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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PKN has an apparent molecular size of 120 kD on SDS–polyacrylamide gel electrophoresis (PAGE), which is close to that of p128. Immunoblot analysis demonstrated that p128 was recognized by antibody to PKN (Fig. 2). We therefore conclude that p128 is the bovine PKN.

We tested whether GTP-RhoA could act as a modulator of PKN kinase activity. When purified PKN underwent autophosphorylation in the presence of [γ-^{32}P]ATP, (adenosine triphosphate), GTP-γ-S–GST–RhoA stimulated this reaction four- to five-fold, whereas GDP-GST–RhoA, GTP-γ-S–GST–Rac, and GTP-γ-S–GST–H-Ras had no effect (Fig. 3A). PKN phosphorylates exogenous substrates such as myelin basic protein and serine-containing synthetic peptides based on the pseudosubstrate site of protein kinase C (PKC) (*11*). We examined phosphorylation of the PKCα peptide [which corresponds to amino acids 19 to 35 of bovine PKCα, except that Ser is substituted for Ala: RFARKGSLRQKNVHEVK (9)] by PKN and found that GTP-γ-S–GST–RhoA stimulated PKN kinase activity in a dose-dependent manner, whereas GDP-GST-RhoA was ineffective (Fig. 3B). GTP-γ-S–GST–Rac showed only a small effect and GTP-γ-S–GST–H-Ras had no effect (Fig. 3C). A brain serine-threonine kinase, PAK, that interacts with Cdc42 and Rac, members of Rho family, has been identified (*12*). PAK is activated by GTP-γ-S–GST–Cdc42 and GTP-γ-S–GST–Rac but not by GTP-γ-S–GST–RhoA. PKN appears to be distinct from PAK on the basis of its mode of activation and its structural properties.

Because PKN is composed of an NH_2-terminal regulatory domain and a COOH-terminal catalytic domain (*10*), we tested whether RhoA directly binds the NH_2-terminal domain of PKN. The NH_2-terminal domain of PKN (amino acids 7 to 155) was expressed as a maltose-binding protein (MBP) fusion protein and mixed with beads coated with GST-RhoA. The MBP-PKN fusion protein was retained on the GTP-γ-S–GST–RhoA beads and could be eluted by 0.2 M NaCl and eluted with GTP-γ-S–GST-RhoA by addition of glutathione (Fig. 4A). The fusion protein was not retained on beads coated with GST, GDP-GST–RhoA, GTP-γ-S–GST–RhoA, GTP-γ-S–GST–RhoA^{A37}, GTP-γ-S–GST–Rac, or GTP-γ-S–GST–H-Ras. MBP-PKN also interacted with GTP-γ-S–GST–RhoB and GTP-γ-S–GST–RhoC to a similar extent as it did with GTP-γ-S–GST–RhoA (*13*). Scatchard analysis indi-

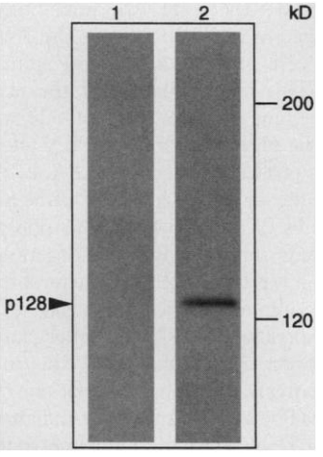

Fig. 2. Identification of p128 as PKN. Immunoblot analysis of p128 was done with preimmune serum (lane 1), or with antibody to PKN as described (lane 2) (*11*).

