IQGAP1 Binds Rap1 and Modulates Its Activity*

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IQGAP1 is a scaffolding protein involved in multiple fundamental cellular activities, including transcription, cell-cell attachment, and regulation of the cytoskeleton. To function in these pathways, IQGAP1 associates with numerous proteins such as actin, calmodulin, E-cadherin, β-catenin, CLIP-170, and components of the mitogen-activated protein kinase pathway. Moreover, IQGAP1 binds to active Cdc42 and Rac1 but not RhoA or Ras. Here we show that IQGAP1 also binds to the small GTPase Rap1. In vitro analysis demonstrates a direct interaction between Rap1 and IQGAP1, which is augmented by activation (GTP loading) of Rap1. Cdc42 does not modulate the interaction between Rap1 and IQGAP1. In contrast, the association is eliminated by calmodulin both in the absence and presence of Ca2+. The binding of Rap1 to a point mutant IQGAP1 construct that is unable to interact with calmodulin is 2.5-fold more than to wild type IQGAP1. Consistent with these findings, Rap1 binds to the IQ region of IQGAP1. Confocal microscopy demonstrates that Rap1 and IQGAP1 co-localize at the periphery of human epithelial cells but not in the cytoplasm. The interaction has functional sequelae. Overexpression of IQGAP1 substantially reduces adhesion-mediated activation of Rap1. In addition, Rap1 activation by cAMP is attenuated in cells that overexpress IQGAP1 and enhanced in cells lacking IQGAP1. These findings reveal that the interaction of IQGAP1 with Rap1 differs in several respects from its interaction with other small GTPases. Furthermore, our data suggest that IQGAP1 may link the calmodulin and Rap1 signaling pathways.

The Ras superfamily of small GTPases comprises ~150 members in humans (1, 2). Members of the Ras family, which contains 36 proteins, include the Ras proteins (H-Ras, K-Ras, and N-Ras) and several related proteins such as R-Ras proteins (R-Ras, R-Ras2, and M-Ras), Rap1 and Rap (2). Like Ras, Rap1 cycles between an active GTP-bound form and an inactive GDP-bound form. Guanine nucleotide exchange factors (GEFs),2 such as Epac (exchange protein directly activated by cAMP), C3G, and CD-GEF, mediate Rap1 activation through exchange of GDP for GTP (3). Rap1 is inactivated by GTPase-activating proteins (GAPs), which catalyze the hydrolysis of bound GTP to GDP. A wide variety of extracellular stimuli, for example thrombin, growth factors, and interferon, activate Rap1. These effects are mediated by second messengers, including Ca2+, diacylglycerol, and cAMP, which directly stimulate Rap1 GEFs. Ras and Rap1 are very similar at the amino acid level, with identical effector regions (4). Nevertheless, the proteins have distinct functions. Rap1 has been implicated in the modulation of a number of cellular responses ranging from Ca2+ signaling and secretion to neurite outgrowth and cell proliferation (3). A well characterized function of Rap1 is in cell adhesion, both integrin-mediated adhesion and cadherin-mediated cell junction formation (5). Rap1 interacts with a large number of proteins that contribute to regulating these aspects of cell function (5).

IQGAP1 is a scaffolding protein composed of multiple protein recognition motifs through which it interacts with a wide spectrum of binding partners (for review, see Refs. 6–9). In the N-terminal half, the calponin homology domain binds actin (10, 11), the WW domain binds extracellular signal-regulated kinase 2 (12), and the IQ domain mediates interactions with calmodulin (10, 13, 14) and calmodulin-related proteins (15). The C-terminal half of IQGAP1 is reported to bind E-cadherin (16, 17), β-catenin (16, 18), CLIP-170 (19) and adenomatous polyposis coli (20). Through binding to diverse proteins, IQGAP1 functions in many physiological processes in cells, including cell-cell attachment, cell polarization, transcription, regulation of actin and microtubule function, the mitogen-activated protein kinase cascade, and Ca2+/calmodulin signaling (6, 7). In addition, IQGAP1 has a role in cell motility, cell invasion, neuronal migration, and neurite outgrowth (21–23).

In addition to the binding partners mentioned above, IQGAP1 also interacts with selected small GTPases. Direct binding of IQGAP1 to active (GTP-bound) Cdc42 (13, 14), Rac1 (14), and TC10 (24) has been documented. However, IQGAP1 does not bind RhoA or H-Ras (14, 25). In this study we show for the first time that IQGAP1 interacts directly with a GTPase that is not a member of the Ras subfamily. The association of IQGAP1 with Rap1, which differs in several respects from its interaction with other small GTPases, alters the activation of Rap1.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture reagents were from Invitrogen. The anti-IQGAP1 polyclonal and monoclonal antibodies have been

2 The abbreviations used are: GEF, guanine nucleotide exchange factor; 8CPT-2Me-cAMP, 8-(4-chloro-phenylthio)-2′-O-methylenadenosine-3′-5′-cyclic monophosphate; Ni2+/H11001, nickel nitrilotriacetic acid; GST, glutathione S-transferase; GAP, GTPase-activating protein; Epac, exchange protein directly activated by cAMP; BSA, bovine serum albumin; MBP, maltose-binding protein; MEF, mouse embryo fibroblast; GFP, green fluorescent protein; RFP, red fluorescent protein; PBS, phosphate-buffered saline; GTPγS, guanosine 5′-3-O-(thio)triphosphate.
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IQGAP1 was previously characterized (10, 26). Anti-Myc monoclonal antibody (9E10.2) was manufactured by Maine Biotechnology. Antibodies were purchased as follows: monoclonal anti-Cdc42 from BD Transduction Laboratories, polyclonal anti-Rap1 from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-β tubulin from Sigma, and horseradish peroxidase-conjugated secondary antibodies from GE Healthcare. Enhanced chemiluminescence (ECL) reagents were from Millipore (Billerica, MA). 8-(4-Chloro-phenylthio)-2’-O-methyladenosine-3’,5’-cyclic monophosphate (8CPT-2Me-cAMP) was purchased from Tocris (Ellisville, MO). All other reagents were of standard analytical grade.

