Calmodulin regulates numerous fundamental metabolic pathways by binding to and modulating diverse target proteins. In this study, calmodulin-binding proteins were isolated from normal (Hs578Bst) and malignant (MCF-7) human breast cell lines with calmodulin-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis. A protein that migrated at approximately 190 kDa bound to calmodulin in the presence of Ca^{2+} and was the only calmodulin-binding protein detected in the absence of Ca^{2+}. This 190-kDa protein was identified as IQGAP1 by nanoelectrospray mass spectrometry and collision-induced dissociation tandem mass spectrometry. IQGAP1 coimmunoprecipitated with calmodulin from lysates of MCF-7 cells. Moreover, overlay with 125I-calmodulin confirmed that IQGAP1 binds directly to calmodulin. Analysis of the functional effects of the interaction revealed that Ca^{2+}/calmodulin disrupted the binding of purified IQGAP1 to the Ras-related protein Cdc42 in a concentration-dependent manner. These data clearly identify IQGAP1 as the predominant calmodulin-binding protein in Ca^{2+}-free breast cell lysates and reveal that calmodulin modulates the interaction between IQGAP1 and Cdc42.

Calmodulin is an acidic effector protein that regulates multiple processes in eukaryotic cells (1). Upon binding Ca^{2+}, the conformation of calmodulin is altered (2), exposing hydrophobic residues that mediate its interaction with amphiphilic α-helical regions of target proteins (1). Removal of Ca^{2+} reverses binding (1). By this mechanism, Ca^{2+}/calmodulin modulates the activity of several proteins, thereby regulating cellular metabolism including protein phosphorylation, cell cycle progression, DNA synthesis, transcription, and cytoskeletal organization (1).

More recently, a novel interaction of calmodulin with a variety of unconventional myosins (3), neuregulin (4), neurogranin (5), Ras-GRF (6), and IRS-1 (7) was identified. Each of these proteins contains one or more IQ motifs, a sequence of approximately 23 amino acid residues with a consensus XXXXRGXXXR (8). The presence of the distal arginine residue in the IQ consensus motif dictates the Ca^{2+} requirement for binding calmodulin (9). When the arginine residue is present, the IQ motif is said to be complete and Ca^{2+} is not necessary for calmodulin binding. An incomplete IQ motif does not contain the distal arginine residue, and calmodulin requires Ca^{2+} to bind the motif (9).

The gene encoding an IQ motif-containing protein was recently cloned from human osteosarcoma tissue by RNA polymerase chain reaction (10). Designated IQGAP1, the predicted protein has a calculated molecular mass of approximately 189 kDa, and contains four complete IQ motifs as well as a region with considerable sequence similarity to the catalytic domain of Ras-GAP1 (10). Since the original submission of this manuscript, three studies have been published that focus on the interaction between IQGAP1 and the Ras-like proteins, Cdc42 and Rac (11–13). However, when isolated as a Cdc42-binding protein from rabbit liver (12), the amino acid sequence of an internal peptide of the 180-kDa protein was not identical to the previously published sequence of IQGAP1 (10). In addition, an antibody to IQGAP1 failed to recognize this putative IQGAP1 from rabbit liver, and the authors proposed that the 180-kDa protein may represent an IQGAP1 homologue (12). Furthermore, the published studies used recombinant IQGAP1 to examine its effect on Ras activity, which produced somewhat conflicting data (10, 11, 13). It is possible that endogenous IQGAP1 undergoes post-translational modification(s) that are not present in the recombinant protein. Therefore experimentation with purified endogenous IQGAP1 is necessary to resolve these discrepancies.

The presence of IQ motifs in IQGAP1 suggests that it binds to calmodulin. In support of this, it was shown very recently that calmodulin associates with the N-terminal region of IQGAP1, which contains the IQ motifs (11, 13). However, the interaction of calmodulin and IQGAP1 has not been characterized and no analysis of the potential functional sequelae has been performed. Here we use calmodulin affinity chromatography to purify sufficient full-length endogenous IQGAP1 from mammalian cells to perform functional analysis. We demonstrate by nanoelectrospray tandem mass spectrometry that calmodulin binds to IQGAP1 in human breast cell lysates both in the presence and absence of Ca^{2+}, and we document that calmodulin modulates the interaction between IQGAP1 and Cdc42.

EXPERIMENTAL PROCEDURES

Materials—The antibodies to calmodulin (14) and IQGAP1 (11) were described previously. Production of the glutathione S-transferase-Cdc42 fusion protein has been described (11). MCF-7 cells were a gift from Dr. A. Dutta (Brigham and Women's Hospital, Boston, MA).

