IQGAP1 colocalizes with actin filaments in the cell cortex and binds in vitro to F-actin and several signaling proteins, including calmodulin, Cdc42, Rac1, and β-catenin. It is thought that the F-actin binding activity of IQGAP1 is regulated by its reversible association with these signaling molecules, but the mechanisms have remained obscure. Here we describe the regulatory mechanism for calmodulin. Purified adrenal IQGAP1 was found to consist of two distinct protein pools, one of which bound F-actin and lacked calmodulin, and the other of which did not bind F-actin but was tightly associated with calmodulin. Based on this finding we hypothesized that calmodulin negatively regulates binding of IQGAP1 to F-actin. This hypothesis was tested in vitro using recombinant wild type and mutated IQGAP1s and in live cells that transiently expressed IQGAP1-YFP. In vitro, the affinity of wild type IQGAP1 for F-actin decreased with increasing concentrations of calmodulin, and this effect was dramatically enhanced by Ca\(^{2+}\) and required the IQ domains of IQGAP1. In addition, we found that calmodulin bound wild type IQGAP1 much more efficiently in the presence of Ca\(^{2+}\) than EGTA, and all 8 IQ motifs in each IQGAP1 dimer could bind calmodulin simultaneously. In live cells, IQGAP1-YFP localized to the cell cortex, but elevation of extracellular Ca\(^{2+}\) reversibly induced the fluorescent fusion protein to become diffusely distributed. Taken together, these results support a model in which a rise in free intracellular Ca\(^{2+}\) promotes binding of calmodulin to IQGAP1, which in turn inhibits IQGAP1 from binding to cortical actin filaments.
Regulation of IQGAP1 Binding to F-actin by Ca\(^{2+}\)/calmodulin

ulin modestly inhibited binding of F-actin to native IQGAP1 purified from bovine adrenal tissue. In the present report, we describe our more recent efforts to clarify how calmodulin influences the binding of IQGAP1 to F-actin. The protein contents of F-actin-binding and non-binding pools of native IQGAP1 were analyzed in further detail. In addition, recombinant full-length and mutant versions of human IQGAP1 were assayed for interactions with calmodulin in the absence and presence of free Ca\(^{2+}\) and with F-actin in the absence and presence of free Ca\(^{2+}\), calmodulin, and Ca\(^{2+}\)/calmodulin. The net results of this study suggest that local rises in free intracellular Ca\(^{2+}\) stimulate binding of calmodulin to the IQ motifs on IQGAP1, which in turn reduces the affinity of IQGAP1 for actin filaments.

**EXPERIMENTAL PROCEDURES**

**Supplies**—Biochemical, molecular biological, immunochromic, and tissue culture reagents used for this study and their respective vendors are as follows: A23187 Ca\(^{2+}\) ionophore and bovine calmodulin (Calbiochem); S19 cells, insect cell media, insect cell antibiotics, the Bac-to-Bac HT expression system, and the pBAC HT vector (Invitrogen); the bacterial strain, BL21DE3, and the pRSET expression vectors (Invitrogen); Tris, TEMED, 40% acrylamide solution (37.5:1), and 2-mercaptoethanol (Bio-Rad); AlexaFluor 488-pRSET expression vectors (Invitrogen); Tris, TEMED, 40% acrylamide/BamHI insert, and ligated the vector together creating the vector pSLBglI/BamHI plasmid to generate pSLLIQG-5. The pSLLIQG-5 plasmid was then digested with BamHI and SacI, and a 1241-bp insert fragment was removed. The BamHI/SacI digest removed the region of IQGAP1 that encoded the IQ domain region (amino acids 740–862). To restore amino acids 447–862, plasmid pSLIQG-2 and primers 5’-CGCGTACGTCGACAGAGGACGAG-3’ (forward primer) and 5’-CTCATCATGGCATTCAACTGAAT-3’ were used to amplify a DNA fragment that was ligated into the pBSIQG1-MH plasmid. From this plasmid, the 523-bp insert was removed, and the remaining vector fragment was ligated to a similarly digested 371-bp PCR fragment containing 150 mM NaCl and 1% Triton X-100. The flow-through was equilibrated with TENT buffer (TES buffer lacking sucrose, but containing 150 mM NaCl), and 500 nucletides from the 3’-end of IQGAP1 and to introduce 3 stop codons and an XhoI site. The resulting plasmid (pFBIQG1-1) was then used to generate baculovirus encoding IQGAP1-MG (see under “Generation of IQGAP1-containing Baculovirus Particles”).

To generate the IQGAP1–2 (522–522) protein fragment, we utilized a second plasmid given to us by Matt Hart, in which the IQGAP1-coding region had been N-terminally fused in-frame to the Myc epitope tag. In this plasmid the start codon was changed from ATG to GGA to create a BamHI site. Digestion of this plasmid with BamHI and HindIII generated a 1567-bp fragment that was ligated into the pRSET vector to generate pRSETIQG1–2 (522–522). This plasmid was then used to express recombinant protein in bacteria (see under “Expression and Purification of Proteins”).