Plasmid Construction and Preparation of Fusion Proteins—The construction of Myc-tagged IQGAP1, IQGAP1-C (C-terminal region, amino acids 864–1657), IQGAP1-N (N-terminal region, amino acids 2–863), IQGAP1ΔWW (amino acids 643–744 deleted), IQGAP1ΔIQ (amino acids 699–905 deleted), and IQGAP1 IQ3,4R (selected Arg residues in the third and fourth IQ motifs substituted by Gln) have been described previously (10). Where indicated, the GST tag was attached to IQGAP1 IQ and IQGAP1 IQ3,4R using one-Sepharose chromatography (GE Healthcare) essentially as described (10, 12, 27, 28). All plasmids were purified using Qiagen DNA purification kits (Qiagen) according to the manufacturer’s instruction. pET His6-Rap1A, His6-Rap1A-63E, and His6-Rap1A-17N were kindly provided by Xavier Cullere and Tanya Mayadas (Brigham and Women’s Hospital and Harvard Medical School, Boston, MA), and GFP-Rap1A was a kind gift of Yasuuki Fujita (University College London, London, UK). pGEX-Cdc42-Q61L, pGEX-Rap1B, and pGEX-Ral-GDS were generously donated by Darerca Owen (University of Cambridge, Cambridge, UK), Andreas Püschel (Westfälische Wilhelms-Universität, Munster, Germany), and Johannes Bos (University Medical Centre, Utrecht, The Netherlands), respectively. pET His6-Rap1 constructs were expressed in Escherichia coli and purified by nickel affinity chromatography using Ni2+-NTA-agarose (Qiagen). GST-IQGAP1 and pGEX-Cdc42-Q61L were expressed in E. coli and purified by glutathione-Sepharose chromatography (GE Healthcare) essentially as described (10). Where indicated, the GST tag was removed from IQGAP1 by tobacco etch virus protease as previously described (29).

We generated maltose-binding protein (MBP) fusion constructs of wild type Rap1A, Rap1A-63E, and Rap1A-17N. The BamHI-HindIII fragment containing Rap1A was cut from pRSETB-Rap1A and inserted into pMAL-c2X at BamHI and HindIII sites. The proteins migrated to the expected position on SDS-PAGE.

GST-Rap1A was generated by PCR on full-length Rap1A using the primers 5’-CGGGATCCCCGTGAGTTACAAAGTC-TAGTGG-3’ and 5’-CGGAATTCTCAGCAAGCGAGCAT- GATTTC-3’. The PCR product was cut with BamHI and EcoRI and inserted into pGEX4T-1 at the same restriction sites.

IQGAP1 was tagged with red fluorescent protein (RFP) using monomeric mRFP which was generously donated by Roger Tsien (University of California, San Diego, CA). A PCR product of mRFP was generated using primers 5’-GCTCTAGATTGGCCTCTCCGAGGACG-3’ and 5’-GAAAGATCTGGCAGCGC-GTTGAGGCGCGTCT-3’ with pSETB-mRFP as template. The PCR product was cut with XbaI and BglIII, and the insert was replaced with a Nhel-BglII fragment bearing the DsRed gene on pDsRed2-C1 (Clontech). To generate RFP-wild type IQGAP1, pcDNA-Myc-IQGAP1 was cut with XbaI and made blunt end with T4 polymerase then partially digested with BamHI. The BamHI-XbaI fragment bearing the whole IQGAP1 gene was inserted into mRFP-C1 at BglII-Smal site. The RFP tag was attached to IQGAP1ΔIQ and IQGAP1 IQ3,4R using RFP-tagged wild type IQGAP1. The PacI-SpeI fragment was cut from mRFP-IQGAP1(wild type) and replaced with the same fragment from pcDNA-myc-IQGAP1ΔIQ or pcDNA-Myc-IQGAP1 IQ3,4R.

Cell Culture and Transfection—MCF-7 human breast epithelial cells and 293H human embryonic kidney cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. MCF-7 cells, which stably overexpress pcDNA3-Myc-IQGAP1 (termed MCF/I cells), and MCF-7 cells with stable integration of small interfering RNA for IQGAP1 (termed MCF-siIQ8 cells) have been described previously (18, 23, 30). MCF/I cells have 3-fold more IQGAP1 than MCF-7 cells, whereas IQGAP1 protein is reduced by 80% in MCF-siIQ8 cells. Mouse embryo fibroblast (MEF) cells, isolated from IQGAP1−/− mice and normal littermate controls, were immortalized with SV40 large T antigen.

Transient transfections were performed with FuGENE 6 (Roche Diagnostics) or Lipofectamine 2000 (Invitrogen) essentially as described (18, 21, 30). Cells were transfected with 5 μg Myc-IQGAP1, Myc-IQGAP1ΔIQ, Myc-IQGAP1 IQ3,4R, RFP-IQGAP1, RFP-IQGAP1ΔIQ, or RFP-IQGAP1 IQ3,4R and 48 h later were either evaluated in adhesion assays, lysed, or fixed for immunocytochemistry.

Gel Filtration Chromatography—MCF-7 and MCF-siIQ8 cells (8 dishes/10 cm each) were lysed in 2 ml of buffer composed of 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 20% glycerol, and protease inhibitors. The lysate was loaded onto a Superose 6 column (Amersham Biosciences) pre-equilibrated with the same buffer. The sample was fractionated at 1 ml/min (0.5-ml fractions were collected) by fast protein liquid chromatography separation performed on an AKTATMPLC system (Amersham Biosciences) equipped with a UPC-900 monitor and a P-920 pump. The system was monitored and controlled by methods run by the UNICORN control system (Version 5.01). The column was calibrated using apoferritin (443 kDa), β-amylase (200 kDa), and bovine serum albumin (66 kDa) as standards. Forty-microliter aliquots of selected fractions were resolved by Western blotting, then probed for IQGAP1 and Rap1.

Adhesion Assays—To analyze cell adhesion, cells were trypsinized, suspended in complete medium, and plated onto collagen I- or fibronectin-coated culture dishes (BD Bioscience) at 37 °C for the times indicated in Figs. 7 and 8. After washing with PBS (to remove nonadherent cells), adherent cells were lysed with buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 10% glycerol, 1% Nonidet P-40, 2 mM Na2VO4, 10 mM NaF, and 5 mM EGTA). Equal amounts of protein lysates were subjected to pulldown assays.

