Identification of IQGAP as a Putative Target for the Small GTPases, Cdc42 and Rac1*

(Received for publication, June 17, 1996)

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Cdc42 and Rac1 have been implicated in the regulation of various cell functions such as cell morphology, polarity, and cell proliferation. We have partially purified a Cdc42- and Rac1-associated protein with molecular mass of about 170 kDa (p170) from bovine brain cytosol. This protein interacted with guanosine 5'-[3-O-thio]triphosphate (GTP-S)glutathione S-transferase (GST)-Cdc42 and GTPγS-GST-Rac1 but not with the GDP-GST-Cdc42, GDP-GST-Rac1, or GTPγS-GST-RhoA. We identified p170 as an IQGAP, which is originally identified as a putative Ras GTPase-activating protein. Recombinant IQGAP specifically interacted with GTPγS-Cdc42 and GTPγS-Rac1. The C-terminal fragment of IQGAP was responsible for their interactions. IQGAP was specifically immunoprecipitated with dominant-active Cdc42Val12 or Rac1Val12 from the COS7 cells expressing Cdc42Val12 or Rac1Val12, respectively. Immunofluorescence analysis revealed that IQGAP was accumulated at insulin- or Rac1-induced membrane ruffling areas. This accumulation of IQGAP was blocked by the microinjection of the dominant-negative Rac1Asn17 or Cdc42Asn17. Moreover, IQGAP was accumulated at the cell-cell junction in MDCK cells, where α-catenin and ZO-1 were localized. These results suggest that IQGAP is a novel target molecule for Cdc42 and Rac1.

Cdc42 and Rac1, members of the small GTPase Rho family, are known to regulate the cytoskeleton through the actin filament (reviewed in Refs. 1 and 2). Cdc42 and Rac1 are implicated in filopodia (3, 4) and lamellipodia (4, 5) formation, respectively, in Swiss 3T3 cells. Rac1 is shown to be involved in platelet-derived growth factor-induced membrane ruffling (5) and insulin-induced one in KB cells (6, 7). Rac1 is also shown to stimulate phosphatidlyinositol 4-phosphate 5-kinase activity and actin-uncapping in human platelets (8) and arachidonic acid release in Swiss 3T3 cells or in Rat-1 cells (9). Cdc42 and Rac1 are shown to regulate the activities of c-Jun N-terminal kinase and p38 (10–12), members of mitogen-activated protein kinases. In T cells, Cdc42 is shown to regulate the polarization of both actin and microtubules toward antigen-presenting cells (13). In Drosophila, ectopic expression of dominant-active Cdc42 inhibits the dendrite and axon growth of neural cells and normal muscle formation (14), and that of dominant-negative Cdc42 inhibits the apico-basal elongation of wing disc epithelial cells (15). Ectopic expression of dominant-active Rac inhibits only axon growth (14) and assembly of actin at adherence junction of wing disc epithelial cells (15), and that of dominant-negative Rac causes disruption of cell shape changes in the lateral epidermis (16). Ectopic expression of dominant-active Rac1 in mice results in the reduction of Purkinje cell axon terminals (17).

Cdc42 and Rac1 have two interconvertible forms: GDP-bound inactive forms and GTP-bound active forms (reviewed in Refs. 1 and 18) and their GTP-bound forms interact with their target molecules and exert their biological functions. The target molecules of Cdc42 and Rac1 have been identified to be serine/threonine kinase PAK (19–21), phosphatidylinositol 3'-kinase (22, 23), and WASP1 (24, 25); the target molecule of Cdc42 is ACK (26), and the target molecule of Rac1 in neutrophils is p67 phox (27, 28). To understand the pleiotropic functions of Cdc42 and Rac1, it is important to identify their novel targets.

We have previously purified target molecules for Rho by the Rho affinity column chromatography and identified them as protein kinase N (29), Rho-kinase (30), and myosin-binding subunit of myosin phosphatase (31). In the present study, we purified a putative target for Cdc42 and Rac1 with a molecular mass of 170 kDa and identified it as an IQGAP, which is originally isolated as a Ras GAP family (32, 33).