**PCR Amplification of DNA**—By using pBSIQG1-MH as a template and primers 5’-CGCGTACGTCGACAGAGGACGAG-3’ (forward primer) and 5’-CTCATCATGGCATTCAACTGAAT-3’ (reverse primer), PCR was used to generate a DNA fragment that introduced an XhoI site 5’ to the start codon and to amplify the 5’-365-bp coding region. Plasmid pBSIQG1-MH and primers 5’-CGAGGTACCCGACGACGAGGAAAGGCC-3’ (forward primer) and 5’-GCTCTAGACTCATCATTACTCCGTTAAGACTCT-3’ (reverse primer) were used to amplify 500 nucleotides from the 3’-end of IQGAP1 and to introduce 3 stop codons and an XhoI site 3’ to the coding region. Finally, to restore the region of IQGAP1 encoding amino acids 447–862, plasmid pSLLIQG-2 and primers 5’-CGCGTACGTCGACAGAGGACGAG-3’ (forward primer) and 5’-CGCGGTACCTCATGGGTAGGACGACGAGTCTG-3’ (reverse primer) were used to generate a DNA fragment that contained a SacI site at its 5’ end and introduced a BamHI site at its 3’ end by creating a silent mutation in the codon for Gln-746 (GAA was changed to GAC). All PCRs used the reagents and protocols of the Elongase Amplification System. The size of each PCR product was verified by agarose gel electrophoresis and gel-purification with gel-purification chemicals from Sigma.

**Expression of IQGAP1**—A plasmid containing the human cDNA for IQGAP1 (pBSIQG1-MH) was kindly provided by Dr. Matt Hart of Onyx Pharmaceuticals. This plasmid was digested with XhoI, and the 574-bp insert containing a portion of the 3’-UTR sequence was removed, and the remaining cDNA was ligated together to form pBSIQG1-2. Next the 5’-UTR was removed by digesting pBSIQG1-2 with XbaI and XhoI. This produced a 523-bp insert fragment that contained only the 5’-UTR but also 365 nucleotides of the 5’-coding region. The 523-bp insert was removed, and the remaining vector fragment was ligated to a similarly digested 371-bp PCR fragment (see under “PCR Amplification of DNA”) that restored the 365 nucleotides of the coding region and introduced an XhoI restriction site 5’ to the start codon. We took the resulting plasmid (pBSIQG-10) and performed a Xhol/XbaI double digest, purified the ~5000-bp fragment, and ligated it into a similarly digested pSL1180 vector generating the pSLLIQG-1 plasmid. Next, to remove the remaining 3’-UTR, the pSLLIQG-1 plasmid was digested with KpnI and XhoI, and the vector was purified away from an ~1100-bp insert. Because this digest removed not only the 3’-UTR but also 500 nucleotides of C-terminal coding region, the purified vector fragment was ligated onto a similarly digested 500-bp PCR fragment (see under “PCR Amplification of DNA”) to restore the missing coding region and to introduce 3 stop codons and an XhoI site (pSLLIQG-2). Finally, pSLLIQG-2 was digested with XhoI and XbaI; the 6500-bp insert was purified and then ligated into a pFastBAC HT vector that had been digested with SalI and XhoI. The resulting plasmid (pFBIQG1-1Q) was then used to generate baculovirus expressing IQGAP1-MQ, the full-length wild type protein (see under “Generation of IQGAP1-containing Baculovirus Particles”).

To generate the IQGAP1-MQ mutant, which lacks the four contiguous IQ motifs but is otherwise identical to IQGAP1-MH, we first digested the pSL1180 vector with BglII and BamHI, removed the 31-nucleotide insert, and ligated the vector together creating the vector pSLBglI/BamHI. Next, pSLIQG-2 was digested with XhoI and XbaI, and the resulting 6500-bp IQGAP1 fragment was purified and ligated into sim-
and bound protein was eluted with TENS buffer (10 mM sodium borate, 1 mM NaSCN, 0.5% Triton X-100, 15% glycerol, 1 mM CaCl₂, pH 8.0). The eluted fractions were monitored by SDS-PAGE and immunoblotting (5). The modification was to use a Umax (Freemont, CA) Astra 2200 scanner with a transparency adapter and 12-bit grayscale depth, instead of a CCD camera, to capture digital images of gels and chemiluminescent Western blots. Concentration series of the following reagents were used to establish standards for quantitation: native bovine adrenal IQGAP1, recombinant IQGAP1ΔG1, recombinant IQGAP1ΔG1-L isoforms of bovine calmodulin, recombinant Hiss-Cdc42, and recombinant His-C-Rac1.

IQGAP1 was immunoprecipitated using the polyclonal IQGAP1 antiserum or IgG purified from the antiserum by protein A-Sepharose affinity chromatography. The supernatant containing IQGAP1, and the resulting mixtures were incubated for 1 h at 27 °C. Next, protein A-Sepharose was added and incubated for an additional hour at 27 °C. After their incubation, the samples were centrifuged for 5–10 s, and the supernatants were removed. The remaining resin was then washed several times with TN buffer. Finally, the beads were suspended in 1× SDS-PAGE sample buffer, heated, and then analyzed by SDS-PAGE and Western blotting.

