Self-association of IQGAP1
CHARACTERIZATION AND FUNCTIONAL SEQUELAE

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The scaffolding protein IQGAP1 participates in numerous cellular functions by binding to target proteins such as actin, calmodulin, E-cadherin, β-catenin, Cdc42, Rac1, and CLIP-170. IQGAP1 regulates the cytoskeleton, promotes cell motility, and modulates E-cadherin-mediated cell-cell adhesion. However, how IQGAP1 exerts its functions in vivo is still unclear. In this study we investigate the self-association of IQGAP1 and its role in IQGAP1 function. Endogenous IQGAP1 co-immunoprecipitated from MCF-7 cells with IQGAP1 tagged with enhanced green fluorescent protein, indicating that IQGAP1 self-associates in cells. In vitro assays confirmed that IQGAP1 can self-associate and that this effect is mediated by the N-terminal half of the protein. Gel filtration analysis suggested that full-length IQGAP1 exists as a combination of monomers, dimers, and larger oligomers. Analysis performed with multiple fragments of IQGAP1 narrowed the self-association region to amino acids 763–863. In support of this observation, a peptide comprising residues 763–863 disrupted self-association of full-length IQGAP1 in a dose-dependent manner. Similarly, deleting this sequence from IQGAP1 abolished binding to full-length IQGAP1. In addition, the ability of IQGAP1 to increase the amount of active Cdc42 in cells is abrogated upon removal of this region. Consistent with these findings, transfection into cells of a peptide containing the self-association domain significantly reduced the amount of active Cdc42 in cell lysates. These observations define a sequence of IQGAP1 that is necessary for its oligomerization and demonstrate that self-association is required for the normal cellular function of IQGAP1.

IQGAP1, identified by Weissbach et al. (1) as a 1657-amino acid protein, is a complex multidomain molecule (for reviews see Refs. 2–4). The name is derived from the presence of an IQ domain and a region with extensive sequence similarity to the catalytic domain of Ras GTPase-activating proteins (GAPs). IQGAP1 interacts with diverse targets. In the N-terminal half, the calponin homology domain (CHD) binds actin (5, 6); the WW domain binds ERK2 (7), and the IQ domain mediates interactions with calmodulin (5, 8, 9) and calmodulin-related proteins (2). The C-terminal half of IQGAP1 is reported to bind active Cdc42 (8, 9) and Rac1 (9), E-cadherin (10, 11), β-catenin (10, 12), and CLIP-170 (13). Through interactions with these and other proteins, IQGAP1 participates in multiple fundamental cellular activities, including transcription, cell-cell attachment, and regulation of the cytoskeleton (2).

Although it is involved in many important activities, how IQGAP1 exerts its function in vivo is unclear. Accumulating evidence supports the hypothesis first proposed by Ho et al. (5) that IQGAP1 functions as a scaffold. Proteins in biological systems rarely act in isolation but bind other biomolecules to elicit specific cellular responses. The self-association of proteins to form dimers and higher oligomers is a very common phenomenon (for a review see Ref. 14). Recent structural and biophysical studies show that dimerization or oligomerization is a key factor in the regulation of proteins such as enzymes, ion channels, receptors, and transcription factors (14). There is some indirect evidence implying that IQGAP1 self-associates. For example, sedimentation equilibrium of IQGAP1 obtained from bovine adrenal tissue suggested an estimated molecular weight of 358,653 to 401,864 (15). Another study inferred from gel filtration analysis that IQGAP1 may form oligomers (16). However, no data have been published to unequivocally document that IQGAP1 can self-associate. Moreover, although it is commonly stated that IQGAP1 dimerizes via multiple putative coiled-coil repeats in the N-terminal half of the molecule (4, 6), direct evidence to bolster this claim is lacking. For these reasons, we set out in this study to establish whether IQGAP1 self-associates and, if so, which region is responsible. Our data reveal that IQGAP1 self-associates both in vitro and in cells. Most unexpectedly, the oligomerization site was not via the coiled-coil repeats, but localized to amino acids 763–863. Deletion of this sequence disrupted self-association of IQGAP1. Moreover, a mutant IQGAP1 construct lacking this sequence failed to increase active Cdc42 in the cells, suggesting that oligomerization is important for IQGAP1 function.

EXPERIMENTAL PROCEDURES

Materials—Lipofectamine 2000, tissue culture reagents, Pfu DNA polymerase, and AcTEV (tobacco etch virus) protease were purchased from Invitrogen. All restriction enzymes were obtained from New England Biolabs. Glutathione-Sepharose beads, protein G-Sepharose, and protein A-Sepharose were from Amersham Biosciences. Ni2+-NTA-agarose and the QIAprep spin miniprep kit were from Qiagen. Fetal bovine serum was obtained from Invitrogen. FuGENE 6 transfection reagent kit was purchased from Roche Applied Science. The transcription and translation (TNT) T7 quick-coupled transcription/translation system was obtained from Promega. Blue dextran, apoferritin, β-amylose, alcohol dehydrogenase, and albumin markers for gel filtration analysis were purchased from Sigma. Anti-Myc monoclonal antibody (9E10.2) was manufactured by Maine Biotechnology. The anti-GFP and anti-Cdc42 antibodies were purchased from Zymed Laboratories Inc. and Gene Transduction, respectively. The anti-IQGAP1 polyclonal 