Assay for Activation of Rap1 and Cdc42—Active Cdc42 (31) and active Rap1 (32) were assessed essentially as described pre--

3 Ren, J. G., Li, Z., and Sacks, D. B. (2007) Proc. Natl. Acad. Sci. U. S. A., in press.
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viously. Briefly, cells were washed with ice-cold PBS and lysed with buffer A. Lysates were cleared by centrifugation, and active Rap1 and Cdc42 were precipitated with glutathione-Sepharose beads precoupled to a GST fusion protein of the Rap1 binding domain of RalGDS for Rap1 and the GTPase binding domain of WASP (Wiskott-Aldrich syndrome protein) for Cdc42. Samples were incubated for 40 min (for Rap1) or 30 min (for Cdc42). Beads were sedimented by centrifugation, washed three times with buffer A, and solubilized in SDS sample buffer. A portion of the cell lysate was reserved for analysis of total Rap1 or Cdc42 content. Cdc42 and Rap1 were detected by Western blotting.

To compare binding of Rap1A and Rap1B to IQGAP1, we used GST-tagged proteins, MBP-Rap1A was loaded with either GDP or GTP$\gamma$S essentially as previously described (13, 33). Briefly, MBP-Rap1A was incubated in buffer B (50 mM Tris, pH 7.4, 150 mM NaCl, and 1% Triton X-100) with 1 mM EDTA. After 10 min at 22 °C, 3 mM MgCl$_2$ and 140 μM GTP$\gamma$S or GDP were added, and samples were incubated for 30 min at 22 °C. Samples were precleared with 40 μl of glutathione-Sepharose for 1 h, and equal amounts of GST-IQGAP1 in 500 μl of buffer B containing 1 mM EGTA were added for 3 h at 4 °C. Complexes were isolated with glutathione-Sepharose, washed three times in buffer B, and resolved by SDS-PAGE. Western blots were probed with anti-Rap1 antibodies.

For binding assays with MBP-tagged proteins, equal amounts of wild-type MBP-Rap1A (loaded with GTP$\gamma$S or GDP), dominant-negative Rap1A-17N, or constitutively active Rap1A-63E were incubated with purified IQGAP1 in 500 μl of buffer B for 3 h at 4 °C. Complexes were isolated with amyllose beads, washed, and resolved by SDS-PAGE, and blots were probed with anti-IQGAP1 antibodies (26).

To compare binding of Rap1A and Rap1B to IQGAP1, we used GST-tagged Rap1 proteins. IQGAP1 was cleaved from GST using tobacco etch virus protease as described (29). GST, GST-Rap1A, and GST-Rap1B were loaded with GTP$\gamma$S as described above. Equal amounts of purified IQGAP1 were incubated with equal amounts of GST$\gamma$S-loaded GST, GST-Rap1A, or GTP Rap1B in 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 0.2% Triton X-100, 10% glycerol, and 1 mM EGTA for 2 h at 4 °C. Complexes were isolated with glutathione-Sepharose, washed, and resolved by SDS-PAGE. The gel was cut into two pieces; the top portion was transferred to polyvinylidene difluoride membranes, and blots were probed with anti-IQGAP1 antibody. The lower half was stained with Coomassie Blue to visualize GST-tagged proteins.

$[^{35}S]$Methionine-labeled transcription and translation (TNT) products were generated with the TNT Quick Coupled Transcription/Translation system (Promega) according to the manufacturer’s instructions. Briefly, 0.5 μg of the IQGAP1 plasmids was incubated with 40 μl of TNT Quick Master mix and 20 μCi of $[^{35}S]$methionine (PerkinElmer Life Sciences) at 30 °C for 1 h. Products were confirmed by SDS-PAGE and autoradiography. Equal amounts of radiolabeled peptide were incubated with His-Rap1A-63E. Complexes were isolated with Ni$^{2+}$-NTA agarose, resolved by SDS-PAGE, and processed by autoradiography.

Competitive Inhibition Analysis—$[^{35}S]$Methionine-labeled IQGAP1 was preincubated with 5 μg of calmodulin, 5 μg of constitutively active Cdc42 (Cdc42-Q61L), or 5 μg of BSA in 500 μl of buffer B containing 1 mM Ca$^{2+}$ or 1 mM EGTA for 1 h at 4 °C. Equal amounts of His-Rap1A-63E protein were added, and samples were incubated another 1 h. The complexes were washed and isolated with Ni$^{2+}$-NTA agarose. Samples were resolved by SDS-PAGE, and bands were analyzed by autoradiography of the dried gel. For calmodulin-Sepharose chromatography, $[^{35}S]$methionine-labeled IQGAP1 was preincubated with 5 μg of His-Rap1A-63E or 5 μg of BSA in the presence of Ca$^{2+}$ or EGTA for 1 h at 4 °C. Equal amounts of calmodulin-Sepharose or empty Sepharose were added. After 1 h beads were isolated and washed three times in buffer B containing Ca$^{2+}$ or EGTA. Proteins bound to calmodulin were resolved by SDS-PAGE and processed by autoradiography of the dried gel.

Immunofluorescence Staining and Confocal Microscopy—MCF-7 or MCF-silQ8 cells were transiently transfected with GFP-Rap1A alone or in combination with RFP-tagged IQGAP1 constructs. Cells were plated on glass coverslips that had been coated overnight with 10 μg/ml type I collagen or 10 μg/ml poly-L-lysine (Sigma-Aldrich) and blocked with 0.5% BSA. Immunocytochemistry was performed as described (23, 31) with a few modifications. Cells were washed twice with PBS and incubated in 4% paraformaldehyde-PBS for 20 min at 22 °C. After washing twice in PBS, cells were permeabilized in 0.2% Triton X-100 with 3% BSA in PBS for 1 h at 22 °C. Polyclonal anti-IQGAP1 antibody was added in 0.2% Triton, 1% BSA for 16 h at 4 °C followed by Alexa-Fluor 543-labeled anti-rabbit IgG secondary antibody (Molecular Probes) for 1 h. Where appropriate, actin was visualized with Alexa-Fluor 543-conjugated phalloidin. Rabbit IgG was used as a control for the corresponding primary antibody. The stained cells were analyzed using a Zeiss LSM 510 confocal microscope and analyzed with Zeiss LSM software.