Light Microscopic Analysis of IQGAP1-YFP and F-actin in Cultured Cells—By using a pBSIQGAP1-2 template and the oligonucleotides, 5′-GCCGACTATGGCCCGCCGACG-3′ and 5′-CCCCGGGGGTGAAGCTTTTGGTT-3′, the entire IQGAP1 sequence was amplified by PCR using the Expand Long Template PCR System. The size of the PCR product was verified by agarose gel electrophoresis. The PCR fragment was then ligated into the pGEM-T Easy vector to produce pGEM-IQGAP1. Finally the pGEM-IQGAP1 plasmid was sequencially digested with SalI and SmaI, and the IQGAP1 insert was ligated into a similarly digested pEYP-N1 expression vector to produce pYFP-IQGAP1.

NIH-3T3 fibroblasts were transiently transfected for IQGAP1-YFP expression using LipofectAMINE Plus according to the vendor’s instructions (Invitrogen). The cells were maintained in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin sulfate. Live transfected cells were observed and recorded by confocal epifluorescence microscopy on an imaging system containing the following components: a Zeiss Axiovert 100 microscope equipped with a CARV spinning disc confocal head, a temperature-regulated incubator, an automatic shutter, an AutoFluor mercury illuminator, and a Hamamatsu (Bridgewater, NJ) Orca-ER cooled CCD. Images captured by the camera were imported into a Power Macintosh G4 computer (Apple; Cupertino, CA) and processed and analyzed using Open Lab 3.0.3 software (Improvision; Lexington, MA). Cells were maintained on the microscope stage in Attofluor Cell Chambers (Atto Instruments; Rockville, MD) at 37 °C in an atmosphere of 95% air plus 5% CO₂. Removal of Ca²⁺ was achieved by replacing the Ca²⁺-free Hanks’ balanced salt solution (HBSS) supplemented the Ca²⁺-ionophore, A23187, at 5 μM, plus 1 mM CaCl₂. For Ca²⁺ removal, the solution bathing the cells was replaced with Dulbecco’s modified Eagle’s medium plus 10% Cosmic calf serum. A total of 48 images were captured, 44 during exposure of cells to A23187 plus CaCl₂ and 20 during the reversal step. For both the forward and reverse series, all images were collected at 30-s intervals and were exposed for 10 s each using a 63× planachromatophoric Zeiss objective without binning. As controls, comparable experiments were performed using HBSS supplemented with 1 mM CaCl₂ or 5 mM A23187 individually. To visualize F-actin, untreated cells, and cells exposed for 20 min to A23187 in the absence or presence of CaCl₂ were fixed and labeled with AlexaFluor 488-phalloidin as described earlier for bodipy-phalloidin (5).

RESULTS

Native IQGAP1 Consists of Two Operationally Distinct Protein Pools—Our original method for purifying native IQGAP1 from bovine adrenal tissue was based on ion exchange and gel filtration chromatography and demonstrated copurification of one calmodulin molecule per 13 IQGAP1 dimers (4). Addition of a large molar excess of exogenous calmodulin to IQGAP1 purified in this manner increased the binding of IQGAP1 to F-actin (5). These observations prompted us to investigate the mechanism by which calmodulin inhibits the binding activity of IQGAP1.

To begin, we modified a procedure based on calmodulin affinity chromatography (8) to develop a new, more efficient method for purifying bovine adrenal IQGAP1 (see “Experimen-
Regulation of IQGAP1 Binding to F-actin by Ca\textsuperscript{2+} / Calmodulin

Figure 1. Native IQGAP1 consists of two distinct protein pools. Native IQGAP1 was purified from bovine adrenal tissue, mixed with actin filaments, and then centrifuged. The pellet was resuspended to the original sample volume, and equal aliquots of the supernatant (S) and pellet (P) fractions were then analyzed by SDS-PAGE. IQGAP1 was detected by staining the gel with GelCode Blue, and calmodulin was detected by anti-calmodulin immunoblotting, and the partitioning of both proteins between the supernatant and pellet fractions was determined by quantitative densitometry. Although approximately 75% of the IQGAP1 was in the pellet, nearly all of the calmodulin was in the supernatant when IQGAP1 blocked with IQGAP1 at a 1:1 molar ratio.

Production of Recombinant Wild Type and Mutant Versions of IQGAP1—It is evident from our limited analysis of calmodulin and small G proteins that the content of reversibly associating cofactors varies from molecule to molecule of tissue-derived IQGAP1. When one takes into account its other known cofactors, β-catenin and E-cadherin, and the possibility that IQGAP1 can be post-translationally modified in a variety of ways and at multiple sites, IQGAP1 purified from tissue may represent a heterogeneous collection of individual protein complexes. This suspected molecular diversity compromised our efforts to interpret how calmodulin affects the F-actin binding activity of native IQGAP1.