1 The abbreviations used are: GAP, GTPase-activating protein; PAG, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; m/i, mass/charge; CID, collision-induced dissociation; MS/MS, tandem mass spectrometry; GST, glutathione S-transferase; GTPyS, guanosine 5‘-O-(3-thiotriphosphate); ECL, enhanced chemiluminescence.
Calmodulin Modulates IQGAP1

HaS578Bst cells were from the American Type Culture Collection. Calmodulin-Sepharose was purchased from Pharmacia Biotech Inc. Affi-Gel was from Bio-Rad. Tissue culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum was from BioWhittaker. All other reagents were of standard analytical grade.

Cell Culture and Lysis—MCF-7 and HaS578Bst cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum in a 37 °C humidified incubator. Cells were washed three times in serum-free medium and lysed in buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, and 1% (v/v) Triton X-100) with either 1 mM CaCl2 or 1 mM EGTA. The lysates were then washed in 50% (v/v) acetonitrile, 100 mM NaHCO3, cut into small pieces, and lyophilized. Modified trypsin in 50 mM NaHCO3 was added, and the sample was incubated overnight at 37 °C. Peptides were extracted twice with 0.1% (v/v) trifluoroacetic acid in 60% (v/v) acetonitrile using sonication, lyophilized, and reconstituted in 5% (v/v) formic acid.

The digest was desalted and concentrated on Poros R2 resin (40–60 μm particles, PerSeptive Biosystems), which had been packed into an Eppendorf Gelaso pipette tip (Brinkman Instruments, Westbury, NY), eluted with 1.5 μl of 5% (v/v) formic acid in 70% (v/v) methanol, and loaded into the back of the nanospray needle.

Protein Identification—Electrospray mass spectra were acquired on a modified Perkin-Elmer Sciex API-III triple quadrupole tandem mass spectrometer (Thornhill, Ontario, Canada). This instrument is equipped with a high pressure collision cell (PE-Sciex) and an articulated nanospray interface developed by Wilm and Mann (17), and was built at the European Molecular Biology Laboratory in Heidelberg, Germany. The operation of the API-III in the nanospray mode has been described in detail elsewhere (18,19).

The molecular weights and precursor ion masses for the tryptic peptides in the unfraccionated digest were determined by scanning the first quadrupole (Q1) from m/z 400–1500 in 0.1-Da steps using a dwell time of 8 ms/mass step. The resolution was adjusted to determine the isotopes of a triply charged ion up to m/z 1000. This allowed singly, doubly, and triply charged ions to be distinguished from one another.

Selected precursor ions were caused to undergo collision-induced dissociation (CID) in the second quadrupole (Q2). The resulting CID product ions were mass analyzed by scanning the third quadrupole (Q3) using a mass step of 1 Da and a mass defect of 50 millimass units/100 Da. The mass spectrometer was scanned over the desired mass range using a dwell of 20 ms/mass step. This approach produces analytically useful tandem mass spectrometry (MS/MS) data rapidly, allowing mass spectrometry product ion spectra to be acquired with a single loading of sample. Product ion spectra were interpreted manually. Mass spectrometric based sequence tags (20) were searched against a nonredundant protein database using the PepFinder program written by Mann and Wilm (20). Peptide fragment ion data base maintained by SmithKline Beecham, using the Peptide Product ion spectra were interpreted manually. Mass spectrometric useful tandem mass spectrometry (MS/MS) data rapidly, allowing molecular size standards is depicted on the right. The 190-kDa band is indicated by an arrow. The gel is representative of at least three separate experiments.

FIG. 1. Identification of calmodulin-binding proteins from human breast cell lines. MCF-7 and HaS578Bst cells were lysed, and equal amounts of protein were incubated with calmodulin-Sepharose in the presence (+) or absence (−) of Ca2+. Samples were washed five times in buffer B, 4 μCi of 125I-calmodulin-Sepharose was added and the blot was incubated for 2 h at 22°C. The membrane was washed five times in buffer B, dried, and exposed to x-ray film. As a positive control, 0.5 μg of purified calcineurin was processed in parallel.