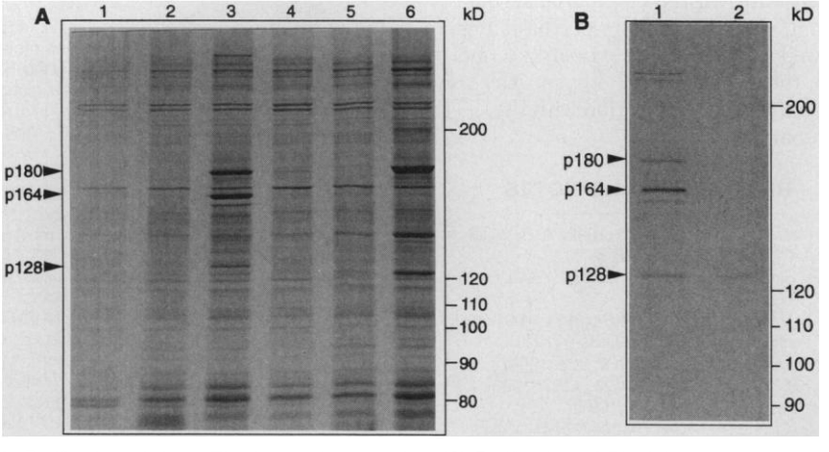

Fig. 1. Purification of three Rho-interacting proteins. (**A**) Purification of RhoA-interacting proteins by GST-RhoA affinity-column chromatography (*15*). The crude membrane extract was loaded on a glutathione-Sepharose column containing GST (lane 1), GDP-GST-RhoA (lane 2), GTP-γ-S–GST–RhoA (lane 3), GTP-γ-S–GST–RhoA^{A37} (lane 4), GDP-GST-Rac (lane 5), or GTP-γ-S–GST–Rac (lane 6). Bound proteins were eluted with the respective GST fusion proteins by addition of glutathione. Portions (45 μl each) from the glutathione-eluted fractions were subjected to SDS-PAGE followed by silver staining. (**B**) Purification of p128 by DEAE-Sepharose column chromatography (*16*). The silver-stained gel shows eluates obtained from the GST-RhoA affinity column with buffer containing 0.2 M NaCl (lane 1) and from the DEAE-Sepharose column with buffer containing 75 mM NaCl (lane 2). The results shown are representative of three independent experiments.

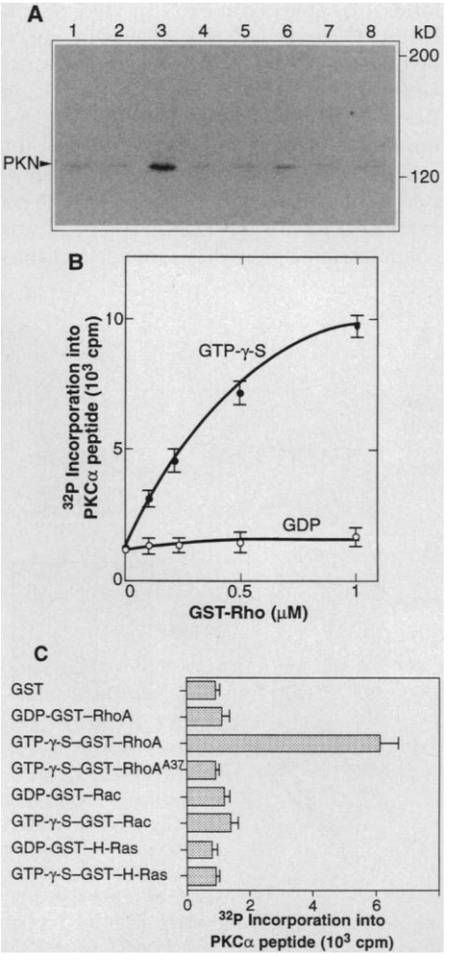

Fig. 3. Autophosphorylation of PKN and phosphorylation of an exogenous substrate by PKN. (**A**) Autophosphorylation of PKN. PKN was autophosphorylated in the presence of various small GTPases (50 pmol each) (*17*). Lanes are as follows: GST control (lane 1), GDP-GST-RhoA (lane 2), GTP-γ-S–GST–RhoA (lane 3), GTP-γ-S–GST–RhoA^{A37} (lane 4), GDP-GST-Rac (lane 5), GTP-γ-S–GST–Rac (lane 6), GDP–GST–H-Ras (lane 7), and GTP-γ-S–GST–H-Ras (lane 8). The results shown are representative of three independent experiments. (**B**) Dose-dependent activation by RhoA of PKN kinase activity on a PKCα peptide. The kinase reaction was carried out with PKCα peptide (40 μM) in the presence of the indicated amounts of GDP-GST-RhoA or GTP-γ-S–GST–RhoA (*17*). The values shown are means ± SEs of triplicates. (**C**) Effects of various small GTPases on the kinase activity of PKN. The kinase reaction was done with PKCα peptide (40 μM) in the presence of various small GTPases (50 pmol each) as indicated (*17*). The values shown are means ± SEs of triplicates. Because purified PKN is labile, we used it for the kinase assay within 1 day after purification.

cated that MBP-PKN formed a 1:1 complex with GTP-γ-S–GST–RhoA. The apparent K_d value for MBP-PKN was estimated to be about 0.4 μM. Identical patterns of retention were observed when a longer NH_2-terminal domain of PKN (amino acids 7 to 540) was used.