To minimize the molecular heterogeneity of IQGAP1 preparations and enable studies using mutated versions of the protein, we produced three recombinant, His\textsubscript{6}-tagged varieties of IQGAP1 (1). IQGAP1\textsubscript{FL}, which corresponds to the full-length protein, IQGAP1\textsubscript{1–522}, which corresponds to the full-length protein and IQGAP1\textsubscript{1–750}, which lacks the four IQ motifs, were expressed in Sf9 insect cells infected with modified baculoviruses. The N-terminal fragment, IQGAP1\textsubscript{1–200}, was expressed in E. coli using a pRSET-derived vector. Several protein interaction domains (shaded regions) are shown and include the calponin homology domain (CHD), the six putative coiled-coil internal repeats (IR), the WW domain (WW), the four calmodulin binding IQ motifs (IQ), and the GAP-related domain (GRD). Numbers below each diagram denote the N- and C-terminal amino acids for each protein and, additionally, in the case of IQGAP1\textsubscript{1–750}, the first and last amino acids flanking the deleted IQ motifs.

Next, falling ball viscometry was used to assay the F-actin cross-linking activities of 200 nM IQGAP1\textsubscript{1–522} and 750 nM IQGAP1\textsubscript{1–750} in the presence of calmodulin, Ca\textsuperscript{2+}, and Ca\textsuperscript{2+} /calmodulin (Fig. 3B). The calmodulin concentrations for these experiments were equimolar to the recombinant IQGAP1 proteins, and Ca\textsuperscript{2+} was used at 1 mM. IQGAP1\textsubscript{1–522} was able to gel 5 μM polymerized actin under all the conditions tested. In the presence of 1 mM EGTA (no Ca\textsuperscript{2+}), IQGAP1\textsubscript{1–522} also formed a gel that was insensitive to calmodulin. In the presence of Ca\textsuperscript{2+}, however, IQGAP1\textsubscript{1–522} was unable to gel F-actin when calmodulin was also present, and the apparent viscosity of such samples was very low, virtually identical to that of F-actin alone. Interestingly, IQGAP1\textsubscript{1–522} did not gel F-
Regulation of IQGAP1 Binding to F-actin by Ca\(^{2+}\)/Calmodulin

Fig. 3. The gelation activity of IQGAP1 is sensitive to Ca\(^{2+}\)/
calmodulin. A, falling ball viscometry was used to measure gelation
dose-response curves for IQGAP1FL and IQGAP1(2–522) in the
presence of 5 \(\mu\)M polymerized actin. A velocity of zero indicates the formation
of an actin filament gel. The critical concentrations for gelation
were determined to be 200 and 750 nM for IQGAP1FL and IQGAP1(2–522), respectively. B, IQGAP1FL (200 nM) or IQGAP1(2–522) (750 nM)
was incubated with 5 \(\mu\)M polymerized actin alone or in the presence
of Ca\(^{2+}\) (1 mM), calmodulin (equimolar to IQGAP1FL or IQGAP1(2–522)),
or Ca\(^{2+}\)/calmodulin as indicated. Samples that lacked Ca\(^{2+}\) (lanes 2, 4,
and 6) contained 1 mM EGTA. Falling ball viscometry was then used to monitor each sample. In each case, sample viscosity is an inverse
function of the measured velocity of the falling ball (y axis). Each point
represents the mean of at least three separate experiments \(\pm\) S.D. Note
that IQGAP1(2–522) formed gels under all conditions tested, but gelation
by IQGAP1FL was inhibited completely by Ca\(^{2+}\)/calmodulin, but only slightly by Ca\(^{2+}\) alone and not at all by calmodulin. C, both
IQGAP1FL and IQGAP1(2–522) are able to bundle actin filaments,
as shown in these negative stain electron micrographs of samples prepared
in the absence of Ca\(^{2+}\) or calmodulin.

Fig. 4. Actin filament binding by IQGAP1FL, but not
IQGAP1(1–522), is sensitive to Ca\(^{2+}\)/calmodulin. A, 0.5 \(\mu\)M IQGAP1FL
was incubated with the indicated amounts of calmodulin in the presence
of 220 \(\mu\)M Ca\(^{2+}\) or EGTA (no Ca\(^{2+}\)) and then was combined with 1
\(\mu\)M polymerized actin. The reaction mixtures were then centrifuged.
The pellets were then resuspended to the initial sample volumes, and
equal aliquots of the supernatant (S) fractions and pellet (P) fractions
were subjected to SDS-PAGE. The gel was stained with GelCode
Blue. B, quantitative densitometry was used to measure the proportion
of IQGAP1FL that pelleted out of comparable starting samples. The
numbers below the x axis refer to the corresponding samples in A, and
the error bars indicate the S.D. for data collected from four experiments.
The S.D. for sample 5 is not visible on this graph because it was negligible.

As a complement to falling ball viscometry, a high speed
pelleting assay was used to monitor the ability of IQGAP1FL
and IQGAP1(1–522) to bind F-actin. We incubated 0.5 \(\mu\)M
IQGAP1FL or IQGAP1(1–522) with 1 \(\mu\)M F-actin in the absence or
presence of calmodulin, Ca\(^{2+}\), or Ca\(^{2+}\)/calmodulin, centrifuged
the samples, and then analyzed the supernatant and pellet fractions by quantitative SDS-PAGE. When they were present,
calmodulin was equimolar (0.5 \(\mu\)M) or in 10-fold molar excess (5
\(\mu\)M) to IQGAP1FL dimers, and the Ca\(^{2+}\) concentration was fixed
at 220 \(\mu\)M.