In Vitro Binding Assay—Glutathione S-transferase (GST) linked to Cdc42 (GST-Cdc42) was treated with 1 mM EDTA for 10 min to remove bound guanine nucleotides, and then incubated with 75 μl GDP or GTP-S in 5 mM MgCl2 for 30 min to reoad. Purified IQGAP1 was incubated with 1 μg of GST-GTPyS, GST-Cdc42-GDP, or GST-Cdc42-GTPyS in buffer A containing different concentrations of CaCl2 as indicated in the figure legends for 30 min at 22°C. Where indicated, calmodulin was included in the assay. Complexes were isolated with glutathione-Sepharose for 30 min at 22°C while rotating, resolved by SDS-PAGE, and transferred to PVDF membrane. Blots were cut in half and the appropriate pieces were probed with anti-calmodulin or anti-IQGAP1 antibodies. Antigen-antibody complexes were visualized with horseradish peroxidase-conjugated secondary antibody developed by ECL.

Miscellaneous Methods—Protein determinations of the cells lysates were performed using the DC Protein Assay from Bio-Rad. Densitometry of the protein bands on the gels, the ECL signals, and autoradiographs was performed with NIH Image.

RESULTS AND DISCUSSION

Calmodulin-binding proteins were isolated from a normal human breast cell line, Hs578Bst, and a malignant human breast cell line, MCF-7, with calmodulin-Sepharose in the presence and absence of Ca2+, resolved by SDS-PAGE, and stained with Coomassie Blue. As a control (C), an equal amount of MCF-7 cell lysate was incubated with glutathione-Sepharose and processed as described above. The migration of molecular size standards is depicted on the right. The 190-kDa band is indicated by an arrow. The gel is representative of at least three separate experiments.

brane was blocked in buffer B (50 mM Tris, pH 7.8, 200 mM NaCl, and 0.05% (v/v) Tween 20), supplemented with 1 mM CaCl2 or 1 mM EGTA containing 5% nonfat dry milk. After washing in buffer B, 4 μCi of 125I-calmodulin-Sepharose was added and the blot was incubated for 2 h at 22°C. The membrane was washed five times in buffer B, dried, and exposed to x-ray film. As a positive control, 0.5 μg of purified calcineurin was processed in parallel.

In Vitro Binding Assay—Glutathione S-transferase (GST) linked to Cdc42 (GST-Cdc42) was treated with 1 mM EDTA for 10 min to remove bound guanine nucleotides, and then incubated with 75 μl GDP or GTP-S in 5 mM MgCl2 for 30 min to reoad. Purified IQGAP1 was incubated with 1 μg of GST-GTPyS, GST-Cdc42-GDP, or GST-Cdc42-GTPyS in buffer A containing different concentrations of CaCl2 as indicated in the figure legends for 30 min at 22°C. Where indicated, calmodulin was included in the assay. Complexes were isolated with glutathione-Sepharose for 30 min at 22°C while rotating, resolved by SDS-PAGE, and transferred to PVDF membrane. Blots were cut in half and the appropriate pieces were probed with anti-calmodulin or anti-IQGAP1 antibodies. Antigen-antibody complexes were visualized with horseradish peroxidase-conjugated secondary antibody developed by ECL.

Miscellaneous Methods—Protein determinations of the cells lysates were performed using the DC Protein Assay from Bio-Rad. Densitometry of the protein bands on the gels, the ECL signals, and autoradiographs was performed with NIH Image.

RESULTS AND DISCUSSION

Calmodulin-binding proteins were isolated from a normal human breast cell line, Hs578Bst, and a malignant human breast cell line, MCF-7, with calmodulin-Sepharose in the presence and absence of Ca2+, resolved by SDS-PAGE, and stained with Coomassie Blue. As a control (C), an equal amount of MCF-7 cell lysate was incubated with glutathione-Sepharose and processed as described above. The migration of molecular size standards is depicted on the right. The 190-kDa band is indicated by an arrow. The gel is representative of at least three separate experiments.

The 190-kDa band was not present in the absence of Ca2+. The 190-kDa band was not dissociated from calmodulin-Sepharose in a Ca2+-dependent manner and were dissociated from calmodulin when EGTA was added to the lysate (Fig. 1). However, one protein with a molecular mass of approximately 190 kDa bound calmodulin both in the presence and absence of Ca2+. In both cell lines the amount of the 190-kDa protein detected in the presence of Ca2+ was 2-fold greater than in the absence of Ca2+. The 190-kDa band was not dissociated from calmodulin-Sepharose by buffer containing 1 mM NaCl and 1% Triton X-100 (data not shown), indicating high affinity binding. Compared with normal cells, the majority of calmodulin-binding proteins were present at higher levels in tumor cells (Fig. 1). By contrast, half as much of the 190-kDa protein from malignant cells bound to calmodulin. Three uni-
dentified bands of approximately 47, 55, and 65 kDa were present in all lanes, even when lysates were incubated with glutathione-Sepharose, suggesting that they bind nonspecifically to the Sepharose beads (Fig. 1).

