Rhotekin, a New Putative Target for Rho Bearing Homology to a Serine/Threonine Kinase, PKN, and Rhophilin in the Rho-binding Domain*

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Using a mouse embryo cDNA library, we conducted a two-hybrid screening to identify new partners for the small GTPase Rho. One clone obtained by this procedure contained a novel cDNA of 291 base pairs and interacted strongly with RhoA and RhoC, weakly with RhoB, and not at all with Rac1 and Cdc42Hs. Full-length cDNAs were then isolated from a mouse brain library. While multiple splicing variants were common, we identified three cDNAs with an identical open reading frame encoding a 61-kDa protein that we named rhotekin (from the Japanese “teki,” meaning target). The N-terminal part of rhotekin, encoded by the initial cDNA and produced in bacteria as a glutathione S-transferase fusion protein, exhibited in vitro binding to [35S]labeled guanosine 5′-O-(thio)triphosphate-bound Rho, but not to Rac1 or Cdc42Hs in ligand overlay assays. In addition, this peptide inhibited both endogenous and GTPase-activating protein-stimulated Rho GTPase activity. The amino acid sequence of this region shares ~30% identity with the Rho-binding domains of rhophilin and a serine/threonine kinase, PKN, two other Rho target proteins that we recently identified (Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakuzuka, A., and Narumiya, S. (1996) Science 271, 645-648). Thus, not only is rhotekin a novel partner for Rho, but it also belongs to a wide family of proteins that bear a consensus Rho-binding sequence at the N terminus. To our knowledge, this is the first conserved sequence for Rho effectors, and we have termed this region Rho effector motif class 1.

The Ras superfamily of small GTPases encompasses a group of ubiquitous regulatory proteins related by both structure and function. The products of such genes are involved in a plethora of intracellular signaling processes (1). These proteins are generally regarded as being activated in the GTP-bound form. The intrinsic hydrolytic activity of these proteins is responsible for the reversion to the resting GDP-bound form. Proteins of the Ras subfamily play a pivotal role in the regulation of cytoskeletal organization and the determination of cell polarity. Strongly linked to the formation of stress fibers and focal adhesions (2), regulation of cell motility (3), aggregation (4, 5), cell cycle progression (6), and contractile ring formation and cytokinesis (7, 8), the Ras proteins occupy key positions in many fundamental cellular processes.

A large number of regulatory proteins for Ras have been characterized, including nucleotide exchange proteins (9–11), GTPase-activating proteins (GAPs) (12, 13), and guanine nucleotide dissociation inhibitors (14, 15). In contrast, there has been surprisingly little information available on the nature of the molecules that are directly regulated by Ras. Recently, Ras has been proposed to regulate phosphatidylinositol 4-5-phosphate 5-kinase and to regulate actin polymerization through increases in phosphatidylinositol 4,5-bisphosphate levels (16). However, a direct interaction with a regulatory element that may give rise to this effect has yet to be demonstrated.

The two-hybrid system was used successfully to demonstrate in vivo interactions between Ras and its downstream effectors, Byr-1 (17), Raf-1, and CYR1 (18). More recently, this system has been used to dissect precisely which features of Ras are involved in interactions with multiple effectors and how this contributes to oncogenesis (19). To isolate and examine downstream Ras targets, we conducted a library screening using the yeast two-hybrid system and complemented our data with in vitro confirmation of the interaction. By these procedures, we have identified a Ser/Thr protein kinase (PKN), a PKN-related protein (rhophilin), and a 180-kDa coiled coil-containing protein (citron) as potential Rho target molecules (20, 21). We have also isolated a novel Ser/Thr protein kinase (p160ROCK), as a potential Rho effector (22) that displays a structural similarity to citron. The present report describes the isolation of a new putative target protein that binds to the GTP-bound form of Ras and inhibits its GTPase activity. The Rho-binding region of this protein appears to be related to those of PKN and rhophilin.