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†The abbreviations used are: GAP, GTPase-activating protein; Ni2+-NTA, nickel-nitrilotriacetic acid; GST, glutathione S-transferase; CHD, calponin homology domain; GRD, RasGAP-related domain; GBD, GTPase-binding domain; WASP, Wiskott-Aldrich syndrome protein; EGFP, enhanced green fluorescent protein; TEV, tobacco etch virus; TNT, transcription and translation; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; FPLC, fast protein liquid chromatography; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
antibody has been characterized previously (5). The anti-IQGAP1 monoclonal antibody was generously provided by Andre Bernard (Massachusetts General Hospital, Boston). Secondary antibodies for enhanced chemiluminescence (ECL) detection were from Amersham Biosciences. All other reagents were of standard analytical grade.

**Cells Lines**—MCF-7 human breast epithelial cells that stably overexpress pcDNA3-myc-IQGAP1 (termed MCF-1 cells) have been described previously (12, 17). MCF-1 cells have 3-fold more IQGAP1 than the parent MCF-7 line (17). HEK-293T and MCF-7 cells were purchased from Invitrogen and American Type Culture Collection, respectively.

**Cell Culture and Transfection**—HEK-293T, MCF-7, and MCF-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 units penicillin, and 100 µg/ml streptomycin and grown at 37 °C in 5% CO2. MCF-7 and MCF-1 cells were transfected using FuGENE 6, whereas Lipofectamine 2000 was used to transfect HEK-293T cells (17).

**Plasmid Construction**—Myc-tagged human IQGAP1, IQGAP1-N (amino acids 2–863) or IQGAP1-C (amino acids 864–1657), in pcDNA3 vector (5) was used. IQGAP1 lacking the CHD domain (IQGAP1ΔCHD, amino acids 35–365 deleted), IQGAP1ΔWW (amino acids 643–744 deleted), IQGAP1ΔGRD (amino acids 1122–1324 deleted), IQGAP1ΔC (amino acids 1502–1657 deleted), IQGAP1-N1 (amino acids 2–431), and IQGAP1-N2 (amino acids 432–863) were described previously (7, 17, 18). IQGAP1-(amino acids 2–431), and IQGAP1-N2 (amino acids 432–863) were deleted, respectively. Each reverse primer included a stop codon directly following the codon corresponding to the last amino acid of the appropriate IQGAP1 sequence. For the construction of IQGAP1 (2–746), PCR was performed on full-length IQGAP1 using forward primer 5′-GCTGGCC-TACCTCTGACCGG-3′ and reverse primer 5′-GCTCTAGATGATCATCCTGTCCTCCT-3′ flanking nucleotides 1763 and 2702, which contains a PacI site. The reverse primer has an XbaI site at the 3′ end. PCR products were digested with PacI and XbaI, and the resultant fragment were inserted into pcDNA3-IQGAP1 cut with PacI-XbaI. The sequence of all constructs was confirmed by both restriction mapping and DNA sequencing. All peptides are Myc-tagged. Plasmids were purified with a QIAprep Spin miniprep kit according to the manufacturer’s instructions.