To identify the 190-kDa protein, the Coomassie Blue-stained band from one lane (MCF-7, \( \text{Ca}_2^+ \)) was digested in situ with trypsin and analyzed by electrospray mass spectrometry using a nanoliter flow interface (17). The nanospray source introduces samples into the mass spectrometer at flow rates on the order of 25–40 nL/min, thus allowing up to 40 min of data collection time for a single 1-\( \mu \)L loading of sample. These long sampling periods allow ample time for multiple experiments in which analysis parameters are optimized, and spectra can be accumulated to obtain useful data even from very weak signals. However, all electrospray methods are sensitive to the type and concentration of salts and buffers present in the sample. In addition, because signal response in an electrospray ion source is concentration-dependent, it is beneficial to have the sample in the smallest possible volume. For nanospray, this is ideally 1–2 \( \mu \)L. Therefore, to desalt and concentrate unfracionated in-gel protein digests, we have developed a simple method that uses gel loading pipette tips packed with a 3–4-mm height bed of reverse phase chromatographic material (22). After washing away the salts and buffers, the peptides can be eluted with 1.5 \( \mu \)L of solvent. This preparation step is critical to the success of the nanospray experiment. Typically, the entire sample is loaded into the nanospray needle. A similar sample preparation approach has been described by Wilm and co-workers (23).

The identity of a protein present in a proteolyzed gel slice can be determined using the sequence tag approach of Mann and Wilm (20). Briefly, an electrospray ionization mass spectrum of the entire mixture is recorded and, based on these data, peptide precursor ions are chosen to be sequenced by CID MS/MS in the triple quadrupole mass spectrometer. Partial sequences as short as 2–3 amino acids can then be used to identify proteins in a data base by the inclusion in the search of the peptide molecular weight and the masses of the portions of the peptide that precede and follow the determined partial sequence.

The mass spectrum of the unfraccionated digest of the 190-kDa band exhibited a broad distribution of peptide molecular ions with charge states varying from 1+ to 4+ (Fig. 2A). Initially, a doubly charged ion at \( m/z \) 617.3 was selected for CID MS/MS in the triple quadrupole mass spectrometer. Partial sequences as short as 2–3 amino acids can then be used to identify proteins in a data base by the inclusion in the search of the peptide molecular weight and the masses of the portions of the peptide that precede and follow the determined partial sequence.

The mass spectrum of the unfractionated digest of the 190-kDa band exhibited a broad distribution of peptide molecular ions with charge states varying from 1+ to 4+ (Fig. 2A). Initially, a doubly charged ion at \( m/z \) 617.3 was selected for CID MS/MS in the triple quadrupole mass spectrometer. Partial sequences as short as 2–3 amino acids can then be used to identify proteins in a data base by the inclusion in the search of the peptide molecular weight and the masses of the portions of the peptide that precede and follow the determined partial sequence.
an in-house nonredundant protein data base modified for the program Peptide Search (20). A single peptide sequence, FPDAGEDELLK (M_r = 1232.6), from IQGAP1 (10) was matched (Table I). Other major ions in the CID spectrum could now be assigned as b_5 and y_8 fragments that are predicted to be formed based on the indicated sequence. In this manner, confidence is gained that the correct sequence has been identified. During the analysis of the CID spectrum from m/z 617.3 precursor, a second IQGAP1 sequence was discovered, which could be assigned to the peptide, YQELINDIAR (M_r = 1233.6). The doubly charged ion for this peptide [M + 2H]^{2+} = 617.8 would overlap the ^13C peak of the first peptide, and so be included in the precursor ion selection window (which has a width of approximately 3 Da). Ions belonging to this second sequence are indicated by ● in Fig. 2, but are not otherwise labeled for the sake of clarity. Two additional doubly charged precursors from the unfraccionated digest were sequenced by CID MS/MS and sequence tags from their spectra also matched tryptic peptides from IQGAP1 (Table I). Moreover, 18 other peptide ions matched tryptic peptides from IQGAP1 based on molecular weight alone (Table I). These data establish that the 190-kDa calmodulin-binding protein is IQGAP1.