EXPERIMENTAL PROCEDURES

Materials—[35S]GTP-γ-S (1000 Ci/mmol), [35S]GDP-β-S (1000 Ci/mmol), and [32P]GTP (6000 Ci/mmol) were obtained from DuPont NEN. Plasmids pGEX-rhoA (23), pGEX-rac1 (24), and pGEX-CDC42Hs (25) (gifts of Dr. Yoshimi Takai, Osaka University, Osaka, Japan) and pGEX-KG-rhoGAP (26) (a gift of Dr. Alan Hall, University College, London, UK) were used as expression vectors.


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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U54638.

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Yeast Two-hybrid System Screening—Two-hybrid system screening was conducted essentially as described previously (18), except that strain AM70 was transformed in conjugation and in trans 10 times in the mating strategy. The initial screening was conducted with a RhoC mutant with a deletion at residue 181, lacking the CAAX box and the polybasic region. Deletion was carried out through PCR amplification using the original rhoC cDNA (27) as a template. This PCR, using a 5′-end primer of AGCGGATCCATGCGGCCATCCGCAAAGAAG and a 3′-end primer of CCAGAATTCAGACCTGGAGGCCAGCCCGAG, introduced a BglII site at the 5′ end before codon 1 and an EcoRI site at the 3′ end after codon 181. This cDNA was then subcloned into the multiple cloning site of a modified pBTM116 plasmid (pBTM116M) (21). This vector was called pBTM116M-rhoCJC and drove the expression of a LexA-RhoCJC fusion protein. Similar deletions were made also by PCR for rhoA using a 5′-end primer of AGCGGATCCCTAGGTGCGAGCATCATCCGCAAAGAAG and a 3′-end primer of CCAGAATTCAGACCTGGAGGCCAGCCCGAG, introduced a BamHI site at the 5′ end before codon 1 and an EcoRI site at the 3′ end after codon 181. This cDNA was then subcloned into the multiple cloning site of a modified pBTM116 plasmid (pBTM116M) (21). This vector was called pBTM116M-rhoAJC and drove the expression of a LexA-RhoAJC fusion protein.

RESULTS AND DISCUSSION

We conducted a library screening with the yeast two-hybrid system in order to identify new partners for Rho. The bait vector pBTM116 drives the expression of the LexA transcription factor fused to a bait protein. The complementary plasmid, pVP16, drives the expression of a nuclear localization sequence and the VP16 transcription activation domain (VAD) fused to a random-primed day 10.5 murine embryonic cDNA library. Positive interactions between the bait and proteins expressed from the library plasmid led to the assembly of a transcriptionally active complex driving the expression of yeast HIS3 and bacterial lacZ genes. An initial screening bait was constructed by deleting the C-terminal polybasic region and the CAAX box of human RhoC (LexA-RhoCJC). This strategy was followed on the premise that this region strongly directs Ras and related proteins to the plasma membrane (31, 32) and would interfere with the nuclear localization required for transcriptional activation of the reporter genes in this assay.

We identified several clones displaying the same interaction profile. They appeared to bear the same cDNA in pVP16. Sequencing this insert revealed a novel 291-base pair cDNA, which we called C21. In the two-hybrid system, VAD-C21 was positive with rhoGAP and rhoC, and negative with rhoA, rhoB, and rhoD-J (Fig. 1A). Truncation of the C-terminal domain of Rho proteins gave rise to a far stronger interaction than did the full-length forms, possibly either because of a tendency for the CAAX box to favor localization to the plasma membrane or through an increased preference of the full-length baits to interact with endogenous yeast proteins. We presume that this reduces the amount of bait protein available for the formation of transcriptionally active complexes. There appeared to be no interaction with either LexA-Rac1 or LexA-Cdc42.

