Rac1 small GTPase plays pivotal roles in various cell functions such as cell morphology, cell polarity, and cell proliferation. We have previously identified IQGAP1 from bovine brain cytosol as a target for Rac1 by an affinity purification method. By using the same method, we purified a specifically Rac1-associated protein with a molecular mass of about 140 kDa (p140) from bovine brain cytosol. This protein interacted with guanosine 5′-3(3-O-thio)triphosphate (GTPγS)-glutathione S-transferase (GST)-Rac1 but not with the GDP-GST-Rac1, GTPγS-GST-Cdc42, or GTPγS-GST-RhoA. The amino acid sequence of this protein revealed that p140 is identified as a product of KIAA0068 gene. We denoted this protein as Sra-1 (Specifically Rac1-associated protein). Recombinant Sra-1 interacted with GTPγS-GST-Rac1 and weakly with GDP-Rac1 but not with GST-Cdc42 or GST-RhoA. The N-terminal domain of Sra-1 (1–407 amino acids) was responsible for the interaction with Rac1. Myc-tagged Sra-1 and the deletion mutant capable of interacting with Rac1, but not the mutants unable to bind Rac1, were colocalized with dominant active Rac1Val12 and cortical actin filament at the Rac1Val12-induced membrane ruffling area in KB cells. Sra-1 was cosedimented with filamentous actin (F-actin), indicating that Sra-1 directly interacts with F-actin. These results suggest that Sra-1 is a novel and specific target for Rac1.

Rac1, a member of the Rho small GTPases, has been shown to regulate actin filament reorganization and cell substratum adhesion such as focal contact (for a review, see Ref. 1). Rac1 has been shown to be involved in platelet-derived growth factor-induced membrane ruffling (2) and insulin-induced membrane ruffling in KB cells (3). In addition to the regulation of actin cytoskeleton, Rac1 has been shown to regulate multiple cellular processes; Rac1 has been shown to stimulate the arachidonic acid release in Swiss 3T3 cells or in Rat-1 cells (4) and to regulate the activities of c-Jun N-terminal kinase and p38 (5–7), members of mitogen-activated protein kinases. Ec-topic expression of dominant active Rac1 in mice results in the reduction of Purkinje cell axon terminals (8).

Rac1 has two interconvertible forms as follows: GDP-bound inactive form and GTP-bound active form (for reviews, see Refs. 1 and 9), and the GTP-bound form interacts with its target molecules and exerts its biological functions. The target molecules for Rac1 have been identified to be serine/threonine kinase PAK (10, 11), WASP (12, 13), POR1 (14), and p67phox (15, 16) in neutrophil. Recently, we and other groups (17–20) have identified IQGAP as a target for Cdc42 and Rac1. Among these targets, POR1 has been shown to specifically interact with Rac1 but not with Cdc42. POR1 has been shown to be involved in the Rac1-induced membrane ruffling formation (14). However, it still remains to be clarified how Rac1-specific function is achieved. Therefore, it is important to identify its novel targets.

In the present study, we purified putative targets for Rac1 with molecular masses of 140 kDa and 120 kDa and identified them as KIAA0068 gene product (Sra-1) (21) and HEM-2 (22), respectively. We found here that Sra-1 directly interacts with activated Rac1 and F-actin, suggesting that Sra-1 is a novel target for Rac1.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—The cDNA of human Sra-1 (KIAA0068) was identified as described (21). Actin was prepared from rabbit skeletal muscle as described (23). KB cells were kindly provided from Dr. Y. Miyata (Kyoto University, Japan). Anti-Myc polyclonal antibody and anti-HA monoclonal antibody from Santa Cruz Co. (Santa Cruz, CA) and Boehringer Mannheim, respectively. Reticulocyte in vitro translation system kit was purchased from Promega Co. (Madison, WI). Other materials and chemicals were obtained from commercial sources.