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Anti-α-catenin antibody and anti-ZO-1 antibody were kindly provided by Drs. A. Nagafuchi and S. Tsukita (Kyoto University, Kyoto, Japan), and Drs. M. Itoh and S. Tsukita (Kyoto University, respectively). KB cells and MDCK cells were provided from Health Science Research Resources Bank (Osaka, Japan). C3 exoxygenase was kindly provided by Dr. Ohashi (Nihon Shering, Osaka, Japan). Other materials and chemicals were obtained from commercial sources.

Plasmid Construction—pGEX2T-Cdc42, pGEX2T-Rac1, and pGEX2T-RhoA were constructed as described previously (29, 34). Cdc42Val12 and Rac1Val12, or Cdc42Asn17 and Rac1Asn17 cDNAs were generated by the site-directed mutagenesis of Gly12 and Thr17 to Val12 and Asn17, respectively. Recombinant wild-type and mutant small GTPases were expressed as GST-fusion proteins and purified as described (29). For microinjection, they were cleaved with thrombin, purified to remove the GST, and concentrated (5, 35). For expression in COS7 cells, pEF-BOS-HA small GTPases were constructed as described (29, 36). To obtain the
IQGAP (521–914 amino acids) as an antigen, a cDNA fragment corresponding to the IQGAP fragment was subcloned into pGEX-4T-2. For in vitro translation of IQGAP, the cDNA fragments corresponding to the indicated IQGAP fragments were subcloned into pBlueScript KS(−) having a sequence encoding a myc epitope tag (MEQKLISEEDL).

GST-Cdc42 and GST-Rac1 Affinity Column Chromatography—The affinity purification was performed essentially as described (29). Briefly, bovine brain cytosol was passed through glutathione beads to remove endogenous GST. Then, the pass fraction was loaded on glutathione beads containing respective GST-small GTPases preloaded with guanine nucleotides as described (29, 37). After washing the columns, bound proteins were coeluted with respective GST-small GTPases by the addition of reduced glutathione.

Interaction of in Vitro Translated IQGAP with Small GTPases—The interactions of indicated fragments of in vitro translated IQGAP with GST-small GTPases were examined as described (38). Briefly, indicated fragments of in vitro translated IQGAP were mixed with affinity beads coated with the respective guanine nucleotide-bound GST-small GTPases. After washing the beads, the bound proteins were coeluted with respective small GTPases by the addition of glutathione. The eluates were resolved by SDS-PAGE, and radioactivities were detected using the bioimaging analyzer BAS2000 (Fuji Film, Tokyo, Japan).

Overlay Assay—The overlay assay was performed as described (19, 30). Briefly, an aliquot of the eluate fraction of GST-S-GST-Cdc42 affinity column was subjected to SDS-PAGE and blotted onto nitrocellulose membrane. After the denaturation with guanidinium hydrochloride and the subsequent renaturation, the membrane was probed with [35S]GTPyS-GST-small GTPases. The bound small GTPases to IQGAP were visualized using bioimaging analyzer.

Coimmunoprecipitation of IQGAP with HA-Small GTPases—COS7 cells were transfected with pEF-BOS-HA-small GTPases by use of the DEAE-dextran method (39). Immunoprecipitation of HA-tagged small GTPases by anti-HA antibody was performed as described (29). The immunocomplex was subjected to SDS-PAGE, followed by immunoblotting using anti-IQGAP antibody.

Microinjection and Immunofluorescence Analysis—KB cells were cultured, seeded, and starved for 36 h as described (6). Microinjection of small GTPases (1 mg/ml) or C3 (200 μg/ml) followed by the stimulation of insulin was performed as described (6). MDCK cells were cultured and seeded as described (40). Immunofluorescence analysis with anti-IQGAP antibody was carried out essentially as described (41).

Other Procedures—The peptide sequence of p170 was determined as described (37, 42). Anti-IQGAP polyclonal antibody was raised by standard procedures using GST-IQGAP (521–914 amino acids) as an antigen.

