Published in final edited form as:

Biochemistry. 2016 November 22; 55(46): 6433–6444. doi:10.1021/acs.biochem.6b00745.

The IQGAP1 N-terminus Forms Dimers and the Dimer Interface is Required for Binding F-actin and Ca²⁺/calmodulin

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Abstract

IQGAP1 is a multi-domain scaffold protein involved in many cellular processes. We have determined the crystal structure of an N-terminal fragment spanning residues 1-191 (CHDF hereafter) which contains the entire calponin homology domain. The structure of the CHDF is very similar to other Type 3 calponin homology domains like those from calponin, Vav, and the yeast IQGAP1 ortholog Rng2. However, in the crystal two CHDF molecules form a “head-to-head” or parallel dimer through mostly hydrophobic interactions. Binding experiments indicate that the CHDF binds to both F-actin and Ca²⁺/calmodulin, but binding is mutually exclusive. Based on the structure, two dimer interface substitutions were introduced. While CHDFL157D disrupts the dimer in gel filtration experiments, oxidized CHDFK161C stabilizes the dimer. These results imply that the CHDF forms the same dimer in solution that is seen in the crystal structure. The disulfide-stabilized dimer displays reduced F-actin binding in sedimentation assays, and shows no binding to Ca²⁺/calmodulin in isothermal titration calorimetry (ITC) experiments indicating that interface residues are utilized for both binding events. The Calmodulin Target Database predicts that residues ⁹³KK⁹⁴ are important for CaM binding, and indeed the ⁹³EE⁹⁴ double mutation displays reduced binding to Ca²⁺/calmodulin in ITC experiments. Our results indicate that the CHDF dimer interface is used for both F-actin and Ca²⁺/calmodulin binding, and ⁹³KK⁹⁴, near the interface, are also used for Ca²⁺/calmodulin binding. These results are also consistent with full-length IQGAP1 forming a parallel homodimer.

Graphical Abstract

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Human IQGAP1 has been shown to bind to a number of proteins including F-actin, calmodulin (CaM), Cdc42, Rac1, β -catenin, E-cadherin, CLIP-170 and ezrin¹⁻³. Through dynamic interaction with these binding partners, IQGAP1 coordinates cellular signaling, cytoskeletal rearrangements, and cell-cell adhesion to define cell polarization, facilitate cell migration and influence other important processes⁴. Over-expression of IQGAP1 has been observed in various cancer types, and *in vitro* models indicate that over-expression and abnormal localization of IQGAP1 can destabilize cell-cell contacts and promote cell migration and invasion^{5, 6}. IQGAP1 binds to and cross-links F-actin to form a gel-like meshwork and recently Pelikan *et al.* found that IQGAP1 can cap the barbed end of F-actin, thereby regulating actin polymerization⁷⁻⁹. *In vitro* studies have shown that IQGAP1 N-terminal fragments (residues 2-210, 1-216 and 1-232) can bind to filamentous actin (F-actin)^{7, 10, 11}. In addition, a fragment including residues 1-232 has also been shown to bind to calcium and also calcium-bound calmodulin ($\text{Ca}^{2+}/\text{CaM}$). Heretofore, little was known about the molecular details of the IQGAP1 N-terminus and its interactions with F-actin and $\text{Ca}^{2+}/\text{CaM}$.

Calponin homology (CH) domains are found in numerous signaling and cytoskeleton proteins [Reviewed in^{12, 13}]. In general, the ~110 residue CH domains have relatively low sequence conservation; however existing structural information indicates that they share a conserved fold. The best understood function for CH domains is their ability to bind to F-actin. Based on sequence similarity, CH domains have been divided into several major types. Type 1 CH domains (CH1) and Type 2 CH domains (CH2) exist in tandem and form actin-binding domains (ABDs). Previous studies have demonstrated that the CH1 domain of ABDs is essential and sufficient for binding to F-actin and the presence of the adjacent CH2 domain serves to increase the binding affinity¹⁴. Type 3 CH domains (CH3) exist in proteins as a single copy and are found in many signaling proteins such as calponin, the Vav proteins, and IQGAPs. Recent studies indicate that CH domains, especially type 3 CH domains, may have additional functions besides actin binding. For example, the eponymous CH domain from calponin cannot bind to F-actin, but instead interacts with other molecules such as phospholipids, extracellular signal-regulated kinases, and calmodulin¹⁵⁻¹⁹. The molecular mechanism of interaction between CH domains and F-actin is still unclear. Several studies using mutagenesis, cross-linking and NMR spectroscopy have proposed three actin binding sites (ABS) on ABDs: ABS1 and ABS2 locate to the first and last helix of CH1, respectively, and ABS3 is located on the linker between CH1 and CH2 and part of the first helix of CH2 [Reviewed in¹²].

The ~ 17 kDa calmodulin belongs to the family of EF-hand calcium-binding proteins which mediate intracellular Ca^{2+} signaling²⁰. IQGAP1 contains four isoleucine- and glutamine-containing motifs (IQ motifs) which bind to both apo-CaM and Ca^{2+} /CaM¹⁰. Binding of CaM to the IQ motifs has been suggested to negatively regulate IQGAP1's binding to other partners such as Cdc42 and F-actin¹⁰. Since IQ motifs are known for their calmodulin-binding function, the biological significance of the interaction between the IQGAP1 N-terminus and Ca^{2+} /CaM is not clear. Ho et al. found that F-actin displaces Ca^{2+} /CaM from IQGAP1 N-terminal residues 1-232. Studies have shown that the CH domains from dystrophin, filamin A, calponin and Vav1 require the presence of Ca^{2+} to bind tightly to CaM. Accumulating evidence indicates that Ca^{2+} /CaM has a regulatory role for these proteins. For instance, Ca^{2+} /CaM inhibits F-actin binding to calponin and filamin A, whereas interaction of the CH domain of Vav1 with Ca^{2+} /CaM is required for T-cell mediated calcium mobilization^{14, 15, 21}.

It has been previously reported that full-length IQGAP1 forms dimers both *in vitro* and *in vivo* and dimerization is crucial for its normal functions; including binding to Cdc42•GTP and actin crosslinking^{7, 8, 22}. Despite the importance of dimer formation by IQGAP1, it is not known how the monomers are arranged in the dimer. Ren et al. found that the region encompassed by residues 763-863 is crucial for IQGAP1 dimerization²². This region overlaps with the calmodulin-binding IQ motifs, hinting that calmodulin may have a role in modulating IQGAP1 dimerization.