**Preparation of Fusion Proteins**—The construction of glutathione S-transferase (GST)-IQGAP1, GST-IQGAP1-N (N-terminal half, amino acids 2–863), GST-IQGAP1-C (C-terminal half, amino acids 864–1657), and GST-WASP-GBD has been described previously (5, 19, 20). A TEV cleavage site was inserted between GST and IQGAP1 to allow removal of the GST tag. A linker containing the TEV protease recognition site (TEV1, 5′-GAAGATCTGATATCATATCCCAAGACCCGG-CTGTATTTCTGGAGCCGGCGGCATCCCGC-3′, and TEV2, 5′-GGGAATCCGCCCGCCTAATTATCCGATGATCCTGG-3′) was inserted into pGEX4T1 to generate pGEX4T1-TEV-IQGAP1. pcDNA3-IQGAP1 was digested with XbaI, and a blunt end was made with T4 DNA polymerase. Following partial digestion with BamHI, the resulting fragment of IQGAP1 was inserted into pGEX4T1-TEV-IQGAP1. A BamHI site was inserted in the 5′ end and a stop codon and a XbaI site at the 3′ end. Digestion with BamHI and XbaI generated a fragment that was subcloned into the BamHI-XbaI site of pcDNA3-myc yielding a plasmid containing the cDNA for the 202-amino acid residues of IQGAP1 fused in-frame at its N terminus to a Myc epitope tag. A similar cloning strategy was employed to construct myc-IQGAP1-(216–683) (containing amino acids 216–683), myc-IQGAP1-(717–916) (containing amino acids 717–916), myc-IQGAP1-(763–863) (containing amino acids 763–863), and myc-IQGAP1-(864–964) using primers flanking nucleotides 1111 and 2514, 2614 and 3213, 2752 and 3054, and 3055 and 3357, of IQGAP1 respectively. The forward primers 5′-CGGATCC-GGATTCTAGGCTCAGCTGTTATT-G-3′, 5′-GGGATCCGCGACCCGTTCCAG-3′, and 5′-CGGATCCGCCCGCCTAATTATTTTCTGGAGCCGGCGGCATCCCGC-3′, and the reverse primers 5′-GCTCTAGATGATCATCCTGTCCTCCT-TATTATCTCTGAGCCCTGTCCTCCTT-3′ were designed to generate the appropriate IQGAP1 sequences (17). PCR was performed on full-length IQGAP1 using forward primer 5′-GCTGGCC-TACCTCTGACCGG-3′ and reverse primer 5′-GCTCTAGATGATCATCCTGTCCTCCT-3′ flanking nucleotides 1763 and 2702, which contains a PacI site. The reverse primer has an XbaI site at the 3′ end. PCR products were digested with PacI and XbaI, and the resultant fragment were inserted into pcDNA3-IQGAP1 cut with PacI-XbaI. The sequence of all constructs was confirmed by both restriction mapping and DNA sequencing. All peptides are Myc-tagged. Plasmids were purified with a QIAprep Spin miniprep kit according to the manufacturer’s instructions.
into the BamHI-Xmal site of pGEX-2T. A similar method was used to construct GST-IQGAP1-(717–916), using the primers 5’-CGGGATCC-TCTATGCGCTTCTGCGAGG-3’ (forward, BamHI site included) and 5’-GCTCTAGACCGGTCATTTGATATCG-AGTTGATT-3’ (reverse, Xmal site and a stop codon included). To obtain His-IQGAP1-(763–863), PCR was performed on full-length IQGAP1 using primers 5’-CGGGATCCCGAGGACTTGGAGGA-3’ (forward, BamHI site included) and 5’-GCTCTAGACCGGTCATTTGATATCG-AGTTGATT-3’ (reverse, Xmal site and a stop codon included) flanking nucleotides 2725 and 3054. The resulting product was gel-purified and cut with Xbal. After making a blunt end with T4 DNA polymerase, the resultant product was cut with BamHI to give a 303-bp fragment. The product was subcloned into the BamHI-EcoRI site of pRSET A containing a His tag, in which EcoRI cleavage was then filled with T4 DNA polymerase, producing pRSET-His-IQGAP1-(763–863). Similar strategy was taken to construct His-IQGAP1-(717–916). Briefly, PCR was performed on full-length IQGAP1 flanking nucleotides 2614 and 3213 using primers 5’-CGGGATCCCGAGGACTTGGAGGA-3’ (forward, BamHI site included) and 5’-GCTCTAGACCGGTCATTTGATATCG-AGTTGATT-3’ (reverse, Xmal site and a stop codon included). The resulting 600-bp products were gel-purified and cut with BamHI and Xmal. The product was subcloned into the BamHI-SmaI site of pRSET A containing a His tag, in which EcoRI cleavage was then filled with T4 DNA polymerase, producing pRSET-His-IQGAP1-(717–916). pRSET-His-IQGAP1 was transformed into E. coli and isolated with glutathione-Sepharose, as described previously (5).

**Immunoprecipitation**—Immunoprecipitation was performed as described previously (7) with minor modifications. Briefly, subconfluent MCF-7 cells were transfected with pEGFP2-IQGAP1, IQGAP1-N, or IQGAP1-C. Twenty four hours later, cells were washed twice with ice-cold phosphate-buffered saline, lysed in buffer A containing PMSF and protease inhibitors mixture, and subjected to centrifugation at 15,000 × g for 10 min at 4 °C to remove the debris. Supernatants were precleared with protein A-Sepharose beads, were incubated for 3 h at 4 °C with 40 μl of GST-WASP-GBD for 2 h at 4 °C. Complexes were collected with glutathione-Sepharose beads, were incubated for 2 h at 4 °C with 40 μl of GST-WASP-GBD and protease inhibitor mixture. Equal amounts of protein lysate, precleared with glutathione-Sepharose beads, were incubated with 40 μg of GST-WASP-GBD for 2 h at 4 °C. Complexes were collected with glutathione-Sepharose, washed, and resolved by SDS-PAGE. The resultant Western blots were probed with anti-Cdc42 antibody.

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**In Vitro Binding Assays**—Equal amounts of [35S]methionine-labeled IQGAP1 constructs described in the previous paragraph were incubated for 3 h at 4 °C with GST-IQGAP1, GST-IQGAP1-N, GST-IQGAP1-(717–916), His-IQGAP1-(763–863), or His-IQGAP1-(717–916) in 1 ml of buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100) containing 1 mM PMSF and protease inhibitors mixture. After centrifugation, samples were washed six times with buffer A and resolved by SDS-PAGE. TNT products were detected by autoradiography of the dried gel. For the competition assays, [35S]methionine-labeled IQGAP1-(763–863) or IQGAP1-(717–916) was preincubated with GST-IQGAP1 for 1 h at 4 °C in buffer A containing PMSF and protease inhibitors, and then equal amounts of [35S]methionine-labeled full-length IQGAP1 was added. After a 2-h incubation, samples were washed six times with buffer A, resolved by SDS-PAGE or Tricine/SDS-PAGE, and followed by autoradiography.

