the Rho signaling was then examined in Swiss 3T3 cells stimulated with lysophosphatidic acid (LPA), which activates Rho in these cells (*1*, *6*), and with botulinum C3 exoenzyme, which specifically adenosine diphosphate–ribosylates and inactivates Rho (*17*). LPA stimulated phosphorylation of endogenous PKN in the cells, and this phosphorylation was inhibited by the prior treatment of the cells with C3 exoenzyme, which suggests that PKN was phosphorylated by a Rho-dependent mechanism in these cells.

The above results taken together suggest that PKN and Rhophilin can serve as targets for Rho. PKN may then phosphorylate the cytoskeletal components or may initiate a kinase cascade (*4*, *6*). In contrast to PKN, no enzymatic activity was found for Rhophilin. It may interact with some cytoskeletal component upon Rho binding or relay a Rho signal to other molecules. Activation of several proteins after addition of Rho to cell homogenates has been reported (*7*). Rho is involved not only in organization of specific actin cytoskeletons (*1*, *2*) but also in transcriptional control and cell cycle progression (*4*, *5*). This may indicate that Rho drives multiple signaling pathways in the cell and that several molecules either alone or in combination serve as Rho effectors (*27*). We have identified an amino acid sequence motif for GTP-Rho binding. This sequence motif may be shared by a family of proteins that work as various Rho effectors.

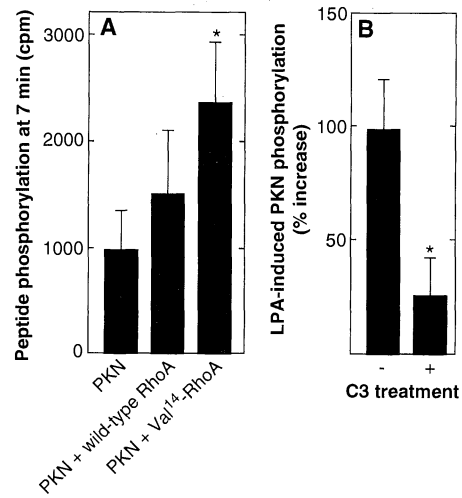


Fig. 4. Rho-dependent activation and phosphorylation of PKN in cultured cells. (**A**) PKN activation by coexpressed Rho. Myc-tagged PKN was expressed in COS-7 cells with wild-type RhoA, Val^{14}-RhoA, or vector alone (*22*), and the PKN activity in immunoprecipitates with antibody to Myc was determined with a PKCδ peptide as a substrate. Reaction proceeded linearly with time, and amounts of phosphorylation were compared at 7 min. Values shown are the mean ± SEM of four experiments. An asterisk denotes significant differences ($P < 0.02$) between the results of peptide phosphorylations with Val^{14}-RhoA and with vector alone. (**B**) LPA-induced phosphorylation of PKN and inhibition by C3 exoenzyme. Swiss 3T3 cells treated with or without C3 exoenzyme were incubated with [^{32}P]Pi and stimulated with LPA. After 20 min, cells were lysed and phosphorylation of PKN was determined (*26*). Results are expressed as LPA-induced percent of increase in PKN phosphorylation compared with the value in the control cells at 0 time, and are shown as the mean ± SEM of three experiments. Asterisk indicates $P < 0.02$ compared to control cells.

