Ca^{2+}-dependent cell-cell adhesion is mediated by the cadherin family of transmembrane proteins. Adhesion is achieved by homophilic interaction of the extracellular domains of cadherins on adjacent cells, with the cytoplasmic regions serving to couple the complex to the cytoskeleton. IQGAP1, a novel RasGAP-related protein that interacts with the cytoskeleton, binds to actin, members of the Rho family, and E-cadherin. Calmodulin binds to IQGAP1 and regulates its association with Cdc42 and actin. Here we demonstrate competition between calmodulin and E-cadherin for binding to IQGAP1 both in vitro and in a normal cellular milieu. Immunocytochemical analysis in MCF-7 (E-cadherin positive) and MDA-MB-231 (E-cadherin negative) epithelial cells revealed that E-cadherin is required for accumulation of IQGAP1 at cell-cell junctions. The cell-permeable calmodulin antagonist CGS9343B significantly increased IQGAP1 at areas of MCF-7 cell-cell contact, with a concomitant decrease in the amount of E-cadherin at cell-cell junctions. Analysis of E-cadherin function revealed that CGS9343B significantly decreased homophilic E-cadherin adhesion. On the basis of these data, we propose that disruption of the binding of calmodulin to IQGAP1 enhances the association of IQGAP1 with components of the cadherin-catenin complex at cell-cell junctions, resulting in impaired E-cadherin function.

The cadherins are a family of cell surface adhesion molecules that participate in Ca^{2+}-dependent cell-cell adhesion (for reviews see Refs. 1 and 2). E-cadherin is an epithelial cell transmembrane protein with conserved repeated amino acid sequences (cadherin repeats) in the extracellular domain. Ca^{2+}-dependent cell-cell contact induces the localization of E-cadherins to the region of contact, where homophilic interactions with E-cadherin on adjacent cells are thought to act as a cell adhesion zipper (3). The cytoplasmic domain of E-cadherin binds directly to either β-catenin or γ-catenin (also called plakoglobin), and this complex is coupled to the cytoskeleton by α-catenin via its association with actin and β-catenin or γ-catenin (1). Disruption of either the E-cadherin-catenin interaction or the association of the cadherin-catenin complex with actin results in loss of cell adhesion. The cadherin-catenin complex suppresses tumor invasion and metastasis (1). For example, several studies (for review, see Ref. 1) have revealed that the expression of E-cadherin and α-catenin is reduced in many types of tumors.

A novel RasGAP^{1}-related protein, designated IQGAP1, was cloned by polymerase chain reaction from human osteosarcoma tissue (4). The protein has significant sequence similarity to the catalytic domains of all previously reported RasGaps (4). In addition, IQGAP1 contains several other motifs that mediate its interaction with a variety of proteins. These targets include actin, which binds to the calponin homology domain (5, 6); calmodulin, which binds to both the calponin homology domain (5) and IQ motifs (5, 7); activated Rac and Cdc42, which bind to the RasGAP-related domain (7); and E-cadherin and β-catenin (8). Several other potential protein-binding motifs, including a coiled coil region and poly-proline binding domain, are present (4, 7), leading to the suggestion that IQGAP1 is a scaffolding protein (5).

The normal cellular function(s) of IQGAP1 are unknown but are under investigation by several groups. IQGAP1 has been shown to bind to and cross-link actin filaments in vitro (6, 9, 10). In Saccharomyces cerevisiae, IQGAP family members Iqg1 and Cyk1p participate in cytokinesis (11, 12). Mammalian IQGAP1 binds to active Cdc42 and Rac (small GTPases), but the functional significance of these interactions has not been established.

Calmodulin is the primary mediator of Ca^{2+}-dependent signaling in eukaryotic cells (13). Essential cellular processes in which calmodulin is a critical regulatory component include Ca^{2+} transport, cell motility, cytoskeletal assembly (13), and DNA synthesis (14). Calmodulin mediates these diverse effects by binding to and modulating a large number of different targets, ranging from protein kinases to structural proteins to signaling proteins. This list of calmodulin targets now includes IQGAP1, which is the major calmodulin-binding protein in Ca^{2+}-free lysates of human breast epithelium (15). Binding of calmodulin to IQGAP1 may affect IQGAP1 function by altering its interaction with other proteins. For example, calmodulin inhibited the binding of Cdc42 (15) and actin (9) to IQGAP1, suggesting a possible role for the interaction in cytoskeletal function.