The mass spectrometry-based analytical approaches used in the present study for identifying proteins from gels are both sensitive and fast. After enzymatic digestion and desalting of the peptides, identification of the 190-kDa protein as IQGAP1 using data base searching of mass spectrometrically derived partial amino acid sequences took approximately 20 min. Forty percent of the sample was used to record the molecular weights of the tryptic peptides and sequence the three precursor ions. This remaining 60% of the digest was stored frozen in the nanospray needle in the event further characterization (such as characterizing suspected post-translational modifications) was necessary.

Additional verification that the 190-kDa band was IQGAP1 was obtained by transferring to PVDF proteins that bound to calmodulin-Sepharose and probing the membrane with anti-IQGAP1 antibody. A single band migrating at 190 kDa was detected (Fig. 3A), confirming its identity as IQGAP1. Analogous to the results obtained in the Coomassie Blue-stained gels (see Fig. 1), 2-fold more IQGAP1 bound calmodulin when Ca^{2+} was included in the incubation buffer (Fig. 3A). Previous studies with immunoprecipitation of lysates from 3T3 cells with anti-IQGAP1 antibody revealed calmodulin, demonstrating that IQGAP1 and calmodulin form a complex in vivo (11). We performed the reverse experiment. Endogenous calmodulin was isolated with anti-calmodulin antibody, and immunoblots were probed with anti-IQGAP1 antibody. A single band migrating at 190 kDa was detected (Fig. 3B), indicating that the interaction occurs in MCF-7 cells.

It is possible that IQGAP1 binds in a complex and does not associate directly with calmodulin. To resolve this issue, [125I]-calmodulin overlay was performed after proteins were isolated from MCF-7 cells by calmodulin-Sepharose and transferred to PVDF. [125I]-calmodulin binding was evaluated both in the presence (+) or absence (−) of Ca^{2+}, or anti-calmodulin antibody (αCaM) (B) as described under “Experimental Procedures.” Proteins were separated by SDS-PAGE, transferred to PVDF, and blots were probed with an antibody to IQGAP1. The data are representative of two independent experiments. The position of migration of IQGAP1 is indicated.

![Figure 3](image.png)

**Fig. 3. Binding of IQGAP1 to calmodulin.** MCF-7 cell lysates were incubated with calmodulin-Sepharose (CaM-Sepharose) (A), in the presence (+) or absence (−) of Ca^{2+}, or anti-calmodulin antibody (αCaM) (B) as described under “Experimental Procedures.” Proteins were separated by SDS-PAGE, transferred to PVDF, and blots were probed with an antibody to IQGAP1. The data are representative of two independent experiments. The position of migration of IQGAP1 is indicated.
Calmodulin Modulates IQGAP1

Ca\(^{2+}\)-containing or Ca\(^{2+}\)-free calmodulin-Sepharose (Fig. 4A, lanes 1 and 2). In addition, an unidentified protein that migrated on SDS-PAGE slightly further than IQGAP1 bound 125\(^{I}\)-calmodulin only when lysates were incubated with Ca\(^{2+}\)-free calmodulin-Sepharose (Fig. 4A, lane 2). It is possible that this band is IQGAP2, which migrates at 175 kDa on SDS-PAGE (12). Purified calcineurin, a well characterized Ca\(^{2+}\)-dependent calmodulin-binding protein (24), was used as a positive control. 125\(^{I}\)-Calmodulin bound to calcineurin when the overlay was performed in the presence of Ca\(^{2+}\) (Fig. 4A, lane 3), but not in its absence (Fig. 4B, lane 3). Although IQGAP1 bound to calmodulin-Sepharose both in the presence and absence of Ca\(^{2+}\), 125\(^{I}\)-calmodulin did not bind IQGAP1 appreciably when the overlay buffer contained EGTA (Fig. 4B, lanes 1 and 2). Although not visible in Fig. 4B, a longer exposure of the autoradiograph revealed IQGAP1 when the overlay buffer lacked Ca\(^{2+}\) (data not shown). While the IQ motifs of some proteins bind calmodulin in the presence of Ca\(^{2+}\), others have higher affinity when Ca\(^{2+}\) is absent (3). For example, rat myr 4 has two IQ motifs; one binds Ca\(^{2+}\)/calmodulin, and the other binds Ca\(^{2+}\)-free calmodulin (25). IQGAP1 has four IQ motifs (10), and the Ca\(^{2+}\) dependence for calmodulin binding to individual IQ motifs is unknown. Therefore, it is possible that some of the IQ motifs in IQGAP1 are unable to bind calmodulin in the overlay procedure. This result is not surprising, since approximately 50% of calmodulin-binding proteins are not detected by the overlay method (26). Note also that none of the other proteins that bind to Ca\(^{2+}\)-free calmodulin-Sepharose (Fig. 4A, lane 2) was detected by 125\(^{I}\)-calmodulin in the absence of Ca\(^{2+}\) (Fig. 4B).