Here, we report the crystal structure of an N-terminal fragment of IQGAP1 encompassing the entire CH domain which will be referred to as the CHDF (CH domain fragment). The CHDF exists as a “head-to-head” or parallel dimer in the crystal and the dimer is mainly stabilized through hydrophobic interactions across a shape-complementary interface. We find that the CHDF can form a dimer in solution, and site-directed mutagenesis studies based on the dimer seen in the crystal indicate that the solution dimer is very similar to the crystal dimer. Our results, using a fragment representing only ~ 12% of the intact protein, suggests that full-length IQGAP1 forms parallel dimers *in vivo* and in doing so, juxtaposes its common domains and motifs. A structure-based sequence alignment reveals a potential CH1-like ABS2 which in the structure exists at the CHDF dimer interface. A dimer interface substitution K161C, when oxidized, shows significantly reduced binding to F-actin suggesting that this ABS2 is required for F-actin binding. Our ITC data modeling indicates that the wild-type CHDF binds to Ca^{2+} /CaM in a “two-site sequential” mode. Oxidized CHDFK161C shows a complete loss of binding to Ca^{2+} /CaM indicating that complete dissociation of the CHDF dimer is required for CaM binding. The Calmodulin Target Database predicts a CaM binding site within CHDF residues 91-103 which do not lie at the dimer interface. Two consecutive lysines within this putative CaM-binding site are critical to the Database prediction, and the ⁹³KK⁹⁴→⁹³EE⁹⁴ double mutation in the CHDF results in a significant reduction in binding to Ca^{2+} /CaM. In conclusion, our results suggest that IQGAP1 forms a parallel dimer, and the N-termini of the dimer, containing the CH domain, must dissociate to allow Ca^{2+} /CaM or F-actin binding.

Experimental Procedures

Generation of CHDF expression constructs

A cDNA encoding residues 1-191 of human IQGAP1, which encompasses the entire calponin homology domain (residues 46-156), was generated via PCR and ligated into the pETtrx vector (A kind gift from Dr. Gunter Stier, EMBL, Heidelberg). This vector encodes *E. coli* thioredoxin followed by a short Gly-Ser linker, 6 consecutive histidines and a tobacco etch virus (TEV) protease cleavage sequence all in frame with and N-terminal to the protein of interest. After tag removal, each protein has a Gly-Ala artifact preceding Met¹. Constructs of CHDF including ⁹³KK⁹⁴→⁹³EE⁹⁴, L157D and K161C were generated via site-directed mutagenesis using the QuickChange (Stratagene) methodology. All DNA constructs were fully sequenced to ensure the proper introduction of mutations.

Expression and purification of proteins

CHDF, CHDF⁹³EE⁹⁴, and CHDFL157D were expressed in Rosetta 2[™] (DE3) pLysS cells (EMD4Biosciences) growing in LB medium. The cells were grown at 37 °C until the OD₆₀₀ reached ~0.8 and then the temperature was reduced to 27 °C and cells were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 5 hours induction, the cells were collected by centrifugation, resuspended in 300 mM NaCl, 15 mM imidazole, and 20 mM Tris-HCl pH 8.0, and 5% glycerol (N1 buffer) and then stored at -80 °C. Thawed cells were lysed at 4 °C using an Emulsiflex-C5 high pressure homogenizer (Avestin) operating at 15,000 p.s.i. The lysate was immediately centrifuged for 45 min at 260,000 g and 4 °C. The supernatant was then passed through a 5μm syringe filter (Gelman Laboratories) and then loaded onto a 5ml HisTrap[™] HP column (GE Healthcare) equilibrated with N1 buffer. After extensive washing with N1 buffer, the protein was eluted with N1 buffer containing an additional 300 mM imidazole. The 6xHis-tag was removed by TEV protease during overnight dialysis against N1 buffer containing 2mM β-mercaptoethanol (BME) at 4 °C. Dialyzed proteins were then applied to a 5ml HisTrap[™] HP column equilibrated with N1 and the flow-through, containing untagged CHDF, was collected and immediately concentrated to ~ 10 ml prior to loading onto a Hiload 26/60 Superdex 75 column (GE Healthcare) equilibrated with 300 mM NaCl, 20 mM Tris-HCl pH 8.0, 5 % glycerol and 2 mM BME. Superdex 75 fractions were analyzed using SDS-PAGE with Coomassie Brilliant Blue staining and fractions containing purified CHDF were pooled, concentrated to 20 mg/mL, and stored at -80 °C for future use. L-selenomethionine-substituted (SeMet) CHDF was produced in the methionine auxotrophic strain B834 (DE3) in minimal media (M9) supplemented with 42 mM glucose, 3 mM of each amino acid except methionine (Sigma), 10 ml of 100xMEM vitamins (Sigma) and 100 mg SeMet (Acros Organics) per liter. Cells were grown at 37 °C until the OD₆₀₀ reached 0.6 and then the temperature was reduced to 27 °C and the cells were induced with 0.5 mM IPTG for 8 hours. Purification of SeMet protein followed an identical procedure to that described above for CHDF and mutants. To facilitate formation of the disulfide bonded-dimer, CHDFK161C was expressed in Rosetta Gami[™] 2(DE3) pLysS cells (EMD4Biosciences). The cells were grown in TB medium at 37 °C until the OD₆₀₀ reached ~ 0.8 and then the temperature was reduced to 18 °C and protein expression was induced with 0.5 mM IPTG overnight. The purification of

CHDFK161C is identical to other CHDF proteins except no reducing agent was used during the purification process.

The gene for human calmodulin (CaM) was purchased from Open Biosystems (GE Healthcare). The CaM coding sequence was amplified by PCR and ligated into pET28. CaM protein was expressed in Rosetta 2[™] (DE3) pLysS cells (EMD4Biosciences) growing in LB medium. The cells were grown at 37 °C until OD₆₀₀ reached 0.6–1.0 and were then induced with 0.5 mM IPTG for 2–3 h. Cells were then harvested by centrifugation and resuspended in 100 mM NaCl, 20 mM Tris-HCl pH 8.0 and 5% Glycerol. The cells were lysed, centrifuged, and the supernatant was collected as described in the previous paragraph. EDTA was added to a final concentration of 10 mM and then the supernatant was loaded onto a 24 ml 10/300 Q Sepharose HP column equilibrated to 50 mM NaCl, 20 mM Tris-HCl pH 8.0, 5% Glycerol, 1 mM EDTA and 2 mM BME (Buffer A). After extensive washing with buffer A, the protein was eluted with a 0% – 40% gradient of Buffer B (Buffer A containing 1 M NaCl). Fractions containing CaM were identified with SDS-PAGE and pooled and adjusted to be 500 mM NaCl, 50 mM Tris-HCl pH8.0, and 10 mM CaCl₂. The sample was then loaded onto 3 × 5ml Phenyl HP columns equilibrated to 500 mM NaCl, 50 mM Tris-HCl pH 8.0, 5% Glycerol, 2mM CaCl₂ and 2mM BME (buffer PA). After extensively washed with buffer PA, the protein was then eluted with buffer containing 50 mM Tris-HCl pH 8.0, 10 mM EDTA and 2 mM BME. The eluate was adjusted to 300 mM NaCl, concentrated and stored at –80 °C for future use.