RESULTS AND DISCUSSION

To identify Cdc42- and Rac1-interacting molecules, the bovine brain cytosol was loaded onto a GST-Cdc42 affinity column. The proteins bound to the affinity column were coeluted with GST-Cdc42 by the addition of glutathione. A protein with molecular mass of about 170 kDa (p170) was detected in the glutathione-eluate from GTPyS-GST-Cdc42 affinity column but not from GST or GDP-GST-Cdc42 affinity column (Fig. 1). Less amount of p170 was detected in the eluate from the GTPyS-GST-Rac1 affinity column but not from the GDP-GST-Rac1 affinity column. To further confirm the specificity of the interaction, affinity column chromatography using GST-RhoA was performed (29). p170 was not eluted from the GTPyS-GST-RhoA affinity column. p170 was eluted from neither GST-HaRas nor GST-RalA affinity columns (data not shown). Proteins with molecular masses of 62 kDa (p62), 90 kDa (p90), and 110 kDa (p110) were also detected in both of the eluates from GTPyS-GST-Rac1 and GTPyS-GST-Cdc42 affinity columns. p62 was recognized by anti-PAK antibody (data not shown). Proteins with molecular masses of 122 kDa (p122) and 140 kDa (p140) were specifically detected in the eluate of GTPyS-GST-Rac1. Identifications of p90, p110, p122, and p140 are currently under investigation.

To identify p170, it was subjected to amino acid sequencing as described (42). Three peptide sequences derived from p170 were determined. The amino acid sequences of the peptides are YGEQVDYYK, IFYPETTDIYDRK, and RLIVDVIRTE. All of the three peptide sequences obtained are displayed.

Fig. 1. Purification of Cdc42- or Rac1-interacting proteins. The bovine brain cytosol was loaded onto a glutathione-agarose column containing the indicated GST-small GTPases. The bound proteins were coeluted with the respective GST-small GTPases by the addition of glutathione. Aliquots of the eluates were resolved by SDS-PAGE, followed by silver staining. Lane 1, GDP-GST-Cdc42; lane 2, GTPyS-GST-Cdc42; lane 3, GDP-GST-Rac1; lane 4, GTPyS-GST-Rac1; lane 5, GDP-GST-RhoA; lane 6, GTPyS-GST-RhoA; lane 7, GST. An arrowhead denotes the position of p170. Arrows from the top denote the positions of p140, p122, p110, and p90, respectively. The results shown are representative of three independent experiments.
GTPγS-GST-Rac1 (Fig. 2A). It was slightly retained on and eluted from the GDP-GST-Cdc42 affinity beads, but it was not detected in the eluate of GDP-GST-Rac1, GST-RhoA, or GST affinity beads.

To determine the binding domain of IQGAP to Cdc42 and Rac1, the N-terminal (1–863 amino acids) and the C-terminal (764–1657 amino acids) fragments of IQGAP were in vitro translated, and their interactions with Cdc42 and Rac1 were examined. The C-terminal fragment of IQGAP interacted with GTPγS-Cdc42 or GTPγS-Rac1 (Fig. 2B), whereas the N-terminal fragment did not (data not shown). Both fragments interacted with neither GST, GDP-Cdc42, nor GDP-Rac1.

Furthermore, direct interaction of the purified IQGAP with GTPγS-GST-Cdc42 or GTPγS-GST-Rac1 was examined using the overlay assay method (19, 30). [35S]GTPγS-GST-Cdc42 and [35S]GTPγS-GST-Rac1 bound to the purified IQGAP, whereas [35S]GTPγS-GST-RhoA did not (Fig. 2C). The consensus sequence of the Cdc42- or Rac1-binding domain of target proteins, such as PAK or WASP, has been determined and was termed as CRIB (25, 43). There is no CRIB domain in the fragment (764–1657 amino acids). Therefore, Cdc42 or Rac1 interacts with at least two distinct target interfaces.

We next examined whether IQGAP interacts with Cdc42 or Rac1 in vivo. Cdc42Val12 and Rac1Val12, structurally equivalent to RasVal12 (18), are thought to be a constitutively GTP-bound form in vivo as a result of defective GTPase activity (3–5), whereas Cdc42Asn17 and Rac1Asn17, structurally equivalent to RasAsn17 (44), are thought to be a constitutively GDP-bound form in vivo as a result of preferential binding to GDP relative to GTP and to specifically interfere in the activation of endogenous respective small GTPases. When HA-tagged Cdc42Val12, Cdc42, Cdc42Asn17, Rac1Val12, or RhoAVal114 was transiently transfected into COS7 cells and HA-small GTPases were immunoprecipitated with anti-HA antibody, IQGAP was coimmunoprecipitated with Cdc42Val12 or Rac1Val12 (Fig. 3). It coimmunoprecipitated with neither Cdc42, Cdc42Asn17, nor RhoAVal114. Almost the same amounts of the HA-small GTPases were precipitated (data not shown). Therefore, it is most likely that IQGAP specifically interacts with GTP-Cdc42 and GTP-Rac1 in vivo.