After the original submission of this manuscript, it was shown that IQGAP1 in cell lysates and in vitro translated IQGAP1 associated with the activated form of GST-Cdc42 (11–13). Similarly, we isolated IQGAP1 from MCF-7 cell lysates with GST-Cdc42 but not with GST alone (data not shown). To extend these findings, we examined the effect of calmodulin on the interaction between IQGAP1 and Cdc42. Full-length purified human IQGAP1 was incubated with either GST preloaded with GTP\(\gamma\)S (GST-GTP\(\gamma\)S), or GST-Cdc42 loaded with GDP (GST-Cdc42-GDP) or GTP\(\gamma\)S (GST-Cdc42-GTP\(\gamma\)S), and complexes were isolated with glutathione-Sepharose. IQGAP1 was present only in samples containing GST-Cdc42-GTP\(\gamma\)S (Fig. 5A), confirming previous observations that purified IQGAP1 bound to the activated form of GST-Cdc42 (11, 13). Binding was specific as minimal IQGAP1 was detected in the samples incubated with GST-GTP\(\gamma\)S. Preincubation of IQGAP1 with calmodulin prior to the addition of Cdc42 to the assay disrupted the interaction between IQGAP1 and Cdc42 (Fig. 5, A and B). In contrast, calmodulin was unable to displace IQGAP1 that was already bound to Cdc42 (Fig. 5B).

The inhibition of the binding of IQGAP1 to Cdc42 by calmodulin was characterized. The extent of disruption of the IQGAP1-GST-Cdc42 complex was proportional to the concentration of calmodulin added. With approximately 4 \(\mu\)M IQGAP1 in the assay, calmodulin concentrations of 10 \(\mu\)M or greater completely prevented binding, while 2 \(\mu\)M calmodulin had no effect (Fig. 6A). Furthermore, the calmodulin-dependent modulation of IQGAP1 binding to Cdc42 was regulated by Ca\(^{2+}\). In the absence of added Ca\(^{2+}\), calmodulin did not interfere with the association of IQGAP1 with Cdc42 (Fig. 6B). In the presence of calmodulin, Ca\(^{2+}\) produced a dose-dependent inhibition of binding of IQGAP1 to Cdc42 (Fig. 6B). The effect of Ca\(^{2+}\) was mediated via calmodulin as addition of Ca\(^{2+}\)-free calmodulin did not impair the binding of IQGAP1 to Cdc42 (Fig. 6B). These data indicate that Ca\(^{2+}\)/calmodulin prevents the binding of IQGAP1 to Cdc42, while Ca\(^{2+}\)-free calmodulin does not (data not shown). No calmodulin was detected on the blots (data not shown), confirming that, as observed previously (11), calmodulin does not associate directly with Cdc42. Cdc42 binds to the Ras-GAP-related domain in the C-terminal region of IQGAP1 (residues 915–1657) (11), while calmodulin binds to the N terminus (11), which contains the IQ motifs. Therefore, the effect of calmodulin on the interaction between Cdc42 and IQGAP1 is probably not direct competition for binding, but is
Calmodulin Modulates IQGAP1

The binding of calmodulin to IQ motifs results in diverse effects on the target proteins. For example, Ca\(^2\+\)/calmodulin promotes the binding of Ras-GRF to Ras thereby activating GTPase activity (6). In contrast, while binding of calmodulin typically activates target proteins, the interaction of calmodulin with the IQ motif of p68 RNA helicase decreases enzyme activity (27). We observed a novel effect, where the binding of Ca\(^2\+\)/calmodulin, but not Ca\(^2\+\)-free calmodulin, prevented the association of IQGAP1 with Cdc42. Since IQGAP1 alters the GTPase activity of Cdc42 (11), our data suggest that Ca\(^2\+\)/calmodulin regulates Cdc42 by modulating its interaction with IQGAP1. This may occur in intact cells as the effects were observed at physiological concentrations of Ca\(^2\+\) and calmodulin. In support of this hypothesis, it has been shown that sustained elevation of intracellular Ca\(^2\+\) dissociates Cdc42 from the cytoskeleton of activated platelets (28). The authors also demonstrated that chelation of Ca\(^2\+\) with BAPTA-AM preserved the interaction of Cdc42 with the cytoskeleton, but the role of calmodulin was not evaluated (28).