**Gel Filtration Chromatography**—GST-IQGAP1 (containing the TEF protease recognition site) was immobilized on glutathione-Sepharose and incubated with 5 μl of TEV protease at 4 °C for 16 h. The GST was pelleted by centrifugation, and the supernatant was passed through a 0.45-μm filter. Fast protein liquid chromatography (FPLC) separation was performed on an AktaTM FPLC 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 (5.01 version). A Superose 6 10/300 GL column (Amersham Biosciences) was pre-equilibrated with 4-column volumes of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, and 0.1% Triton X-100 (equilibration buffer). After loading the sample (30 μg of IQGAP1 in 500 μl of equilibration buffer containing 1% Triton X-100) onto the column at a flow rate of 0.5 ml/min, the equilibration buffer was passed through the column, and 0.5-mL fractions were collected. An aliquot (450 μl) of each fraction was precipitated with trichloroacetic acid, solubilized, and subjected to SDS-PAGE followed by Western blotting. The column was calibrated using blue dextran (2,000 kDa), apoferritin (443 kDa), β-amylose (200 kDa), alcohol dehydrogenase (150 kDa), and albumin (67 kDa) as standards. IQGAP1 was also chromatographed on a Tosoh Biosciences) G4000SW (600 × 7.5 mm inner diameter) column operated at 0.5 ml/min with 50 mM sodium phosphate, 150 mM NaCl, pH 6.5. Fractions were collected at 0.5-min intervals and analyzed as described above.

**Measurement of Activated Cdc42**—Active Cdc42 was measured with a GST fusion construct of the GTPase-binding domain (GBD) of Wiskott-Aldrich syndrome protein (WASP) as described previously (20). The GST-WASP-GBD binds only GTP-Cdc42 (20). Briefly, HEK-293T cells were transfected with selected constructs (indicated in the legend to Fig. 11), followed by lysis with buffer B (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 1 mM MgCl2) containing 20 μM GTP and protease inhibitor mixture. Equal amounts of protein lysate, precleared with glutathione-Sepharose beads, were incubated with 40 μg of GST-WASP-GBD for 2 h at 4 °C. Complexes were collected with glutathione-Sepharose, washed, and resolved by SDS-PAGE. The resultant Western blots were probed with anti-Cdc42 antibody. Equal amounts of protein lysate were also subjected to SDS-PAGE, and blots were probed with anti-β-tubulin, anti-IQGAP1, and anti-Myc antibodies.

**Miscellaneous**—Protein assays were performed using the DC protein assay from Bio-Rad. Densitometry of ECL signals were analyzed with Un-scan-it software (Silk Scientific Corp.). Statistical analysis was assessed by Student’s t test with Excel (Micro software).
RESULTS

IQGAP1 Self-Associates in Cells—Co-immunoprecipitation was used to investigate whether IQGAP1 self-associates in cells. IQGAP1, tagged at its N terminus with two molecules of EGFP (termed EGFP₂-IQGAP1), was transfected into MCF-7 cells. Examination of the lysate demonstrated expression of transfected EGFP₂-IQGAP1, which migrates to the expected position on SDS-PAGE (Fig. 1A, lane 2, top band). To evaluate self-association, cells transfected with EGFP₂-IQGAP1 were lysed and immunoprecipitated with anti-EGFP antibody. Probing the resultant immunoblots with anti-IQGAP1 antibody revealed that endogenous IQGAP1 co-immunoprecipitated with EGFP₂-IQGAP1 (Fig. 1A, bottom band, lane 4). Note that two bands of EGFP-tagged IQGAP1 are visible in the immunoprecipitates (Fig. 1A, lane 4). This is most likely caused by the removal of one molecule of EGFP from the dual EGFP-tagged IQGAP1 during the immunoprecipitation. Probing the PVDF membrane with anti-EGFP antibody confirms this hypothesis as two bands are visible in the immunoprecipitates at the predicted molecular weights (Fig. 1B, lane 4, upper panel). To exclude the possibility that the bottom band in Fig. 1A, lane 4, results from the degradation of EGFP₂-IQGAP1 by removal of both molecules of EGFP, we repeated the analysis in MCF/l cells. These cells stably express myc-IQGAP1 (Fig. 1C) (17), permitting discrimination from the GFP-tagged IQGAP1, which lacks the Myc tag. Lysates from MCF/l cells, transfected with EGFP₂-IQGAP1, were immunoprecipitated with anti-EGFP antibody. Probing the Western blots with anti-Myc antibody revealed that myc-IQGAP1 co-immunoprecipitated with EGFP₂-IQGAP1 (Fig. 1C). The specificity of the interaction is validated by the absence of IQGAP1 from immunoprecipitates derived from samples transfected with GFP alone (Fig. 1). Collectively, these data indicate that IQGAP1 self-associates in cells.

Identification of the Self-Association Domain of IQGAP1—In order to identify the region(s) that mediate IQGAP1 self-association, pulldown assays with portions of IQGAP1 labeled with [³⁵S]methionine were performed. Selected constructs of IQGAP1 (depicted in Fig. 2A) were labeled with [³⁵S]methionine in a reticulocyte lysate and incubated with full-length GST-IQGAP1 immobilized on glutathione-Sepharose beads. Constructs that bound to GST-IQGAP1 were resolved by SDS-PAGE and identified by autoradiography. Radiolabeled full-length IQGAP1 bound to full-length GST-IQGAP1 (Fig. 2B, upper panel), demonstrating that IQGAP1 self-associates in vitro. Specificity of bind-
IQGAP1 Self-association

![FIGURE 3. Specificity of anti-IQGAP1 antibodies.](Image)

