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Interaction of the Rho Family Small G Proteins with Kinectin, an Anchoring Protein of Kinesin Motor[1]

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The Rho family small G proteins are implicated in various cell functions, such as cell morphological change, cell motility, and cytokinesis. However, their modes of action in regulating these cell functions remain to be clarified. In the present study, we have isolated a cDNA encoding a protein which interacts with the GTP-bound form, but not with the GDP-bound form, of the Rho family members, including RhoA, Rac1, and Cdc42, by the yeast two-hybrid method. This protein is kinectin, known to be a vesicle membrane anchoring protein of kinesin, which is an ATPase motor transporting vesicles along microtubules.

The Rho family belongs to the small G protein superfamily and consists of the Rho, Rac, and Cdc42 subfamilies in mammal [for reviews, see (1,2)]. The Rho subfamily consists of three members, RhoA, -B, and -C (Rho); the Rac subfamily consists of two members, Rac1 and -2 (Rac); and the Cdc42 subfamily consists of one member (Cdc42). This family of small G proteins regulates various cell functions, such as cell morphological change, cell motility, cytokinesis, and smooth muscle contraction in various types of cells. In budding yeast, *Saccharomyces cerevisiae,* there is also the Rho family consisting of the Rho and Cdc42 subfamilies [for a review, see (3)]. The Rho subfamily consists of four members, *RHO1, -2, -3,* and *-4,* whereas the Cdc42 subfamily consists of one member, *CDC42.* This family is essential for cell growth by budding.

Evidence is accumulating that the Rho family members regulate reorganization of actin filaments, but it has not been clarified how they regulate it. Several direct target molecules have recently been isolated both in mammal and yeast: Protein kinase N (4,5), Rho kinase (6-8), and Citron (9) are found for Rho; PAK protein kinase is for Rac and Cdc42 (10,11); WASP is for Cdc42 (12-14) in mammal; whereas Protein kinase C1 (15,16), glucan synthase (17,18), and Bnil[3] are found for Rho1; Ste20 is for Cdc42 (19,20) in yeast. On the other hand, the Rac subfamily has been shown to regulate NADPH oxidase-dependent superoxide formation in neutrophils (21-23). These results indicate that each Rho family member has multiple target molecules.

In this study, we attempted to isolate a target molecule of Rho and succeeded in identifying

Abbreviations used: *E. coli, Escherichia coli;* PCR, polymerase chain reaction; ER, endoplasmic reticulum.

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it to be kinectin (24), known to be a vesicle membrane anchoring protein of kinesin (25), an ATPase motor transporting vesicles along microtubules (26). In subsequent analyses, it turned out that the GTP-bound form of the Rho family members, including Rho, Rac1, and Cdc42, bound to kinectin.

MATERIALS AND METHODS

Materials and chemicals. 3-Amino-1,2,4-triazole was from Nacalai Tesque, Japan, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was from Wako Pure Chemical Industries, Ltd., Japan.

Strains, media, and yeast transformations. Yeast strain L40 (*MATa trp1 leu2 his3 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was used as a host for the two-hybrid screening. Yeast cells were grown on rich media that contained 2% glucose (YPDAU), 2% Bacto-peptone (Difco Laboratories, Detroit, MI), 1% Bacto-yeast extract (Difco), 0.04% adenine sulfate, and 0.02% uracil. Yeast transformations were performed by the lithium acetate methods (27). Transformants were selected on SD medium that contained 2% glucose and 0.7% yeast nitrogen base without amino acids (Difco) and amino acids were supplemented to SD medium when required. An *E. coli* strain DH5α was used for construction and propagation of plasmids.

Molecular biological techniques. Standard molecular biological techniques were used for construction of plasmids, DNA sequencing, and PCR (28). DNA sequences were determined using ALFred DNA sequencer (Pharmacia Biotech, Inc.) and PCRs were performed using GeneAmp PCR System 2400 (Perkin Elmer). Point mutations were introduced by the PCR mutagenesis methods (29).

Plasmid construction. Various DNA fragments encoding a wild type or a mutant form of the Rho family members were cloned into the two-hybrid vector, pBTM116, as described (15).

Two-hybrid method. A strain L40 carrying pBTM116-RhoA (G14V) was transformed with a human B cell cDNA library made in pACT (kindly provided by Stephen J. Elledge). Approximately 7×10^6 transformants were screened for the growth on SD plate media lacking tryptophan, leucine, and histidine, but containing 5 mM 3-amino-1,2,4-triazole, which is a specific inhibitor of the *HIS3* gene product. His^+ colonies were then placed on the nitrocellulose filter and stained with 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside for β-galactosidase activity as described (30). From the His^+ and $lacZ^+$ positive clones obtained with this screening, library plasmids were recovered through *E. coli* transformation. The recovered plasmids were transformed again into L40 containing pBTM116-RhoA (G14V). The nucleotide sequences of plasmid DNAs which conferred the His^+ phenotype on L40 containing pBTM116-RhoA (G14V) were determined. For quantitative assay for β-galactosidase activity, cells of each transformant were cultured in SD-trp-leu medium and the β-galactosidase activity was measured according to the ONPG assay method described (31).

