A Novel Serine/Threonine Kinase Binding the Ras-related RhoA GTPase Which Translocates the Kinase to Peripheral Membranes*

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We previously reported the cloning of a serine/threonine kinase, PAK (for p21 (Cdc42/Rac)-activated kinase), which binds to the Ras-related GTPases Cdc42Hs and Rac1 (Manser, E., Leung, T., Salihuddin, H., Zhao, Z-s., and Lim, L. (1994) Nature 367, 40–46). These p21 proteins together with RhoA comprise the Rho subfamily of proteins that are involved in morphological events. We now report the isolation of a rat cDNA encoding a 150-kDa protein, which specifically binds RhoA in its GTP form and contains an N-terminal serine/threonine kinase domain highly related to the human myotonic dystrophy kinase and a cysteine-rich domain toward the C terminus. The RhoA binding domain is unrelated to other p21 binding domains. Antibody raised against the kinase domain of the predicted protein, termed ROKα (for ROKα, RhoA-binding kinase), recognized a ubiquitous 150-kDa protein. The brain p150 purified by affinity chromatography with RhoA exhibited serine/threonine kinase activity. In cultured cells, immunoreactive p150 was recruited to peripheral membranes, which is consistent with the kinase being a specific target for RhoA.

MATERIALS AND METHODS

Expression Screening—A rat brain cZAP library (Stratagene) was used for the expression screening. Plates containing phages 6 h after plating were induced for 16 h with nitrofurazone filters (20×20 cm) previously soaked with 10 mM isopropanol-β-thiogalactoside. Filters were denatured with 6 M guanidine chloride in decreasing concentrations and returned to a renaturation buffer containing phosphate-buffered saline with 1% bovine serum albumin, 0.5 mM MgCl2, 0.1% Triton X-100 and 5 mM dithiothreitol. Filters were probed with solution containing [32P]labeled glutathione S-transferase (GST)/RhoA and washed as described (7). Plaque showing binding were detected by autoradiography. Six overlapping clones (rbf-1 to rbf-6) were obtained from 5×107 plaques after secondary and tertiary screening.

Sequence Analysis and Construction of Expression Vectors—Sequencing of exonuclease III/S1 nuclease nested-deleted subclones was carried out in both directions using the Sequenase sequencing kit (U. S. Biological Corp.). The construct rbf-7 was derived from a HindIII digest of rbf-1 and cloned in the expression vector pMAL (New England Biolabs) encoding maltose-binding protein (Mpl) from vector polylinker site. Likewise, rbf-8 was derived from a SpeI HindIII fragment of rbf-1. For raising antibodies, a 5‘ HindII fragment (corresponding to amino acid residues 33–316) was subcloned in frame into the pMAL vector for expression. Fusion proteins of rbf-7 and rbf-8 in pMAL vector were prepared according to the recommended protocol, separated on 9% SDS-polyacrylamide gels, blot-transferred to nitrocellulose membrane, and renatured for p21 binding assays (8).

Tissue and Cell Preparation, Detection of p21 Binding Activities, and Western Blotting—Rat C6 glial, human SK-N-SH neuroblastoma, mouse NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/liter glucose and maintained with 5% CO2. Rat cerebellar granule cells were cultured as described by Leung (11). Adult Sprague-Dawley rat tissues and cultured cells were homogenized in extraction buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.1% Triton X-100, 2 μg/ml each of leupeptin, pepstatin, and apronin. Soluble extracts (150 μg) obtained by centrifuging at 100,000×g for 30 min at 4 °C were separated on 9% SDS-polyacrylamide gels, blot-transferred to nitrocellulose membrane, and renatured for RhoA binding assays as described earlier. For immunological analysis, blots were first blocked with 5% skim milk before using mouse polyclonal antiserum or a monoclonal antibody 1A1 raised against the N-terminal kinase domain. To determine the specificity and nucleotide dependence of p21 binding, GST/RhoA and GST/Cdc42 (8) were phosphorylated using chicken-mediated formation of stress fibers (6). In searching for potential targets of different p21 proteins of the Rho family, we developed methods for detecting direct interaction of the p21 proteins with putative target proteins and isolated cDNAs encoding the tyrosine kinase ACK1 (7) and the serinethreonine kinase PAK (8). ACK and PAK have related sequences responsible for the interaction with Cdc42 and Cdc42/Rac, respectively (8). We have also demonstrated that there are cellular proteins that can specifically interact with RhoA, with a p350 being ubiquitously present in all tissues examined (8). We now report the isolation and characterization of a cDNA encoding this RhoA binding protein. The deduced amino acid sequence predicts a N-terminal serinethreonine kinase domain homologous to myotonic dystrophy kinase (9, 10). A 90-amino acid region contains a distinctive motif that binds RhoA in the GTP-bound form. Overexpression of RhoA results in the recruitment of ROKα to peripheral membranes, which is consistent with the kinase being a specific target for RhoA.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U38481. § To whom correspondence should be addressed: Glaxo-IMCB Group, Institute of Molecular and Cell Biology, National University of Singapore, Kent Ridge, Singapore 0511. Tel.: 65-772-6167; Fax: 65-774-0742.