Miscellaneous—Densitometry of enhanced chemiluminescence (ECL) signals was analyzed with UN-SCAN-IT software (Silk Scientific Corp.). Statistical analysis was assessed by Student’s $t$ test with Instat software (GraphPad Software, Inc.). Protein concentrations were determined with the DC protein assay (Bio-Rad).

RESULTS

Direct Interaction between IQGAP1 and Rap1—In vitro analysis was conducted to examine a possible association between IQGAP1 and Rap1. Purified MBP-Rap1A was loaded with either non-hydrolyzable GTP$\gamma$S to form active Rap1A or with GDP to produce inactive Rap1A. Equal amounts of the Rap1A constructs were incubated with purified GST-IQGAP1. Pulldown with glutathione-Sepharose beads revealed that Rap1A binds to IQGAP1. IQGAP1 binds more efficiently to GTP-Rap1A than to the inactive form of Rap1A.
Identification of the Rap1A Binding Domain on IQGAP1

To ascertain the region of IQGAP1 to which Rap1 binds, pulldown assays with \[^{35}S\]methionine-labeled IQGAP1 were performed. We sought to determine whether IQGAP1 interacts with both isoforms, we evaluated in vitro binding. Equal amounts of GST-Rap1A and GST-Rap1B (Fig. 1C) were loaded with GTP\_S and incubated with purified IQGAP1. The amounts of IQGAP1 bound to Rap1A and Rap1B were approximately equal (Fig. 1C), implying that the binding domain on Rap1 lies outside the hypervariable region. All subsequent in vitro analyses were conducted with Rap1A.

Calmodulin Abrogates the Binding of Rap1A to IQGAP1—Ca\(^{2+}\)/calmodulin is known to influence the binding of IQGAP1 to several target proteins, most likely by altering the conformation of IQGAP1 (15). Therefore, we investigated the effect of calmodulin on the interaction between IQGAP1 and Rap1A. \[^{35}S\]Methionine-labeled IQGAP1 was preincubated with calmodulin, and purified His-Rap1A-63E was subsequently added. Complexes were isolated with Ni\(^{2+}\)-NTA-agarose, and the association was detected by autoradiography. Calmodulin abrogates binding of IQGAP1 to Rap1A (Fig. 2A). The effects of calmodulin are independent of Ca\(^{2+}\), as disruption occurred both in the presence of Ca\(^{2+}\) and when Ca\(^{2+}\) was chelated with EGTA. Disruption was specific for calmodulin because an equivalent amount of BSA did not reduce binding (Fig. 2A).

To extend these findings, we evaluated the effect of Rap1A on the binding of IQGAP1 to calmodulin. (Fig. 1A). Binding was specific, as no Rap1A associated with GST alone. Coomassie staining reveals that equal amounts of GST-IQGAP1 were present in the assay (Fig. 1A).

To confirm these findings, the reverse experiment was done. MBP-Rap1A was incubated with purified IQGAP1. Pulldown with amylase beads reveals that more IQGAP1 binds to GTP-loaded Rap1A than to GDP-Rap1A (Fig. 1B). Similar analysis was conducted with MBP-tagged forms of the constitutively active Rap1A-63E and dominant negative Rap1A-17N. Consistent with the data obtained with guanine nucleotide loading, IQGAP1 associates with Rap1A-63E but not with Rap1A-17N (Fig. 1B). The amounts of MBP-Rap1A present in the assay were equivalent (Fig. 1B). Collectively, these data reveal that Rap1A interacts directly with IQGAP1 in a GTP-regulated manner.

Rap1 has two isoforms, Rap1A and Rap1B, which differ in only a few amino acids, predominantly in the hypervariable region (3). To determine whether IQGAP1 interacts with both...
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**Figure 3. Identification of amino acid residues of IQGAP1 necessary for Rap1 binding.**

A schematic representation of IQGAP1 constructs. Full-length and deletion mutants of IQGAP1 are depicted. The identified protein interaction motifs and the specific amino acid residues absent from each mutant are indicated. WW, polyproline binding domain; IQ, Ras-GAP-related domain; GRD, Ras-GAP-related domain; IQGAP1-N, N-terminal half of IQGAP1; IQGAP1-C, C-terminal half of IQGAP1. 8 and C. 

Selected constructs of IQGAP1 (depicted in Fig. 3A) were labeled with [35S]methionine in a reticulocyte lysate and incubated with His-tagged Rap1A-63E. Constructs that bound to Rap1A were identified by autoradiography. As shown in Fig. 2, full-length IQGAP1 binds Rap1A-63E (Fig. 3B). The N-terminal half (comprising amino acids 2–863) of IQGAP1 also binds to Rap1A, but no binding of the C-terminal half of IQGAP1 was detected. To further narrow the binding site, selected IQGAP1 constructs lacking specific protein interaction motifs in the N-terminal half were used. Analysis showed that the WW domain residue (643–744) was not necessary for IQGAP1 to bind Rap1A (Fig. 3C). In contrast, absence of the IQ domain that includes the IQ domain (717–916) completely abrogates IQGAP1 binding (28). IQGAP1 that is unable to bind calmodulin exhibits increased Rap1 binding. IQGAP1 was isolated with Ni2+ -NTA-agarose beads (Pulldown). Samples were transferred to polyvinylidene difluoride membranes, and the blots were probed with anti-Myc antibody. Data are representative of three independent experiments.