We examined whether activated RhoA forms a complex with PKN in vivo. When $RhoA^{V14}$ (activated RhoA) was immunoprecipitated from COS-7 cells transfected with complementary DNAs encoding $RhoA^{V14}$ and PKN, some PKN was also immunoprecipitated (Fig. 4B). On the other hand, when wild-type RhoA was immunoprecipitated from the COS-7 cells overexpressing RhoA and PKN, little PKN was coimmunoprecipitated. Coimmunoprecipitation of PKN with RhoA was only detected if $RhoA^{V14}$ or RhoA was overexpressed in COS-7 cells. Some $RhoA^{V14}$ was also coimmunoprecipitated with PKN when PKN was immunoprecipitated from the COS-7 cells overexpressing $RhoA^{V14}$ and PKN (*13*). Overexpression of $RhoA^{V14}$ with PKN stimulated the kinase activity of PKN 2.2 ± 0.4–fold.

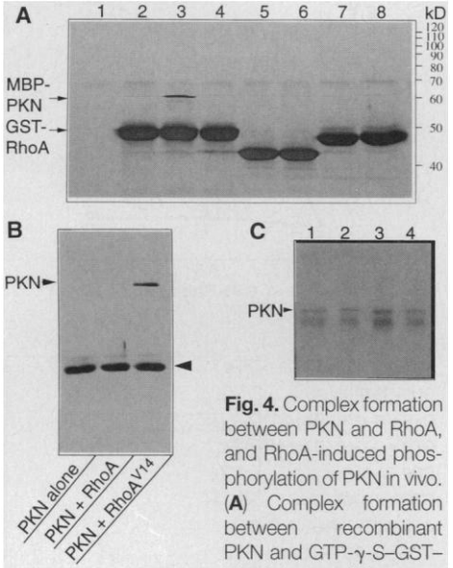

Fig. 4. Complex formation between PKN and RhoA, and RhoA-induced phosphorylation of PKN in vivo. (**A**) Complex formation between recombinant PKN and GTP-γ-S–GST–RhoA in a cell-free system. MBP-PKN was mixed with glutathione-Sepharose beads coated with GST (lane 1), GDP-GST-RhoA (lane 2), GTP-γ-S–GST–RhoA (lane 3), GTP-γ-S–GST–$RhoA^{A37}$ (lane 4), GDP-GST-Rac (lane 5), GTP-γ-S–GST–Rac (lane 6), GDP–GST–H-Ras (lane 7), or GTP-γ-S–GST–H-Ras (lane 8), and MBP-PKN was eluted by addition of glutathione (*18*). (**B**) Complex formation between RhoA and PKN in COS-7 cells. RhoA or $RhoA^{V14}$ was immunoprecipitated from COS-7 cells overexpressing RhoA or $RhoA^{V14}$ and PKN (*19*). The washed immunoprecipitates were immunoblotted for the presence of PKN (*11*). Arrowhead denotes the position of the mouse immunoglobulin G heavy chain. (**C**) Stimulation of PKN phosphorylation by LPA. Swiss 3T3 cells were seeded in a 35-mm dish and treated with bolulinum C3 exoenzyme (10 μg/ml, lanes 2 and 4) or without it (lanes 1 and 3) as described (*20*). The cells were labeled with 18.5 MBq of [^{32}P]orthophosphate for 2 hours and treated with LPA (200 ng/ml, lanes 3 and 4) or without it (lanes 1 and 2) for 10 min. The cells were then lysed and PKN was immunoprecipitated. The washed immunoprecipitates were subjected to SDS-PAGE for autoradiography. The faster-migrating band may be degraded PKN. The results shown are representative of three independent experiments.

We tested whether LPA activates PKN in vivo. When Swiss 3T3 cells were stimulated by LPA, stress fibers formed (*2*). We measured the kinase activity of PKN immunoprecipitated from Swiss 3T3 cells that were stimulated by LPA and could not find that PKN was reproducibly activated. We assume that PKN was activated by GTP-Rho during the action of LPA, but that GTP-Rho was immediately converted to GDP-Rho during the disruption of the cells and the immunoprecipitation of PKN. This may explain why the activated PKN became inactive. Alternatively, we measured the phosphorylation of PKN after stimulation of Swiss 3T3 cells by LPA. LPA stimulated the phosphorylation of PKN in Swiss 3T3 cells 2.0 ± 0.4–fold. This phosphorylation was inhibited when endogenous Rho function was blocked by treatment of the cells with botulinum C3 exoenzyme (Fig. 4C). The phosphorylation of PKN may reflect autophosphorylation as stimulated by Rho in vitro. Alternatively, the phosphorylation may be catalyzed by a distinct Rho-dependent kinase.

