# Actin Filament Binding by a Monomeric IQGAP1 Fragment With a Single Calponin Homology Domain

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IQGAP1 is a homodimeric protein that reversibly associates with F-actin, calmodulin, activated Cdc42 and Rac1, CLIP-170, β-catenin, and E-cadherin. Its F-actin binding site includes a calponin homology domain (CHD) located near the N-terminal of each subunit. Prior studies have implied that medium- to high-affinity F-actin binding (5–50 $\mu M$ $K_d$) requires multiple CHDs located either on an individual polypeptide or on distinct subunits of a multimeric protein. For IQGAP1, a series of six tandem IQGAP coiled-coil repeats (IRs) located past the C-terminal of the CHD of each subunit support protein dimerization and, by extension, the IRs or an undefined subset of them were thought to be essential for F-actin binding mediated by its CHDs. Here we describe efforts to determine the minimal region of IQGAP1 capable of binding F-actin. Several truncation mutants of IQGAP1, which contain progressive deletions of the IRs and CHD, were assayed for F-actin binding in vitro. Fragments that contain both the CHD and at least one IR could bind F-actin and, as expected, removal of all six IRs and the CHD abolished binding. Unexpectedly, a fragment called IQGAP1$_{2\text{-}210}$, which contains the CHD, but lacks IRs, could bind actin filaments. IQGAP1$_{2\text{-}210}$ was found to be monomeric, to bind F-actin with a $K_d$ of ~47 $\mu M$, to saturate F-actin at a molar ratio of one IQGAP1$_{2\text{-}210}$ per actin monomer, and to co-localize with cortical actin filaments when expressed by transfection in cultured cells. These collective results identify the first known example of high-affinity actin filament binding mediated by a single CHD. Cell Motil. Cytoskeleton 58:231–241, 2004. © 2004 Wiley-Liss, Inc.

**Key words:** cell motility; lammelipodium; Cdc42; Rac1; calmodulin

## INTRODUCTION

IQGAP1 is a widely expressed mammalian protein that co-localizes in the cell cortex with actin filaments and intercellular adhesion sites, and reversibly binds several other proteins, among them activated Cdc42 and Rac1, E-cadherin, β-catenin, calmodulin, S100B, and CLIP-170 [Fukata et al., 2002; Hart et al., 1996; Joyal et al., 1997; Kuroda et al., 1996, 1998; Mbele et al., 2002; McCallum et al., 1996]. Although its functions remain only partially understood, it is becoming increasingly apparent that IQGAP1 modulates cellular motility and morphogenesis through its interactions with this diverse collection of signaling, regulatory, cytoskeletal, and cell adhesion proteins [Mateer and Bloom, 2003]. In aqueous solution, native IQGAP1 is a dimer comprising two identical 190-kDa subunits [Bashour et al., 1997]. Each subunit contains several protein motifs, including an N-terminal calponin homology domain (CHD) involved in F-actin binding, six putative coiled-coil IQGAP re-peats (IRs) that enable dimerization of the protein, a WW domain of unknown significance, four IQ motifs that can

bind calmodulin and S100B, and a GTPase activating protein (GAP) related domain, or GRD, that apparently lacks GAP catalytic activity. IQGAP1 cross-links F-actin into bundles and gels in vitro [Bashour et al., 1997; Fukata et al., 1997], and its F-actin binding activity is negatively regulated by calcium/calmodulin [Mateer et al., 2002]. Furthermore, calcium/calmodulin induces the dissociation of Cdc42 from IQGAP1 [Ho et al., 1999], indicating that IQGAP1 serves as a point of integration between calcium/calmodulin signaling and Cdc42-mediated processes, such as actin filament assembly and re-organization.

The CHD is a 100–amino acid residue motif found in several actin-binding and signaling proteins [Castresana and Saraste 1995]. Based on sequence heterogeneity, several different classes of CHDs have been defined, but they all share numerous invariant residues [Gimona et al., 2002]. According to this classification system, IQGAP1 and other members of the IQGAP protein family contain type 3 CHDs, which are also found in the actin-binding proteins, calponin and SM22, the Rho guanine nucleotide exchange factor, Vav, and several additional proteins.

Most known F-actin binding proteins, including those that rely on CHDs for this activity, have dissociation constants for F-actin in the range of 5–50 μM [Gimona et al., 2002]. So far, however, all proteins that have been reported to bind actin filaments via CHDs contain multiple CHDs. This has led to the hypothesis that two or more CHDs are required to form an F-actin-binding site on a protein [Gimona et al., 2002]. For instance, the sequentially arranged type 1 CHD and a type 2 CHD located near the N-termini of α-actinin, filamin, and β-spectrin enable those proteins to bind F-actin [Gimona et al., 2002], whereas IQGAP1 is a homodimeric protein, each of whose subunits contains a single N-terminal type 3 CHD [Bashour et al., 1997; Fukata et al., 1997]. Although the importance of type 3 CHDs for actin filament binding has come into question [Gimona and Mital, 1998; Gimona and Winder, 1998], we reasoned that the single type 3 CHD present on each of the two IQGAP1 subunits function together as the high-affinity F-actin binding site on the native protein.

Supporting that model was the finding of F-actin binding activity for recombinant fusion proteins of glutathione-S-transferase ([1]GST) coupled to various IQGAP1 fragments that contained its N-terminal 216 amino acids, which includes the CHD. In contrast, IQGAP1-GST fusion proteins that lacked the CHD of IQGAP1 did not bind actin filaments [Fukata et al., 1997]. These results localized the F-actin binding domain of IQGAP1 to a CHD-containing N-terminal region slightly more than twice as large as the CHD itself. Moreover, they reinforced the notion that at least two CHDs are required

for F-actin binding activity, because of fusion protein dimerization caused by the GST moiety [Guthenberg and Mannervik, 1981].

The present report describes our recent studies of the F-actin binding activities of a series of N-terminal IQGAP1 fragments that were not fused to GST. To our surprise, we found that a monomeric N-terminal fragment extending to amino acid residue 210 bound to actin filaments in vitro and co-localized with cortical F-actin in transiently transfected cultured cells. To the best of our knowledge, these results represent the first examples of medium- to high-affinity F-actin binding activity for a protein containing only one CHD, and of a type 3 CHD being necessary for such activity.