**Figure 4. Mutant IQGAP1 that is unable to bind calmodulin exhibits increased Rap1 binding.**

A, 293H cells were transiently transfected with pcDNA3 vector (V), wild type IQGAP1 (WT), or IQGAP1 IQ3,4R (IQ3,4R), a mutant IQGAP1 construct lacking calmodulin binding. After lysis, equal amounts of protein were resolved by SDS-PAGE and blotted with anti-Myc antibody (all IQGAP1 constructs are Myc-tagged) (Lysate). Equal amounts of protein lysate were incubated with His-Rap1A-63E or Ni2+ -NTA-agarose beads (Pulldown). Samples were transferred to polyvinylidene difluoride membranes, and the blots were probed with anti-Myc antibody. Data are representative of three independent experiments. B, the relative amounts of IQGAP1 bound to Rap1 were quantified by densitometry. Data, expressed relative to the amount of wild type IQGAP1, are the means ± S.D. (n = 3).

IQGAP1, incubated with lysates from 293H cells transfected with IQGAP1, was isolated with Ni2+ -agarose. Probing the resultant Western blots revealed that IQGAP1 binds specifically to Rap1A (Fig. 4A). We were unable to reproducibly and specifically co-immunoprecipitate Rap1 and IQGAP1 from cell lysates. The reason for this is unknown. This may be due to masking of the antibody recognition epitope in the complex or comodulin binding to IQGAP1 facilitates the binding of Rap1. IQGAP1 IQ3,4R and wild type IQGAP1 were transiently transfected to equivalent levels in 293H cells (Fig. 4A, upper panel). Analysis reveals that the amount of IQGAP1 IQ3,4R pulled down by Rap1A is 2.5-fold greater than that of wild type IQGAP1 (Fig. 4B).

Equal amounts of His-Rap1A were present in each sample (data not shown). These data demonstrate that elimination of calmodulin binding to IQGAP1 facilitates the binding of Rap1.

Rap1A-63E, incubated with lysates from 293H cells transfected with IQGAP1, was isolated with Ni2+ -agarose. Probing the resultant Western blots revealed that IQGAP1 binds specifically to Rap1A (Fig. 4A). We were unable to reproducibly and specifically co-immunoprecipitate Rap1 and IQGAP1 from cell lysates. The reason for this is unknown. This may be due to masking of the antibody recognition epitope in the complex or comodulin binding to IQGAP1 facilitates the binding of Rap1. IQGAP1 IQ3,4R and wild type IQGAP1 were transiently transfected to equivalent levels in 293H cells (Fig. 4A, upper panel). Analysis reveals that the amount of IQGAP1 IQ3,4R pulled down by Rap1A is 2.5-fold greater than that of wild type IQGAP1 (Fig. 4B).

**Binding of Mutant IQGAP1 to Rap1**—The binding of IQGAP1 to Rap1 in a normal cell milieu was examined. His-
ing RNA specifically targeted to IQGAP1 (23). Termed MCF-siIQ8, these cells have 80% reduction in IQGAP1 levels (23). When lysates from MCF-siIQ8 cells were resolved by gel filtration chromatography, the elution of Rap1 was changed (Fig. 5A). Virtually no Rap1 eluted in fractions 24–30, which corresponds to ~150–300 kDa. Moreover, the amount of Rap1 that eluted in fractions 16–22 was substantially reduced, with a concomitant increase in the amount of Rap1 detected at lower molecular masses. Interestingly, the elution of IQGAP1 in MCF-siIQ8 cells was different from that in MCF-7 cells (Fig. 5A). The IQGAP1 shifted to a lower molecular mass, perhaps due to attenuated oligomerization when the IQGAP1 concentration is reduced. Taken together, the gel filtration data suggest that Rap1 associates with IQGAP1 in MCF-7 cells.

**Rap1 Co-localizes with IQGAP1 at the Cell Periphery**—We next examined the localization of Rap1 and IQGAP1 in MCF-7 cells transfected with GFP-Rap1. In agreement with previous reports (17, 30), endogenous IQGAP1 is diffusely distributed throughout the cytoplasm, with accumulation at cell-cell contacts in MCF-7 cells (Fig. 5B). The distribution of transfected Rap1 is similar. GFP-tagged Rap1 is found throughout the cell with some accumulation at the plasma membrane (Fig. 5B). Analysis with specific co-localization software revealed that IQGAP1 and Rap1 co-localize exclusively at the cell periphery; essentially no co-localization is detected in the cytoplasm (Fig. 5B). We were unable to obtain specific immunostaining with commercially available antibodies to Rap1, precluding analysis of the endogenous protein by immunocytochemistry. Similar limitations have been reported by other investigators (34). The GFP tag does not affect Rap1 activity (35).

Based on our analyses in this study, one would anticipate that IQGAP1ΔIQ, which does not bind Rap1, will exhibit less co-localization with Rap1 than that seen with wild type IQGAP1. Moreover, Rap1 should have greater co-localization with IQGAP1Δ3,4R than with wild type IQGAP1. To test these hypotheses, we transfected GFP-Rap1A and RFP-tagged versions of the pertinent IQGAP1 constructs. As seen with endogenous IQGAP1, transfected wild type IQGAP1 co-localizes with Rap1A predominantly at the cell periphery (Fig. 5C). As anticipated, Rap1A has weaker co-localization with IQGAP1ΔIQ than with wild type IQGAP1. Note that in cells transfected with Rap1A and IQGAP1ΔIQ, the two proteins co-localize in a perinuclear region (Fig. 5C). Also consistent with our prediction, Rap1A exhibits enhanced co-localization with