Recent analysis revealed that IQGAP1 binds to E-cadherin and β-catenin in vitro and in vivo (8). In the present study we investigated the potential participation of calmodulin in modulating the interaction between IQGAP1 and E-cadherin. We

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1 The abbreviations used are: GAP, GTPase-activating protein; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; PBS, phosphate buffered saline; GST, glutathione S-transferase; PVDF, polyvinylidene difluoride; HBS, HEPES-buffered saline; PAGE, polyacrylamide gel electrophoresis.
observed that calmodulin and E-cadherin compete for binding to IQGAP1. Inhibition of calmodulin with structurally diverse antagonists impaired E-cadherin function, resulting in significantly decreased E-cadherin homophilic adhesion. Moreover, we show by immunochemistry that calmodulin antagonism altered the amount of IQGAP1 and E-cadherin at sites of cell-cell contact, implying that calmodulin regulates the in vivo association between IQGAP1 and E-cadherin.

**EXPERIMENTAL PROCEDURES**

**Materials—**Tissue culture reagents were obtained from Life Technologies Inc. Fetal bovine serum was from Biowhittaker, and insulin was purchased from Life Technologies, Inc. MCF-7 and MDA-MB-231 human breast epithelial carcinoma cells were obtained from American Type Culture Collection. Permanox plastic 8-chamber culture slides were from Fisher. CGS9343B was generously donated by Dr. E. Moret and B. Schmid (Novartis, Switzerland). All other reagents were of standard analytical grade.

**Antibodies—**Anti-calmodulin monoclonal (16), anti-IQGAP1 polyclonal (5), and E4.6 anti-E-cadherin monoclonal (17) antibodies have been previously characterized. Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG were from Sigma, and anti-IgG polyclonal antibody was from Zymed Laboratories Inc.

**Generation of EL and L Fibroblasts—**A full-length human E-cadherin cDNA was generated by ligation of the EcoRV-PmlI fragment encoding the first four domains of E-cadherin from the vector pERF-1 (kindly provided by Dr. David Rimm) to a PmlI-Xhol fragment encoding the fifth cadherin repeat, transmembrane and cytoplasmic regions of human E-cadherin obtained by polymerase chain reaction amplification from 16E6.A5 human epithelial cell cDNA. The complete EcoRV-Xhol coding sequence was introduced into the expression vector pCEP4 (Invitrogen Corp.) cleaved with PvuII and XhoI. Murine L fibroblasts were stably transfected with 20 μg of pCEP4/E-cadherin plasmid by calcium phosphate precipitation (Stratagene Inc.) and selected on G418-containing 400 μg/ml hygromycin B for 4 weeks. Clones expressing E-cadherin were isolated by staining with anti-E-cadherin antibody using an EPICS Elite ESP flow cytometer equipped with an Autoclone module (Coulter Corp.). L cells transfected with pCEP4 alone were used as controls.

**Cell Culture—**MCF-7 and MDA-MB-231 breast carcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum in a 5% CO2 incubator at 37 °C. MCF-7 cells were grown in the same medium as the breast epithelial cells, with the addition of 400 μg/ml hygromycin B. MTSV1–7 breast epithelial cells (kindly donated by Dr. Joyce Taylor-Papadimitriou) were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 10 μg/ml insulin, and 5 μg/ml hydrocortisone.

**Adhesion assays were conducted essentially as described previously (19). The wells of Linbro 96-well microtiter plates (ICF-Laboratories) were coated directly with 100 μl/well of 0.1% (w/v) poly-l-lysine or 0.2 mg/ml human umbilical cord hyaluronan. Alternatively, 1 μg/well goat anti-human IgG polyclonal antibody in 100 μl of Tris-buffered saline (20 mM Tris, pH 7.4, and 150 mM NaCl) was added to wells, followed by blocking with 1% (w/v) bovine serum albumin in Tris-buffered saline for 2 h at 22 °C, with the subsequent addition of 0.05 μg/ml human E-cadherin–Fc (19) or human IgG1 (Calbiochem) in 50 μl/well Tris-buffered saline, pH 7.4, containing 1 mM CaCl2 for 18 h at 4 °C. The wells were subsequently washed three times with 20 mM HEPES, pH 7.4, 137 mM NaCl, and 3 mM KCl (HBS) with 1 mM CaCl2 prior to adhesion assays.

**MCF-7, MDA-MB-231, or MTSV1–7 cells were released from culture dishes using 0.02% (w/v) trypsin, 2 mM CaCl2, and HBS to minimize proteolysis of E-cadherin. After adding 2 volumes of 0.04% (w/v) soy bean trypsin inhibitor in HBS and washing twice with HBS, the cells were resuspended in HBS containing 0.1% (w/v) bovine serum albumin and 1 mM CaCl2 with the appropriate concentration of calmodulin inhibitor or vehicle, added to coated microtiter plate wells, and incubated for 40 min at 37 °C. After washing twice with HBS and 1 mM CaCl2, the number of bound cells was determined using a fluorigenic assay of endogenous cellular phosphatase activity (20). In each assay, the percentage of adherent cells was determined by comparison with wells containing cells treated identically but not subjected to the washing procedure.