Our results provide evidence that PKN is a putative target for Rho and may serve as a mediator of the Rho-dependent signaling pathway. Although the catalytic domain of PKN is highly related to that of PKC, the NH_2-terminal domain is distinct from those of other protein kinases (*10*). Rho interacts directly with this NH_2-terminal regulatory domain, which contains a polybasic region followed by a leucine zipper-like motif. Thus, the NH_2-terminal domain may confer specificity for interaction with the GTP-Rho complex.

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15. The crude membrane fraction was prepared from bovine brain gray matter (200 g) (*14*). The proteins of the membrane fraction were extracted by addition of an equal volume of homogenizing buffer [25 mM tris-HCl (pH 7.5), 5 mM EGTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 10% sucrose] containing 4 M NaCl (100 ml), and the extract was dialyzed against buffer A [20 mM tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and 5 mM $MgCl_2$]. Solid ammonium sulfate was added to a final concentration of 40% saturation. The precipitate was dissolved in 16 ml of buffer A, dialyzed against buffer A, and then passed over a 1-ml glutathione-Sepharose column. One-eighth of the fraction that passed through the column (2 ml) was loaded onto a 0.25-ml glutathione-Sepharose column containing 6 nmol of respective small GTPases preloaded with guanine nucleotides as indicated. The columns were washed with 2.5 ml of buffer A and bound proteins were eluted with respective small GTPases by addition of 0.825 ml of buffer A containing 10 mM glutathione.
16. The fraction that passed through the glutathione-Sepharose column (16 ml) was loaded onto a 1-ml glutathione-Sepharose column containing 24 nmol of GTP-γ-S–GST–RhoA. p128 was eluted by addition of buffer A containing 0.2 M NaCl. The sample was dialyzed against buffer A and applied to a 0.3-ml DEAE-Sepharose column equilibrated with buffer A. The column was washed with 1.5 ml of buffer A containing 50 mM NaCl, and proteins were eluted with 1.5 ml of buffer A containing 75 mM NaCl. Fractions of 0.3 ml were collected, and portions (30 μl each) were subjected to SDS-PAGE followed by silver staining. p128 appeared as a single peak in fractions 1 through 3.
17. The kinase reaction was carried out in 50 μl of kinase buffer [50 mM tris-HCl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, and 0.06% CHAPS detergent] containing 2 μM [γ-^{32}P]ATP [600 to 800 gigabecquerel (GBq) per millimole and purified PKN (10 ng of protein) with or without 40 μM PKCα peptide. After incubation for 10 min at 30°C, the reaction mixtures were boiled in SDS sample buffer and subjected to SDS-PAGE for the autophosphorylation assay. The radiolabeled bands were visualized by autoradiography. The reaction mixtures were spotted onto a piece of Whatman p81 paper for the kinase assay (*11*). Incorporation of ^{32}P into the PKCα peptide was assessed by scintillation counting.
18. The NH_2-terminal domain of PKN (amino acids 7 to 155) was expressed as an MBP fusion protein and purified with amylose resin (New England Biolabs). MBP-PKN (0.2 nmol) was mixed with glutathione-Sepharose beads (30 μl) containing 0.75 nmol of GST, GDP-GST-RhoA, GTP-γ-S–GST–RhoA, GTP-γ-S–GST–$RhoA^{A37}$, GDP-GST-Rac, GTP-γ-S–GST–Rac, GDP-GST–H-Ras, or GTP-γ-S–GST–H-Ras in 0.8 ml of buffer A. MBP-PKN was eluted three times by addition of 0.1 ml of buffer A containing 0.2 M NaCl and then three times by addition of 0.1 ml of buffer A containing 10 mM glutathione. Portions (30 μl each) of the first fraction of the glutathione eluate were subjected to SDS-PAGE followed by silver staining.
19. The plasmids pMh-PKN7 and pTB701-HA-RhoA or pTB701-HA-$RhoA^{V14}$ were transfected into COS-7 cells to express PKN and RhoA or $RhoA^{V14}$ tagged with hemagglutinin (HA) (*11*). After 48 hours, the cells were harvested, suspended in lysis buffer [30 mM tris-HCl (pH 7.5), 0.5 mM Na_3VO_4, 5 mM NaF, leupeptin (2.5 μg/ml), 0.05% NP-40, and 0.05 M NaCl], and homogenized in a Dounce homogenizer. The cytosol was subjected to immunoprecipitation by antibody to HA (12CA5).
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