**Amino Acids 763–863 of IQGAP1 Are Necessary for Self-Association**

Further analysis was performed to narrow the self-association domain of IQGAP1. Because the N-terminal half of IQGAP1 is responsible for self-association, it was divided into two equal portions, termed N1 and N2 (Fig. 5A). In addition, a fragment comprising amino acids 763–964 was constructed to investigate the possibility that the self-association region may overlap the junction between the N- and C-terminal halves of IQGAP1. Pull down with GST-IQGAP1 of [35S]methionine-labeled fragments revealed binding of N2 and IQGAP1-(763–964) but not of N1 (Fig. 5B). These data indicate that the self-association site is between residues 763 and 863 of IQGAP1. To confirm this site, the IQGAP1-(763–964) peptide was divided into two equal halves, termed IQGAP1-(763–863) and IQGAP1-(864–964) (Fig. 5A). Consistent with the data in Fig. 5B, IQGAP1-(763–863), but not IQGAP1-(864–964), bound to full-length IQGAP1 (Fig. 5C). Similarly, pull down with GST-IQGAP1-N showed specific binding of N2 (Fig. 5B), IQGAP1-(763–964) (Fig. 5, B and C), and IQGAP1-(763–863) (Fig. 5C) to the N-terminal half of IQGAP1 (Fig. 5, B and C). These data reveal that amino acids 763–863 of IQGAP1 mediate self-association.

**The Putative Coiled-coil Region Does Not Mediate Self-Association of IQGAP1**

It is widely recognized that the α-helical coiled-coil structural motif mediates oligomerization of a large number of proteins (for a review see Ref. 21). The IQGAP1 repeat region has six putative coiled-coils located between amino acids 168 and 601 as predicted by the COILS program ([www.ch.embnet.org/software/COILS_form.html](http://www.ch.embnet.org/software/COILS_form.html)). However, our data indicate that the segment containing the self-association domain resides in amino acids 763–863, which is outside the coiled-coil region. It is conceivable that the N1 and N2 constructs described above have disrupted the coiled-coil region, thereby impeding binding. To establish whether the coiled-coil also participates in the

![FIGURE 4. IQGAP1-N co-immunoprecipitated with endogenous IQGAP1. A. Myc-IQGAP1-N (N), myc-IQGAP1-C (C), and pcDNA3 vector (V) were transfected into MCF-7 cells. Equal amounts of protein lysate were immunoprecipitated (IP) with anti-IQGAP1 polyclonal (pAb) or monoclonal (mAb) antibodies. Complexes were isolated and washed as described under “Experimental Procedures.” The samples were resolved by SDS-PAGE, transferred to PVDF, and probed with anti-Myc antibody. Open arrowhead, IQGAP1-C; filled arrowheads, IQGAP1-N. B, the PVDF membrane shown in A was stripped and re-probed with anti-IQGAP1 monoclonal antibody. C, the PVDF membrane shown in A was stripped and re-probed with anti-IQGAP1 polyclonal antibody. The data are representative of at least three independent experiments. WB, Western blot.)

self-association of IQGAP1, we constructed a peptide encompassing amino acids 2-746, which contains the entire putative coiled-coil region (Fig. 6A). [35S]Methionine-labeled IQGAP1-(2-746) was incubated separately with GST-IQGAP1 and GST-IQGAP1-N on glutathione-Sepharose beads. Constructs that bound to the GST fusion proteins were resolved by SDS-PAGE and identified by autoradiography. No binding of IQGAP1-(2-746) was detected to either fusion construct (Fig. 6B, right panel). Note that both the positive and negative controls (IQGAP1-N2 and IQGAP1-N1, respectively) gave the expected results (Fig. 6B). These findings suggest that the putative coiled-coil region of IQGAP1 is not capable of binding to IQGAP1 and therefore does not participate in self-association.

A Peptide Comprising Amino Acids 763–863 of IQGAP1 Inhibits the Self-association of Full-length IQGAP1—To determine whether amino acids 763–863 are sufficient for self-association, a His-tagged fusion

A schematic representation, nomenclature, and amino acids boundaries of the fragments of human IQGAP1-(Δ746–860) (Δ746–860), B, GST-IQGAP1 or GST alone immobilized on glutathione-Sepharose beads was incubated with equal amounts of [35S]methionine-labeled wild type IQGAP1 (WT), IQGAP1-N1 (N1), and IQGAP1-(Δ746–860) (Δ746–860). Complexes were washed, resolved by SDS-PAGE, and processed by autoradiography. The input is equivalent to 10% of the amount that was subjected to pull down. Data are representative of two independent experimental determinations.

construct comprising these residues was generated. The His-tagged peptide, immobilized on Ni2+–NTA beads, was incubated with [35S]methionine-labeled IQGAP1-(763–863). Radiolabeled IQGAP1-(763–863) was retained on beads loaded with His-IQGAP1-(763–863) but not on Ni2+–NTA beads alone (Fig. 7A), demonstrating that amino acids 763–863 of IQGAP1 can self-associate. Further validation that this region mediates self-association of full-length IQGAP1 was obtained from in vitro competition analysis. IQGAP1-(763–863) inhibited in a dose-dependent manner the self-association of full-length IQGAP1 (Fig. 7B). Specificity is revealed by the observation that a large excess of bovine serum albumin did not reduce self-association. Collectively, these data reveal that amino acids 763–863 of IQGAP1 are responsible for self-association.