## MATERIALS AND METHODS

### Mutagenesis of IQGAP1

The pRSETIQGAP1$_{2\text{-}522}$ mutant construct described previously [Mateer et al., 2002] was used as a template to generate a series of truncation mutants by polymerase chain reaction ([1]PCR), using the eLONGase Amplification System (Invitrogen; Carlsbad, CA). The forward primer 5′-GATATACATATGCGGGGTTCT-CATC-3′, and the following reverse primers were used to generate the IQGAP1$_{2\text{-}71}$, IQGAP1$_{2\text{-}210}$, and IQGAP1$_{2\text{-}371}$ mutants, respectively: 5′-CCCCCCAAGCT-TACCCCTCCTCCAGTTCTGTGG-3′, 5′-CCCCCC-AAGCTTACAGTTCATTAGCCAAGATGCCC-3′, and 5′-TCTCTCAAGCTTACAGCTCCTCCTTCTG-CAGGG-3′.

Each reverse primer included a stop codon and HindIII site directly following the codon corresponding to the last amino acid found in the appropriate IQGAP1 truncation mutant.

The PCR amplification products were precipitated with cold sodium acetate at pH 5.2 and 2 volumes of 100% ethanol. The pellets were then washed with 70% ethanol, air dried, and finally resuspended in a small volume of Tris-HCl at pH 8.5. The PCR fragments, as well as the pRSET-IQGAP1$_{2\text{-}522}$ plasmid, were then double digested with BamHI and HindIII (New England BioLabs; Peabody, MA). The resulting digested pRSET vector and PCR products were verified by agarose gel electrophoresis, and subsequently gel purified using a Qiagen (Valencia, CA) Gel Extraction Purification Kit. Each purified PCR product was then ligated into the pRSET vector (Invitrogen; Carlsbad, CA) using T4 Ligase (New England BioLabs) in a 16-h incubation. Ligation reactions were then transformed into DH5α *Escherichia coli*, and positive transformants were confirmed by restriction endonuclease digest.

## Protein Expression, Purification, and Radioiodination

Rabbit skeletal muscle actin was purified as described earlier [Bashour et al., 1997], and recombinant bacterially expressed IQGAP1 fragments were expressed and purified using a modified version of a procedure we used in a prior study [Mateer et al., 2002]. Modifications included inoculation of 2–3 one liter LB/Amp (100 mg/ml) with overnight bacterial suspensions containing the desired plasmid. Following appropriate growth and protein expression induction, the cultures were centrifuged at 5,000$g$ for 10 min, combined, and either resuspended in lysis buffer (50 mM $NaH_2PO_4$, 10 mM imidazole pH 8.0, 300 mM NaCl, 5 mM 2-mercaptoethanol) for purification or stored at $-80°C$. Slide-A-Lyzer cassettes (Pierce; Rockford, IL) with 3,000 molecular weight cut-off were used for concentration and buffer exchange of $IQGAP1_{2-71}$. IodoBeads (Pierce) were used to label purified $IQGAP1_{2-210}$ with $^{125}I$ (Amersham; Piscataway, NJ) according to instructions supplied by the IodoBeads vendor. The average labeling efficiency was measured to be less than one $^{125}I$ per $IQGAP1_{2-210}$, and radioiodination did not appear to affect the F-actin binding properties of $IQGAP1_{2-210}$.

## F-Actin Pelleting Assay, Gel Electrophoresis, and Immunoblotting

Pelleting assays for solutions of F-actin and $IQGAP1_{2-210}$ were performed exactly as described earlier for solutions of F-actin and other recombinant versions of IQGAP1 [Mateer et al., 2002]. Proteins were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis, or SDSPAGE [Laemmli 1970], and transferred to nitrocellulose membranes [Towbin et al., 1979] using a Mini-Protean II apparatus (Bio-Rad, Hercules, CA). Following electrophoresis, gels were transferred at 4°C either at 400 mAmps for 2 h or at 35 V overnight. Membranes were then blocked with Blotto (5% powdered milk plus 0.1% Tween 20 in Tris buffered saline, or TBS) for 1 h before being probed with antibodies. All antibodies were diluted in Blotto, and TBS/0.1% Tween 20 was used for washing steps. Primary antibodies included mouse monoclonal anti-$his_6$ (Molecular Probes; Eugene, OR) and mouse monoclonal anti-human actin (Cedar Lane; Hornby, Ontario, Canada). Horseradish peroxidase-labeled goat anti-mouse IgG (Kirkegaard & Perry; Gaithersberg, MD) was used as a secondary antibody, and SuperSignal luminol chemiluminescence reagents (Pierce; Rockford, IL) were used for visualizing immunoreactive protein according to the vendor's instructions.

## Disulfide Bond Assay

Purified $IQGAP1_{2-210}$ and rabbit IgG were diluted into SDSPAGE sample buffers containing or lacking the reducing agent, β-mercaptoethanol, and then were resolved by SDSPAGE on a 12% polyacrylamide gel. The gel was stained using Gelcode Blue (Pierce).

## Analysis of $IQGAP1_{2-210}$ by Gel Filtration Chromatography

A Superdex 200 HR 10/30 column (Amersham; Piscataway, NJ) was equilibrated with buffer (50 mM Tris pH 7.4, 1 mM EGTA, 300 mM NaCl), and calibrated with the following molecular weight standards for gel filtration chromatography (Sigma; St. Louis, MO): Blue Dextran ($\sim$2,000,000) to determine the void volume ($V_O$), sweet potato β-amylase (200,000), bovine serum albumin (66,200), bovine erythrocyte carbonic anhydrase (29,000), and horse heart cytochrome C (12,400). The elution volume ($V_E$) for each globular protein standard was measured, and a standard curve was generated by plotting $V_E/V_O$ versus the logarithm of molecular weight. The $V_E$ for $IQGAP1_{2-210}$ was then measured, and its apparent molecular weight was determined from the standard curve [Whitaker, 1963].

## Quantitation of the Binding of $IQGAP1_{2-210}$ to F-Actin

Samples (100 μl) containing 2.5 μM polymerized actin and 2.5–160 μM radioiodinated $IQGAP1_{2-210}$ were spun for 20 min at 40,000 rpm (64,000 $g_{max}$) using a TLA 100.3 rotor in an Optima TLX tabletop ultracentrifuge (Beckman; Palo Alto, CA). In each sample, the amounts of $^{125}I$ that pelleted or remained in the supernatant were measured using a gamma counter, and plotted as bound versus free $IQGAP1_{2-210}$, respectively, using Prism software for Macintosh OS X (GraphPad; San Diego, CA). For each data point, the bound counts were corrected for the amount of $IQGAP1_{2-210}$ that pelleted in the absence of F-actin, and the curve shown in Figure 6 was generated by Prism based on the assumption of a single class of binding site on F-actin for $IQGAP1_{2-210}$.