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8. A yeast two-hybrid system was done essentially as described (*18*). Human *rhoA* cDNA truncated at Ala^{181} was prepared by polymerase chain reaction and ligated into the vector pBTM116. The *Saccharomyces cerevisiae* strain L40 harboring this plasmid was transformed with pVP-16 fused with a library of cDNA fragments from mouse embryos. From approximately 8×10^6 transformants, 89 His^+LacZ^+ colonies were isolated. They were subjected to segregation of pLexA-truncated *rhoA* in tryptophan-supplemented medium. The resultant 85 Trp^-Leu^+ isolates were mated with yeast strain AMR 70 transformed with pLexA-lamin, and 75 $LacZ^+$ colonies were eliminated. Of the remaining colonies, four carried the same cDNA and showed a strong interaction with Val^{14}-*rhoA*. A representative clone, clone 79, was used for characterization.
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10. Clone 79 cDNA was ligated into pGEX and expressed in *Escherichia coli*. Proteins from cell lysates were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. GST-RhoA, GST-Rac1 (provided by Y. Takai), and GST-Cdc42Hs (provided by P. Polakis) were prepared (*19*) and used in an overlay assay (*20*).
11. λZAPII cDNA libraries of mouse embryo and brain were screened with the clone 79 cDNA as a hybridization probe. Of the four independent clones obtained, a 2.3-kb clone from embryo was fully sequenced in both strands.
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22. *hPKN* was ligated in frame to the Myc epitope sequence inserted in pCMX. 1.5 μg of the resultant plasmid was transfected into 2×10^5 COS-7 cells per 6-cm dish with 1.5 μg each of pEF-BOS (*21*) harboring Val^{14}-*rhoA*, wild-type *rhoA*, or vector alone. Cells were cultured in Opti-MEM (Gibco-BRL) for 48 hours and lysed in 0.2% Triton X-100 in the washing buffer [20 mM tris-HCl (pH 7.5), 1 mM

EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1 μM okadaic acid, 10 mM β-glycerophosphate, 25 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.2 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol]. Immunoprecipitation was done with 9E10 antibody to Myc conjugated with protein G–Sepharose. The immunocomplex was washed three times with the above buffer containing 0.5 M NaCl and twice with kinase buffer [20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, and 1 μM okadaic acid] containing 10 mM β-glycerophosphate and 10 mM sodium pyrophosphate. The complex was finally suspended in the kinase buffer and incubated with 50 μM [γ-^{32}P]ATP and 100 μM PKCδ peptide (*15*) at 30°C.

23. PKN was partially purified from rat testis by chromatography on a Hi-Trap Heparin and a Mono-Q column (Pharmacia) (*15*). The PKN fraction (25 μg of protein) was incubated with GST-RhoA (1.3 nmol) loaded either with GTP-γ-S or GDP at 30°C for 30 min, and precipitated with glutathione-agarose. The precipitates were washed twice with the washing buffer [25 mM sodium 2-(*N*-morpholino)ethanesulfonate (pH 6.5), 50 mM NaCl, 5 mM $MgCl_2$, and 0.05% Triton X-100] and subjected to SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane and probed with antibody to PKN (*15*). For autophosphorylation, the PKN fraction (2.2 μg of protein) was incubated with GST-RhoA (160 pmol) at 30°C for 2 min in a total volume of 11 μl. [γ^{32}P]adenosine triphosphate (ATP) was then added (0.3 μM) and the reaction was continued for 0.5 or 1.5 min at 30°C. The reaction was terminated by the addition of 50 mM EDTA and 200 μM ATP. Antibody to PKN conjugated with protein A–Sepharose was then added. The mixture was stirred at 4°C for 2 hours and centrifuged. The precipitates were washed and subjected to SDS-PAGE. The radioactive bands corresponding to PKN were excised and the incorporated radioactivity was determined.
24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
25. *hPKN* (*15*) was digested with Sma I to isolate a fragment corresponding to amino acids 30 to 365 or with Bam HI to isolate a fragment encoding amino acids 1 to 538. The Bam HI fragment was then digested with Bal I to yield two fragments encoding amino acids 1 to 135 and 137 to 538, respectively. Each fragment was ligated to pGEX and expressed.
26. Swiss 3T3 cells were cultured with C3 exoenzyme (30 μg/ml) for 3 days (*17*). Cells were then incubated with [^{32}P]Pi (0.5 mCi/ml) in Hepes-buffered Krebs-Ringer solution without sodium phosphate for 2 hours. They were then stimulated with LPA (5 μM) for 0 or 20 min and lysed with 1% Triton X-100 in the washing buffer (*22*). PKN was precipitated and the incorporated radioactivity was measured (*23*). The amount of immunoprecipitated PKN examined by immunoblot (*23*) did not differ between the control and C3 exoenzyme–treated cells.
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Identification of a Putative Target for Rho as the Serine-Threonine Kinase Protein Kinase N