Complexes of IQGAP1 and Ca\(^{2+}\)/Calmodulin Bind Poorly to
F-actin—The data presented to this point favor the hypothesis

Fig. 4, A and B, summarizes the results for IQGAP1FL. In
controls, as well as in samples supplemented with Ca\(^{2+}\)
or equimolar calmodulin alone, ~65% of the IQGAP1FL pelleted
with the actin filaments. The amount of pelleted IQGAP1FL dropped to ~40% when either a 10-fold molar excess of calmodulin
or Ca\(^{2+}\) plus equimolar calmodulin was present. Finally,
in the presence of Ca\(^{2+}\) plus a 10-fold molar excess of calmodulin,
just ~20% of the IQGAP1FL was pelleted. Thus, calmodulin
inhibited binding of IQGAP1FL to F-actin, and Ca\(^{2+}\)
potentiated this activity of calmodulin.

Analogous experiments using IQGAP1(1–522) demonstrated that
deletion of the high affinity calmodulin binding region from
IQGAP1 abolished the ability of Ca\(^{2+}\)/calmodulin to inhibit its
F-actin binding activity (Fig. 5A). In the absence of F-actin,
~10% of IQGAP1(1–522) was pelleted by the centrifugation
conditions used for these experiments, but addition of actin filaments
caus ed a nearly 4-fold increase in the amount of
IQGAP1(1–522) found in the pellet fraction. Although IQGAP1(1–522)
ever bound F-actin as efficiently as IQGAP1FL or native
bovine adrenal IQGAP1, its affinity for actin filaments was not
sensitive to 220 \(\mu\)M Ca\(^{2+}\), 5 \(\mu\)M calmodulin, or a combination
of Ca\(^{2+}\) plus calmodulin.

The low efficiency binding was evidently due to an inherently
reduced affinity for F-actin of IQGAP1(1–522), as compared with
native IQGAP1, IQGAP1FL, or IQGAP1(2–522). This was
determined by demonstrating that the amount of IQGAP1(1–522)
that could be pelleted in the presence of F-actin steadily increased
as the F-actin concentration increased (Fig. 5B). Consistent
with the evidence that the F-actin binding activity of
IQGAP1(1–522) was insensitive to calmodulin was the finding that
IQGAP1(1–522) could not coimmunoprecipitate with calmodulin
in either the absence or presence of Ca\(^{2+}\) (Fig. 5C). Taken
altogether, these results led us to conclude that the IQ motifs of
IQGAP1, which correspond to the high affinity calmodulin
binding region of the protein (9), account for the F-actin binding
sensitivity of IQGAP1 to Ca\(^{2+}\)/calmodulin.
that binding of Ca$^{2+}$/calmodulin to one or more of the eight IQ motifs present in an IQGAP1 dimer can substantially decrease the affinity of that dimer for F-actin. It follows naturally that motifs present in an IQGAP1 dimer can substantially decrease the affinity of that dimer for F-actin. As can be seen, the vast majority of the calmodulin partitioned in the supernatant and calmodulin exhibited a Ca$^{2+}$-dependent gel mobility shift. B, supernatants from samples similar to those shown in A were immunoprecipitated with a monoclonal antibody against IQGAP1, and the resulting immunoprecipitates were immunoblotted with monoclonal antibodies to either IQGAP1 or calmodulin. Note that IQGAP1 was tightly associated with a much higher level of calmodulin when Ca$^{2+}$ was present, as opposed to absent. C, supernatants from samples similar to those shown in A were immunoprecipitated with a monoclonal antibody against the His$_6$ tag present on IQGAP1FL. The resulting immunoprecipitates were then analyzed for IQGAP1FL by quantitative SDS-PAGE and for calmodulin by quantitative immunoblotting with anti-calmodulin. The measured molar ratios of calmodulin to IQGAP1FL in the presence or absence of Ca$^{2+}$ are indicated, and each point represents the mean ± S.D. of three experiments. Note that ~3 times as much calmodulin was associated with IQGAP1FL in the presence of Ca$^{2+}$ than in its absence. Furthermore, when Ca$^{2+}$ was present, most IQGAP1FL dimers apparently bound a calmodulin molecule to each of their 8 IQ motifs.