## Transfection and Immunofluorescence

A 1,586-bp cDNA fragment of IQGAP1 was used as a template to amplify the coding region for $IQGAP1_{2-210}$. The forward primer 5′-GATAAGGATCGATTC-CCATGTCCGCC-3′ and the reverse primer 5′-CCCG-GTACCTTATTCATTAGCCAAGAT-3′ were designed to generate a 648-bp DNA product containing a start codon and a HindIII site at the 5′ end, and a stop codon and a KpnI site at the 3′ end. Digestion with HindIII and KpnI generated a fragment that was ligated into the pFLAG-CMV-5a expression vector (Sigma), yielding a
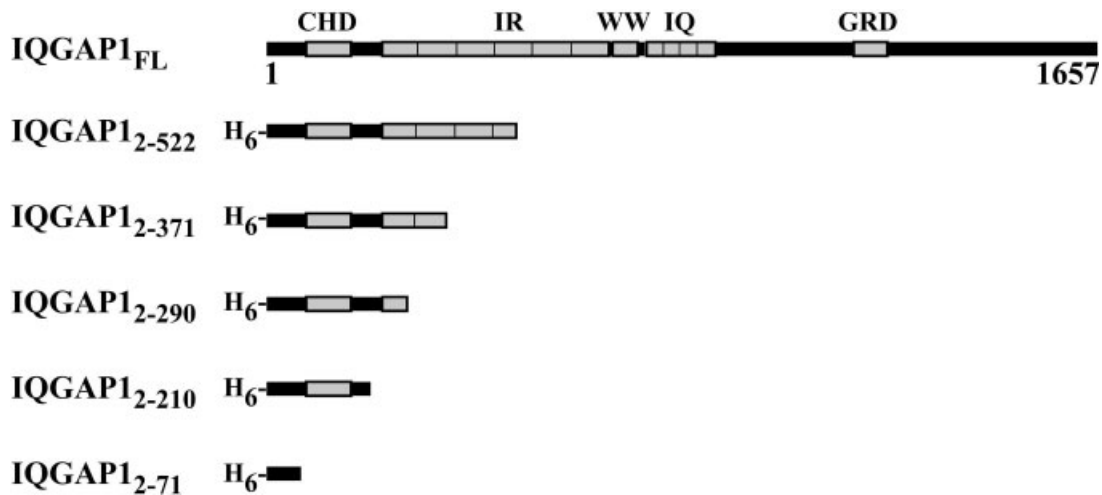
Fig. 1.   Recombinant IQGAP1 fragments. Shown here are the domain structures of the five his$_6$-tagged IQGAP1 fragments used in this study and the full-length protein from which they were all derived (IQGAP1$_{FL}$). CHD: calponin homology domain (implicated in actin filament binding); IR: IQGAP coiled-coil repeats (dimerization domains); WW: protein-protein interaction motif of unknown signifi-cance for IQGAP1; IQ: calmodulin-binding and S100B-binding IQ motifs; GRD: GTPase activating protein (GAP) related domain in-volved in binding to activated Cdc42 and Rac1. The *subscripts* at the end of each fragment name indicate the first and last amino acids, relative to the sequence of human IQGAP1, that are linked to the N-terminal his$_6$ tags.

plasmid containing the cDNA for the N-terminal 210– amino acid residues of IQGAP1 fused in frame at its N-terminal to the FLAG epitope tag. Turbo Pfu polymer-ase and 10X Pfu Buffer were provided by Stratagene (La Jolla, CA), dNTPs were obtained from Gibco (Grand Island, NY), and primers were from Integrated DNA Technology (Skokie, IL). In contrast to the plasmid for his$_6$-tagged IQGAP1$_{2-210}$, the plasmid for the FLAG-tagged fragment encoded the methionine that corre-sponds to the first amino acid of full-length IQGAP1, so we refer to the latter fragment henceforth as FLAG-IQGAP1$_{1-210}$. This plasmid was transfected into Rat-1, HeLa, and Cos-7 cells using Lipofectamine 2000 (In-vitrogen; Carlsbad, CA) by a slightly modified version of the manufacturer's instructions. Briefly, cells were main-tained in Dulbecco's MEM (Gibco) supplemented with cosmic calf serum (HyClone; Logan, UT) and 50 μg/ml gentamicin sulfate (Sigma). Cells were cultured without serum or antibiotics for 1 h prior to transfection, which was accomplished by addition to the medium of Lipo-fectamine 2000 plus 1 μg of FLAG-IQGAP1$_{1-210}$ plas-mid or vector, followed by a 4-h incubation in a $CO_2$ incubator at 37°C. Controls included equivalent incuba-tions with serum-free medium plus or minus Lipo-fectamine 2000. Following the 4-h incubations, serum was added to obtain a final concentration of 10%. Twenty-four hours after transfection, cells were fixed with 4% ρ-formaldehyde, solubilized with 0.05% Triton X-100, and stained for immunofluorescence using mouse monoclonal anti-FLAG M2 IgG1 (Sigma) followed by FITC-labeled goat-anti mouse IgG (Southern Biotech-

nology; Birmingham, AL), plus Alexa 568-phalloidin (Molecular Probes; Eugene, OR) to visualize F-actin [Mundy et al., 2002]. Cells were observed on a Zeiss (Thornwood, NY) Axiovert microscope using a 63X planapochromat Zeiss objective, and digital micrographs were captured using a Hamamatsu (Bridgewater, NJ) Orca-ER cooled CCD.