**Immunoprecipitation—**Cells were washed three times in phosphate-buffered saline (PBS) (145 mM NaCl, 12 mM Na2HPO4, and 4 mM NaH2PO4, pH 7.2), lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, and 1% (v/v) Triton X-100 (buffer A) with 1 mM CaCl2 or 1 mM EGTA and quick frozen in methanol/solid CO2. Samples were clarified by centrifugation at 15,000 × g for 5 min at 4 °C and preincubated with protein A-Sepharose. Immunoprecipitation with anti-IQGAP1 antisemur or nonimmune rabbit serum was performed for 4 h at 4 °C and immune complexes were collected with protein A-Sepharose. After sedimentation by centrifugation, samples were washed five times with buffer A, resolved by SDS-PAGE, and transferred to PVDF. Blots were probed with anti-IQGAP1, anti-calcmodulin, or anti-Myc primary antibodies, followed by the appropriate horseradish peroxidase-conjugated secondary antibody and detected by ECL.

**IQGAP1 Plasmid Construction—**Myc-tagged wild type IQGAP1 in pcdNA3 vector was used (5, 7). IQGAP1 lacking the IQ region (IQGAP1-IQ, amino acids 699–905 deleted) was generated by digesting of full-length IQGAP1 with PflMI. Blunt ends were generated by T4 polymerase and allowed to undergo self-ligation. Deletion of the C-terminal region (IQGAP1-ΔC, amino acids 1502–1657 deleted) was performed by digestion of IQGAP1 with XhoI and partial digestion with SpeI. The resulting ~10-kilobase product was gel purified and allowed to undergo self-ligation. The sequence of both constructs was confirmed by restriction mapping and DNA sequencing. Plasmids were purified with a QIAprep Spin Miniprep Kit (Qiagen) following the instructions provided by the manufacturer. Both mutant proteins migrated to the expected position on SDS-PAGE (Fig. 4).

**Transient Transfection—**Transient transfection was performed with wild type IQGAP1, IQGAP1-IQ, and IQGAP1-ΔC using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, EL cells were grown to 50–70% confluence in a 35-mm dish. FuGENE 6 (6 μl) was mixed with 2 μg of plasmid DNA and added to the cells. After 48 h, cells were harvested, lysed, and processed for analysis as described above.

**Construction of GST Fusion Proteins—**The cytoplasmic domain of human E-cadherin (amino acids 732–882) was generated by polymerase chain reaction using a forward BamHI-flanked primer, CGCG-CGATCCGAGGAGCGCTGG, and a reverse XhoI-flanked primer, CTCGAGTCCTAATGCT. The polymerase chain reaction product was digested with BamHI and XhoI and then inserted into pGEX4T. The sequence of the construct was confirmed by DNA sequencing. GST-E-cadherin was expressed in Escherichia coli. Full-length IQGAP1 was excised from pcDNA3, inserted into pFastBac (Life Technologies, Inc.), and transfected into Sf9 cells to obtain recombinant baculovirus. GST fusion proteins were isolated with glutathione-Sepharose as described previously (5). The identities of the purified fusion proteins were confirmed by SDS-PAGE (data not shown).

**Competitive Inhibition Analysis—**Competition with purified proteins was performed by incubating 2 μg of GST-IQGAP1 with different amounts of GST-E-cadherin. GST, without E-cadherin, was used as a control. After 30 min at 4 °C, 4 μg of calmodulin was added, and the incubation was continued for another 30 min. Samples were immunoprecipitated with anti-IQGAP1 antibody and processed by Western blotting as described above.

To perform competition assays in a normal cell milieu, clarified L and EL fibroblast cell lysates, equalized for protein concentration, were incubated with 50 μg of GST or different amounts of GST-E-cadherin (as indicated in the figure legend) for 60 min at 4 °C. Calmodulin-Sepharose chromatography was performed as described previously (15). After five washes with buffer A, samples were resolved by SDS-PAGE, and immunoblotting was performed as described above.

**Immunofluorescence Staining—**Cells were removed from 100-mm culture dishes with trypsin, washed twice with PBS, and allowed to attach to Permanox plastic slides overnight at 37 °C in Dulbecco's modified Eagle's medium supplemented with 1% (v/v) fetal bovine serum. Where indicated, cells were incubated with C6S9343B or an equal volume of vehicle at 37 °C for 30 min. All subsequent processing was performed at 4 °C. Cells were immediately cooled on ice, washed with ice-cold PBS, and fixed with methanol for 15 min. Methanol was removed by two washes with PBS, and cells were incubated for 1 h with anti-E-cadherin monoclonal antibody (mouse) (17) or anti-IQGAP1 polyclonal antibody (rabbit) (5). Unbound antibody was removed by four washes in PBS, following incubation for 2 h with the appropriate secondary antibody: FITC-labeled goat anti-mouse IgG or TRITC-labeled goat anti-rabbit antibody. After four washes with PBS, slides were mounted with Aqua Polymount (Polysciences, Inc.). The specificity of staining was established by omitting the primary antibody in each case.