immunoprecipitated with a polyclonal antibody to IQGAP1, and the immunoprecipitates were then analyzed by immunoblotting with monoclonal antibodies to calmodulin and IQGAP1. As can be seen in Fig. 6B, the relative levels of calmodulin to IQGAP1 were much greater in the presence of Ca$^{2+}$ than EGTA. Quantitative analysis of such Western blots indicated that addition of Ca$^{2+}$ caused the amount of calmodulin able to coprecipitate with IQGAP1 to increase 3-fold (Fig. 6C), rising from ~2.5 mol of calmodulin per IQGAP1 dimer in the presence of EGTA to ~7.5 mol in the presence of Ca$^{2+}$. This result compares well with a prior report that Ca$^{2+}$ caused a 2-fold increase in the binding of IQGAP1 to calmodulin-Sepharose beads (8). Taken together, these results demonstrate that Ca$^{2+}$ stimulates the binding of calmodulin to IQGAP1 and lends further support to the concept that binding of calmodulin to IQGAP1 inhibits the latter's affinity for actin filaments.
Regulation of IQGAP1 Binding to F-actin by Ca\(^{2+}\)/Calmodulin

### Table

| Sample |
|--------|
| Calcium | + | - | + | - | + | - |
| Actin   | + | + | 1st | 1st | 2nd | 2nd |
| Calmodulin | - | 2nd | 2nd | 1st | 1st | 1st |

### Localization of IQGAP1-YFP in the Cell Cortex Is Reversibly Disrupted by Ca\(^{2+}\)—Prior immunofluorescence studies (4, 7) from several laboratories had indicated that IQGAP1 is typically most concentrated in the cell periphery, where it colocalizes with cortical actin filaments (2, 5). A key prediction of the in vitro biochemical data described here is that a rise in intracellular Ca\(^{2+}\) should remove IQGAP1 from the cortex. To test that prediction, NIH-3T3 cells were transiently transfected for expression of an IQGAP1-YFP fusion protein and exposed to the Ca\(^{2+}\) ionophore, A23187 and CaCl\(_2\), alone or in combination. When used individually, neither the ionophore nor the CaCl\(_2\) had any noticeable effect on the distribution of IQGAP1-YFP (not shown). When cells were exposed simultaneously to both agents, however, IQGAP1-YFP was rapidly removed from the cell cortex, an effect that was quickly reversed upon removal of the A23187 and CaCl\(_2\).

These effects are illustrated in Fig. 8, which shows several frames from a time lapse movie of a single field of view photographed following addition of A23187 plus CaCl\(_2\) and their subsequent removal. The movie was recorded using confocal imaging, and the plane of focus was near the lower surface of the cells. Cortical fluorescence decreased markedly within 5 min of exposure of the cells to A23187 and CaCl\(_2\), especially at cell-cell contact sites, and recovered almost completely within 10 min after the A23187 and CaCl\(_2\) were removed. QuickTime movies of both the forward and reverse reactions of the cells are available in the Supplemental Material. It is noteworthy that cortical F-actin remained intact during comparable exposures of cells to A23187 plus CaCl\(_2\), as determined by staining of cells with AlexaFluor 488-phalloidin (Fig. 9). Thus, the displacement of IQGAP1-YFP from the cell cortex in response to elevated Ca\(^{2+}\) did not reflect a loss of cortical actin filaments.

One unexpected observation of these experiments was of intranuclear IQGAP1-YFP in occasional cells, an example of which is shown in Fig. 8 and the QuickTime movies (see the Supplemental Material). We do not yet understand the significance of this observation, but perhaps it reflects an association of IQGAP1 with intranuclear \(\beta\)-catenin. Regardless of what the explanation may prove to be, two other groups have published immunofluorescence micrographs of endogenous IQGAP1 that apparently was in the nucleus (2, 18).

### DISCUSSION

The initial report (5) that IQGAP1 binds to actin filaments included preliminary evidence for regulation of this activity by calmodulin, but the regulatory mechanism remained undefined. The study described here explains the broad details of that mechanism and suggests that the ability of IQGAP1 to bind actin filaments can be fine-tuned by calmodulin, acting through Ca\(^{2+}\). We found that as the level of calmodulin associated with IQGAP1 increased, the F-actin binding activity of IQGAP1 progressively decreased and that Ca\(^{2+}\) potently enhanced the affinity of calmodulin for IQGAP1. Considering that IQGAP1 is most abundant intracellularly on cortical actin filaments (2, 4, 5, 7), these in vitro biochemical findings suggest that elevation of intracellular Ca\(^{2+}\) should promote reversible binding of calmodulin to IQGAP1 and, by extension, reversible withdrawal of IQGAP1 from the cortex. That is exactly what we observed for IQGAP1-YFP by time lapse fluorescence microscopy of live transfected cells whose intracellular Ca\(^{2+}\) levels were raised or decreased by the respective addition or removal of Ca\(^{2+}\) plus A23187. Fig. 10, which is based on the collective data presented here, summarizes how Ca\(^{2+}\)/calmodulin might negatively regulate interactions between IQGAP1 and cortical actin filaments in a reversible manner.

The question of how the other known cofactors for IQGAP1 impact on the regulation of its actin filament binding activity...
must await further experimentation. In the meantime, an emerging theme from studies of IQGAP1 is how its binding partners compete with each other for association with their common ligand. For example, there are reports that Ca\(^{2+}\)/calmodulin disrupts the association between IQGAP1 and E-cadherin (19) or activated Cdc42 (8) and that activated forms of Cdc42 or Rac1 inhibit binding of IQGAP1 to \(\beta\)-catenin (21).