We then expressed the C21 peptide as a bacterial GST fusion protein and examined its interaction with various small GTPases in vitro by the ligand overlay assay (Fig. 1B). A specific interaction was seen with GTP-RhoA and GTP-RhoB, but not with GDP-RhoA, GTP-Rac1, or GTP-Cdc42. Our attempts to express RhoC as a GST fusion protein were unsuccessful. Taken together, these results are in agreement with the interaction profile observed in the two-hybrid system and clearly demonstrate a specific interaction with GTP-bound Rho proteins. However, due to the method of construction of the library, which included a PCR step, C21 contained one nonsense PCR mutation and 24 base pairs of the 5′-noncoding region as deduced from the full-length cDNA for rhoC (see below). To ensure that these did not contribute to or interfere
with the Rho binding properties of this peptide, we expressed a fragment of the full-length rhotekin containing the N-terminal coding sequence (amino acids 7–113). This peptide was also found to be positive in the overlay assay with GTP-RhoA (Fig. 1B). Moreover, when the full-length rhotekin coding region was introduced into pVP16, this, too, displayed an identical interaction profile in the two-hybrid system as did the original cDNA clone (Fig. 1A). This indicated that in vivo Rho binding activity is a property of the full-length protein as well as the restricted N-terminal fragment.

GST-C21 could be purified from E. coli as a soluble protein, allowing us to investigate its effect upon the endogenous and GAP-stimulated GTPase activity of RhoA in vitro. We found that this peptide inhibited both endogenous and GAP-stimulated GTP hydrolysis (Fig. 2A), and this inhibition occurred in a dose-dependent manner (Fig. 2B), indicating that not only does this protein inhibit the interaction of a GAP with Rho, but that it can also modify the inherent hydrolytic activity of the cognate GTPase. Similar interactions between a small GTPase, its effector, and GAP have been reported for Rac/Cdc42 and p120AK (29), and these interactions also occurred in vitro.

Screening a mouse brain cDNA library using C21 cDNA as a hybridization probe yielded 15 positives from 1.13 × 10⁶ independent clones. Three 2.7-kilobase cDNAs were found to be identical and presumed to be full-length (type 1 cDNA) (Fig. 3A). Type 2 and thymus. The size of the transcript appeared to be different in some tissues, and there appeared to be multiple mRNA species in kidney. Consistent with this finding, multiple splicing arrangements were detected also in the brain library, and these inserts appeared also to be full-length (Fig. 3A). Type 2
cDNA contains two exon changes at nucleotide 376 (sequence GAG/GC) and at nucleotide 1910 (sequence ATG/GC). The former was localized in the 5'-noncoding region, and the latter caused a 185-base pair insert in the 3'-region of type 1 cDNA. The third splicing variant showed an exon change at nucleotide 662 (sequence GAG/GA) of type 1 cDNA and had a different 5'-end (type 3 cDNA; data not shown). As only one cDNA clone was obtained for each of types 2 and 3, they were not fully characterized. Type 1 cDNA featured a single open reading frame starting at the ATG codon at base 591 and encoding a protein of 551 amino acid residues with a calculated molecular mass of 61 kDa, which we named rhotekin (Fig. 3B). Two proline-rich motifs were found toward the C terminus of rhotekin (amino acids 421–427 (PAPRKPP) and amino acids 525–533 (PLPPQRSPK)). Such regions have recently been described as general cognate ligands for numerous SH3 groups (34). C21 cDNA covers nucleotides 567–858, which encodes the rhotekin N-terminal peptide (amino acids 1–89). This, together with the finding obtained with the rhotekin fragment (amino acids 7–113), could locate the Rho-binding domain within amino acids 1–113.
This region specifically binds GTP-Rho and may constitute the first consensus effector sequence for Rho small GTPases.

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Fig. 4. Tissue distribution of the rhotekin transcript. Poly(A)+ RNA was prepared from murine tissues, and 2 μg of each sample was loaded. Full-length rhotekin cDNA was used as template for the probe. Lanes are as follows: B, brain; H, heart; T, thymus; Lu, lung; L, liver; SI, small intestine; LI, large intestine; K, kidney; S, spleen; Te, testis; and SM, skeletal muscle.