**Confocal Laser Scanning Microscopy—**Digital micrographs were acquired using a Zeiss Axiovert 510 microscope with the MRC-1024.
Confocal Imaging System (Bio-Rad). Excitation and emission wavelengths were 494 and 520 nm, respectively, for FITC and 505 and 533 nm, respectively, for TRITC. Images were imported into a Dell PowerEdge 2200 computer and processed using the Lasersharp 3.0 program (Bio-Rad).

Confocal data were quantified by comparing the intensity of fluorescence at cell-cell contact sites with that in the cytoplasm of the same cell. Data were obtained from multiple cells in at least five different fields, each from at least two independent experimental observations performed on different days. In all cases, the fluorescence of IQGAP1 and E-cadherin was analyzed concurrently in the same cells.

**RESULTS**

**Calmodulin Antagonism Impairs E-Cadherin Function—** Both fluorescence-activated cell sorting (100% positive) (data not shown) and Western blotting with monoclonal antibody (see Fig. 2A) revealed that MCF-7 cells contain E-cadherin. This observation is consistent with published data (21). E-cadherin function in MCF-7 cells was evaluated with a specific cell-to-substrate adhesion assay using binding of MCF-7 cells to recombinant E-cadherin-Fc (19). In this assay, MCF-7 cells adhered to E-cadherin-Fc (Fig. 1A). Specificity of binding was demonstrated by the absence of cell binding to hyaluronan or human IgG1 (Fig. 1A). Moreover, MDA-MB-231 cells, which do not contain E-cadherin (Fig. 2A) (21), did not bind to E-cadherin-Fc (Fig. 1A). Finally, anti-E-cadherin antibodies abrogated the attachment of MCF-7 cells to E-cadherin-Fc (data not shown).

Ca²⁺/calmodulin competes with Cdc42 (5, 15) and actin (9) for binding to IQGAP1, suggesting that IQGAP1 may link Ca²⁺/calmodulin to cytoskeletal function. Therefore, we examined the effect of calmodulin on E-cadherin function. The cell-permeable calmodulin antagonist W7 significantly decreased adhesion of MCF-7 cells to E-cadherin-Fc (Fig. 1A). Inhibition of cell adhesion was demonstrated by the absence of cell binding to hyaluronan or human IgG1 (Fig. 1A). Moreover, MDA-MB-231 cells, which do not contain E-cadherin (Fig. 2A) (21), did not bind to E-cadherin-Fc (Fig. 1A). Finally, anti-E-cadherin antibodies abrogated the attachment of MCF-7 cells to E-cadherin-Fc (data not shown).

To test the hypothesis that calmodulin and E-cadherin compete for binding to IQGAP1, as was observed for calmodulin and Cdc42 (5, 15), increased concentrations of E-cadherin should attenuate the association between calmodulin and IQGAP1. This hypothesis was evaluated by both co-immunoprecipitation and competitive inhibition. Prior to conducting these analyses, we determined the percentage of total protein immunoprecipitated by the anti-IQGAP1 antibody. As depicted in Fig. 2B, 50–60% of the total IQGAP1 is immunoprecipitated from MCF-7 cells under routine assay conditions. A second immunoprecipitation resulted in essentially complete immunodepletion, with no IQGAP1 detectable in subsequent immunoprecipitations or in the final lysate. Thus, the co-immunoprecipitation approach can be used because the results reflect a significant component of the IQGAP1 in the lysate.

Using the co-immunoprecipitation strategy, we observed that the amount of calmodulin that co-immunoprecipitated with IQGAP1 following solubilization in 1% Triton X-100 from MDA-MB-231 (E-cadherin negative) cells was 3.13 ± 0.61-fold (mean ± S.E., n = 3), greater than that from MCF-7 (E-cadherin positive) cells (Fig. 2, C and D). This finding suggested a reciprocal relationship between the amount of calmodulin and E-cadherin bound to IQGAP1 because there was no significant difference between MCF-7 and MDA-MB-231 cells in the level of expression of IQGAP1 and calmodulin (Fig. 2A). Note that both of these cell lines express α- and β-catenin (21).