## RESULTS

### An F-Actin Binding Fragment of IQGAP1 Lacks IR Domains

In a recent study, we demonstrated that IQGAP1$_{2-522}$, an N-terminal IQGAP1 fragment that contains ~3.5 of the 6 IRs present in the full-length protein, can cross-link actin filaments into gels and bundles [Mateer et al., 2002]. IQGAP1$_{2-522}$ is, therefore, a dimer, and as few as three complete coiled-coil IR domains may thus be suf-ficient to cause dimerization of the protein. To determine if fragments containing fewer IRs than IQGAP1$_{2-522}$ can also bind F-actin, we designed, expressed, and purified a new series of progressively shorter N-terminal fragments of IQGAP1 (Fig. 1). The N-terminal methionine on each of the new recombinant proteins was replaced by a histidine$_6$ tag to facilitate purification by nickel affinity chromatography. All of these proteins, except IQGAP1$_{2-71}$, contained at least one complete CHD, and IQGAP1$_{2-371}$, IQGAP1$_{2-290}$, and IQGAP1$_{2-210}$ contained ~2, ~1, and zero IRs, respectively.

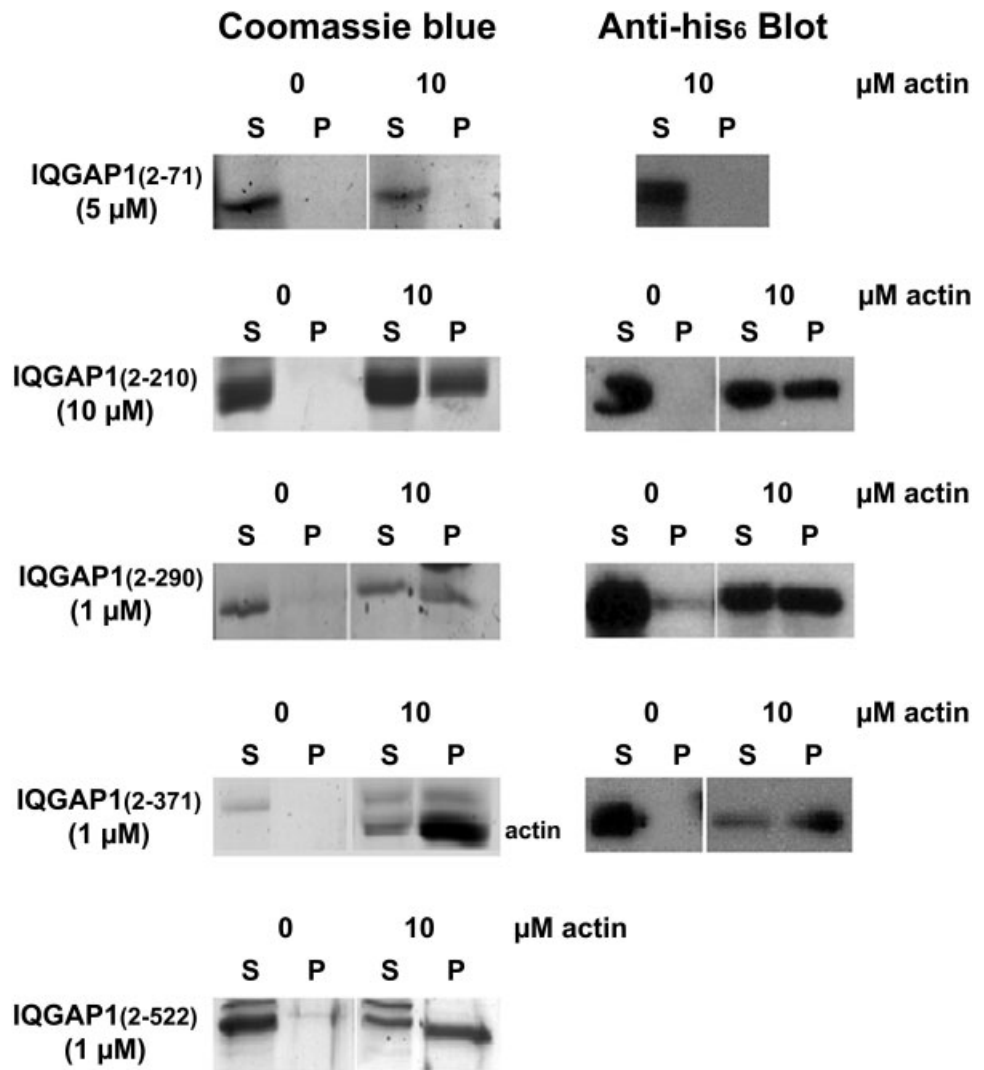Based on the assumption that IQGAP1 requires two CHDs to bind F-actin [Gimona et al., 2002] and the

Fig. 2. IQGAP1 fragments containing a CHD bind to actin filaments in vitro. Fragments at the indicated concentrations were centrifuged in the absence or presence of 10 μM polymerized actin, the resulting pellets were then resuspended to the original volumes, and equal aliquots of the supernatants (S) and pellets (P) were analyzed by SDSPAGE (GelCode Blue Stained Gels) or immunoblotting with an anti-his$_6$ antibody. F-actin was able to bind all fragments except IQGAP$_{2-71}$, which was the only fragment that lacked a CHD.

evidence that its IR domains cause the protein to dimerize [Mateer et al., 2002], we anticipated that any truncated protein that would be able to bind F-actin would contain at least one IR and would be dimeric in aqueous solution. As shown in Figure 2, we found that IQGAP1$_{2-371}$, which contains two IRs, and IQGAP1$_{2-290}$, which includes just a single IR, were able to bind F-actin in a high-speed pelleting assay. Not surprisingly, IQGAP1$_{2-71}$, which does not contain any IRs and also lacks the F-actin binding CHD, did not co-sediment with actin filaments. In contrast to these expected results, we also found that IQGAP1$_{2-210}$, which contains the CHD but does not have any IRs, was able to bind F-actin.