![FIGURE 5. Co-localization of Rap1 and IQGAP1. A, lysates from MCF-7 (upper two panels) and MCF-siIQ8 (lower panels) cells were fractionated on a Superose 6 gel filtration column as described under “Experimental Procedures.” Fractions (1 ml) were collected. A 40-μl aliquot of the indicated fractions was analyzed by Western blotting and probed for IQGAP1 and Rap1. The positions of elution of known markers are depicted. Data are representative of three independent analyses. B, MCF-7 cells, transiently transfected with GFP-Rap1, were plated on coverslips coated with poly-L-lysine and cultured for 12 h. IQGAP1 was stained with anti-IQGAP1 antibody. The colocalization of GFP-Rap1 (green) and IQGAP1 (red) was analyzed by confocal microscopy. Data are representative of three experiments. C, MCF-7 cells were transiently transfected with GFP-Rap1 and RFP-tagged constructs of wild type IQGAP1 (WT), IQGAP1ΔIQ (ΔIQ), or IQGAP1IQ3,4R (IQ3,4R). After 48 h cells were fixed and processed for immunocytochemistry. Merge represents a composite of the two channels, with yellow indicating co-localization of IQGAP1 and Rap1. Colocalization of GFP-Rap1 (green) and each RFP-IQGAP1 construct (red) was analyzed with Zeiss LSM software on the confocal microscope. Representative images are depicted. Scale bar, 10 μm.](http://www.jbc.org/content/jbc/282/28/20757)
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Effects of IQGAP1 on Activation of Rap1 by Extracellular Matrix—Rap1 is activated during cell adhesion (36, 37). We used this knowledge to investigate whether interaction with IQGAP1 alters Rap1 function. MCF-7 cells that stably overexpress IQGAP1 were used. Termed MCF/I, these cells contain 3-fold more IQGAP1 than MCF-7 cells (Fig. 7, A and C) (18, 23). Probing the blots for tubulin verifies equal loading. MCF/I and MCF-7 cells were seeded on plates coated with fibronectin or collagen I, and adherent cells were harvested at different time intervals. Adhesion to fibronectin increases the amount of the active, GTP-bound form of Rap1 in MCF-7 cells in a time-dependent manner, reaching 13.7-fold at 90 min (Fig. 7, A and B). Total Rap1 is not altered. Overexpression of IQGAP1 reduces the amount of GTP-Rap1 in non-adherent cells (Fig. 7, A and B). The effect of increasing IQGAP1 on activation of Rap1 by adhesion is even more dramatic. Rap1 activation is markedly blunted in MCF/I cells in response to adhesion. Maximum levels of GTP-Rap1 in MCF/I cells are 14.3% of these in MCF-7 cells (Fig. 7, A and B). The data strongly suggest that IQGAP1 attenuates extracellular matrix-induced Rap1 activation.

To validate this hypothesis, we examined the effect of IQGAP1 on activation of Rap1 by cell adhesion to collagen I. Adhesion of MCF-7 cells to collagen I increases the amount of GTP-Rap1, although the magnitude of stimulation is substantially less than that seen with fibronectin (Fig. 7, C and D). Analogous to the findings with fibronectin, overexpression of IQGAP1 attenuates activation of Rap1 by adhesion to collagen I (Fig. 7, C and D). Although the variation among experiments with collagen I is large, the patterns are virtually identical to those seen with fibronectin. Collectively these data reveal that overexpression of IQGAP1 reduces activation of Rap1 by cell adhesion.

To confirm that the observed effect is mediated by Rap1 binding to IQGAP1, we repeated the analysis with cells transfected with IQGAP1IQ, which does not interact with Rap1 (see Fig. 3). In contrast to the results obtained with wild type IQGAP1, IQGAP1IQ neither reduces the amount of GTP-Rap1 in cells in suspension nor impairs the increase in GTP-Rap1 on cell adhesion (Fig. 8, A and B).

Cdc42 activation is different to that of Rap1. Adhesion of MCF-7 cells to fibronectin had little effect on Cdc42 activation (Fig. 7, A and B). A slight increase in active Cdc42 was evident only at 90 min. This is most likely due to the development of E-cadherin-mediated adherens junctions, which are known to augment GTP-Cdc42 (31). As we documented previously (23, 30), overexpression of IQGAP1 significantly increases the amount of GTP-Cdc42 in cells (Fig. 7, A and B). Adhesion to fibronectin does not substantially alter active Cdc42 in MCF/I cells (Fig. 7, A and B).

Blockade of calmodulin has been reported to affect the GTP loading of Rap1 (38). It is conceptually possible that when overexpressed, IQGAP1 could perturb calmodulin signaling pathways by acting as a calmodulin “sink.” To test this hypothesis, we used IQGAP1 IQ3,4R which does not bind calmodulin. MCF-7 cells were transiently transfected with IQGAP1 IQ3,4R, and the response of Rap1 to adhesion to fibronectin was examined. Analogous to wild type IQGAP1, IQGAP1 IQ3,4R substantially reduced Rap1 activation in response to adhesion (Fig.

- IQGAP1 IQ3,4R, which is mainly observed at the cell periphery, particularly in areas of ruffling (Fig. 5C).
- Transfection of Rap1A Alters the Morphology of Cells with Reduced IQGAP1—The effects of transfecting GFP-Rap1A on the morphology of MCF-7 cells expressing different IQGAP1 levels was compared. When expressed in MCF-siIQ8 cells, GFP-Rap1A was diffusely distributed (Fig. 6). The cell morphology was altered with the appearance of multiple, elongated, actin-rich processes at the cell periphery (Fig. 6B). It is not possible to discern whether these result from membrane retraction or are extensions from the cell margin. Nevertheless, these structures were seen more frequently when Rap1A was transfected into MCF-siIQ8 cells than into MCF-7 cells. Analysis of >200 cells transfected with GFP-Rap1A revealed that elongated processes were present in 73 and 16% of MCF-siIQ8 and MCF-7 cells, respectively. In addition, there seemed to be less accumulation of GFP-Rap1A at MCF-siIQ8 cell-cell junctions than at junctions of MCF-7 cells (Fig. 6B).

FIGURE 6. Expression of GPRap1A in MCF-7 and MCF-siIQ8 cells. MCF-7 or MCF-siIQ8 (siIQ8) cells were transiently transfected with GFP-Rap1A, and 48 h later were processed for immunocytochemistry. A, cells were incubated with anti-IQGAP1 polyclonal antibody followed by Alexa-Fluor 543-tagged anti-rabbit secondary antibody (red). Merge represents a composite of the two channels, with yellow indicating co-localization. B, cells were incubated with Alexa-Fluor 543-conjugated phalloidin to identify actin (red). Two different images are depicted for MCF-siIQ8 cells. Data are representative of images obtained from two experiments. Scale bar, 10 μm.
IQGAP1 modulates several fundamental cellular functions by associating with multiple proteins including calmodulin, Cdc42, Rac1, actin, β-catenin, E-cadherin, CLIP-170, extracellular signal-regulated kinase 1/2, and MEK1/2 (mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2) (6, 7). Previous studies revealed that IQGAP1 regulates the function of many of its binding partners. For example, IQGAP1 inhibits the E-cadherin and β-catenin complex (16, 17), increases β-catenin-mediated transcriptional activation (18), and modulates mitogen-activated protein kinase signaling (12, 26).