Crystallization conditions

CHDF crystals belonging to spacegroup P1 were grown by vapor diffusion at 4 °C using a 1:1 ratio of protein solution to reservoir solution (20% PEG 3350, 100 mM Tris pH 8.5, 200–300 mM ammonium acetate). Crystals appeared after three or four days and continued to grow for several weeks. The triclinic crystals have unit cell parameters $a = 57 \text{ \AA}$, $b = 66 \text{ \AA}$, $c = 83 \text{ \AA}$ and $\alpha = 99.6^\circ$, $\beta = 104.1^\circ$, $\gamma = 108.7^\circ$. Cell volume calculations suggested that four to six CHDF molecules were likely to be contained within the unit cell. Oxidized CHDFK161C crystals belonging to spacegroup P1 were grown by vapor diffusion at 4 °C using a 1:1 ratio of reservoir solution (18% PEG 3350, 10% glucose, 10% ethylene glycol and 100–160 mM potassium acetate). The triclinic crystals have unit cell parameters $a = 53.75 \text{ \AA}$, $b = 72.46 \text{ \AA}$, $c = 73.27 \text{ \AA}$ and $\alpha = 77.15^\circ$, $\beta = 72.07^\circ$, $\gamma = 86.80^\circ$. Wild-type and K161C CHDF coordinates have been deposited in the Protein Data Bank with IDs 3I6X and 5L0O, respectively.

X-ray data collection and processing

For data collection at 100K, CHDF crystals were cryo-protected by stepwise transfer to 20% PEG 3350, 100 mM Tris-HCl pH 8.5, 200 mM ammonium acetate and 20% glycerol. SeMet P31 crystals were cryoprotected by adjusting the solution containing the crystals to be 200 mM KCl, 50 mM HEPES pH 7.5, and 35% pentaerythritol propoxylate. CHDFK161C crystals were grown in cryo-solution, and so these could be flash-frozen immediately. In each case, cryoprotected crystals were suspended in a rayon loop (Hampton Research) and flash-frozen in liquid nitrogen. Triclinic CHDF data were collected at the 31-ID beamline at the Advanced Photon Source, Argonne, Illinois. Data were collected at wavelength 0.9397 Å

on a Mar 165 CCD detector. The diffraction intensities were integrated with DENZO, and scaled and merged with SCALEPACK²³. Data for the crystals of oxidized CHDFK161C was collected at 100K on a Bruker Microstar generator equipped with Helios Optics and a Proteum 4K Platinum 135 CCD camera. Proteum 2 software (Bruker AXS) was used to integrate and scale the data.

Structure determination and refinement

The CHDF structure was determined for the P1 crystal form using molecular replacement and the program AMoRe²⁴. A search model was derived from the *S. pombe* Rng2 structure (Protein Databank ID 1P2X) where all non-glycine residues in the Rng2 structure were truncated beyond the C β atom. Using data in the range 8 Å – 3.5 Å for the rotation function, four solutions were obtained that had Patterson correlation coefficients at least 25% higher than the next highest solutions. The translation function and subsequent rigid body refinement in AMoRe confirmed that these were the correct angular orientations yielding a crystallographic R-factor of 0.495 for this polyalanine model. At this point, the automated refinement program CNS²⁵ and the interactive graphics program O were used for model refinement and model building, respectively. To help avoid model bias, simulated annealing omit maps were calculated during model building. Because we had non-crystallographic symmetry (NCS), we used the CCP4 program ACT to identify CHDF residues that were in similar packing environments in order to apply NCS restraints during refinement. In all, NCS restraints were applied to 82 of 164 ordered residues of each monomer. Twenty-seven IQGAP1 residues at the N-terminus are disordered in the crystal and were not modeled. In addition, the electron density for the sidechains of residues 29 – 34 and residue 191 is incomplete, so the final model does not contain atoms in these sidechains that were not supported by electron density. The current refinement statistics for all data with $|F| > 0$ in the range 25 Å – 2.5 Å are $R_{\text{cryst}} = 0.235$, $R_{\text{free}} = 0.263$ (see Table 1). The model has 94.6% and 5.4% of residues in the most favored and additionally allowed regions of the Ramachandran plot, respectively. As a check to ensure that NCS was properly applied, the coordinates were subjected to a 5000° simulated-annealing slowcool refinement using torsion angle dynamics and no NCS restraints. At the end of the slowcool refinement, the $R_{\text{cryst}} = 0.242$, $R_{\text{free}} = 0.274$ and the mean pairwise r.m.s.d. for superimposing 164 Ca atoms of each CH domain onto the other molecules is 0.191 Å (6 superpositionings using LSQKAB of the CCP4 suite of programs²⁶).

AMoRe and molecular replacement using wild-type CHDF as a search model was used to determine the CHDFK161C (oxidized) structure. Statistics for the input CHDFK161C coordinates used to calculate the simulated annealing total omit 2Fo-Fc map are: $R_{\text{cryst}} = 0.262$, $R_{\text{free}} = 0.286$ for all $|F| > 0$ in the data range 15 Å – 2.36 Å. This model includes four CHDF molecules arranged as two parallel dimers that are essentially identical to the wild-type CHDF described above, however no water molecules have been added and all sidechain atoms are included in this model (Table 1).

Dimer interface analysis

The programs NACCESS, 2P2I inspector and PDBePISA online server were used to analyze the CHDF dimer interface^{27–29}. NACCESS was used with default settings. The shape complementarity statistic was calculated using SC in CCP4 suite with default settings³⁰.

Gel-filtration chromatography

A Superdex 200 10/300GL (GE Healthcare) was equilibrated with 100 mM NaCl, 100 mM KCl, 50 mM HEPES pH 7.5 and 5% glycerol, 2 mM CaCl₂, 2 mM BME (ITC buffer) and then calibrated with the MWGF200 kit (Sigma) which contains blue dextran (2,000 kDa), β -Amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). A flow rate of 0.3 ml/min was used. The phase distribution coefficient (K_{av}) was calculated from $K_{av} = (V_e - V_0)/(V_c - V_0)$ where V_e is elution volume, V_0 is void volume (blue dextran) and V_c is column volume (24 ml). The standard calibration curve is the plot of logMW (ordinate) versus K_{av} (abscissa). 100 μ l of 100 or 680 μ M of CHDF (or CHDF mutants) was loaded onto the column using and eluted at 0.3 ml/min. The apparent molecular weight of each sample was calculated by their K_{av} using the standard curve. Values are listed in Table 2.

Isothermal titration calorimetry

A MicroCalTM ITC₂₀₀ calorimeter (GE Healthcare) was used for the binding studies. CHDF, CHDF mutants and calmodulin were dialyzed extensively against 100 mM NaCl, 100 mM KCl, 50 mM HEPES pH 7.5, 5% glycerol, 2 mM CaCl₂ and 2 mM BME at 4 °C to eliminate heat changes caused by buffer effects. After dialysis, the concentrations of the proteins were determined using light absorbance at 280 nm and the calculated extinction coefficients for the proteins as provided by ProtParam (Expasy Bioinformatics Research Portal). The concentration of CaM was calculated by Bradford assay using lyophilized CaM as the standard. For apo-calmodulin binding experiments, 10 mM EGTA was used instead of 2mM CaCl₂. CHDFs were placed in the calorimeter cell and CaM was loaded in the syringe. ITC₂₀₀ data collection consisted of eighteen 2 μ l aliquots of titrant injected into the sample cell at time intervals of 120 s. The experiments were conducted at 30 °C with the cell contents stirred at 1500 rpm to facilitate immediate mixing of injectant. The power required to maintain the cell temperature at 30 °C was monitored over time and the peaks observed in the power vs. time plot are shown in the upper panel of our ITC figures. In every experiment, a control titration of injectant into sample cell containing only dialysis buffer was subtracted from the original data to eliminate potential heats of dilution. The raw ITC data were integrated and analyzed using Origin 7.0 software (MicroCal, GE Healthcare). The lower panel of the ITC figures shows integrated heats. Binding parameters are listed in Table 4. CHDFK161C used in the experiments was first incubated on ice for 48 hours in buffer containing either 10mM Tris (2-carboxyethyl) phosphine (TCEP) or 15 mM oxidized glutathione (Sigma) in an attempt to acquire homogeneous populations of either reduced or oxidized species (respectively) and then further dialyzed with CaM against the suitable ITC buffer overnight.