### IQGAP1$_{2-210}$ Does Not Dimerize Via Interchain Disulfide Bonds

IQGAP1$_{2-210}$ contains cysteines at positions 45, 57, and 151 relative to the sequence of full-length human

IQGAP1, raising the possibility that the truncated protein is a dimer held together by covalent inter-chain disulfide bonds. To test that hypothesis, we used SDSPAGE under reducing and non-reducing conditions. As shown in Figure 3, IQGAP1$_{2-210}$ migrated in SDSPAGE as an M$_r$ ~30,000 protein in either the presence or absence of the reducing agent, β-mercaptoethanol. To verify the efficacy of this method, we performed a similar analysis of rabbit IgG, which contains two ~50-kDa heavy chains, each of which is covalently cross-linked by interchain disulfide bonds to the other heavy chain and to one of two ~20-kDa light chains. As predicted, rabbit IgG migrated in SDSPAGE as an ~140-kDa band in the absence of β-mercaptoethanol, but the heavy chains became detectable when the reducing agent was present (the light chains were not visible at the protein load used for the experiment). We, therefore, concluded that IQGAP1$_{2-210}$ does not dimerize via inter-chain disulfide bonds. How-
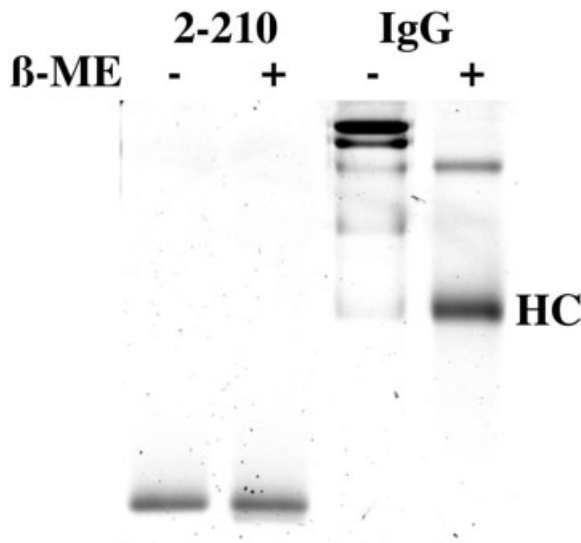
Fig. 3. IQGAP1$_{2-210}$ is not a multimeric protein whose subunits are linked covalently by disulfide bonds. IQGAP1$_{2-210}$ (2-210) and rabbit IgG were analyzed by SDSPAGE in the absence (−) and presence (+) of the reducing agent, β-mercaptoethanol (β-ME). Note that the rabbit IgG heavy chain (HC) was resolved only when β-ME was present (the light chains were not visible at this protein load). In contrast, the electrophoretic mobility of IQGAP$_{2-210}$ was unaffected by the reducing agent, indicating that IQGAP$_{2-210}$ does not form interchain disulfides.

ever, this experiment did not exclude the possibility that IQGAP1$_{2-210}$ dimerizes by another mechanism.

### F-Actin Binding Fragment, IQGAP1$_{2-210}$, Is a Monomer

Gel filtration chromatography, which separates molecules based on their Stokes radii, can be used to measure an apparent molecular weight of a protein by comparing its elution profile to the elution profiles of a series of globular protein standards of known molecular weight, which are used to calibrate the column [Whitaker, 1963]. If the apparent molecular weight of a protein measured by this method proves to be close to the molecular weight calculated from its amino acid composition, the protein must therefore be monomeric. Gel filtration chromatography was used in this manner to analyze IQGAP1$_{2-210}$. As shown in Figure 4, IQGAP1$_{2-210}$ eluted from a Superdex 200 HR gel filtration column as a very sharp peak with an apparent molecular weight of ∼33,400. Because that value is only 21.5% larger than the molecular weight of an IQGAP1$_{2-210}$ monomer, we concluded that the protein is a slightly asymmetric monomer in aqueous solution. When considered collectively with the F-actin binding results (Fig. 2), these data indicate that monomeric IQGAP1$_{2-210}$ can bind actin filaments even though it contains just one CHD, and that residues 72–210, which encompasses a portion of IQGAP1's type 3 CHD and is barely larger than the CHD itself, contains a region necessary for F-actin binding. (Fig. 5).

### Saturation Binding of IQGAP1$_{2-210}$ to F-Actin

To characterize its binding to F-actin quantitatively, 2.5–160 μM IQGAP1$_{2-210}$, a portion of which was covalently labeled with $^{125}$I, was mixed with 2.5 μM polymerized actin and centrifuged. The amount of pelleted (actin filament-associated) and soluble (non-bound) IQGAP1$_{2-210}$ was determined by gamma counting, and bound versus free IQGAP1$_{2-210}$ was then plotted. Figure 6 illustrates the combined results of three such experiments, all of which were corrected for the amount of IQGAP1$_{2-210}$ that pelleted in the absence of F-actin at the indicated concentrations of IQGAP1$_{2-210}$. Assuming a one site binding mechanism, saturation was achieved at 1.9 ± 0.3 μM IQGAP1$_{2-210}$, and the dissociation constant, $K_d$, was found to be 47 ± 18 μM. We conclude that IQGAP1$_{2-210}$, which contains just a single CHD, can saturate F-actin at a molar ratio of one IQGAP1$_{2-210}$ molecule per actin subunit in a filament, and that its affinity for F-actin is comparable to that of other proteins that bind actin filaments via multiple CHDs.