Although IQGAP1-(763–863) expressed well in reticulocyte lysate, analysis by Western blotting indicated that it is unstable in mammalian cells and does not express well. This may be caused by disruption of domains necessary for proper folding. Therefore, we produced a larger peptide comprising residues 717–916 of IQGAP1 (Fig. 7C), which expresses well in cells. Analogous to the data obtained with IQGAP1-(763–863), IQGAP1-(717–916) can self-associate and also disrupted the self-association of full-length IQGAP1 (Fig. 7, D and E).

Deletion of Amino Acids 746–860 Abrogated Self-association of IQGAP1—The data in Fig. 7A reveal that amino acid residues 763–863 of IQGAP1 are sufficient for self-association. If this sequence is necessary for binding, one would predict that deletion of this region would eliminate IQGAP1 self-association. It is noteworthy that amino acids 763–863 are located in the IQ domain. Because the IQ domain begins at residue 746, a little proximal to the self-association region, we deleted amino acids 746–860 from IQGAP1. IQGAP1-(Δ746–860) (Fig. 8A) was labeled with [35S]methionine and incubated with GST-IQGAP1 on glutathione-Sepharose. IQGAP1-(Δ746–860) was incapable of binding to full-length IQGAP1 (Fig. 8B, right panel). These data reveal that amino acids 763–863 of IQGAP1 are both necessary and sufficient for self-association of IQGAP1.

A Peptide Comprising Amino Acids 216–683 of IQGAP1 Does Not Self-associate—A prior publication (16) suggested that IQGAP1 forms oligomers through amino acids 216–683. Because these findings differ from our observations, we examined a peptide corresponding to amino acids 216–683 of IQGAP1 in our assay system. The binding of IQGAP1-(216–683) was compared with that of IQGAP1-(717–916) using radiolabeled peptides. Consistent with the data in Fig. 7D that it can self-associate, [35S]methionine-labeled IQGAP1-(717–916) bound specifically to GST fusion proteins of full-length IQGAP1 and IQGAP1-(717–916). In contrast, IQGAP1-(216–683) interacted with neither full-length IQGAP1 nor GST-IQGAP1-(717–916). Similarly, GST-IQGAP1-(216–683) bound neither IQGAP1-(216–683) nor IQGAP1-(717–916) (Fig. 8). In addition, GST-IQGAP1-(216–683) did not pull down endogenous IQGAP1 from MCF-7 breast epithelial cell lysates (data not shown). Therefore, we did not detect any binding of IQGAP1-(216–683) to full-length IQGAP1 or to itself under our assay conditions. Note that GST IQGAP1-(216–683) bound to IQGAP1 in bovine brain cytosol (16). Although the reasons for the different results are not known, it is possible that the binding partners of endogenous IQGAP1 in human breast epithelial cells are different from those in bovine brain cytosol.

Oligomerization of IQGAP1—Our analysis reveals that IQGAP1 is capable of self-association. In order to determine whether IQGAP1 exists exclusively as a dimer or whether it is a mixture of monomers, dimers, and larger oligomers, we performed gel filtration chromatography. When applied to a Superose 6 10/300 GL column purified IQGAP1 eluted in several fractions, with peaks corresponding to ~800, 400, and 200 kDa (Fig. 10). These peaks may contain tetramers, dimers, and monomers, respectively, of IQGAP1. Similarly, despite a different buffer and packing material, chromatographic separation of IQGAP1 on a Tosohaas G4000SW column yielded essentially identical results (data not shown). Collectively, these findings support the concept that IQGAP1 can form multimers.

Oligomerization Is Necessary for IQGAP1 to Increase Active Cdc42—The observations that IQGAP1 self-associates both in vitro and in cells leads to the question of whether the formation of multimers is important for the biological function of IQGAP1. Previous work from our laboratory documented that overexpression of full-length IQGAP1 increases active Cdc42 in cells (17). Therefore, active Cdc42 was measured in cell lysates using GST–WASP–GDB (which binds only to active, GTP-Cdc42 (17, 20)) to investigate the possible functional sequelae of disrupting IQGAP1 self-association. Two complementary strategies were used. In the first, cells were transfected with the mutant IQGAP1-
IQGAP1 increased GTP-Cdc42 by expression of wild type IQGAP1. Transient transfection of wild type effect on the amount of active Cdc42 in HEK-293T cell lysates. Note our prior observations (17). In contrast, IQGAP1-(717–916) resolved by SDS-PAGE, and blots were probed with anti-Cdc42 (8). Equal amounts of protein were also subjected to SDS-PAGE, and Western blots were probed with anti-Myc antibody revealed that the peptide was expressed well and migrated to the expected position on SDS-PAGE (Fig. 11A). More importantly, transfection of the peptide reduced by 60% the amount of active Cdc42 in cell lysates (Fig. 11). Collectively, these data reveal that self-association of IQGAP1 is necessary for it to modulate Cdc42.