Multiple sequence alignment

In order to reduce the potential for bias, a structure-based sequence alignment was derived using the PROMALS3D online server³¹. The IQGAP1 CHDF was pairwise aligned with crystal structures of the CH1 domains from α -actinin (1TJT), utrophin (1QAG), dystrophin (1DXX) and filamin B(2WA5).

F-actin co-sedimentation assay

Lyophilized G-actin from rabbit muscle (Sigma) was resuspended in G buffer (0.2 mM CaCl_2 , 0.2 mM DTT, 0.2 mM ATP and 5.0 mM Tris-HCl pH7.5) to a final concentration of 40 μM and stored at -80°C . For experiments, 76 μl of this G-actin stock was polymerized at room temperature for 1 h by the addition of 15.2 μl 10x AP buffer (1 M NaCl, 1 M KCl, 20 mM MgCl_2 , and 5 mM ATP 200 mM Tris-HCl pH 7.5). Then 7.6 μl of 10x G buffer and 53.2 μl ddH₂O were added to the polymerized F-actin to make a final concentration of 20 μM actin. 1x F-buffer was made from dilution of 10x G-buffer and 10x AP buffer. CHDF and CHDFK161C were diluted with buffer (300 mM NaCl, 20 mM Tris-HCl pH 8.0 and 5% glycerol to a final concentration of 97 μM with or without 9mM TCEP and incubated on ice for 48 h. 0.5 μl of 97 μM CHDF and CHDFK161C was incubated with 19.5 μl 10 μM F-actin or 1xF-buffer at RT for 30 min. Samples were then centrifuged at 18,600g at 25°C for 20 min. and the supernatants and pellets were examined by electrophoresis on an 15% SDS-PAGE at 120V for 2.5 h followed by Coomassie brilliant blue staining.

Results

Structure description

The IQGAP1 CHDF is a small globular molecule composed of 5 major α -helices (I, II, III, IV and V), 3 minor α -helices (α_a - α_c), and four short 3_{10} helices (3_{10a} - 3_{10d} ; Figure 1A left). Helices II and IV have approximately parallel axes and can be considered to form the core of the domain with helices I and III packing on opposite sides of these core helices with relative angles to the core helices of approximately -45° and 40° , respectively. Even though the axes of helices I and III are tilted with respect to the core helices (II and IV), the C-termini of all four helices are near each other indicating that the CH domain is essentially a parallel helical bundle. A long loop (residues 162 – 178) which wraps around helix I, connects helices IV and V. The CHDF is very similar to the structure of its yeast IQGAP ortholog Rng2 (Figure 1). Whereas both the CHDF and Rng2 possess an equivalent helix V, the structures of canonical CH domains such as calponin and those within the actin-binding domains (ABDs) of dystrophin, filamin, utrophin, α -actinin and others, do not have a structurally equivalent helix at this position. Therefore helix V and the loop that connects it to helix IV are extraneous to canonical CH domains and appear to be IQGAP-specific structural features.

CHDF dimer in the crystal

The P1 crystal unit cell contains four CHDF molecules arranged as two essentially identical dimers (Figure 2A). Within each dimer, the two CHDFs are arranged in parallel (“head-to-head”) with the N- and C-termini of each monomer located at one end of the dimer. The

r.m.s.d using all the Ca atoms between the two monomers in a dimer is 0.2 Å. Overall, calculation with NACCESS shows that a total of 1,347 Å² of solvent accessible surface area is buried upon dimer formation and 1,138 Å² of this area is nonpolar. The 2P2INSPECTOR online server finds no hydrogen bonds, salt bridges, or disulfide bonds at the dimer interface. Therefore, hydrophobic and Van der Waals interactions appear to be responsible for dimer stabilization. According to NACCESS, fifteen residues from each monomer have lost >10 Å² of buried surface area at the dimer interface. The ten of these that appear to be most significant in terms of % buried surface area, are shown in Figure 2B. The average solvation free energy gain upon formation of the interface calculated by PDBePISA is -12.8 kcal/mol with a P-value of 0.052 which indicates an unexpected higher-than-average hydrophobicity, suggesting that dimer formation is highly specific²⁹. The shape complementary (S_c) value for the interface is 0.635 (a value of 1.0 indicates perfect complementarity). The S_c value of CHDF dimer is not particularly high compared to constitutive protein oligomers or protein-inhibitor pairs where S_c values range from 0.7 to 0.76³⁰. However, a 0.635 S_c value is an indicator of significant surface-shape complementarity when considering that the two CHDFs that form the interface must dissociate in order to perform their F-actin and CaM-binding functions (discussed below). Yeast Rng2 has conserved dimer interface residues when compared to the CHDF, however, Rng2 is a monomer in the crystal because that protein fragment has 11 extra residues at the C-terminus which engage the potential dimer interface thereby preventing dimer formation. Presumably, the 19 extra C-terminal residues of IQGAP1 fragment 1–210 also interfere with dimerization and this is why that structure presents as a monomer in solution (NMR structure: 2RR8)¹¹.

CHDF dimer

We have seen that the CHDF crystallizes as a parallel dimer, and during gel filtration purification we found that the CHDF elutes at volumes consistent with dimers and monomers. The dimer dissociation constant (K_d) derived from an ITC dilution experiment is 22.9 μM (Figure 3). Previous studies have shown that full length IQGAP1 is able to form dimers or even higher molecule weight oligomers, and dimerization is required for its proper functions^{7, 8, 22}.