RESULTS

Molecular cloning of kinectin as a potential target molecule of RhoA. To search for a gene which encodes a potential target molecule of RhoA, the two-hybrid method was used. Since it was presumed that a target molecule of RhoA specifically binds to the GTP-bound form of RhoA, a dominant active form of RhoA, RhoA (G14V), carrying an amino acid substitution which is likely to keep RhoA in the GTP-bound form, was used as a bait to screen a human B cell cDNA library. Among 7×10^6 total transformants, 30 positive clones (His^+ and $lacZ^+$) were identified and the library plasmids were recovered from these clones. Among these 30 plasmids, 2 clones were found to confer both the His^+ and $lacZ^+$ phenotypes on L40 containing pBTM116-RhoA (G14V). Among these 2 clones, one clone, MI-1, conferred the clearest His^+ phenotype on L40 containing pBTM116-RhoA (G14V) (Fig. 1). DNA sequencing of the insert DNA of MI-1 revealed that MI-1 encoded amino acid positions from 630 to 935 of kinectin (GenBank accession number Z22551).

Kinectin has originally been identified to be a protein which binds to a kinesin ATPase motor (24) and subsequent analyses have suggested that kinectin is a vesicle membrane anchoring protein of kinesin (25). Kinectin is a protein of 1,356 amino acids in length and the structural feature of kinectin is that it contains a potential transmembrane region at its N-terminus and the leucine zipper region in amino acid positions from 934 to 962 (32) (Fig. 2). Interestingly, the RhoA-binding region (amino acid positions from 630 to 935) is located in a region predicted to form a coiled-coil structure.

Specific interaction of the GTP-bound form of the Rho family members with kinectin. In the

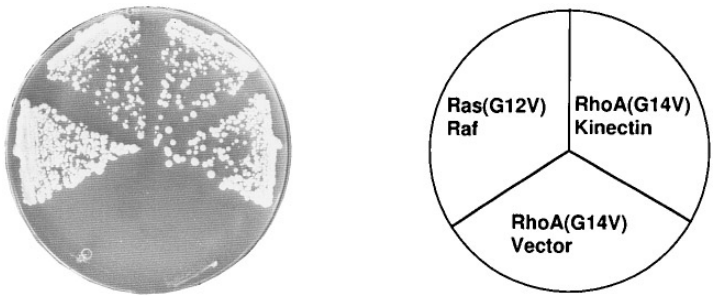

FIG. 1. Binding of RhoA (G14V) with kinectin in the two-hybrid method. Cells of yeast strains were streaked on SD-Trp-Leu-His medium containing 2 mM 3-amino-1,2,4-triazole and incubated for 3 days at 30°C. Yeast strains used were L40 containing pBTM116-Ras (G12V) and VP16-Raf [Ras (G12V), Raf], pBTM116-RhoA (G14V) and pACT-kinectin [RhoA (G14V), Kinectin], or pBTM116-RhoA (G14V) and pACT [RhoA (G14V), Vector].

next set of experiments, the specificity and extent of the interaction of the Rho family members with kinectin were investigated. As shown in Table 1, RhoA (G14V) interacted with kinectin to an extent similar to that of the interaction of Ras (G12V) with Raf, that is a target molecule of Ras (30). In contrast, the wild type of RhoA (the GDP-bound form) did not interact with kinectin. A dominant negative form of RhoA, RhoA (G17A) (the GDP-bound form or the guanine nucleotide-free form), interacted with kinectin very weakly. Moreover, RhoA (G14V, T37A), carrying an amino acid substitution in the effector domain, which interacts with its target molecule, did not interact with kinectin. These results suggest that kinectin is a target molecule of RhoA. However, surprisingly, as shown in Table 2, the dominant active form of Rac1 and Cdc42, but not the wild type of Rac1 or the dominant negative form of Rac1 and Cdc42, interacted with kinectin, although the wild type of Cdc42 weakly interacted with kinectin. These results indicate that the GTP-bound form of the Rho family members, including RhoA, Rac1, and Cdc42, specifically interacts with kinectin.