In a second approach, competitive inhibition was examined. *In vitro* analysis with purified proteins revealed that incubation of GST-E-cadherin (the cytoplasmic domain) with IQGAP1 inhibited in a dose-dependent manner calmodulin binding to IQGAP1 (Fig. 3A). A 2-fold molar excess of GST alone slightly decreased binding, but the magnitude was substantially less than that produced by GST-E-cadherin. To characterize this interaction in a normal cellular milieu, L and EL fibroblasts were used. The amount of endogenous IQGAP1 bound to calmodulin-Sepharose from mouse L fibroblasts, which lack endogenous E-cadherin, was 2-fold greater than that from E-cadherin-transfected L fibroblasts (EL cells) (Fig. 3B). Moreover, addition of GST-E-cadherin decreased the amount of IQGAP1 that bound to calmodulin in both cell lines. The inhibition produced by GST-E-cadherin was dose-dependent in both EL (Fig. 3C) and L (data not shown) fibroblasts. By contrast, addition of an equal amount of GST did not significantly alter the interaction between calmodulin and endogenous IQGAP1 (Fig. 3B). These data support the conclusion that the cytoplasmic domain of E-cadherin competes with IQGAP1 for binding to calmodulin-Sepharose. Note that expression of E-cadherin did not significantly alter the amount of IQGAP1 or calmodulin in L fibroblasts (Fig. 2A).

**Mutations of IQGAP1 Alter Calmodulin Binding—** To further test the hypothesis that calmodulin and E-cadherin compete for binding to IQGAP1, two mutant IQGAP1 proteins were generated: IQGAP1 ΔIQ, which lacks the major calmodulin-binding region (5), and IQGAP1 ΔC, which is unable to bind E-cadherin (8). Cells were transiently transfected to the same level with Myc-tagged IQGAP1-ΔIQ, IQGAP1-ΔC, and wild type IQGAP1. All transfected proteins migrated to the expected position on SDS-PAGE (Fig. 4A). Longer exposure of the blots...
probed with anti-IQGAP1 antibody revealed the presence of endogenous IQGAP1 in the cells transfected with IQGAP1-DIQ and IQGAP1-DIC (data not shown). Probing samples immunoprecipitated with anti-IQGAP1 antibody for calmodulin revealed that mutation of IQGAP1 altered calmodulin binding.

**FIG. 1.** Calmodulin antagonists inhibit E-cadherin-mediated homophilic adhesion. A, microtiter plates were coated with E-cadherin-Fc, human IgG1, or the CD44 ligand hyaluronan, and the adhesion of E-cadherin-expressing MCF-7 cells or E-cadherin negative MDA-MB-231 was determined in the presence of 100 μM W5 or W7 or vehicle alone as described under "Experimental Procedures." Means ± S.D. are shown (n = 3). Data are representative of three separate experiments. B, MCF-7 cells were incubated in wells coated with E-cadherin-Fc or poly-L-lysine with the indicated concentrations of CGS9343B. After washing, the number of adherent cells was determined with a fluorogenic assay. Data are expressed as the percentages of adherent cells after background adhesion to poly-L-lysine was subtracted. The results are the means and S.D. (n = 3) and are representative of five experimental determinations. C, the adhesion of MTSV-7 cells to E-cadherin-Fc or poly-L-lysine was determined in the presence of the indicated concentrations of W5 or W7. For clarity, the results are plotted as the percentages of adherent cells measured in the presence of vehicle alone (32% of vehicle-treated cells adhered to E-cadherin-Fc and 22% adhered to poly-L-lysine). Background adhesion to human IgG1 (3%) was subtracted and means ± S.D. are shown (n = 3). The absence of error bars indicates that the range is smaller than the symbols. Similar results were obtained in two separate experiments. *, significantly different from vehicle alone (p < 0.02); **, significantly different from vehicle alone (p < 0.005).