Plasmid Construction—Recombinant wild-type and mutant small GTPases were expressed as GST fusion proteins and purified as described (20). For expression in COS7 cells, pEF-BOS-HA-small GTPases were prepared as described (20). To obtain pBluescript SK(−) full-length cDNA of Sra-1, cDNA of Sra-1 subcloned into EcoR1 and NcoI sites of pBluescript SK(−) was digested with SacII, blunted, and inserted into ClaI-digested and blunt-ended pBluescript SK(−). To produce pGEX4T-1-Sra-1 (1–407 aa), the cDNA fragment corresponding
Sra-1 as a Novel Target for Rac1

1–407 aa was ligated into EcoRI and Smal sites of pGEX4-T1. For the
in vitro translation, the cDNA fragments encoding Sra-1 N-1 (1–859
aa), Sra-1 N-2 (1–632 aa), Sra-1 N-3 (1–407 aa), and Sra-1 C-1 (659–
1253 aa) were inserted into pBlueScript SK (→). To produce pBluescript
KS (→)-Myc-Sra-1, the full-length cDNA of Sra-1 was inserted into
EcoRV and XhoI sites of pBlueScript KS (→)-Myc (20). To express the
various mutants Myc-Sra-1 in mammalian cells, the respective cDNA
fragments were inserted into XhoI site of pEF-BOS. To produce Myc-
Sra-1 in insect cells, the cDNA of Myc-Sra-1 was inserted into XhoI sites
of pAcYM1.

GST-Rac1 Affinity Column Chromatography—The affinity purifica-
tion was performed essentially as described (20). Briefly, 30 ml of bovine
brain cytosol (25 mg/ml protein) was passed through glutathione beads
(1 ml) to remove endogenous GST. Then, the pass fraction was loaded
on glutathione beads (1 ml) coated with GST-small GTPases pre-
generated with guanine nucleotides as described (20). After washing the columns, bound proteins were coeluted with GST-small GTPases by the addition of 500
mM guanine nucleotides as described (20). After washing the columns, bound proteins were coeluted with GST-small GTPases by the addition
of 500 mM of reduced glutathione. After p140 was resolved by SDS-PAGE, the protein was transferred to polyvinylidene difluoride membrane and
subjected to amino acid sequencing as described (24). The eluates (40
μl) were subjected to SDS-PAGE followed by immunoblotting using anti-Sra-1 antibody.

Interaction of Sra-1 with Small GTPases—The insect cells overex-
pressing Myc-Sra-1 were homogenized with buffer containing 20 mM
Tris/HCl at pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, and 10 mM
(p-amidinophenyl)methanesulfonyl fluoride on ice and centrifuged at
100,000 × g for 1 h at 4 °C. The supernatant and the pellet were then subjected to SDS-PAGE followed by immunoblotting using anti-Sra-1 antibody. The results are representative of three independent
experiments.

Cosedimentation Assay—Cosedimentation assay was performed as des-
cribed (25). Myc-Sra-1 (280 nm) and F-actin (3 μm) were mixed and
incubated for 2 h at 4 °C. The mixture (50 μl) was loaded onto 20% (w/v)
sucrose layer (100 μl) and then centrifuged at 200,000 × g for 1 h at
4 °C. The supernatant and the pellet were then subjected to SDS-PAGE
followed by immunoblotting with anti-Sra-1 antibody, and the amounts
of Sra-1 were quantified.

Other Procedures—Anti-Sra-1 polyclonal antibody was raised against GST-Sra-1 (1–407 aa) by standard procedures.

RESULTS AND DISCUSSION

To identify Rac1-interacting molecules, the bovine brain cy-
tosol was subjected to GST-Rac1 affinity columns as described (20). The proteins bound to the affinity column were coeluted with GST-Rac1 by the addition of glutathione. The eluates were subjected to SDS-PAGE followed by silver staining. Lane 1, GST; lane 2, GDP-GST-Rac1; lane 3, GTPyS-GST-Rac1. The predicted amino acid sequence of human Sra-1. Sequences derived from bovine p140 were determined. The determined sequences identical to human Sra-1 were boxed. These sequence data are available from DDBJ/EMBL/GenBank under accession number D38549.

FIG. 1. Predicted amino acid sequence of p140. A, affinity purification of p140 and p120. The bovine brain cytosol was subjected to GST,
GDP-GST-Rac1, or GTPyS-GST-Rac1 affinity column, and the bound proteins were eluted with GST-small GTPases by the addition of glutathione
(20). The eluates were subjected to SDS-PAGE followed by silver staining. Lane 1, GST; lane 2, GDP-GST-Rac1; lane 3, GTPyS-GST-Rac1. The
predicted amino acid sequence of human Sra-1. Sequences derived from bovine p140 were determined. The determined sequences identical to
human Sra-1 were boxed. These sequence data are available from DDBJ/EMBL/GenBank under accession number D38549.