Since our small N-terminal fragment forms a dimer both in crystals and solution, and other considerations (hydrophobicity of the dimer interface, shape complementarity), we hypothesized that the dimer seen in solution is identical to that seen in crystal structure. In order to verify our hypothesis, two dimer interface substitutions were created based on analysis of the crystal structure. One substitution L157D was created to specifically disrupt dimer formation. The leucine-to-aspartate substitution not only removes two important hydrophobic residues from the dimer interface, but also introduces destabilizing charge repulsive forces. We then sought to stabilize the dimer by introducing a disulfide bond. The Ca-Ca distance of cysteines involved in a disulfide bond is usually less than 7.5 Å³². Two pairs of residues at the dimer interface satisfied this requirement: K161-K161 (6.7 Å) and A154-A154 (7.1 Å). We first made the A154C substitution, but this variant was not stable during the purification (data not shown). We next introduced the K161C substitution which can be stably expressed and can be oxidized to form a disulfide bond across the dimer interface. Oxidized glutathione can facilitate formation of the disulfide bond whereas

addition of TCEP results in reduction of the disulfide. To verify that introduction of the disulfide bond has not led to aberrant folding of that mutant, we have determined the crystal structure of CHDFK161C in the disulfide-bonded form (Figure 4). The overall structure of the CHDFK161C disulfide dimer is essentially identical to wild type dimer. In fact, the calculated average r.m.s.d. value for 328 C α atoms from each disulfide-bonded dimer vs. each wildtype CHDF dimer (4 comparisons) is 0.439 Å with no local regions exhibiting large differences (largest difference = 1.88 Å occurring at the N-terminus). In our simulated-annealing composite omit map, we see clear evidence of a disulfide bond between cysteines 161 of molecules A and B (dimer 1; see Figure 4). However, electron density between these cysteines in molecules C and D is less convincing (not shown), indicating that our crystal is heterogeneous; consisting of one oxidized dimer and one reduced or partially reduced CHDFK161C dimer.

The molecular weight of the CHDF monomer is 22.2 kDa. Gel filtration was used to monitor the monomer-dimer state of CHDFs at two different loading concentrations (100 μ M and 680 μ M). The apparent molecular weights of the CHDFs at these concentrations are reported in Table 2. L157D eluted at a volume consistent with ~ 29 kDa at both concentrations and this corresponds to the monomeric form. Wild-type CHDF eluted at 34.9 kDa at the lower concentration and 43 kDa at the higher protein concentration indicating a monomer-to-dimer equilibrium that is concentration dependent. At 100 μ M, the oxidized CHDFK161C was eluted as a dimer (54.3 kDa) as expected. The reduced form of CHDFK161C also eluted at an apparent molecular weight that corresponded to the dimeric form. This result indicates that although there is no disulfide bond, the lysine-to-cysteine substitution itself appears to stabilize the dimer state. This can be understood by inspection of the two lysines at position 161 in the CHDF structure. Apparently these two large sidechains must be in specific rotamer conformations in order to be accommodated at the dimer interface. These steric considerations would not be an issue when a cysteine occupies position 161. The observation of a dimeric species in gel filtration that is affected by specific mutations based on the crystal structure, suggests that the CHDF forms the same dimer in solution as seen in the crystal structure.

Strong F-actin binding requires exposure of the dimer interface

Since a single CH1 has been shown to bind to F-actin and two proposed F-actin binding sites (ABS1 and ABS2) are located on CH1s, we decided to compare the CH domain in the CHDF with the CH1 domains from α -actinin, dystrophin, utrophin and filamin B¹². Our structure-based sequence alignment did not reveal conserved residues that corresponded to the ABS1 in these CH1 domains, but several residues within helix IV (located at the dimer interface) are very similar to those seen in CH1 ABS2s (Figure 5A). We therefore hypothesized that dimer interface residues are important for F-actin binding and preventing access to these residues would inhibit F-actin binding. The CHDFK161C purified from overnight expression in Rosetta Gami2 typically contained ~ 40% disulfide-bonded dimer. 48 h of air oxidation increases the disulfide-bonded dimer population to ~ 70% while a 48 h incubation with 9 mM TCEP in the buffer results in 0% disulfide-bonded dimer as estimated from optical densitometry analysis of non-reducing SDS-PAGE gels (data not shown). F-actin co-sedimentation assays show that the air oxidized CHDFK161C sample

shows a decreased amount of protein in the pellet fraction (with filamentous actin) as compared to the TCEP treated sample. Importantly, the wild-type samples treated with 48 h air oxidation or 9 mM TCEP, show no difference in the amount of CHDF protein found in the pellet with F-actin (Figure 5B, 5C). This result indicates that formation of disulfide bonded dimer decreases the F-actin binding ability of the CHDF, and therefore, residues at the dimer interface are used in F-actin binding. Leu157 is a conserved residue in the ABS2 which is likely a critical residue for F-actin binding. However surprisingly, the L157D substitution does not noticeably affect CHDFL157D affinity for F-actin (data not shown).

ITC data shows that the CHDF binds to Ca^{2+} /CaM in a two-site sequential model

A previous study has shown that residues 1-232 of IQGAP1 interact with CaM in the presence of Ca^{2+} ¹⁰. We decided to use ITC to determine whether our smaller fragment (CHDF, residues 1-191) could bind to CaM. Both CaM and the CHDF were dialyzed extensively against the same buffer (containing either 10 mM EDTA or 2 mM CaCl_2) prior to use in the ITC experiments to eliminate heat changes due to unmatched buffer. In the presence of EDTA, no heat changes were detected when CaM was titrated into CHDF (data not shown). In the presence of CaCl_2 , endothermic peaks were observed during titration (Figure 6A) indicating that CaM binding to CHDF requires the presence of Ca^{2+} . Surprisingly, the ITC data could not be well fit to a one-site model (one molecule of Ca^{2+} /CaM binding to one molecule of CHDF). However, we were able to fit the data to a two-site sequential binding model, where two molecules of Ca^{2+} /CaM bind to one molecule of CHDF in an ordered fashion (see Table 3). Note, in one-site binding, the largest binding heats will occur in the first full injection – when all of the binding sites in the calorimetry cell are available for binding (by the injectant). Subsequent injections will produce smaller heats as the binding sites are saturated. In contrast, the raw data and integrated heats shown in Figure 6 are clearly not representative of single-site binding, and are instead consistent with the more complex binding scenario of two-site sequential.

CaM interaction also requires exposure of the dimer interface

Data acquired when Ca^{2+} /CaM was titrated into the cell containing CHDFL157D can also only be fitted to the two-site sequential model of binding. The binding constants were similar to wild type CHDF (Table 3). However, the thermal pattern differs: the first few peaks of the experiment were clearly exothermic as opposed to the endothermic peaks observed in wild type CHDF (Figure 6B, top panel). One possible explanation for this difference may be explained by considering the possibility that dimer dissociation is required in order for binding to occur, and the heat associated with dimer dissociation was masking these exothermic peaks in the case of the wild-type protein. This idea is supported by our dimer dilution experiment which shows endothermic heats associated with dissociation of the dimer (Figure 3). To determine if dimer dissociation is required for CaM binding, we titrated Ca^{2+} /CaM into disulfide-bonded CHDFK161C. In this experiment, no significant heats are observed indicating that the dimer interface, especially in the direct vicinity of lysine 161, is critical for CaM binding (Figure 6C). When TCEP-reduced CHDFK161C is used in ITC with CaM, we still see two-site sequential binding, albeit with a decrease in association constants (Figure 6D). This observed decrease in affinity of reduced

CHDFK161C for $\text{Ca}^{2+}/\text{CaM}$ indicates that the particular residue identity at interface position 161 is also important for strong CaM binding.