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The activation of cells by a number of growth factors through their surface receptors specifically affect the cytoskeleton. Some of these effects such as the formation of stress fibers (1, 2) lamellipodia (3), and filopodia (4, 5) can be achieved by direct microinjection into cells of specific p21 proteins of the Rho subfamily, indicating that these p21 proteins act downstream of these growth factor receptors. Very little information is available concerning the signaling pathways beyond this point. A recent report has implicated a tyrosine kinase in the RhoA-

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1 The abbreviations used are: ACK, activated Cdc42Hs-associated kinase; PAK, p21 (Cdc42/Rac)-activated kinase; ROKα, RhoA-binding kinase; GST, glutathione S-transferase; MEF, 4-morpholinoolactonesulfonic acid; HA, hemagglutinin; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; GTPγS, guanosine 5′-3-O-(thiotri)phosphate; rbf, Rho-binding DNA fragment.
protein kinase A (Sigma) and [γ-32P]ATP (Amersham). The resulting labeled p21 proteins (about 106 cpm/μg protein) were probed with either GDP or GTP-γ-S (Boehringer Mannheim) as described (17) for probing filters containing maltose-binding protein/rbl-7 and maltose-binding protein/rbl-8 fusion proteins (0.1 μg/lane). The concentration of [32P]-labeled GST/RhoA and GST/Cdc42 used in the binding assay was 0.1 μM. After 30 min at 4°C, filters were washed three times with "wash buffer" (12) and exposed to Hyperfilm for 4–6 h.

Purification of a p150 Kinase from Rat Brain—Rat brain cytosol from 20 adult male rats (about 40 g) in Q buffer containing 25 mM Tris-HCl, pH 8.0, 0.5 mM MgCl2, 0.05% Triton X-100, and 0.1 mM EDTA was loaded onto an 80-ml Sepharose-4-C column (Pharmacia Biotech Inc.), washed with 120 ml of buffer Q containing 0.1 mM NaCl. The p150-enriched 0.3 M NaCl eluent, as determined by RhoA binding and immuno blotting assays, was diluted to 0.1 M NaCl with S buffer containing 25 mM MES-NaOH, pH 6.0, 0.5 mM MgCl2, 0.05% Triton X-100 and loaded onto a 20-ml column with Sepharose-S (Pharmacia). This was washed with 50 ml of S buffer with 80 mM NaCl and eluted at 0.2 M NaCl. This p150-enriched fraction was then loaded onto a 1-ml glutathione-Sepharose column preloaded with 5 mg/ml GST/RhoA. After washing with three column volumes of S buffer, binding proteins were eluted with Q buffer of increasing pH (pH 6–9) before finally debinding with 5 mM glutathione. The presence of p150 ROKα was confirmed by immunoblotting with the specific antibody with the fraction eluted at pH 8 (E3) giving the best recovery of purified p150. Kinase assays and subsequent phosphoamino acid analysis were performed as described previously (8).