### Transfected IQGAP1$_{1-210}$ Co-Localizes With Actin Filaments in Cells

The data shown In Figures 2 and 6 clearly demonstrate the ability of IQGAP1$_{2-210}$ to bind directly to actin filaments in vitro, but do not address the more physiologically relevant issue of whether such binding can also occur in the cell. To resolve that issue, we transfected cultured Cos-7, Hela, and Rat-1 cells with a vector encoding the first 210 amino acid residues of IQGAP1 fused at its N-terminal to the FLAG epitope tag. Cells were then stained with a monoclonal antibody to the FLAG tag followed by FITC-labeled goat anti-mouse IgG to visualize the transfected protein, and with Alexa 568-labeled phalloidin to detect polymerized actin. Protein expression was confirmed by Western blotting using an anti-FLAG antibody (not shown). As illustrated in Figure 7, FLAG-IQGAP1$_{1-210}$ was targeted primarily to the cell cortex, where it colocalized extensively with polymerized actin. Figure 7 also demonstrates that, like endogenous IQGAP1 [Bashour et al., 1997], FLAG-IQGAP1$_{1-210}$ is not associated with the bundled actin filaments present in stress fibers, which were most readily visible in the Rat-1 cells. By immunofluorescent labeling of transfected cells with our monoclonal anti-IQGAP1, which reacts with full-length IQGAP1, but not with IQGAP1$_{2-210}$, we found that IQGAP1$_{2-210}$ expression did not obviously alter the steady-state distribution of endogenous IQGAP1. Preliminary studies have suggested, however, that IQGAP1$_{2-210}$ expression inhibits lamellipodial motility (data not shown).
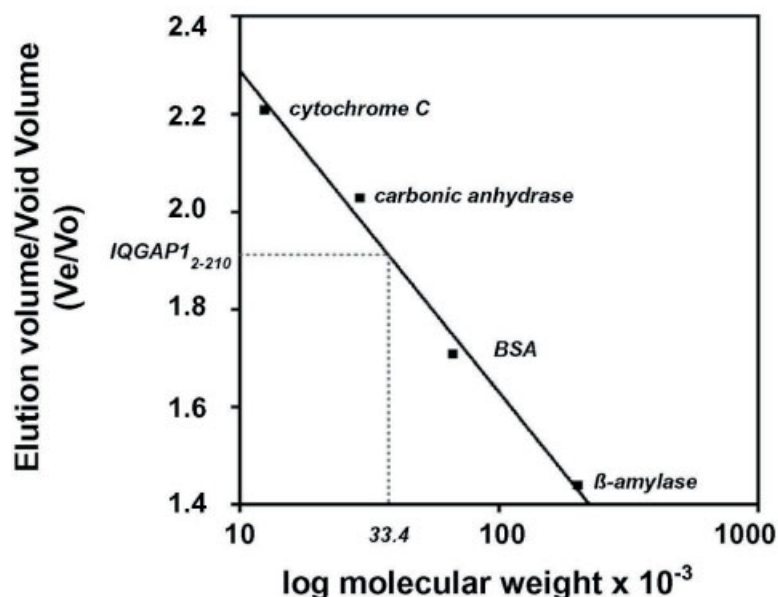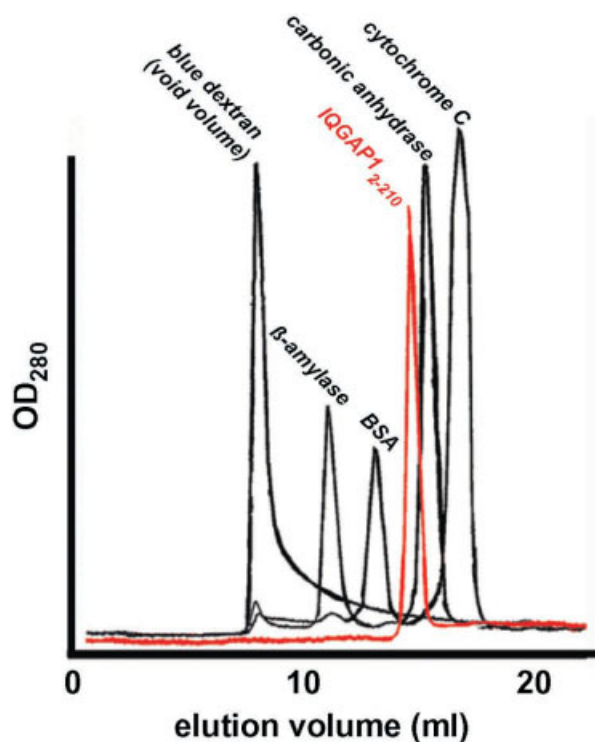
Fig. 4. IQGAP1$_{2\text{-}210}$ is a monomer in aqueous solution. IQGAP1$_{2\text{-}210}$ was run through a Superdex 200 HR gel filtration column that had been calibrated with the indicated globular molecular weight standards, and with blue dextran to mark the void volume. The elution profiles for the markers and IQGAP1$_{2\text{-}210}$ are shown (**top**) and a graph showing column calibration is illustrated (**bottom**). Note that IQGAP1$_{2\text{-}210}$ eluted from the column with an apparent molecular weight of 33,400, which is only 21.5% larger than the molecular weight of the protein calculated from its amino acid composition. We, therefore, conclude that IQGAP1$_{2\text{-}210}$ is a slightly asymmetric monomer in solution.

## DISCUSSION

The CHDs are defined as 100-amino-acid-long modules that contain a number of invariant residues, but can be classified into as many as eight structurally distinct groups [Gimona et al., 2002]. Most, if not all CHDs are found on proteins that can bind F-actin, but studies of numerous CHD-containing proteins have consistently reinforced the hypothesis that a single CHD is not sufficient for this activity. Prior efforts to demonstrate medium- to high-affinity (5–50 $\mu$M K$_d$) F-actin binding [Gimona et al., 2002] attributable to individual CHDs have been unsuccessful. Moreover, without exception, proteins that bind F-actin in that affinity range in a CHD-dependent manner have been found to contain multiple CHDs that either are arranged in tandem on a single polypeptide, or are present as a single copy on each of at least two subunits of a multimeric protein [Gimona and Winder, 1998; Stradal et al., 1998].
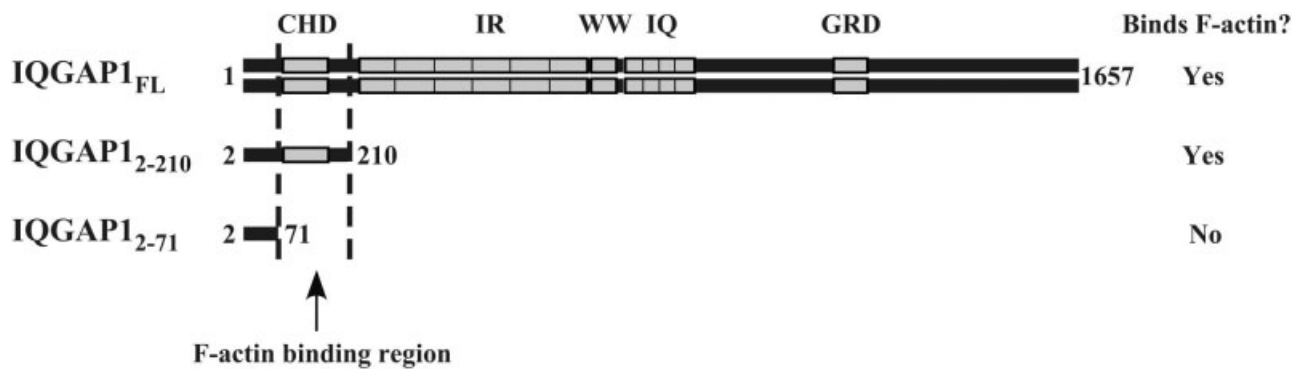
Fig. 5.    The F-actin binding domain of IQGAP1$_{2-210}$. When considered collectively, the F-actin binding data shown in Figure 2 and the solution behavior of IQGAP1$_{2-210}$ shown in Figure 4 demonstrate that monomeric IQGAP1$_{2-210}$ binds F-actin by a domain no larger than the region between amino acid residues 72–210, relative to the sequence of human IQGAP1. Note also that whereas IQGAP1$_{2-210}$ is monomeric, full-length IQGAP1 is a dimer, and IQGAP1$_{2-71}$ cannot bind actin filaments.