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Rho, a Ras-like small guanosine triphosphatase, has been implicated in cytoskeletal responses to extracellular signals such as lysophosphatidic acid (LPA) to form stress fibers and focal contacts. The form of RhoA bound to guanosine triphosphate directly bound to and activated a serine-threonine kinase, protein kinase N (PKN). Activated RhoA formed a complex with PKN and activated it in COS-7 cells. PKN was phosphorylated in Swiss 3T3 cells stimulated with LPA, and this phosphorylation was blocked by treatment of cells with botulinum C3 exoenzyme. Activation of Rho may be linked directly to a serine-threonine kinase pathway.

Rho is a small guanosine triphosphatase (GTPase) that exhibits both guanosine diphosphate (GDP)–GTP binding and GTPase activities (*1*). Rho has GDP-bound inactive and GTP-bound active forms that are interconvertible by GDP-GTP exchange and GTPase reactions (*1*). Rho has been implicated in appropriate responses of the cytoskeletal network to extracellular signals such as LPA and certain growth factors. Rho participates in signaling pathways that lead to formation of stress fibers and focal contacts (*2*) and to regulation of cell morphology (*3*), cell aggregation (*4*), cell motility (*5*), and cytokinesis (*6*). Rho also regulates the synthesis of phosphatidylinositol 4,5-bisphosphate (*7*) and the transcription controlled by the c-*fos* serum response element (*8*). When cells are stimulated with certain extracellular signals, GDP-Rho is converted to GTP-Rho, which binds to specific targets and exerts its biological functions. However, specific targets for Rho have not yet been identified.

To enrich RhoA-interacting proteins, we fractionated crude extracts of a bovine brain membrane fraction by adding ammonium sulfate (40%). The precipitated proteins were loaded onto one of four glutathione-Sepharose affinity columns on which the following recombinant proteins were immobilized: glutathione-S-transferase (GST); GDP-GST–RhoA; GTP-γ-S–GST–RhoA, where GTP-γ-S is guanosine 5′-(3-O-thio) triphosphate, a nonhydrolyzable GTP analog; or GTP-γ-S–GST–RhoA^{A37}, which contains an amino acid substitution in the effector domain. Proteins bound to the affinity columns were then eluted with GST-RhoA by addition of glutathione (Fig. 1A). Three proteins of 128, 164, and 180 kD (named p128, p164, and p180, respectively) were identified in the eluate from the GTP-γ-S–GST–RhoA affinity column but not from the GST or GDP-GST-RhoA affinity columns, which indicates that these proteins specifically interacted either directly or indirectly with GTP-γ-S–GST–RhoA. These proteins appeared to show weaker affinity for the effector domain mutant GST-RhoA^{A37} because less protein was retained on the GTP-γ-S–GST–RhoA^{A37} affinity column than on the GTP-γ-S–GST–RhoA column. The specificity was further examined with affinity-column chromatography with GST-Rac and GST–H-Ras as probes. Three proteins of 122, 140, and 182 kD, distinct from the above RhoA-interacting proteins, were eluted from the GTP-γ-S–GST–Rac affinity column but not from the GDP-GST-Rac affinity column (Fig. 1A). We confirmed that the 182-kD Rac-interacting protein was distinguishable from p180 by electrophoresis. Neither p128, p164, nor p180 was eluted from the GTP-γ-S–GST–H-Ras affinity column. We conclude that the three proteins p128, p164, and p180 interact specifically with activated RhoA.

Among the RhoA-interacting proteins identified, we enriched p128 by specific elution from the GTP-γ-S–GST–RhoA affinity column in the presence of 0.2 M NaCl (Fig. 1B). The p128 protein was further purified to near-homogeneity (greater than 95%) by DEAE-Sepharose column chromatography (Fig. 1B). Purified p128 was subjected to amino acid sequencing. Five peptide sequences derived from p128 were determined: QLAIELK, NVLRLL, LGLLREALERRL, SPLTLEDF, and VLLSEFRP (9). They were found within the sequence of the human serine-threonine kinase protein kinase N (PKN), which is also known as PRK1 (*10*).

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