**FIG. 2.** E-cadherin attenuates the association of calmodulin with IQGAP1. A, equal amounts of protein lysate from MCF-7 and MDA-MB-231 cells or from EL and L fibroblasts were resolved by SDS-PAGE and transferred to PVDF as described under "Experimental Procedures." Blots were probed with antibodies to IQGAP1, E-cadherin, and calmodulin (CaM). B, lysate from MCF-7 cells was immunoprecipitated sequentially with anti-IQGAP1 antibody. An aliquot of the lysate before immunoprecipitation (Initial Lysate), sequential immunoprecipitates (IP), and an aliquot of the final lysate were resolved by SDS-PAGE, and the blot was probed with anti-IQGAP1 antibody. Data are representative of nine experimental determinations. C, equal amounts of protein lysate from MCF-7 and MDA-MB-231 cells were immunoprecipitated (IP) with anti-IQGAP1 antibody (αIQGAP1) or nonimmune rabbit serum (NIRS) as described under "Experimental Procedures." Complexes were resolved by SDS-PAGE, transferred to PVDF, and probed with anti-IQGAP1 (top panel) or anti-calmodulin (CaM) (bottom panel) antibodies. Immune complexes were visualized by ECL. In all cases, data are representative of at least two separate experiments. D, the relative amount of calmodulin that co-immunoprecipitated with IQGAP1 in C was quantified. Results expressed relative to MCF-7 cells represent the means ± S.E. from three independent experimental determinations.
Calmodulin-Sepharose was added, and complexes were isolated and resolved by SDS-PAGE as described under "Experimental Procedures." Following transfer to PVDF, blots were probed with anti-IQGAP1 antibody. C, equal amounts of protein lysate from EL cells were incubated with 0, 25, 50, or 75 μg of GST-E-cadherin. Calmodulin-Sepharose was added, and samples were processed as described for B. The relative amount of IQGAP1 protein in each sample was quantified. The results, presented in the lower panel, are expressed as percentages of the value of the control (no GST-E-cadherin added). In all cases, data are representative of at least two independent experimental determinations.

**Fig. 3.** E-cadherin and calmodulin compete for binding to IQGAP1. A, GST-IQGAP1 was incubated with 0, 2, or 4 μg of GST-E-cadherin or 4 μg of GST. Calmodulin was added, and samples were immunoprecipitated with anti-IQGAP1 antibody. Samples were resolved by SDS-PAGE and transferred to PVDF, and the blots were probed with anti-calmodulin (CaM) antibody (upper panel). The relative amount of calmodulin protein in each sample was quantified. The data, presented in the lower panel, are expressed as percentages of the control (no GST proteins added). B, equal amounts of protein lysate from EL and L fibroblasts were incubated with no additions (−), 50 μg of GST or 50 μg of GST-E-cadherin (E-Cad). Calmodulin-Sepharose was added, and complexes were isolated and resolved by SDS-PAGE as described under "Experimental Procedures." Following transfer to PVDF, blots were probed with anti-IQGAP1 antibody. C, equal amounts of protein lysate from EL cells were incubated with 0, 25, 50, or 75 μg of GST-E-cadherin. Calmodulin-Sepharose was added, and samples were processed as described for B. The relative amount of IQGAP1 protein in each sample was quantified. The results, presented in the lower panel, are expressed as percentages of the value of the control (no GST-E-cadherin added). In all cases, data are representative of at least two independent experimental determinations.

**Fig. 4.** Effect of mutation of IQGAP1 on calmodulin binding. Cells were transiently transfected with wild type IQGAP1 (WT), IQGAP1-DIQ (ΔIQ), or IQGAP1-ΔΔC (ΔC) as described under "Experimental Procedures." A, equal amounts of protein lysate were resolved by SDS-PAGE and transferred to PVDF, and blots were probed with anti-IQGAP1 (IQGAP1) antibody (upper panel). Membranes were stripped according to the manufacturer’s protocol, and blots were reprobed with anti-Myc (Myc) antibody (lower panel). B, equal amounts of protein lysate were immunoprecipitated with anti-IQGAP1 antibody (IP: αIQGAP1), and samples were resolved by SDS-PAGE and immunoblotting. The upper and lower halves of the blot were probed with anti-Myc (Myc) and anti-calmodulin (CaM) antibodies, respectively. Data are representative of two independent experiments.

Compared with wild type IQGAP1, the amount of calmodulin that co-immunoprecipitated with IQGAP1-DIQ and IQGAP1-ΔΔC was decreased and increased, respectively (Fig. 4B). Because the anti-IQGAP1 antibody recognizes both endogenous and mutant IQGAP1, the presence of some calmodulin in the IQGAP1-ΔIQ sample is expected. In the absence of a crystal structure, it is not possible to exclude the premise that conformational changes in IQGAP1-ΔΔC are responsible for the enhanced calmodulin binding. However, the data are also consistent with the hypothesis that calmodulin and E-cadherin compete for binding to IQGAP1.

**Fig. 5.** E-cadherin regulates the subcellular location of IQGAP1. MCF-7 (left panels) and MDA-MB-231 (right panels) cells were fixed with methanol and probed with both anti-IQGAP1 (rabbit) and anti-E-cadherin (mouse) antibodies as described under "Experimental Procedures." Primary antibodies were visualized with TRITC-labeled goat anti-rabbit (red) (IQGAP1, top panels) and FITC-labeled goat anti-mouse (green) (E-cadherin, middle panels) antibodies. Superimposed images are presented in the bottom panels (IQGAP1/E-cadherin). Yellow indicates co-localization of IQGAP1 and E-cadherin. A minimal amount of nonspecific staining is visible in some of the MDA-MB-231 cells in the middle panel. Representative fields of at least two independent experimental observations are shown.