Until now, IQGAP1 seemed to be a typical example of a protein that fits the multi-CHD hypothesis. It is homodimeric, and each subunit contains a single type 3 CHD located near its N-terminal [Bashour et al., 1997]. Curiously, proteins such as calponin can still bind actin filaments after their type 3 CHDs are removed, prompting speculation that the type 3 CHD is irrelevant for F-actin binding [Gimona and Mital, 1998]. Nevertheless, a 216–amino acid residue N-terminal fragment of IQ-
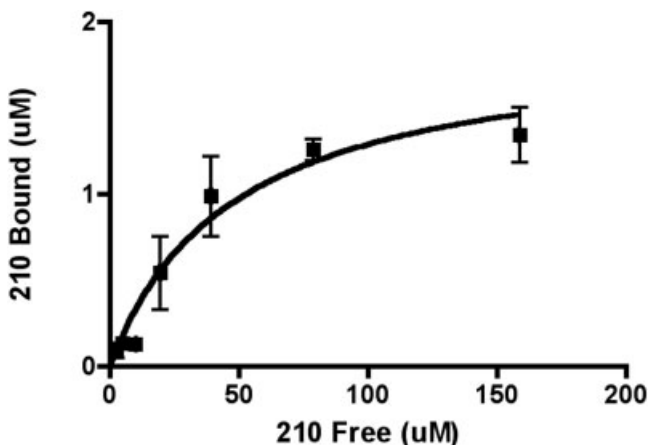


Fig. 6.    Determination of the affinity and saturation binding of IQGAP1$_{2-210}$ to F-actin. IQGAP1$_{2-210}$ (2.5–160 μM) labeled covalently with $^{125}$I was mixed with 2.5 μM polymerized actin and centrifuged. The amounts of bound (pelleted) and free (soluble) IQGAP1$_{2-210}$ were then measured using a gamma counter and plotted versus each other as shown. The results illustrated here were corrected for the amount of IQGAP1$_{2-210}$ that pelleted in the absence of F-actin. Each data point represents the average value for three experiments, and each error bar represents the standard error of the mean. Assuming a single class of binding site, IQGAP1$_{2-210}$ saturated the actin filaments at 1.9 ± 0.3 μM, which was very close to one IQGAP1$_{2-210}$ molecule per actin subunit in a filament, and half maximal binding, which indicates the dissociation constant ($K_d$), occurred at ~47 ± 18 μM IQGAP1$_{2-210}$.

GAP1 that contains a type 3 CHD and presumably cannot dimerize on its own was shown to bind F-actin when it was coupled at its C-terminal to GST [Fukata et al., 1997]. Because GST is a natural dimer [Guthenberg and Mannervik, 1981], however, this F-actin binding fusion protein was likely to be dimeric also, and thus contain a pair of type 3 CHDs.

Here we demonstrate that a slightly smaller fragment of IQGAP1 that is not coupled to GST can bind actin filaments. The fragment, IQGAP1$_{2-210}$, was found to be monomeric in aqueous solution (Fig. 4), and to bind actin filaments not only in vitro (Figs. 2 and 6), but also in cells that expressed the protein by transient transfection (Fig. 7). By assaying F-actin binding for IQGAP1$_{2-210}$, and for both smaller and larger N-terminal fragments of IQGAP1 (Fig. 2), we have identified a region located between residues 72 and 210 that is slightly larger than the CHD and is necessary for F-actin binding (Fig. 5). These collective results indicate the first known examples of medium- to high-affinity F-actin binding mediated by a single CHD, and of actin filament binding activity associated with a type 3 CHD. We measured the dissociation constant of IQGAP1$_{2-210}$ for F-actin to be ~47 μM (Fig. 6). It has been difficult to measure the $K_d$ of full-length IQGAP1 for F-actin, because its dimeric structure enables it to cross-link actin filaments. Nevertheless, the native protein clearly binds much more tightly than IQGAP1$_{2-210}$ to actin filaments, because experiments in which 0.4 μM native IQGAP1 was mixed with an increasing concentration series of actin filaments demonstrated 50% binding of IQGAP1 at an F-actin concentration of 40 nM [Bashour et al., 1997]. This contrast between monomeric IQGAP1$_{2-210}$ and dimeric native IQGAP1 suggests that the two CHDs of the native protein work cooperatively for binding to actin filaments.
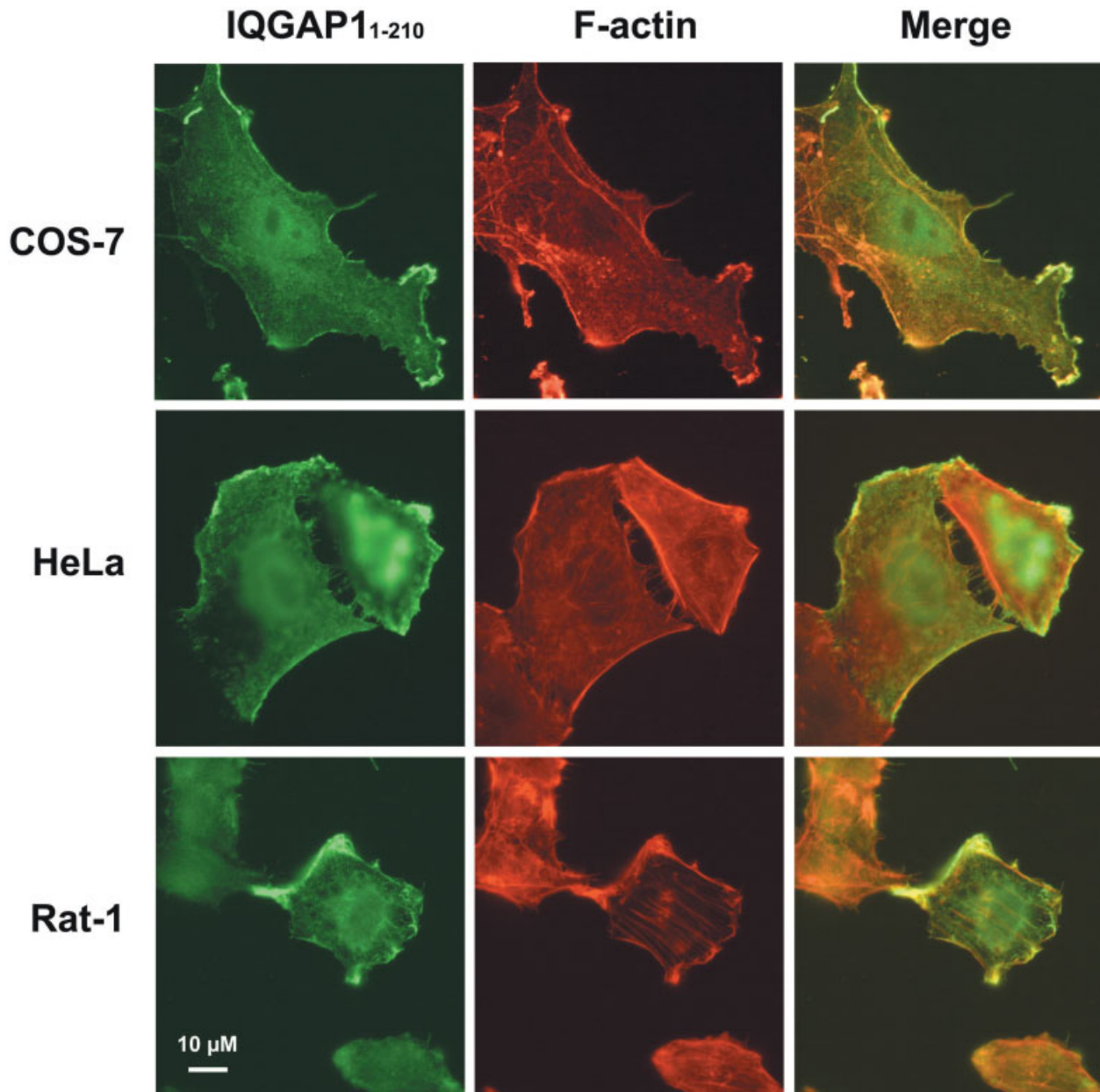
## IQGAP1₁₋₂₁₀    F-actin    Merge