DISCUSSION

Specific protein dimerization, a common physical property of proteins, is integral to biological structure, function, and control (14). Dimerization or oligomerization regulates diverse proteins ranging from enzymes and ion channels to receptors and transcription factors. Homodimerization enables proteins to bind simultaneously to different targets that have overlapping binding sites on an individual subunit and also provides a means to shift the association equilibrium, such that a protein bound to one complex will favor binding to a second complex with a different sequence. IQGAP1 is a scaffold protein that integrates signaling pathways (2). Several published studies have shown that binding of one protein alters the ability of IQGAP1 to bind another target. For example, Ca2+/calmodulin prevents IQGAP1 from binding Cdc42 (8), actin (6), and E-cadherin (11). Similarly, binding of active Cdc42 regulates the association of IQGAP1 with other molecules, such as CLIP-170 (13). Therefore, it seems reasonable to hypothesize that IQGAP1 may self-associate and that this process might regulate its function.

Combining analysis in vitro and in intact cells, we established that IQGAP1 self-associates. To evaluate self-association in cells, we transfected human breast epithelial cells with EGFP–IQGAP1. This approach permits discrimination of the transfected protein from endogenous IQGAP1 by two methods, namely altered migration on SDS-PAGE (the EGFP tags retard mobility) and recognition by anti-GFP antibodies. Endogenous IQGAP1 co-immunoprecipitated with EGFP-tagged IQGAP1, validating that IQGAP1 can self-associate in cells. We verified by co-immunoprecipitation that adding an EGFP tag to the N terminus of IQGAP1 did not alter its interaction with several targets, including calmodulin, Cdc42, β-tubulin, and E-cadherin (3). Others (6) have also reported that an EGFP tag does not alter IQGAP1 function. In vitro analysis similarly demonstrated that IQGAP1 can self-associate. Fusion proteins of full-length IQGAP1 or the N-terminal half of IQGAP1 were able to self-associate and could also bind one another. These data document that IQGAP1 self-associates.

Two prior studies have stated that IQGAP1 self-associates. In the first, IQGAP1 was estimated to have a molecular weight of 358,653 to 401,864 by sedimentation equilibrium (15). The authors concluded that these findings indicated that IQGAP1 comprises exclusively two subunits. The second study identified a very broad peak on gel filtration, which was interpreted to indicate that IQGAP1 existed as monomers, dimers, and trimers (16). The different methods of analysis, coupled with different sources of IQGAP1 protein (purified or expressed in bacteria), may account for the discrepancies between these two studies. Estimation of molecular weight can be correlated with elution on gel filtration only when the unknown protein has the same shape or conformation as the proteins used to calibrate the column (22). Moreover, neither of the prior studies performed detailed in vitro analysis to con-

(Δ746–860), which cannot self-associate, and compared with overexpression of wild type IQGAP1. Transient transfection of wild type IQGAP1 increased GTP-Cdc42 by ~2-fold (Fig. 11), consistent with our prior observations (17). In contrast, IQGAP1-(Δ746–860) had no effect on the amount of active Cdc42 in HEK-293T cell lysates. Note that IQGAP1-(Δ746–860) was expressed at slightly higher levels than wild type IQGAP1 (Fig. 11). The second strategy was to disrupt oligomerization of endogenous IQGAP1 in cells using a peptide. A Myc-tagged peptide corresponding to amino acid residues 717–916 of IQGAP1 was transfected into HEK-293T cells. Probing Western blots with anti-Myc antibody revealed that the peptide was expressed well and migrated to the expected position on SDS-PAGE (Fig. 11A). More importantly, transfection of the peptide reduced by 60% the amount of active Cdc42 in cell lysates (Fig. 11). Collectively, these data reveal that self-association of IQGAP1 is necessary for it to modulate Cdc42.

FIGURE 10. Gel filtration chromatography of IQGAP1. A, GST was removed from GST-IQGAP1 with TEV protease, and 30 μg of IQGAP1 was loaded onto a Superose 6 10/300 GL gel filtration column. Fractions (0.5 ml) were collected. A 450-μl aliquot of each fraction was precipitated with trichloroacetic acid and analyzed by Western blotting. B, the trace depicts the relative amounts of IQGAP1 (in μg) as determined by densitometry and corrected for the amount of active Cdc42 was quantified by densitometry and corrected for the amount of active Cdc42 in the corresponding lysate. Data are expressed relative to V or 717–916 (717–916) as determined by densitometry. The apoptosis of elution of known markers are depicted. Data are representative of two independent experiments.

FIGURE 11. Self-association is necessary for IQGAP1 to increase active Cdc42. A, HEK-293T cells were transiently transfected with 5 μg of either vector (V), wild type IQGAP1 (WT), IQGAP1-(Δ746–860) (Δ746–860), or IQGAP1-(717–916) (717–916). Equal amounts of protein were incubated with GST-WASP-GRD as described under “Experimental Procedures.” Complexes were collected with glutathione-Sepharose and resolved by SDS-PAGE, and Western blots were probed with anti-Cdc42 (top panel, active Cdc42). Equal amounts of protein were also subjected to SDS-PAGE, and blots were probed with anti-β-tubulin and anti-Myc (which detect transfected IQGAP1-(717–916)) antibodies. B, the amount of active Cdc42 was quantified by densitometry and corrected for the amount of β-tubulin in the corresponding lysate. Data are expressed relative to the amount of active Cdc42 in vector-transfected cells and represent the means ± S.D. (n = 3). *, significantly different to vector-transfected cells (p < 0.001); #, significantly different to cells transfected with wild type IQGAP1 (p < 0.01).