IQGAP1 contains four putative IQ motifs (10), and the Ca\(^2\+\) requirement for calmodulin binding to individual IQ motifs is not known. It has been reported recently that calmodulin binding to some IQ motifs requires Ca\(^2\+\), while binding to other IQ motifs occurs in the absence of Ca\(^2\+\) (29). The Ca\(^2\+\) dependence of the interaction of calmodulin with individual IQ motifs depends on the amino acid sequence of the motif, the number of molecules of calmodulin bound to the protein if multiple IQ motifs are present, and the sequence in which IQ motifs become occupied with calmodulin molecules (29). Calmodulin binds to IQGAP1 both in the presence and absence of Ca\(^2\+\), but inhibits the association between IQGAP1 and Cdc42 only in the presence of Ca\(^2\+\) (Figs. 5 and 6). These data are consistent with the model of Houdusse et al. (29) and suggest that occupation of all the calmodulin binding sites in IQGAP1 is not necessary to disrupt its interaction with Cdc42.

Based on its sequence similarity to GAP (10), IQGAP1 was predicted to act as a Ras-GAP. Recently, however, it was shown that recombinant IQGAP1 does not bind Ras, but binds Cdc42, and maintains it in the GTP-bound state (11). Since GTP hydrolysis is necessary to deactivate Ras family proteins (30), IQGAP1 has been hypothesized to function in cell transformation induced by Ras-like proteins (10). Cdc42 participates in cell cycle progression (31) and, with other Ras-related proteins (including Rac and Rho), influences cytoskeletal assembly (32). Both cell cycle progression (33) and cytoskeletal assembly (34) are also modulated by calmodulin. Furthermore, overexpression of calmodulin shortened the cell cycle and altered cell morphology (35). A recent study illustrated that Cdc42 is localized primarily in the Golgi apparatus in mammalian cells, where it may play a role in vesicular transport (36). Similarly, calmodulin has been linked to vesicular transport and calmodulin antagonists interfere with Golgi function (37). Despite these overlapping functions, the role of calmodulin in signaling pathways involving Cdc42 has not been investigated.

Calmodulin levels are significantly increased in malignant cells (38), including breast cancer (39). In concordance with these observations, we observed that calmodulin levels were increased by 2-fold in MCF-7 cells as compared with normal breast cells.\(^2\) It is not known whether the increased calmodulin contributes to neoplastic transformation or is a consequence of the altered cellular homeostasis that occurs during malignancy. Since calmodulin regulates the interaction of IQGAP1 with Cdc42, and Cdc42 participates in cell proliferation, differentiation, and morphology, it is possible that increased levels of calmodulin contribute to breast carcinogenesis by modulating the activity of Cdc42. Understanding the molecular mechanisms by which normal cells become malignant could potentially contribute to improved methods of prevention, detection and treatment of cancer. Therefore, future studies should be directed toward examining the interactions among calmodulin, IQGAP1, and Cdc42 in malignant transformation.

Acknowledgments—We thank Zhiqiang Li for expert technical assistance and Sharon Porter (Washington University Medical Center, St. Louis, MO) for preparing the anti-calmodulin antibody.