Residue cluster $^{93}\text{KK}^{94}$ is also involved in CaM binding

Since the ITC data for the CHDF vs. $\text{Ca}^{2+}/\text{CaM}$ was fitted into a two-site sequential model, we attempted to determine whether there is another binding site distinct from the dimer interface. The Calmodulin Target Database is a web server that can provide information about potential CaM binding sites given a target protein's sequence³³. This database indicated that residues 91-103 (SLKKIYDREQTRY) comprise a potential CaM binding site. One feature of CaM binding sites is basic residues bracketed by hydrophobic residues^{34, 35}. Residue cluster $^{92}\text{LKKI}^{95}$ within the identified site satisfies this requirement so we targeted $^{93}\text{KK}^{94}$ for mutation purposes. When the two adjacent lysines are mutated to glutamates, the Database no longer identifies a CaM binding site within the CHDF. ITC data indicates that $^{93}\text{EE}^{94}$ substitution significantly reduces but does not entirely eliminate CHDF binding to $\text{Ca}^{2+}/\text{CaM}$ (Figure 2, Figure 6E, Table 3).

$\text{Ca}^{2+}/\text{CaM}$ cannot compete with F-actin for binding to CHDF

Our results indicate that both $\text{Ca}^{2+}/\text{CaM}$ and F-actin utilize residues at the CHDF dimer interface for binding. Because $\text{Ca}^{2+}/\text{CaM}$, in general, negatively affects IQGAP1's interactions with other binding partners³⁶, we decided to determine whether $\text{Ca}^{2+}/\text{CaM}$ could prevent F-actin binding to the CHDF. In F-actin co-sedimentation assays, CHDF was incubated for 30 minutes with F-actin in the presence or absence of CaM in buffer containing 1mM CaCl_2 . Samples were centrifuged, and the supernatants and pellets were subjected to SDS-PAGE as described in Experimental Procedures. CHDF alone and F-actin incubated with CaM were used as controls. By comparing lanes 3 and 5, and lanes 4 and 6 in Figure 7, it can be seen that the presence of $\text{Ca}^{2+}/\text{CaM}$ does not appreciably affect the CHDF binding to F-actin. In addition, no $\text{Ca}^{2+}/\text{CaM}$ pelleted with F-actin when the CHDF was not present (Figure 7, lanes 7 and 8). These results suggest that F-actin and $\text{Ca}^{2+}/\text{CaM}$ cannot simultaneously bind to CHDF which is consistent with overlapping binding sites for the two proteins. Through use of sedimentation assays, we estimate the K_d for the F-actin/CHDF interaction to be roughly 5 μM (data not shown). This value is lower than the K_d of $\text{Ca}^{2+}/\text{CaM}$ for the CHDF, which was estimated to be 33.3 μM using ITC ($1/K_1$; see Table 3). This difference in relative affinities might explain the observation that F-actin appears to out-compete $\text{Ca}^{2+}/\text{CaM}$ for binding to the CHDF, although relative K_{on} and K_{off} rates for these interactions, which we are not estimating, should be considered as well.

Discussion

Studies using sedimentation and gel filtration have found that full length IQGAP1 forms dimers and even higher order oligomers^{7, 8, 22}. IQGAP1 can crosslink F-actin to form actin bundles^{8, 22}. Fukuta *et al.* further found that the presence of GST-Cdc42•(GTP γ S) increased oligomerization as well as the F-actin crosslinking activity of IQGAP; supporting the idea that IQGAP1 dimerization is required for crosslinking. In addition, dimerization of IQGAP1 is also required for binding and stabilizing active (GTP-bound) Cdc42²². The formation of different types of actin networks or bundles is dependent on the size, molecular architecture,

and flexibility of actin binding proteins³⁷. Two actin binding domains are required for crosslinking purpose³⁸. Some actin-binding proteins only contain one actin binding domain, therefore dimerization is indispensable to these proteins for the formation of actin bundles. Like IQGAP1, both filamin and α -actinin only have one actin binding domain (CH domain) located at the very N-terminus of each protein. Filamin A is a 280 kDa protein that dimerizes using the very C-terminal end and forms very flexible V-shaped head-to-head dimers that are able to crosslink F-actin to form actin networks^{38, 39}. α -actinin is a 102 kDa protein which uses its central rod-like repeats to form head-to-tail dimers that crosslink F-actin to form actin bundles^{37, 38}. It has been previously shown that IQGAP1 dimerizes via a region very near the IQ motifs. However, to our knowledge there has been no study designed to determine how the IQGAP1 dimer is arranged. Because we see the CHDF as a parallel dimer in three different crystal forms and we have supporting evidence that this dimer is present in solution, we feel that the full-length molecule, like filamin A, likely exists as a parallel dimer. This assertion is further supported by our own recent discovery that the GAP-related domain of IQGAP2 forms a parallel dimer that is stabilized by active Cdc42⁴⁰.

An alignment using the CHD sequences of all 3 human IQGAPs reveals an overall 68.9% identity for residues equivalent to those seen in the IQGAP1 CHDF crystal structure. This very high level of sequence identity indicates a very high structural similarity for these residues. For residues occupying crucial interface positions (highlighted in Figure 2B), the sequences in IQGAP1 and IQGAP2 are identical. This supports the notion that the IQGAP2 CHDF would form the same parallel dimer seen for IQGAP1; again consistent with results obtained for the IQGAP2 GAP-related domain⁴⁰. However, it is not certain that IQGAP3 will also form a parallel dimer since there are some differences for residues that would occupy the interface. While F148 and R151 are conservative substitutions for IQGAP1 residues Y158 and K161, respectively, R36 and T121 are not good mimics for H46 and I131 (respectively) found in IQGAP1. Within the putative ABS2 shown in Figure 5A, 74.2% of residues are identical between the three CHDs with an additional 16% being highly conserved (e.g. isoleucine vs. valine). Even higher conservation is displayed when just IQGAP1 and IQGAP2 are considered (90.3% identical). In addition, all 3 CHDs possess the equivalent of the IQGAP1⁹³KK⁹⁴ motif which we found to be important for CaM binding. Ultimately, experiments with purified proteins will be necessary to verify that these other CHDs dimerize, and bind Ca²⁺/CaM and/or F-actin.

Previous studies have reported that larger IQGAP1 N-terminal fragments are monomeric^{7, 10, 11, 41}. All of these larger fragments contain residues at the C-terminus not found in our construct. Upon examination of the NMR structure of IQGAP1 residues 2-210 and the crystal structure of the yeast ortholog Rng2, one can see that the extra 10–20 amino acids at the C-terminus bind residues that would be at the dimer interface thereby preventing dimer formation. Therefore, the existence of a “free” C-terminal tail could be the reason that these longer N-terminal fragments are monomeric. It seems quite possible that these extra C-terminal residues may be unable to access the dimerization surface of the CHDF in the context of the full-length IQGAP1 protein because they likely are involved in a structured region lying immediately C-terminal to the CHDF. Directly C-terminal of the CHDF in IQGAPs is a region containing 5 – 6 ~50 amino acid sequence repeats. Although we do not have direct structural information regarding the repeat regions of IQGAPs, we have

previously expressed and purified (including gel filtration chromatography) well-behaved protein fragments encompassing all 6 IQGAP repeats from IQGAP1 and IQGAP3, and the 5 IQGAP repeats from IQGAP2³.