IQGAP1 is known to interact with members of the Rho GTPase family, specifically Cdc42, Rac1, and TC10 (6, 14, 23, 24). In contrast, IQGAP1 does not bind RhoA or H-Ras (14), nor have interactions with any other members of the Ras superfamily been previously documented (immunoaffinity purification of proteins cross-linked to constitutively active M-Ras identified 18 proteins including Rap1 and IQGAP1, but no direct binding of IQGAP1 to M-Ras or Rap1 was documented in that study (41)). The interactions of IQGAP1 with Cdc42 and Rac1 have been well characterized by several groups. Direct binding of Cdc42 and Rac1 to IQGAP1 is dependent on GTP (13, 14, 42). IQGAP1 does not function as a GAP; in fact it maintains Cdc42 and Rac1 in the active, GTP-bound form (10, 14). This results in an increase in the GTP-bound forms of the small GTPases in cells that overexpress IQGAP1 (30). Importantly, the interaction has functional significance. Overexpression of IQGAP1 promotes cell motility and invasion at least in part via its association with Cdc42 and Rac1 (23). In this study we document for the first time a direct interaction between IQGAP1 and a GTPase that is not a member of the Rho subfamily.

8, C and D). These data reveal that the inhibitory effect of IQGAP1 on activation of Rap1 is not mediated via calmodulin.

IQGAP1 Regulates cAMP-induced Activation of Rap1—To determine whether IQGAP1 modulates activation of Rap1 by another pathway we examined Epac. Epac is a cAMP target and a Rap1-specific GEF (39). Therefore, we incubated cells with 8CPT-2Me-cAMP, which specifically activates Epac without activating protein kinase A (40). As anticipated, the cAMP compound induces an increase in active Rap1 in MCF-7 cells. GTP-Rap1 was ∼2-fold higher at 30 min than in unstimulated MCF-7 cells (Fig. 9, A and B). By contrast, 8CPT-2Me-cAMP is unable to increase GTP-Rap1 in MCF/I cells. These data demonstrate that IQGAP1 overexpression modulates activation of Rap1 by cAMP.

The involvement of IQGAP1 in cAMP-mediated activation of Rap1 was also analyzed by a second strategy. If IQGAP1 is a negative regulator of Rap1, one would predict that reduction of IQGAP1 levels would enhance the amount of GTP-Rap1. We tested this hypothesis with MEF cells isolated from IQGAP1-null mice. These cells have no detectable IQGAP1 protein (Fig. 9C). The amount of GTP-bound Rap1 in IQGAP1−/− MEFs is 2.3-fold greater than that in control MEFs (Fig. 9, C and D). Moreover, the ability of cAMP to activate Rap1 is significantly enhanced in the cells lacking IQGAP1 when compared with control MEFs.

DISCUSSION

IQGAP1 modulates several fundamental cellular functions by associating with multiple proteins including calmodulin, Cdc42, Rac1, actin, β-catenin, E-cadherin, CLIP-170, extracellular signal-regulated kinase 1/2, and MEK1/2 (mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2) (6, 7). Previous studies revealed that IQGAP1 regulates the function of many of its binding partners. For example, IQGAP1 inhibits the E-cadherin and β-catenin complex (16, 17), increases β-catenin-mediated transcriptional activation (18), and modulates mitogen-activated protein kinase signaling (12, 26).

FIGURE 7. Extracellular matrix-induced activation of Rap1 is attenuated in cells overexpressing IQGAP1. MCF-7 and MCF/I cells were plated on culture dishes coated with fibronectin (A and B) or collagen I (C and D) as described under “Experimental Procedures.” At the times indicated adherent cells were harvested with lysis buffer and analyzed by SDS-PAGE and Western blotting. Equal amounts of protein lysate were also used in a GST-WASP (for active Cdc42) or GST-RalGDS (for active Rap1) pulldown followed by Western blotting with anti-Cdc42 or anti-Rap1 antibody. Protein lysates were also blotted for IQGAP1 and β-tubulin (as loading control). B and D, the relative amounts of active Rap1 (panels B and D) and Cdc42 (panel B) in each sample were quantified by densitometry and corrected for the total Rap1 or Cdc42, respectively, in the corresponding cell lysate. Data, expressed relative to the amount of active GTPase in MCF-7 cells at time 0, represent the means ± S.D. (n = 3).
IQGAP1 Modulates Rap1 Activation

The interaction of Rap1 with IQGAP1 differs in several respects to the interaction of Cdc42 and Rac1 with IQGAP1. For example, although more GTP-Rap1 binds to IQGAP1, GDP-Rap1 also associates with IQGAP1. Moreover, binding to IQGAP1 does not appear to stabilize the GTP-bound form of Rap1 as described for Cdc42 and Rac1 (11, 28). This is substantially different to other binding domains identified for Ras proteins.

A second novel aspect of the interaction of Rap1 with IQGAP1 is the effect of calmodulin. Calmodulin attenuates the interaction of IQGAP1 with all of its binding partners examined in vitro, reducing its ability to bind other proteins (11, 13, 28). Although the mechanism by which calmodulin produces this effect is not known, we have proposed that in the presence of Ca^2+ calmodulin alters the tertiary conformation of IQGAP1, thereby reducing its ability to bind other proteins (6, 13, 15). It was, thus, surprising that calmodulin abrogates Rap1 binding to IQGAP1 both in the absence and presence of Ca^2+. This observation implies that the molecular mechanism is different. Some insight was obtained when the site of Rap1 binding on IQGAP1 was identified. In contrast to Cdc42 and Rac1, which bind to a region that includes the GRD in the C-terminal half of IQGAP1 (10, 14, 43), Rap1 binds to the IQ domain in the N-terminal half of IQGAP1. Because calmodulin also binds to this region (28), it is likely that calmodulin directly competes with Rap1 by sterically hindering access of Rap1 to the IQ region. Thus, apocalmodulin, which binds only to the third and fourth IQ motifs (28), may restrict access of Rap1 without altering the conformation of IQGAP1.