These findings, along with the results presented here, imply that multiple functions of IQGAP1 are regulated by its competitive interactions with a variety of signaling and structural proteins. Such functions may include the formation of filopodia and lamellipodia, which involve assembly and reorganization of actin filaments, and dimerize (25, 26); cell-cell adhesion, which involves E-cadherin and \(\beta\)-catenin (27, 28); and \(\beta\)-catenin-mediated gene activation (27).

The deletion mutant, IQGAP1-(2–522), provides insight into the protein substructure that enables its native full-length counterpart to bind actin filaments and dimerize. The only known structural motifs in IQGAP1-(2–522) are the calponin homology domain, or CHD, and 3.5 of the 6 putative coiled-coil repeats present in the full-length protein. Because IQGAP1-(2–522) was able to cross-link actin filaments into gels (Fig. 3), it must contain at least the minimum structural information for actin filament binding and dimerization. The ability of IQGAP1-(2–522) to bind actin filaments is consistent with an earlier report (21) that an even smaller N-terminal fragment of IQGAP1 is also capable of binding F-actin. This fragment, called IQGAP1(CHD), composed the first 232 amino acids of the full-length protein, extended shortly beyond the CHD, and was expressed as a fusion protein coupled to the C-terminal of GST (21).

Similarly, the lack of detectable effects of Ca\(^{2+}\)/calmodulin on the F-actin binding properties of IQGAP1-(2–522) or and lamellipodia, which involve assembly and reorganization of actin filaments, and are regulated, respectively, by Cdc42 and Rac1 (25, 26); cell-cell adhesion, which involves E-cadherin and \(\beta\)-catenin (27, 28); and \(\beta\)-catenin-mediated gene activation (27).

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IQGAP1 (Figs. 3 and 5) implies that the sensitivity of IQGAP1 to Ca**2*/calmodulin for actin filament binding depends on its IQ motifs. Interestingly, a prior study (9) of IQGAP1 included evidence that IQGAP1**CHD** can bind calmodulin in a Ca**2*-dependent, F-actin-sensitive manner. Although that result may seem at odds with the results presented here, it is noteworthy that the prior study clearly indicated that the affinity of Ca**2*/calmodulin for the IQ motifs was much greater than for IQGAP1**CHD** (9). The apparent discrepancy between our results and those reported earlier may reflect differences in the deletion mutants and actin binding assays used in the two studies. Regardless of what the explanation eventually proves to be, the collective evidence from both studies indicates that the ability of Ca**2*/calmodulin to suppress the F-actin binding activity of IQGAP1 is caused primarily by association of Ca**2*/calmodulin with the IQ motifs.

Calmodulin binding IQ motifs contain the consensus sequence IQXXXRGXXXR (29) and have been proposed to fall into two classes. Incomplete IQ motifs are defined as lacking the C-terminal arginine and reportedly have a much higher affinity for Ca**2*/calmodulin than for calmodulin alone. In contrast, complete IQ motifs are defined as containing the distal arginine and are thought to bind well to free calmodulin (30). By these definitions, each IQGAP1 dimer contains 6 complete and 2 incomplete IQ domains. In the presence of EGTA, a 1:1 molar ratio of calmodulin to IQGAP1**FL** dimer had no detectable effect on the actin gelation or binding activity of IQGAP1**FL** (Fig. 3), and a 10-fold excess of calmodulin caused only a ~20% reduction in the amount of IQGAP1 that could bind F-actin (Fig. 4). In the presence of Ca**2*, however, a 1:1 ratio of calmodulin to IQGAP1**FL** dimer prevented gelation of actin filaments (Fig. 3). Likewise, addition of Ca**2*/actin to mixtures containing either 1:1 or 10:1 ratios of calmodulin to IQGAP1**FL** reduced the amount of IQGAP1**FL** found in actin pellets by ~45 and ~80%, respectively.

The collective data presented here suggest two possible routes, which may not be mutually exclusive, by which calmodulin can suppress the F-actin binding activity of IQGAP1. One possibility is that the affinity of IQGAP1 for F-actin is inversely proportional to the number of its bound calmodulin, at least to a first approximation. Arguing in favor of this hypothesis is the finding that in the presence of Ca**2*, the level of F-actin binding by IQGAP1**FL** was inhibited ~40% by equimolar calmodulin but by ~70% when a 10-fold molar excess of calmodulin was present (Fig. 4). Interestingly, all 8 IQ motifs in IQGAP1 were occupied by calmodulin in the latter case (Fig. 6), indicating that IQGAP1 can retain a low F-actin binding activity even when it is saturated with calmodulin. An alternative model was suggested by the finding that ~25% of purified native IQGAP1 was associated with equimolar calmodulin and did not bind F-actin in vitro (Fig. 1). This result raises the possibility that binding of a single calmodulin molecule to an IQGAP1 dimer can prevent the IQGAP1 from binding to an actin filament. Moreover, it invites speculation that calmodulin must be associated with a particular IQ motif, perhaps an incomplete IQ motif, in order to exert such a drastic effect. Even though the native IQGAP1 was purified from bovine adrenal tissue in the presence of EGTA, it is possible that Ca**2*/calmodulin-IQGAP1 complexes that existed in vivo were able to remain intact throughout the purification procedure and, as a result, retain low F-actin binding activity in vitro. Although both of these models are worthy of consideration in light of the existing data, it is clear that further studies of the IQ motifs of IQGAP1 will be required to determine in greater detail how calmodulin regulates its binding to F-actin.