Transient Transfection and Morphological Analyses—Cos-7 cells were grown in DMEM plus 10% fetal bovine serum (FBS) (Life Technologies, Inc.) in 5% CO2. Cells at 80% confluence were transfected with vector pXj 40 (13) containing the hemagglutinin (HA) tag as control or vectors with wild type RhoA or activated RhoAV14 using Lipofectamine (LifeTechnologies, Inc.) in 5% CO2. Cells at 80% confluence were transfected with pXj40 vector with either RhoAV14 or Cdc42V12, cells were fixed with 4% paraformaldehyde and immunostained for ROKα and p21 proteins (about 108 cpm/g of protein) were resolved on a 10% polyacrylamide gel for autoradiography. Membrane association of RhoA was determined by Western blotting with antibodies to ROKα or to HA (Boehringer, Mannheim, Germany). For cell staining, HeLa cells were grown in multichamber tissue culture slides (Lab-Tek) in DMEM supplemented with 10% FBS and maintained at 37°C in 5% CO2. After 16 h of transfection with pXj40 vector with either RhoA14 or Cdc42Q12, cells were fixed with 4% paraformaldehyde and immunostained for ROKα expression using monoclonal antibody 1A1/fluorescein isothiocyanate-conjugated anti-mouse IgG, and for p21 expression using rabbit anti-HA/horseradish anti-rabbit IgG. Tetramethylrhodamine β-isothiocyanate-conjugated anti-mouse IgG (Sigma; 0.5 μg/ml) was used for staining filamentous actin. Confocal imaging was with a MRC600 system with a Zeiss microscope.

RESULTS

Using GTP-Cdc42 as a probe for expression screening, we previously isolated a novel brain Cdc42-binding tyrosine kinase (7). A similar approach was employed to identify cDNAs encoding proteins binding GTP-RhoA in a rat brain cDNA expression library. Six clones were obtained, which contained overlapping sequences, all of which encoded the RhoA binding domain (Fig. 1A). We then derived a small region (about 90 amino acids; rbf-8) responsible for the p21 binding (Fig. 1B). The sequence was dissimilar to the binding motifs for Cdc42/Rac1 (8). RhoA was only bound in its GTP form. Little or no Cdc42 was bound (Fig. 1C).

The complete sequence of a putative 159-kDa protein was obtained from analysis of the overlapping cDNAs (Fig. 2A). It contained a novel serine/threonine kinase most closely related to the human myotonic dystrophy kinase (9, 10) (53% identity) and a p180 Cdc42-binding kinase (53% identity) recently identified in our laboratory,2 as well as to the product of the fungal cot-1 gene (14) essential for hyphal elongation and the Drosophila “warts” gene (15) implicated in cell growth and morphology (Fig. 2B). The RhoA-binding kinase (termed ROKα) also contains a cysteine/histidine-rich domain at the C terminus (Fig. 2C); this domain had some similarity to those of the PKCε and chimaerin families (16), but the spacing of the invariant cysteines is not consistent with its being a diacylglycerol receptor. Between the kinase and RhoA binding domains, the sequence is predicted to assume an α-helical coiled-coil structure (data not shown).

We have been unable to express full-length recombinant ROKα protein in Escherichia coli. However, the kinase domain could be expressed as a fusion protein with maltose-binding protein which was used to raise polyclonal antiserum. An immunoreactive 150-kDa protein was detected in all tissues, including brain as well as cultured rodent and human cells (Fig. 3A), which appeared to correspond to a ubiquitous p150 RhoA-binding protein previously described (8). The native 150-kDa RhoA-binding protein was then purified from rat brain cytosol by affinity chromatography with GST/RhoA fusion protein. The purified protein bound RhoA and was recognized by the antiserum to ROKα (Fig. 3A, lane E3). The p150 was capable of autophosphorylation and of phosphorylating myelin basic protein at serine and threonine residues (Fig. 3B). Although GTP-RhoA did not appear to stimulate p150 kinase activities toward GTP-γ-S, GDP, or GTP-γ-S, which were exchanged with either GTP-γ-S or GDP, and used for binding to fusion proteins expressed from rbf-7 (upper band) and rbf-8 (lower band).

2 T. Leung and L. Lim, unpublished observations.
either RhoA or RhoAV14, both of which are effective in promotion screening (rbf-1 to rbf-6). Restriction sites refer to those shown in two overlapping clones as well as other cDNAs isolated by expression screening.

domains are shown in bold. The invariant residues present in most CRDs of this class (16) are in bold, and marked with (*). A diagrammatic representation of the CRD(a) of ROKn, human Raf-1, and rat PKCα (accession no. P28867) using the CLUSTAL method (DNASTAR). C, alignment of cysteine-rich domains (CRD) of ROKα, rat PKCα, human Raf-1, and rat n-chimaerin.