3 Z. Li and D. B. Sacks, unpublished observations.
**IQGAP1 Self-association**

Firm dimerization. For these reasons, we conducted the work described here. Coiled-coil proteins are characterized by a repeating pattern of seven residues, (abcdefg)$_n$, with hydrophobic amino acids predominating at positions a and d of the heptad repeat (21). The coiled-coil is thought to be one of the principal motifs, which mediates subunit oligomerization of a large number of proteins (21). The N-terminal half of IQGAP1 contains six tandem repeats (1). It has been assumed previously, without detailed investigation, that this region of IQGAP1 mediates its dimerization (4, 6). On the basis of the putative coiled-coil domains between residues 168 and 601 of IQGAP1 (predicted by the COILS program), we anticipated that this region of IQGAP1 would mediate self-association. Experimental evidence did not support this hypothesis. The N-terminal half of IQGAP1 (amino acids 2–863), which includes the putative coiled-coil region, bound to full-length IQGAP1. However, deletion of the terminal 115 amino acids (residues 746–860, distal to the coiled-coil region) from this construct abrogated binding. Moreover, a peptide comprising the N-terminal 746 amino acids (which contains all the coiled-coils) failed to bind to either full-length IQGAP1 or the N-terminal half of IQGAP1 in vitro. Consistent with these findings, a peptide comprising residues 763–863 of IQGAP1 both bound to full-length IQGAP1 and was capable of self-association. Finally, excision of this region from IQGAP1 abrogated its ability to bind full-length IQGAP1, indicating that residues 763–863 are both necessary and sufficient to mediate the self-association of IQGAP1.

Our findings differ from a prior publication. By using GST fusion constructs of four selected portions of IQGAP1, amino acids 216–683 were reported to pull down endogenous IQGAP1 from bovine brain cytosol, whereas a peptide comprising amino acids 521–914 could not bind IQGAP1 in cell lysates (16). Neither in vitro analysis with pure proteins nor detailed characterization of the binding region was performed in that study. In contrast to that work, we were unable to detect binding of a peptide comprising residues 216–683 to full-length IQGAP1. Similarly, the 216–683 peptide did not self-associate. Several methodological differences may account for the differences between the studies. In the prior work, the buffer consisted of 20 mM Tris-Cl, 1 mM EDTA, and 1 mM dithiothreitol. Our assay conditions were more stringent. The buffer we used contained physiological concentrations of salt (150 mM NaCl) to minimize possible nonspecific electrostatic interactions. The presence of multiple bands visible on the silver-stained gel derived from the pull down with the 216–683 fragment in their study lends credence to this hypothesis.

It is well recognized that self-association regulates protein function (14, 23–25). In particular, the function of several scaffolding proteins is regulated by self-association (26, 27). Therefore, we analyzed the possible functional sequelae of self-association of IQGAP1 by using its effect on Cdc42 as a readout. IQGAP1 binds directly to Cdc42, maintaining Cdc42 in the active GTP-bound form (5, 28). Moreover, overexpression of full-length IQGAP1 and a dominant negative IQGAP1 construct increased and decrease, respectively, activated Cdc42 in cell lysates (17, 29). In contrast, overexpression of IQGAP1∆IQ, which lacks amino acids 699–905, had no effect on the amount of active Cdc42 (17). Here we observed that transfection of IQGAP1-(Δ746–860) into cells failed to augment activated Cdc42 in cell lysates. Congruent with these results, a peptide that disrupted IQGAP1 self-association reduced the amount of activated Cdc42 in cell lysates. Collectively, these data suggest that oligomerization is necessary for IQGAP1 to increase activated Cdc42.

Some caveats of the functional data should be borne in mind. Protein self-association domains are often fairly large (30, 31), necessitating excision of several amino acids to prevent self-association. It is possible that removal of amino acid residues 746–860 from IQGAP1 may alter its conformation. For this reason, we complemented the functional analysis of the IQGAP1 deletion mutant with transfection of the peptide corresponding to the self-association region. The peptide, which specifically inhibited in vitro self-association in a dose-dependent manner, significantly reduced the amount of active Cdc42 in cells. Although it is not possible to unequivocally establish that the 717–916 peptide reduced active Cdc42 via IQGAP1, in combination with results of IQGAP1-(Δ746–860), the findings strongly suggest that oligomerization is necessary for IQGAP1 to increase activated Cdc42. A second consideration is that IQGAP1-(Δ746–860) lacks the major calmodulin binding region (5, 19), preventing it from associating effectively with calmodulin. However, binding to calmodulin abrogates the interaction of IQGAP1 with Cdc42 (8). Thus, one would anticipate that IQGAP1-(Δ746–860) would exhibit an enhanced ability to increase active Cdc42, an effect opposite to what we observed.

In conclusion, our work is the first detailed characterization of IQGAP1 self-association. We document both in vitro and in intact cells that IQGAP1 can self-associate. More importantly, this study demonstrates for the first time that oligomerization of IQGAP1 is important for its function. The identification of the self-association domain presented here is likely to augment the ability of future studies to elucidate the molecular mechanisms and myriad biological functions of IQGAP1, and provides a unique motif for the genetic engineering of protein self-association domains.

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