**E-Cadherin Regulates IQGAP1 Location**—Transfection of mouse L fibroblasts with E-cadherin (EL cells) resulted in the accumulation of IQGAP1 at sites of cell-cell contact (S). No accumulation was observed in nontransfected L cells. The role of endogenous E-cadherin on IQGAP1 subcellular location in epithelial cells was assessed by comparing MCF-7 (E-cadherin positive) cells with MDA-MB-231 (E-cadherin negative) cells by immunocytochemistry (Fig. 5). In MCF-7 cells, IQGAP1 was distributed throughout the cytoplasm, with accumulation at cell-cell junctions where it co-localized with E-cadherin (Fig. 5). By contrast, no significant staining of IQGAP1 was detected at cell-cell contact sites in MDA-MB-231 cells. Although a role for other proteins cannot be excluded, it appears that endogenous E-cadherin is required for the location of IQGAP1 at sites of cell-cell contact in human breast epithelial cells.
investigate the participation of calmodulin in the subcellular location of IQGAP1, the antagonist CGS9343B was used. Immunocytochemistry suggested that antagonism of calmodulin altered the distribution of IQGAP1 in MCF-7 cells; IQGAP1 concentrations at areas of cell-cell contact appeared to be increased (Fig. 6A). To accurately quantify confocal data, the fluorescence signal of IQGAP1 was measured at an emission wavelength of 533 nm in multiple cells. The amount of IQGAP1 at cell-cell contact sites was compared with the IQGAP1 signal in the cytoplasm of the same cell. This strategy eliminates possible variability in antibody decorating that may arise from the experimental technique. As an additional precaution, the E-cadherin fluorescence signal (at excitation and emission wavelengths of 494 and 520 nm, respectively) was quantified concurrently in the same cells used for IQGAP1 examination. Analysis of 48 CGS9343B-treated cells and 48 control cells demonstrated that the calmodulin antagonist increased by 30% the accumulation of IQGAP1 at cell-cell junctions (Fig. 6B). The magnitude of the translocation was proportional to the concentration of CGS9343B used (Fig. 6C). Consistent with the findings on E-cadherin function (Fig. 1), antagonism of calmodulin also had a profound effect on E-cadherin location (Fig. 6A). Analysis of the same cells examined for IQGAP1 revealed that the amount of E-cadherin on the cell surface as judged by fluorescence-activated cell sorting analysis of E-cadherin expression (data not shown) and had no effect on cell viability.

By contrast, CGS9343B did not change calmodulin location, whereas depletion of intracellular free Ca^{2+} with BAPTA caused calmodulin to move out of the nucleus into the cytoplasm (data not shown). The latter observation is concordant with the recent findings that a localized increase in intracellular free Ca^{2+} concentration induces translocation of calmodulin into the nucleus (26). The effect of CGS9343B was not due to a general disruption of the cytoskeleton because no significant change in actin distribution was observed in cells treated with CGS9343B (data not shown).
IQGAP1 binds to signaling proteins and to components of the cytoskeleton. The functional sequelae of these associations in intact cells are unknown, but evidence implicates IQGAP1 as an intersection point that links signaling pathways. Previous studies have demonstrated that calmodulin regulates the binding of IQGAP1 to Cdc42 and actin (5, 9, 15). In this paper, we investigate the interaction among calmodulin, IQGAP1, and E-cadherin in intact cells and assess the effect on E-cadherin function.

Kaibuchi and colleagues (8) demonstrated that IQGAP1 binds directly to E-cadherin. EL fibroblasts (which are L fibroblasts stably transfected with E-cadherin) that overexpressed IQGAP1 were less aggregated in culture than EL cells expressing vector alone (8). Although many adhesion proteins could be involved, Kuroda et al. suggested that IQGAP1 regulates cell-cell adhesion through the cadherin-catenin pathway (8). Our data support this model and extend the findings by demonstrating that translocation of endogenous IQGAP1 to cell-cell junctions coincides with decreased E-cadherin homophilic adhesion. Although the magnitude of the IQGAP1 increase was relatively modest (usually no more than 30%), direct measurement of the adhesion of cells to purified E-cadherin-Fc under these conditions documented that E-cadherin-mediated adhesion was substantially reduced. Thus, the function of endogenous E-cadherin was impaired when the amount of IQGAP1 was increased at cell-cell junctions. The molecular mechanism that underlies the functional impairment can be ascertained from our understanding of cadherin function (3). IQGAP1 presumably alters the interaction between the cytoplasmic filament system and the transmembrane cadherin molecules, thereby dissociating the cell-adhesion zipper (3). This hypothesis is supported by the significant decrease in the amount of E-cadherin at sites of cell-cell attachment (Fig. 6), without a decline in the total cell surface level of E-cadherin. Moreover, calmodulin appeared to be an important element in the interaction between E-cadherin and IQGAP1. Inhibition of calmodulin function induced the translocation of IQGAP1 to cell-cell junctions, resulting in impaired E-cadherin function.