DISCUSSION

We have isolated here a RhoA-interacting molecule by the yeast two-hybrid method from a human B cell cDNA library and identified it to be kinectin. The GTP-bound form of RhoA interacts with kinectin, but the GDP-bound form or the effector domain mutant of RhoA does not interact with kinectin. Moreover, we have shown here that other Rho subfamily members, such as Rac1 and Cdc42, similarly interact with kinectin. These results suggest that kinectin

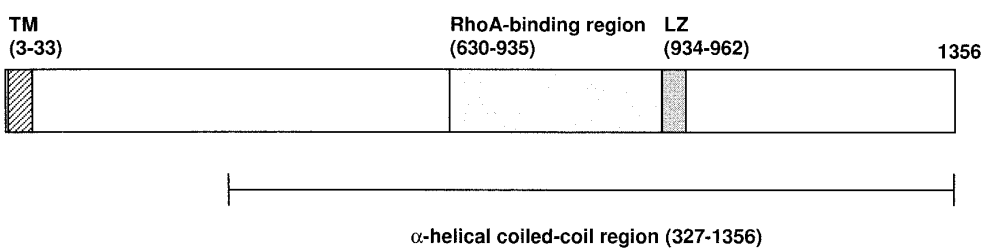

FIG. 2. Schematic representation of the region of kinectin which was cloned as a RhoA-binding region by the two-hybrid screening method. TM, transmembrane region; LZ, leucine zipper region.

TABLE 1
Two-Hybrid Interactions between RhoA and Kinectin

DBD_{LexA} fused to	AD_{GAL4} fused to	β-Galactosidase activity[a] (Units)
RhoA (G14V)	Kinectin[b]	59 ± 18
RhoA (G14V)	Vector	<1
RhoA	Kinectin	<1
RhoA (G17A)	Kinectin	12 ± 5
RhoA (G14V, T37A)	Kinectin	<1
Ras (G12V)	Raf	47 ± 13
Ras (G12V)	Kinectin	<1

[a] The values are average ± SE for three transformants.
[b] A plasmid pACT-kinectin (630–935) was used as a fusion of kinectin with the *GAL4* transcriptional activating domain (AD_{GAL4}).

is another possible downstream target molecule of the Rho family members. Recently, many potential target molecules have been identified for Rho (4-9, 15-18), Rac (10,11), and Cdc42 (10-14,19,20). Of these target molecules, Pkc1p (15,16) and Ste20p (19,20) have been shown genetically and biochemically to function as target molecules of Rho and Cdc42 in intact cells, respectively. However, concerning other target molecules, it remains to be clarified whether they actually function as target molecules of the Rho family members in intact cells. Like other target molecules, it remains to be clarified whether kinectin is a physiological target molecule of the Rho family members.

We have shown here that the amino acid positions 630 to 935 of kinectin contains a domain which interacts with the Rho family members. The primary structure of this region is not apparently homologous to those of the corresponding regions of other target molecules of the Rho family members thus far reported. This result is not surprising, because recent protein crystal structural studies have clarified that proteins without homology in primary structures often show similar three dimensional folding structures (33).

It is generally believed that microtubules do not directly interact with the plasma membrane or the membranes of intracellular organelles through their minus or plus ends. In neuronal cells, for example, vesicles transported along microtubules in an axon are transferred to actin filaments near the plasma membrane of a nerve terminal. By analogy, microtubules between ER and Golgi membranes may be also connected by actin filaments with ER and Golgi. Therefore, it could be speculated that the kinectin-containing vesicles are first formed on ER and then transported to microtubules along actin filaments.

It has been shown that kinectin (24) and the Rho family members[4] are located on ER and ER-Golgi areas in addition to other areas, respectively, and that the Rho family members are involved in reorganization of actin filaments [for reviews, see (1,2)]. The present result that the Rho family members interact with kinectin, together with these earlier observations, suggests that the Rho family-kinectin system is involved in the formation of the kinectin-containing vesicles and their transport to microtubules along actin filaments. This idea is also consistent with the recent findings that Rho and Rac are involved in vesicle transport in mast cells (34,35) and PC12 cells.[5] Further studies are necessary to establish the physiological significance of the interaction of the Rho family members with kinectin.

[4] K. Takaishi, T. Sasaki, and Y. Takai, unpublished results.
[5] R. Komuro, T. Sasaki, S. Orita, K. Takaishi, and Y. Takai, manuscript in preparation.

TABLE 2
Two-Hybrid Interactions between Rac1 or Cdc42 and Kinectin

DBD_{LexA} fused to	AD_{GAL4} fused to	β-Galactosidase activity[a] (Units)
Rac1 (G12V)	Kinectin[b]	46 ± 1
Rac1 (G12V)	Vector	<1
Rac1	Kinectin	<1
Rac1 (T17N)	Kinectin	<1
Cdc42 (G12V)	Kinectin	79 ± 2
Cdc42 (G12V)	Vector	<1
Cdc42	Kinectin	18 ± 3
Cdc42 (T17N)	Kinectin	<1

[a] The values are average ± SE for three transformants.
[b] A plasmid pACT-kinectin (630–935) was used for a fusion of kinectin with the *GAL4* transcriptional activating domain (AD_{GAL4}).

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