REFERENCES

1. Cohen, P., and Klee, C. B. (1988) Calmodulin, Elsevier Science Publishing Co., Inc., New York.
2. Ikura, M., Hiroaki, T., Hikichi, K., Mikumi, T., Yazawa, M., and Yagi, K. (1983) *Biochemistry* 22, 2573–2579
3. Watanuki, J. S. (1995) *Trends Cell Biol.* 5, 310–316
4. Chapman, E. R., Au, D., Alexander, K. A., Nicolson, T. A., and Storm, D. R. (1991) *J. Biol. Chem.* 266, 207–213
5. Baudier, J., Brunner, C., Kligman, D., and Cole, R. D. (1991) *J. Biol. Chem.* 266, 1824–1828
6. Farnsworth, C. L., Freshney, N. W., Rosen, L. B., Ghosh, A., Greenberg, M. E., and Feig, L. A. (1995) *Nature* 376, 524–527
7. Munshi, H. G., Burke, D. J., Joyal, J. L., White, M. F., and Sacks, D. B. (1996) *Biochemistry* 35, 15883–15889
8. Cheney, R. E., and Mooseker, M. S. (1992) *Curr. Opin. Cell Biol.* 4, 27–35
9. Houdusse, A., and Cohen, C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 10644–10647
10. Weissbach, L., Settleman, J., Kalady, M. F., Snijders, A. J., Murthy, A. F., Yan, J. X., and Bernards, A. (1994) *J. Biol. Chem.* 269, 20517–20521
11. Hart, M. J., Callow, M. G., Souza, B., and Palakos, P. (1996) *EMBO J.* 15, 2997–3005
12. McCallum, S. J., Wu, W. J., and Cerione, R. A. (1996) *J. Biol. Chem.* 271, 21732–21737
13. Kuroda, A., Fukuta, M., Kobayashi, K., Nakafuku, M., Nomura, N., Iwamatsu, A., and Kaibuchi, K. (1996) *J. Biol. Chem.* 271, 23863–23867
14. Sacks, D. B., Porter, S. E., Ladenson, J. H., and McDonald, J. M. (1991) *Anal. Biochem.* 191, 369–377
15. Joyal, J. L., and Sacks, D. B. (1994) *J. Biol. Chem.* 269, 30039–30048
16. Rosenfeld, J., Capdevielle, J., Guillomet, J., and Ferrara, P. (1992) *Anal. Biochem.* 203, 173–177
17. Wilm, M., and Mann, M. (1994) *Int. J. Mass Spectrom. Ion Proces.* 136, 167–180
18. Carr, S. A., Huddleston, M. E., and Annan, R. S. (1996) *Anal. Biochem.* 239, 180–192
19. Wilm, M., and Mann, M. (1996) *Anal. Chem.* 68, 1–8

\(^2\) J. L. Joyal and D. B. Sacks, unpublished observations.
20. Mann, M., and Wilm, M. (1994) *Anal. Chem.* **66**, 4390–4399
21. Biemann, K. (1990) *Methods Enzymol.* **193**, 886–888
22. Annan, R. S., McNulty, D. E., Huddleston, M. E., and Carr, S. A. (1996) *Proceedings of the 44th ASMS Conference on Mass Spectrometry and Allied Topics, Portland, OR*, p. 702
23. Wilm, M., Schevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L, Fotis, T., and Mann, M. (1996) *Nature* **379**, 466–469
24. Klee, C. B., Draetta, G. F., and Hubbard, M. J. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* **61**, 149–200
25. Bahler, M., Kroschewski, R., Steffler, H, and Behrmann, T. (1994) *J. Cell Biol.* **126**, 375–389
26. Carr, D. W., and Scott, J. D. (1992) *Trends Biochem. Sci.* **17**, 246–250
27. Buelt, M. K., Glidden, B. J., and Storm, D. R. (1994) *J. Biol. Chem.* **269**, 29367–29370
28. Dash, D., Aepfelbacher, M., and Siess, W. (1995) *FEBS Lett.* **363**, 231–234
29. Houdusse, A., Silver, M., and Cohen, C. (1996) *Curr. Biol.* **4**, 1475–1490
30. Boguski, M. S., and McCormick, F. (1983) *Nature* **366**, 643–654
31. Olson, M. F., Ashworth, A., and Hall, A. (1995) *Science* **269**, 1270–1272
32. Symons, M. (1996) *Trends Biochem. Sci.* **21**, 178–181
33. Reddy, G. P., Reed, W. C., Sheehan, E. L., and Sacks, D. B. (1992) *Biochemistry* **31**, 10426–10430
34. Gratzer, W. B., & Baines, A. J. (1988) in *Calmodulin* (Cohen, P., and Klee, C. B., eds) pp. 329–340, Elsevier Science Publishing Co. Inc., New York
35. Rasmussen, C. D., and Means, A. R. (1992) *Cell. Mol. Cytoskel.* **21**, 45–57
36. Erikson, J. W., Zhang, C., Kahn, R. A., Evans, T., and Cerione, R. A. (1996) *J. Biol. Chem.* **271**, 26850–26854
37. West, D. W., and Clegg, R. A. (1981) *Biochem. Soc. Trans.* **9**, 77
38. Van Eldik, L. J., and Burgess, W. H. (1983) *J. Biol. Chem.* **258**, 4539–4547
39. Singer, A. L., Sherwin, R. P., Dunn, A. S., and Appleman, M. M. (1976) *Cancer Res.* **36**, 60–66