FIG. 2. Direct interaction of Sra-1 with Rac1. A, the bovine brain cytosol was loaded on the glutathione column coated with the indicated
GST-small GTPases. The bound proteins were coeluted with the GST-
small GTPases by the addition of glutathione. Aliquots were resolved by
SDS-PAGE followed by immunoblotting using anti-Sra-1 antibody. Lane 1, GST; lane 2, GTPyS-GST-Rac1; lane 3, GDP-GST-Rac1; lane 4, GTPyS-
GTP-RhoA; lane 5, GDP-GST-Cdc42; lane 6, GTPyS-GST-Rac1; lane 7, GDP-GST-RhoA. The arrowhead denotes the position of Sra-1. B, Myc-
Sra-1 was loaded on the glutathione column coated with the indicated
GST-small GTPases. Myc-Sra-1 was coeluted with GST-small GTPases by the addition of 500 μl of glutathione. Lanes are the same as those
in the legend of Fig. 2A. Forty μl of the eluates were resolved by SDS-
PAGE followed by silver staining. The arrowhead denotes the position
of Sra-1. The results are representative of three independent
experiments.
phosphatidylinositol-3 kinase, respectively (20). To determine the molecular identities of the p140 and p120, they were subjected to amino acid sequencing as described (24). Seven peptide sequences derived from p140 were determined (Fig. 1B). The amino acid sequences of p140 revealed that p140 was identical to those of a putative protein encoded by a human cDNA, KIAA0068 (DDBJ/EMBL/GenBank accession number D38549), which was identified by the cDNA project (21). The amino acid sequences of the p120 are 1) AAEDLFVNIRGY, 2) ELATVLSDQPG, 3) ASLSLADHREL, and 4) RLSSVDSVLK, all of which are identical to the deduced amino acid sequence of rat HEM-2 (22). We have also determined the amino acid sequence of p180 and found that p180 is a novel protein which is highly homologous to myosin heavy chain.3 The molecular weights of a KIAA0068 gene product and HEM-2 are calculated to be 145,180 and 128,250, respectively, which are almost the same as the apparent molecular masses of p140 and p120 estimated by SDS-PAGE. Moreover, anti-Sra-1 antibody recognized p140 in the eluate from GST-GTP\textsubscript{z}Rac1 affinity column (Fig. 2A). Therefore, we concluded that p140 and p120 were the bovine counterparts of a human KIAA0068 gene product and HEM-2, respectively. We also confirmed that p140 was detected specifically in the GST-Rac1-affinity eluate but not in the GST-Cdc42 nor GST-RhoA affinity eluates from bovine brain cytosol (20). Since p140 specifically interacted with Rac1 as described below, we denoted this protein as Sra-1 (Specifically Rac1-interacting protein) and hereafter refer to it as Sra-1.

To examine whether recombinant Sra-1 interacts with GTP\textsubscript{y}S-Rac1, the affinity beads coated with GST-small GTPases were mixed with Myc-Sra-1 purified from overexpressing insect cells. After washing the affinity beads, GST-small GTPases were eluted by the addition of reduced glutathione. Myc-Sra-1 coeluted strongly with GTP\textsubscript{y}S-GST-Rac1 and weakly

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2 Detailed analysis of HEM-2 will be described elsewhere.

3 Detailed analysis of p180 will be described elsewhere.
with GDP-GST-Rac1 (Fig. 2B), indicating that Sra-1 directly interacts with GTP₇S-GST-Rac1. Recombinant Sra-1 was not detected in the eluate of GST-RhoA, GST-Cdc42, or GST affinity beads.

Rac1 and Cdc42 share their targets PAK (10, 11), WASP (12, 13), and IQGAP (17–20). A specific target for Rac1 has been shown to be POR1 (14) and that for Cdc42 to be ACK (26). Our result indicates that Sra-1 is categorized into a specific target for Rac1, such as POR1.