In this study we used pharmacologic agents that bind directly to calmodulin, thereby preventing calmodulin from interacting with its target proteins (27–30). Caution should always be exercised in interpreting results obtained with antagonists. Calmodulin antagonists have been widely used to investigate calmodulin function in cells (31–34), but some of these drugs can affect other targets (27). Several approaches were adopted in this study to minimize the likelihood of inhibiting other pathways. First, CGS9343B is reported to be specific for calmodulin (23, 32) at concentrations as high as 1000 μM and, unlike other antagonists (e.g., trifluoperazine), does not inhibit protein kinase C activity (23). Second, structurally distinct antagonists CGS9343B and W7 were used. W7 has been frequently used to antagonize calmodulin in intact cells and is reported to be highly specific at the concentrations used in this work (34). Third, W5, the nonchlorinated analogue of W7 that has decreased affinity for calmodulin (22), was employed as a control and had no effect on E-cadherin function. Fourth, the antagonists exhibited dose-dependent effects that occurred at concentrations believed to be specific for calmodulin inhibition. Fifth, calmodulin antagonism did not decrease cell adhesion to the CD44 ligand hyaluronan or poly-L-lysine. These precautions, coupled with the competition shown by GST-E-cadherin and the previously documented effects of calmodulin on IQGAP1 function (albeit in vitro) (5, 9, 15), strongly support a role for calmodulin in the interaction between IQGAP1 and E-cadherin in intact cells.

The results obtained with E-cadherin in this study should be interpreted in the context of the interaction of IQGAP1 with Cdc42 and actin, other proteins that couple IQGAP1 to the cytoskeleton. Although calmodulin regulates the interaction of IQGAP1 with Cdc42 in vitro and in cells (5, 15), there are conflicting data for F-actin. Calmodulin partially inhibited the binding of purified IQGAP1 to F-actin in a Ca\(^{2+}\)-independent manner in vitro (9). However, F-actin did not reduce binding to calmodulin-Sepharose of endogenous IQGAP1 in cell lysates (5). Therefore, the role of calmodulin in the association between IQGAP1 and actin in intact cells remains to be established. The molecular interactions of Ca\(^{2+}\)/calmodulin, Cdc42, Rac, actin, E-cadherin, and β-catenin with IQGAP1 and the effects of these associations on cytoskeletal function are likely to be extremely complex. Nevertheless, the data presented here suggest that the regulation of E-cadherin function produced by calmodulin is mediated, at least in part, via IQGAP1.

Based on both our data and those generated by other investigators, we propose a model (Fig. 7) to explain the interactions among IQGAP1, E-cadherin and calmodulin. It is known that a substantial proportion of endogenous IQGAP1 is bound to calmodulin (5). Moreover, binding of calmodulin appears to have an allosteric effect on IQGAP1, changing its tertiary confor-
tion (5, 15). Therefore, disrupting the association between calmodulin and IQGAP1 could enhance the interaction of IQGAP1 with other targets. This hypothesis is supported by the competition between Ca^2+ /calmodulin and Cdc42 (5) or actin (9) for IQGAP1 binding. One might propose a mechanism in which inhibition of calmodulin decreases its binding to IQGAP1, leading to an increase in the association of IQGAP1 with E-cadherin and attenuation of E-cadherin adhesion. It remains to be determined how IQGAP1 modulates E-cadherin function. It is possible that the direct binding of one or multiple IQGAP1 molecules to E-cadherin might sever the association between α-catenin and the E-cadherin-β-catenin complex (Fig. 7B, 1) (8). Alternatively, the increased IQGAP1 that is available may form dimers (Fig. 7B, 2) (9) that could interfere with E-cadherin function by either allosteric or steric effects. A third possibility is that the IQGAP1 released from calmodulin may disrupt the cadherin-catenin complex by binding to β-catenin (Fig. 7B, 3) (8). The last mechanism is most concordant with the immunocytotoxic data, reconciling increased IQGAP1 and decreased E-cadherin at sites of cell-cell contact. Regardless of the mechanism, the result is that E-cadherin homophilic association will diminish, E-cadherin will move away from regions of cell-cell contact, and cell-cell attachment will decrease (Fig. 7B). Thus, IQGAP1 integrates calmodulin signaling, the cytoskeleton, and cell-cell attachment and may function as a critical protein module in the regulation of cytoskeletal function.

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