In the absence of calmodulin, Ca**2* caused a slight inhibition of F-actin gelation by IQGAP1, but not IQGAP1-(2–522) (Fig. 3). Reportedly, Ca**2* can directly associate with IQGAP1**CHD** (9). It is therefore possible that direct binding of Ca**2* somewhere within the N-terminal region of IQGAP1**FL** slightly inhibited its affinity for actin filaments. Why such an effect was not observed for IQGAP1-(2–522) remains a mystery. One possible explanation is that the C-terminal of IQGAP1**FL** is missing in IQGAP1-(2–522), can partially block the CHD when Ca**2* is bound to the N-terminal region of the protein. Another plausible explanation is that the methionine that marks the N-terminal amino acid residue in IQGAP1**FL** and is absent from IQGAP1-(2–522) is required for calmodulin-independent binding of Ca**2*. Although Ca**2* affected IQGAP1**FL** gelation activity, it had no detectable effect in a pelleting assay for F-actin binding (Fig. 4). Because falling ball viscometry is thought to be more sensitive than the pelleting assay, its possible that the slight Ca**2* effect observed in the gelation assay was too small to be detected in the pelleting assay.

There is one discrepancy between the results we present here and those we described earlier (5) for the effects of Ca**2* and calmodulin on IQGAP1. The F-actin binding activity of IQGAP1 was originally reported to be comparably inhibited by calmodulin or Ca**2*/calmodulin (5), but we now present evidence that Ca**2*/calmodulin is much more potent inhibitor than calmodulin alone (see Figs. 3B and 4). This disparity probably reflects two major differences in experimental design between our earlier work (5) and the work described here. First, we used native bovine adrenal IQGAP1 originally (5) and recombinant human IQGAP1 more recently. The recombinant protein consistently appeared to be much purer than its native counterpart by SDS-PAGE (compare Fig. 2 in this paper with Fig. 3A in Ref. 5) and was also demonstrated to contain substantially less calmodulin. Because IQGAP1**FL** was produced by baculovirus-directed overexpression in insect cells, it is likely that the purified recombinant protein also contained lower levels than the native protein of other cofactors that may influence how Ca**2* and calmodulin affect F-actin binding by IQGAP1. Such cofactors are known to include the small G proteins Cdc42 and Rac1 (2–4), β-catenin (18), and E-cadherin (18, 19), and others may remain to be discovered. These considerations lead us to believe that protein-protein interactions involving IQGAP1 can be dissected more systematically and accurately using recombinant, as opposed to native, versions of the protein.

The other substantive difference between our original and present experiments is that calmodulin was previously used at a 5:1 molar ratio to the IQ motifs on IQGAP1 but at only a 1:8 or 5:4 molar ratio for the current study. The new data shown in Fig. 4 illuminates the importance of this difference. In particular, the binding of IQGAP1 to F-actin was not inhibited at a 1:8 ratio of calmodulin to IQGAP1 but was inhibited at a 5:1 ratio (Fig. 4, lane/pellet fraction 2) but was inhibited by ~40% at the same calmodulin concentration in the presence of Ca**2* (lane/pellet fraction 5) or at a 10-fold higher calmodulin concentration in the absence of Ca**2* (lane/pellet fraction 3). Furthermore, the inhibition was even more pronounced at the higher calmodulin concentration when Ca**2* was also present (lane/pellet fraction 6). These data support the view that calmodulin inhibits the binding of IQGAP1 to F-actin and that Ca**2* increases the affinity of calmodulin for IQGAP1. Thus, the effects of calmodulin on the F-actin binding properties of IQGAP1 may be insensitive to Ca**2* when calmodulin is present in vast excess to IQGAP1, as was the case in our earlier study (5) but not our present study.

Now that regulation of the F-actin binding activity of IQGAP1 by Ca**2* and calmodulin is understood in considerable
Regulation of IQGAP1 Binding to F-actin by Ca\(^{2+}\)/Calmodulin

In detail, new questions about the biochemical and cell biological properties of IQGAP1 will rise to the forefront. In the most general sense, it will be important to develop an understanding of how the various binding partners of IQGAP1, individually and collectively, affect its numerous functions. Because the proteins that consort with IQGAP1 have been implicated in activities as diverse as cellular motility and morphogenesis, cell-cell adhesion, and gene regulation, it may be useful to think of IQGAP1 as a signal integration protein that can mount an array of specific responses to diverse extracellular and intracellular signals. The particular combination of signals that is operable at any moment may determine which cofactors bind to IQGAP1 at that time and, by extension, may specify the predominant functions of IQGAP1 while those signals remain strong.

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