The invariant residues present in most CRDs of this class (16) are in bold and marked with asterisks. The position of His as a potential substitution for consensus Cys is marked (+).

transfected with empty vector (Fig. 4A). Upon transfection with either RhoA or RhoAV14, both of which are effective in promoting stress fiber formation when microinjected into cells (1), there was an increase in p150 ROKn binding to membranes. This association was investigated at the cytological level using HeLa cells transfected with the dominant-positive RhoAV14 mutant. In HeLa and other cells examined, ROKn was distributed in the cytoplasm. RhoAV14-transfected cells showed a generally rounded morphology with increased actin microfilaments mainly at the cell periphery (Fig. 4B, panel b), but also present in stress fibers adjacent to the substratum (Fig. 4B, panel c). Much of the endogenous ROKα immunoreactivity co-localized with the actin microfilaments at the peripheral cell membrane (Fig. 4B, panel a) and cultured cells (Fig. 4B, panel f). Neither was membrane translocation occurred with ROKα immunoreactivity being generally cytosolic (Fig. 4B, panel f). Neither was PKCα (analyzed as another control) membrane-associated in RhoAV14-transfected cells (data not shown). We conclude that there is a specific recruitment of ROKα to plasma membrane with activated RhoA.

DISCUSSION

ROKα is a member of a serine/threonine kinase family, which includes the myotonic dystrophy kinase (9, 10) Neurospora cot-1, and the Drosophila warts gene product (15), as well as a recently isolated Cdc42-binding kinase. The myotonic dystrophy kinase may be involved in membrane functions related to ion channels (17, 18) while cot-1 and warts mutants show abnormal cell growth with morphological consequences. It is intriguing that the related mammalian ROKα contains a RhoA binding domain. This domain is unrelated to the other known p21 binding domains and does not affect intrinsic or p190 RhoGAP-stimulated GTPase activities of RhoA (data not shown), unlike PAK or ACK, which inhibit the GTPase activi-
Membrane Association of RhoA-binding Kinase

In the in vivo effects of RhoA in promoting membrane association of ROKα, we have found that HA-tagged ROKα is activated when co-transfected with RhoA on enzymatic analysis of HA-immunoprecipitates (data not shown). Most cells with membrane-associated ROKα showed a more rounded morphology, with the kinase being co-localized with peripheral actin microfilaments. No correlation of ROKα localization with stress fibers was observed, although the latter are increased in RhoA-overexpressing cells. This suggests that ROKα may not be directly involved in regulating or maintaining stress fibers. However, apart from increased stress fibers a frequent consequence of RhoA transfection is the occurrence of cells with rounded morphology. Filamentous actin reorganization is essential in the rounding up process and in the Rho-dependent formation of the contractile ring, which precedes cell division (19), and it is possible that ROKα participates in these events. In addition to morphology and cytokinesis (19, 20), evidence is accumulating for the involvement of Rho proteins in motility (21), transformation (22, 23), and apoptosis (24). Our finding of a RhoA-binding kinase should prove helpful in determining the mechanisms underlying these important cellular activities as well as the pathological basis of myotonic dystrophy because of the similarity of the dystrophic kinase to ROKα.

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FIG. 4. RhoA-dependent membrane association of ROKα. A, Cos-7 cells were transiently transfected with vector containing a HA tag alone (panel 1), or vector with either RhoA (panel 2) or RhoA V14 (panel 3). Soluble (s) and pellet (p) fractions (100 μg) from transfected cells were separated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose for Western blotting using antibodies to ROKα or anti-HA for the p21 proteins. B, in panels a–c, HeLa cells were transiently transfected with the vector containing HA-tagged RhoA V14. After 16 h, cells were fixed and stained with monoclonal antibody 1A1 for ROKα (a). Actin microfilament distribution at the same (confocal) level of the membrane where ROKα was located upon RhoA V14 expression is shown in b. Stress fibers of these cells are located at their base in contact with the substratum (c). Cells expressing RhoA V14 (d and e) or Cdc42 V12 (f and g) were double-stained for ROKα (d and f) and for the HA-tagged p21 proteins (e and g). Low levels of p21 expression were sometimes not detectable with HA staining. Bar = 10 μm.
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