Fig. 7. FLAG-IQGAP1$_{1\text{-}210}$ co-localizes with actin filaments in cultured cells. FLAG-tagged IQGAP1$_{1\text{-}210}$ expressed in COS-7, HeLa, and Rat-1 cells by transient transfection was detected by immunofluorescence microscopy using an antibody to the FLAG epitope tag and a FITC-labeled secondary antibody. The cells were also stained for polymerized actin using Alexa 568-phalloidin. Note that like endogenous IQGAP1 [Bashour et al., 1997] FLAG-IQGAP1$_{1\text{-}210}$ frequently co-localized with actin filaments in cortical regions of transfected cells and was absent from F-actin-rich stress fibers.

The cellular localization of transfected FLAG-IQGAP1$_{1\text{-}210}$ is intriguing. Like full-length IQGAP1 [Bashour et al., 1997], FLAG-IQGAP1$_{1\text{-}210}$ co-localizes extensively with polymerized actin in the cell cortex, but not on stress fibers (Fig. 7). This implies that FLAG-IQGAP1$_{1\text{-}210}$, just like full-length endogenous IQGAP1 [Bashour et al., 1997], contains all of the structural information needed for targeting not only to actin filaments, but also to binding sites located near the cytoplasmic face of the plasma membrane. With one exception, the molecules that constitute those binding sites remain mysterious, but they appear to be essential for the functions of IQGAP1. The exception is VEGFR2, the type 2 receptor for vascular endothelial growth factor (VEGF).

We recently found that binding of VEGF to VEGFR2 in cultured endothelial cells stimulates direct binding of IQGAP1 to the cytoplasmic domain of VEGFR2, and association of activated Rac1 with IQGAP1. Moreover, these biochemical events are followed rapidly by increased endothelial cell motility, which is exquisitely sensitive to siRNA-induced reduction of IQGAP1 protein levels (Yamaoka-Tojo et al., unpublished findings)[1]. This functional connection of IQGAP1, Rac1, and VEGFR2 is reminiscent of evidence that cortically situated IQGAP1 is required for recruitment of GTP-bound Cdc42 to the cortex and downstream signaling of activated Cdc42 [Swart-Mataraza et al., 2002]. The involvement of Rac1 and Cdc42 in myriad functions, including mobilization of the actin cytoskeleton and cell adhesion machinery to enable cellular morphogenesis and motility, is well documented [Etienne-Manneville and Hall, 2002]. It follows naturally that a fuller understanding of how activated Rac1 and Cdc42 accomplish those tasks will require learning more about the molecular basis for targeting IQGAP1 to the cell cortex. It will, therefore, be important to determine the minimal cortical targeting domain of IQGAP1 and cortical molecules other than VEGFR2 with which that domain associates. We are especially interested in learning whether the cortical targeting domain of IQGAP1 can be separated from its actin filament binding region, and what the effects are on endogenous IQGAP1 localization and cellular behavior of overexpressing small, potentially interfering fragments of the protein, like FLAG-IQGAP1$_{1\text{-}210}$.

## CONCLUSIONS

Past studies have questioned the relevance of type 3 CHDs for binding to F-actin, and have implied that CHD-dependent binding requires at least two CHDs, which can be located on an individual polypeptide or on each subunit of a multimeric protein [Gimona and Mital, 1998; Gimona and Winder, 1998]. Here we show that IQGAP1$_{2\text{-}210}$, a monomeric, N-terminal fragment of IQGAP1, binds saturably and with moderate affinity directly to F-actin via a region that apparently includes, and is only slightly larger than, its single type 3 CHD. When expressed in cells by transient transfection, a FLAG-tagged version of the protein, like endogenous IQGAP1 [Bashour et al., 1997], is targeted to the cortex, where it co-localizes extensively with polymerized actin. These collective results identify the first known example of moderate affinity, actin filament–binding mediated by a

single CHD, and establishes that the cortical targeting domain of IQGAP1 is located within its N-terminal 210 amino acids.

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