IQGAP was originally identified as a Ras GAP, but the recombinant IQGAP did not show any GAP activity toward Ras and Rho (33). We also attempted to detect GAP activity using affinity-purified IQGAP toward Ha-Ras, Ki-Ras, R-Ras, RaLa, Cdc42, Rac1, and RhoA but failed (data not shown). We cannot exclude the possibility that the purified IQGAP lost its activity. Further study is necessary to clarify the function of the GAP domain of IQGAP.

Insulin is shown to induce membrane ruffling in KB cells (45, 46), and the insulin-induced membrane ruffling is blocked by the prior microinjection of Rac1Asn17 into the cells but not by that of C3 (6). Microinjection of GTPγS-Rac1 is shown to induce the membrane ruffling in KB cells (6). Then, we examined the localization of IQGAP in KB cells. When KB cells were stimulated with insulin, membrane ruffling was induced, and IQGAP accumulated at the membrane ruffling area (Fig. 4, A and B). The insulin-induced membrane ruffling and IQGAP accumulation at the membrane ruffling area were blocked by prior microinjection of Rac1Asn17 (Fig. 4, C and D). The prior microinjection of Cdc42Asn17 also blocked the insulin-induced membrane ruffling and the IQGAP accumulation in some injected cells (Fig. 4, E and F), whereas both processes weakly occurred in other injected cells. Cdc42Asn17 may not completely block insulin-dependent activation of endogenous Rac1. The prior microinjection of C3 did not block the insulin-induced membrane ruffling and IQGAP accumulation (Fig. 4, G and H). Microinjection of Rac1Val12 induced membrane ruffling and IQGAP accumulation at the membrane ruffling area (Fig. 4, I and J). That of Cdc42Val12 induced filopodia formation, but IQGAP was not accumulated at the filopodia (data not shown).
It should be noted that IQGAP was accumulated at the cell-cell junction of KB cells.

To further examine the IQGAP accumulation at the cell-cell junction, we examined the localization of IQGAP in MDCK cells. IQGAP was specifically accumulated at the cell-cell junction (Fig. 5, B and E). IQGAP showed similar distribution as α-catenin and ZO-1, which are marker molecules of adherence junction and tight junction, respectively, at the cell-cell junction (Fig. 5, C and F). It seems that IQGAP is well colocalized with α-catenin, whereas IQGAP is not completely colocalized with ZO-1. It remains to be clarified whether IQGAP is localized at the adherence junction or tight junction in MDCK cells. This observation suggests that IQGAP regulates the cell-cell junction, or that the cell-cell junctional signal leads to the IQGAP-accumulation. This accumulation was not affected by the addition of insulin or by microinjection of various small GTPases. It is possible that Rac1 or Cdc42 is not necessary for the localization of IQGAP at the cell-cell junction once IQGAP is complexed with the cell-cell junctional cytoskeleton, or that activation of Rac1 or Cdc42 at the cell-cell junction is blocked by neither Rac1Asn17 nor Cdc42Asn17. When actin filament was visualized with rhodamine-labeled phalloidin, IQGAP was observed in association with cortical actin at the membrane ruffling area in KB cells (Fig. 6).

We here identified the Cdc42- or Rac1-interacting p170 protein as IQGAP. We showed that recombinant IQGAP directly interacts with active forms of Rac1 and Cdc42 in vitro and in vivo. Moreover, we found that IQGAP is accumulated at the insulin- or Rac1-induced membrane ruffling area, and that the insulin-induced membrane ruffling and the IQGAP accumula-

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A detailed analysis will be described elsewhere.
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J. Biol. Chem. 1996, 271:23363-23367.
doi: 10.1074/jbc.271.38.23363

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