The indicated mutants of Sra-1 were translated in vitro, and their interactions with GST-Rac1 were examined. The in vitro translated full-length Sra-1 interacted with GTP₇S-GST-Rac1 and slightly with GTP₇G-GST-Rac1 (Fig. 3). Sra-1 N-1 (1–959 aa) interacted with GTP₇S-GST-Rac1, whereas Sra-1 C-1 (659–1253 aa) did not. The shorter fragments of Sra-1, N-2 (1–632 aa), and N-3 (1–407 aa) were also interacted with GTP₇S-GST-Rac1. This result indicates that the N-terminal domain of Sra-1 (1–407 aa) is responsible for the interaction with Rac1. The consensus sequence of the Rac1-binding domain of the target proteins such as PAK and WASP has been determined, and it was designated as CRIB (13, 27). The shortest fragment Sra-1 N-3 did not contain the CRIB domain. Therefore, Sra-1 is categorized into the target group that does not have the CRIB domain.

Rac1 has been shown to regulate membrane ruffling (2, 3). To explore whether Sra-1 is also involved in the Rac1-mediated regulation of actin cytoskeleton, we microinjected cDNAs of Myc-Sra-1 with HA-Rac1Val-12, structurally equivalent to RasVal-12 (9), into serum-starved KB cells. Membrane ruffling was observed in the injected cells as described (3) (Fig. 4A). Sra-1 specifically accumulated at Rac1-induced membrane ruffling area and colocalized with Rac1Val-12 (Fig. 4, B and C). Myc-Sra-1 was also colocalized with cortical actin filaments (Fig. 4, E and F). We also attempted to examine whether Sra-1 interacts with Rac1 in COS7 cells overexpressing Sra-1 and Rac1Val-12, but we failed to do so (data not shown). When Sra-1 was coexpressed with Rac1, most Sra-1 was recovered in the membrane fraction and hardly extracted by detergents. However, colocalization of Sra-1 with Rac1 strongly suggests that these proteins interact in vivo.

To explore further the interaction of Sra-1 with Rac1, we microinjected various deletion mutants of Sra-1 with Rac1Val-12 into serum-starved KB cells and analyzed their distributions. When the deletion mutants were microinjected with Rac1Val-12 into the cells, the membrane ruffling was elicited (Fig. 5, A, D, G, and J), and Rac1Val-12 was accumulated at the membrane ruffling area (Fig. 5, C, F, I, and L). The deletion mutants capable of interacting with Rac1Val-12 were accumulated at the membrane ruffling area (Fig. 5, B, E, and H), whereas the mutant unable to interact with Rac1Val-12 was not (Fig. 5K), suggesting that the interaction of Sra-1 with Rac1 is necessary for the accumulation of Sra-1 at the membrane ruffling area. This result is consistent with the in vitro interaction result and suggests that Rac1 may recruit Sra-1 to the membrane ruffling area.

Activated Rac1 and Cdc42 induce distinct phenotypes, membrane ruffling, and filopodia formation, respectively (2, 28, 29). It is likely that the distinct phenotype elicited by Cdc42 and Rac1 reflects their specific target activities. POR1 enhances the Ha-RasVal-12-induced membrane ruffling, and truncated POR1 inhibited the Rac1-induced membrane ruffling (14). Although this suggests that POR1 is required for the membrane ruffling formation, it is possible that other targets are also required for the membrane ruffling formation elicited by Rac1. Our results suggest that Sra-1, in addition to POR1, is involved in the membrane ruffling formation.

We explored the relationship between Sra-1 and actin filament. When Myc-Sra-1 was incubated with F-actin and centrifuged, Myc-Sra-1 was cosedimentated with F-actin (Fig. 6). Myc-Sra-1 was not cosedimentated in the absence of F-actin. This result indicates that Sra-1 directly interacts with F-actin. It is difficult to examine whether Rac1 affects the interaction of Sra-1 with F-actin, because Sra-1 was sedimentated even in the absence of F-actin when it was mixed with GST-Rac1 for an unknown reason. Taken together, it is likely that Sra-1 is involved in the regulation of membrane ruffling formation induced by Rac1 through the interaction with F-actin.

Recently, it is reported that introduction of activated PAK, a common target for Rac1 and Cdc42, resulted in the formation of filopodia in Swiss 3T3 cells (30) and induces the morphological change of HeLa cells (31). We have recently found that IQGAP1, another common target for Rac1 and Cdc42, directly interacts with F-actin (32). It is likely that these targets may be required for alteration of actin filament necessary for both filopodia and membrane ruffling formation. Our results suggest that Sra-1 is the specific machinery necessary for the membrane ruffling formation. Taken together, it is plausible that Sra-1 can function in concert with other targets such as PAK, IQGAP1, and POR1, resulting in the formation of membrane ruffling.

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