There is no guarantee that this region of IQGAP1 (CHDF) is necessarily binding its counterpart in the full-length protein. However, masking a hydrophobic surface until it is required would eliminate potentially non-relevant interactions and would seem to be at least entropically favorable. Since our results indicate that complete exposure of the dimer interface is required for binding to Ca^{2+} /CaM and for full F-actin binding activity, controlled exposure and concealment of this hydrophobic surface would be a way to regulate these IQGAP1 activities. A previous study has shown that an IQGAP1 N-terminal fragment (residues 1-522) could interact with the IQGAP1 C-terminus (residues 956-1675) suggesting that this intramolecular interaction plays a regulatory role - in this case, preventing the IQGAP1 C-terminus from binding to N-WASP⁴². Two other IQGAP1 fragments (residues 956-1274 and 1276-1657) from the C-terminal half of IQGAP1 also have been shown to bind to each other; likely to effect another level of regulation⁴³. Since residues 1-522 contain the CHDF, it would be interesting to determine whether the CHDF directly contributes to binding to the IQGAP1 C-terminus, and if so, use mutagenesis to determine if the surface corresponding to the dimer interface is necessary for this activity.

Our structure-based sequence alignment reveals a potential ABS2 at the CHDF dimer interface. To our knowledge, this is the first identification of an actin binding site on a Type 3 CH domain. The fact that there is no sequence similarity to an ABS1 or an ABS3 on the CHDF implies that only an ABS2 is required to bind to F-actin. But the situation may not be this simple. A recent study has found that the isolated utrophin CH1 domain has a similar binding affinity as the intact utrophin ABD (CH1 and CH2 domains) while the isolated utrophin CH2 domain shows only very weak binding⁴⁴. The utrophin CH1 has both the ABS1 and ABS2 which is consistent with a requirement for the ABS2 for F-actin binding. However, in dystrophin the ABS3 alone (residues preceding and just within the N-terminus of CH2) was shown to interact with F-actin with a dissociation constant comparable to the intact dystrophin ABD (284 nM vs 129 nM)⁴⁵. Barring other factors that may influence binding (buffer, pH, etc.) these results might suggest that the role of particular ABS sequences in F-actin binding depends on their particular sequences and the protein (context) they reside in.

Site-directed mutagenesis of the ABS2 of α -actinin has revealed that substitutions of M127A, I128A, W129A, I131A and I132A significantly reduce F-actin binding while the mutation, D118A, has a less significant effect⁴⁶. From these results it would seem that hydrophobic interactions are the predominant driving force in ABS2-mediated F-actin binding. In our study, the L157D substitution, which converts an ABS2-conserved leucine to a charged aspartate, does not appear to affect F-actin binding (Figure 5A). We don't have a ready explanation for this result. A leucine and an aspartate are to some degree "isosteric" in that they have the same number of atoms in roughly the same configuration. Also, the L157D mutation disrupts CHDF dimer formation. Perhaps an increase in the availability of the dimer interface (afforded by L157D) and some positive Van der Waals interactions by

the isosteric aspartate balances out the negative implications of swapping a hydrophobic residue for a polar/charged residue?

Our disulfide-bonded dimer shows only partially impaired F-actin binding. One possible reason for this observation is that the disulfide bond is located at one end of the dimer interface and through a hinge-like mechanism, the disulfide-bonded form still allows some exposure of interface residues. Another possibility is that there are residues outside the putative ABS2 at the dimer interface which contribute to F-actin binding.

Several calmodulin-binding cytoskeleton proteins have shown a “flip-flop” regulation mechanism wherein in the presence of Ca^{2+} , the cytoskeletal protein will bind to CaM while in the absence of Ca^{2+} , the protein will bind to F-actin or tubulin⁴⁷. For example, dystrophin contains two putative Ca^{2+} /CaM binding sites which overlap with the ABS1 and ABS2 sites within its CH1 domain, and the presence of Ca^{2+} /CaM impairs F-actin binding to either site⁴⁵. Also, the CH1 domain of filamin interacts with Ca^{2+} /CaM and this interaction has been shown to cause the entire filamin ABD to dissociate from F-actin¹⁴. Although our results indicate that F-actin and Ca^{2+} /CaM both use residues at the dimer interface for binding, Ca^{2+} /CaM cannot displace F-actin from the CHDF. This is consistent with a previous report that F-actin can inhibit Ca^{2+} /CaM binding to IQGAP1 residues 1-232¹⁰. What might be the biological significance of Ca^{2+} /CaM binding to IQGAP1 CHDF? Some calponin homology domains have more functions than merely F-actin binding and it has been suggested that Ca^{2+} /CaM may play a role in regulating some of these functions. For instance, the single Type 3 CH domain from calponin does NOT account for the binding of calponin to F-actin, but instead, this CH3 interacts with phospholipids and the extracellular regulated kinase^{15–19}. Both Ca^{2+} /CaM and Ca^{2+} /S100 (another EF-hand Ca^{2+} binding protein) inhibit the binding of calponin to phospholipids¹⁵. Vav family CH domains are involved in the regulation of guanine-nucleotide exchange factor (GEF) activity of these proteins and participate in regulating $[\text{Ca}^{2+}]$ levels⁴⁸. The CH3 from Vav1 has been shown to be associated with Ca^{2+} /CaM using a pulldown assay, and this interaction is apparently required by T-cell mediated Ca^{2+} signaling²¹. An IQGAP1 N-terminal fragment (residues 1-160) has been shown to directly interact with the chemokine receptor CXCR2⁴⁹. Perhaps Ca^{2+} /CaM is involved in regulating the CXCR2 interaction with IQGAP1? S100P, is reported to interact with both the IQ-motif region and the IQGAP1 N-terminus (residues 1-232) in a Ca^{2+} dependent manner⁵⁰. Can S100P displace F-actin from the IQGAP1 N-terminus? Does S100P play a role in regulating IQGAP1 binding to other proteins via the N-terminus? With the continuous discovery new IQGAP1 binding partners, it is quite possible that Ca^{2+} /CaM regulates binding of an as yet unidentified protein partner

Acknowledgments

Funding Information:

This work was supported by NIH grant R01 GM084072 to DKW.

This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

Use of the Lilly Research Laboratories Collaborative Access Team (LRL-CAT) beamline at Sector 31 of the Advanced Photon Source was provided by Eli Lilly Company, which operates the facility.

Abbreviations

CaM	calmodulin
CH domain	calponin homology domain
CHDF	IQGAP1 residues 1-191 containing the entire CH domain
ITC	isothermal titration calorimetry
CH1/2/3	type 1/2/3 calponin homology domain
ABS	actin binding site
ABD	actin binding domain
S_c	shape complementary

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Figure 1. Overall structure of the CHDF is very similar to its yeast ortholog Rng2
(A) (Left to right) Crystal structure of the IQGAP1 CHDF (left) with its five major helices colored (N-to-C) blue, red, skyblue, green, and purple. Superimposed crystal structures of the CHDF (grey) and Rng2 (salmon with C-terminus in yellow), and Rng2 alone. (B) Structure-based sequence alignment of the IQGAP1 CHDF and Rng2.