The IQ motif contains 20–25 amino acids with a core consensus of IQGAP1. Because calmodulin also binds to this region (28), it is noteworthy that Rap1 does not interfere with calmodulin binding to IQGAP1, possibly because the affinity of IQGAP1 for Rap1 is lower than its affinity for calmodulin. Additional support for this mechanism was obtained by examining the mutant construct IQGAP1 Q3,4R. This construct contains replacement of selective hydrophobic residues in the third and fourth IQ motifs, which specifically abrogates binding to apocalmodulin (28). Rap1 binds more efficiently to IQGAP1 Q3,4R in cell lysates than to wild type IQGAP1. It is noteworthy that Rap1 does not interfere with calmodulin binding to IQGAP1, possibly because the affinity of IQGAP1 for Rap1 is lower than its affinity for calmodulin. Finally, the different binding sites for the GTPases explain why Cdc42 did not attenuate the interaction between Rap1 and IQGAP1. Based on these in vitro data, it seems likely that IQGAP1 may bind simultaneously to Rap1 and Cdc42. Collectively, our findings suggest that the association of calmodulin with IQGAP1 is a critical regulatory factor in the interaction of Rap1 with IQGAP1.

Rap1 binds to the IQ region of IQGAP1. This is substantially different to other binding domains identified for Ras proteins. The IQ motif contains 20–25 amino acids with a core consensus...
sus IQXXRGXXXR (where X is any amino acid) (44). A recent search identified IQ domains in 2479 proteins in the SMART non-redundant data base. Calmodulin, myosin light chains, and a few other Ca\(^{2+}\)-binding proteins associate with IQ motifs. The crystal structures of IQ motifs of myosin V bound to calmodulin (45) and to myosin light chain 1 (46) have been solved recently. There are no published structures of the IQ domains of IQGAP1. It is not known how Rap1 associates with the IQ motifs of IQGAP1. Differences in the amino acid composition and size between the IQ domains of IQGAP1 and myosin V preclude extrapolation of the myosin V data to IQGAP1. Therefore, solving the structure of Rap1 bound to the IQ motifs of IQGAP1 is necessary to provide insight into the nature of this unusual interaction.

We (present study) and others (34, 35) observed that Rap1 is found in the cytoplasm, particularly in the perinuclear area and at the plasma membrane where it is enhanced in ruffles. IQGAP1 has a similar distribution, with enhanced localization in the perinuclear area and at the plasma membrane (17, 23, 47). Notwithstanding the localization of both IQGAP1 and Rap1 in the cytoplasm, we observed co-localization exclusively at the cell periphery. This limited and specific co-localization suggests that functional interactions between IQGAP1 and Rap1 are restricted to the cell periphery. The reason for this is unknown. Nevertheless, this limited area of interaction may contribute to our inability to co-immunoprecipitate the endogenous proteins from cell lysates. Based on the region of co-localization, we speculate that IQGAP1 may serve to integrate Rap1 with the cytoskeleton at sites of cell contact both with other cells or potentially with the substratum. Support for the former hypothesis is that both IQGAP1 (17) and Rap1 (35) are known to participate in adherens junctions. Rap1 was shown to be recruited to mature epithelial cell-cell contact sites (35). In that study the authors raised the question as to which molecules are involved in anchoring Rap1 to cell-cell contacts (35). Based on our data, it is tempting to speculate that IQGAP1 binds GDP-Rap1 at adherens junctions, perhaps participating in anchoring the Rap1. A functional interaction between Rap1 and integrins is well recognized (5). However, an interaction of IQGAP1 with integrin is less well documented and is controversial (6). Further studies are necessary to determine whether the IQGAP1-Rap1 interaction contributes to the regulation of adherens junction formation and/or to the attachment of cells to the substratum.

Binding of Rap1 to IQGAP1 has functional sequelae as IQGAP1 modulates activation of Rap1. We observed that overexpression of IQGAP1 reduces the amount of active Rap1 in cells in suspension and substantially impairs the activation of Rap1 on cell adhesion. Consistent with these findings, a mutant IQGAP1 that lacks Rap1 binding did not attenuate activation of Rap1. Similarly, overexpression of IQGAP1 reduces the ability of cAMP to increase active Rap1. Moreover, cells lacking IQGAP1 have increased amounts of GTP-bound Rap1 and display an enhanced response to activation by cAMP. Collectively, these data imply that IQGAP1 acts as a negative regulator of Rap1. The molecular mechanism underlying these observations is unknown. One possibility is that binding to IQGAP1 attenuates the interaction of Rap1 with GEFs, impairing the exchange of GDP for GTP. This could occur by altering Rap1 conformation or by changing the subcellular localization of Rap1 to microdomains lacking the GEFs. Another possibility is that IQGAP1 may facilitate the interaction of Rap1 with GAPs, resulting in enhanced GTP hydrolysis. These postulates are not mutually exclusive, and more than one may be operative. Regardless of the mechanism, the result is that IQGAP1 reduces GTP-Rap1, an effect opposite that produced on Rac1 and Cdc42, which are maintained in the GTP-bound form when bound to IQGAP1 (10, 14). These differences most likely are the result of different modes of interaction of the GTPases with IQGAP1. Cdc42 and Rac1 bind to the GTPase regulatory domain (GRD) of IQGAP1, whereas Rap1 binds to the IQ domain.

In summary, our data document for the first time a direct interaction between a member of the Ras subfamily and IQGAP1. These findings extend the repertoire of binding partners for both IQGAP1 and Ras GTPases. We propose that IQGAP1 is a target for Rap1. Based on its well documented interaction with the cytoskeleton, we hypothesize that IQGAP1 integrates Rap1 with actin dynamics and cell-cell attachment. Finally, because calmodulin abrogates IQGAP1 binding to Rap1, it is tempting to speculate that IQGAP1 may serve as a link between the calmodulin and Rap1 signaling pathways.

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IQGAP1 Binds Rap1 and Modulates Its Activity
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