Figure 2. CHDF dimer

(A) Representative "head-to-head" or parallel CHDF dimer seen in the crystal with participating monomers colored grey and light blue. Shown in magenta are $^{93}\text{KK}^{94}$ which contribute to CaM binding. (B) Common residues involved in non-bonded dimer interactions for the four monomers in the unit cell.

Figure 3. CHDF ITC dilution experiment

CHDF was dialyzed extensively against 50mM Tris pH 8.0, 100mM NaCl, 100mM KCL, 5% glycerol, 2mM β -mercaptoethanol and 0.5mM CaCl_2 at 4°C. For ITC, $29 \times 10 \mu\text{l}$ of 750 μM CHDF was injected into the VP-ITC cell containing the same buffer used in dialysis. The experiment was conducted at 20°C. Data were fitted to the “dissociation model” in Origin 7.0. χ^2 is 754.2 and the calculated dissociation constant is 0.0229 mM or 22.9 μM .

Figure 4. “Omit” 2Fo-Fc density for oxidized CHDK161C dimer 1

The CNS program ‘Composite_omit_map’ was used with torsion dynamics, 3% of the model omitted per cycle, and 500 degree starting temperature for each cycle (25 degree cooling rate) followed by 30 steps of gradient minimization before map calculation of the omitted residues. The map is contoured at 1.0σ .

Figure 5. Decreased dimer interface exposure results in decreased F-actin binding
 (A) The structure-based sequence alignment with CH1s suggests that dimer interface residues 127-157 may constitute a novel ABS2. Alignment residues colored orange or red are indicative of highly conserved or identical residues, respectively. (B) CHDF and CHDFK161C were incubated on ice for 48 h in buffer containing 0 mM TCEP or 9 mM TCEP. The treated samples were then used in F-actin co-sedimentation assays as described in Methods. The upper SDS-PAGE gel shows CHDFK161C and the lower gel is the CHDF control. Lanes 1–4 are the samples incubated in buffer without TCEP and lanes 5–8 are the samples incubated in buffer containing 9 mM TCEP. P = Pellet. S = supernatant. (C) Percentage of CHDFs in the pellet in the presence of F-actin estimated using optical densitometry (n=3). Mean values $\pm$ standard deviation (error bars) for the 3 trials are plotted. Note, 100% of WT CHDF pelleted under both the reducing and oxidizing conditions in each trial.

Figure 6. Representative ITC data for CHDFs vs. $\text{Ca}^{2+}/\text{CaM}$

Titration were conducted at 30°C using an ITC₂₀₀ calorimeter. CaM was the titrant and CHDFs were in the cell. (A) CHDF. (B) CHDFL157D. (C) oxidized CHDFK161C. (D) reduced CHDFK161C (E) CHDF^{93EE94}. (A–E) Top panels are the raw data; bottom panels are the integrated heats after subtraction of the heats of CaM titrated into buffer alone.

Figure 7. Ca^{2+} /CaM does not compete with F-actin for binding to the CHDF

(Lanes 1 and 2) CHDF incubated in 1×F-Buffer. (Lanes 3 and 4) CHDF incubated with F-actin. (Lanes 5 and 6) CHDF, CaM and F-actin incubated together. (Lanes 7 and 8) CaM incubated with F-actin. P = pellet. S = supernatant.

Table 1

CHDF data collection and refinement statistics

Data collection	WT	K161C
Space group	P1	P1
Unit cell a, b, c (Å)/ α , β , γ (°)	57.43, 66.42, 83.01/99.55 104.07 108.69	53.75, 72.46, 73.27/77.15, 72.07, 86.8
Wavelength (Å)	0.9397	1.5418
Resolution range (Å)	25–2.5	68.06–2.36
Total reflections	134,286	130,500
Unique reflections	36,983	41,528
Redundancy	3.6(3.3) ^a	3.13(1.79)
Completeness (%)	98.5 (96.9)	98.4(94.8)
I/σ ^b	17.5 (2.7)	8.63(2.14)
R_{symm} (%) ^c	9.0 (49.5)	8.47(35.04)
Refinement Statistics	WT	K161C
Resolution (Å)	25 – 2.5	15–2.36
R_{cryst} ^d (%)	23.6	26.2
R_{free} ^e (%)	26.4	28.6
No. of atoms (protein/solvent)	5336/38	5436/0
Average B factor protein atoms (Å ²)	46.0	33.7
R.m.s.d bond length (Å)	0.008	0.008
R.m.s.d bond angle (°)	1.28	1.24
ϕ/ψ (most favored/additional allowed)	94.6%/5.4%	94.6%/5.4%

^aValues in parenthesis are for the highest-resolution shell (2.59Å - 2.50Å).

^b $I/\sigma I$, mean signal to noise, where I is the integrated intensity of a measured reflection and σI is the estimated error in the measurement.

^c $R_{\text{symm}} = 100 \times \sum |I| - \langle I \rangle / \sum I$, where I is the integrated intensity of a measured reflection.

^d $R_{\text{cryst}} = \sum |F_{oh} - F_{ch}| / \sum F_{oh}$, where F_{oh} and F_{ch} are observed and calculated structure factors. amplitudes for the reflection h . The summations are over all reflections.

^eR-factor calculated for 5% of randomly chosen reflections not included in the refinement.

Table 2
Apparent molecular weight (MW) of CHDFs estimated using gel filtration chromatography

Protein concentration	wtCHDF	L157D	oxidized K161C	reduced K161C	Apparent molecular weight (kDa)
100 μ M	34.9	28.7	54.3	53.1	ND
680 μ M	43	29.1	ND	ND	

Oxidized and reduced K161C were produced via 48 h incubation with either 10 mM oxidized glutathione or 9 mM TCEP on ice to promote the complete formation or disruption of the interface disulfide bond.

Table 3ITC thermodynamic parameters of CHDFs vs Ca^{2+} CaM

Parameters	CHDF	CHDFL157D	Reduced CHDFK161C	CHDF ^{93EE} ⁹⁴
K1 ($10^4 \times \text{M}^{-1}$)	3.02±0.2	1.07±0.023	0.79±0.07	0.07±0.01
H1 ($10^4 \times \text{cal/mol}$)	0.77±0.02	−0.22±0.01	−0.19±0.02	2.11±0.29
S1 (cal/mol/deg)	45.9	11.3	11.3	82.7
K2 ($10^4 \times \text{M}^{-1}$)	2.52±0.16	1.27±0.07	0.45±0.03	0.54±0.06
H2 ($10^4 \times \text{cal/mol}$)	1.36±0.04	1.54±0.02	1.69±0.04	7.83±0.58
S2 (cal/mol/deg)	64.8	69.6	73.6	275