On the interaction between human IQGAP1 and actin

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Abstract

IQGAPs are eukaryotic proteins which integrate signals from various sources and pass these on the cytoskeleton. Understanding how they do this requires information on the interfaces between the proteins. Here, it is shown that the calponin homology domain of human IQGAP1 (CHD1) can be crosslinked with α -actin. The stoichiometry of the interaction was 1:1. A molecular model was built of the complex and associated bioinformatics analyses predicted that the interaction is likely to involve an electrostatic interaction between Lys-240 of α -actin and Glu-30 of CHD1. These residues are predicted to be accessible and are not involved in many intra-protein interactions; they are thus available for interaction with binding partners. They are both located in regions of the proteins which are predicted to be flexible and disordered; interactions between signalling molecules often involve flexible, disordered regions. The predicted binding region in CHD1 is well conserved in many eukaryotic IQGAP-like proteins. In some cases (e.g *Dictyostelium discoideum* and *Saccharomyces cerevisiae*) protein sequence conservation is weak, but molecular modelling reveals that a region of charged, polar residues in a flexible N-terminus is structurally well conserved. Therefore we conclude that the calponin homology domains of IQGAP1-like proteins interact initially through the electrostatic interaction identified here and that there may be subsequent conformational changes to form the final complex.

Key words: α -actin; protein flexibility; protein-protein crosslinking; calponin homology domain; CHD; IQGAP-like protein

Introduction

The IQGAPs are a family of cytoskeletal scaffolding proteins in fungi and animals [1-10]. The name IQGAP is derived from two of the domains present in this group of proteins: the IQ, and the Ras GTPase-activating (GAP) related domains. They link a diverse range of signalling pathways, including calcium-calmodulin, small GTPases, kinases and growth factor receptors to the actin cytoskeleton [11-24]. In addition, they also help control the microtubule system indirectly through CLIP-170 and APC [25, 26]. Not surprisingly, since they sit at the interface of many cellular processes, they have been implicated in diseases including cancer, heart disease and infection by bacteria and viruses [27-33]. In the last decade, considerable advances have been made in the understanding of the IQGAP family's cellular roles. This is especially true of the best characterised member of the family, human IQGAP1. However, many molecular-level details remain to be discovered. Although high resolution structures are available for some domains, there is no structure of a complete IQGAP; therefore there is little information about how the various domains in the protein communicate [34-37]. There is also only limited data available on how the protein interacts with its many binding partners. Such information would illuminate cellular level studies and may be of value in the event that it is shown to be desirable to disrupt these interactions for the development of novel anti-cancer or anti-infective drugs.

It is known that interaction with actin occurs through the calponin homology domain (CHD), located at the N-terminus of the protein [13, 38, 39]. The functional consequence of this interaction is the bundling of actin filaments [40-42]. The CHD also provides an interaction site for calmodulin, although this protein interacts primarily through IQ-motifs located towards the middle of the primary sequence [13, 43, 44]. Here a crosslinking protocol was used to confirm the actin/CHD interaction and determine its stoichiometry. Molecular

modelling of the complex between the two molecules was undertaken to predict the site of interaction.

Materials and Methods

Proteins

A protein containing residues 2-215 from human IQGAP1 (which encompass the CHD and is referred to as CHD1 hereafter) was expressed in, and purified from, *Escherichia coli* as described previously [44]. Rabbit skeletal muscle actin was purchased from Cytoskeleton Inc. (Denver, CO, USA) as freeze dried powder. The powder was reconstituted in G-actin buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM CaCl₂, 0.2 mM ATP, 0.5 mM DTT) to a final concentration of 1 mg ml⁻¹, placed on ice for 60 min and then centrifuged at 13,000 g at 4 °C for 15 min. The supernatant was removed and assumed to be G-actin. Protein concentrations were estimated by the method of Bradford [45], using BSA as a standard.

Crosslinking

Actin (23 µM) and CHD1 (27 µM) were mixed and incubated at 37 °C for 60 min. After this time, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was added to a final concentration of 70 mM and the reaction allowed to proceed for 60 min. The reaction was stopped by the addition of SDS-PAGE loading buffer (120 mM TrisHCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 5% (w/v) bromophenol blue, 1% (w/v) DTT) and heating at 95 °C for 5 min. Results were analysed by SDS-PAGE.

Mass spectrometry

The gel band was excised and cut into 1 mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot, using standard protocols.

Briefly the gel cubes were destained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37°C. The peptides were extracted with 10% formic acid and concentrated down to 20 µL (SpeedVac, ThermoSavant). They were then separated using an UltiMate nanoLC (LC Packings, Amsterdam) equipped with a PepMap C18 trap & column, using a 60 min gradient of increasing acetonitrile concentration, containing 0.1 % formic acid (5-35% acetonitrile in 35 min respectively, 35-50% in a further 20 min, followed by 95% acetonitrile to clean the column). The eluant was sprayed into a Q-Star XL tandem mass spectrometer (Applied Biosystems, Foster City, CA) and analysed in Information Dependent Acquisition (IDA) mode, performing 1 sec of MS followed by 3 sec MSMS analyses of the 2 most intense peaks seen by MS. These masses are then excluded from analysis for the next 60 sec. MS/MS data for doubly and triply charged precursor ions was converted to centroid data, without smoothing, using the Analyst QS1.1 mascot.dll data import filter with default settings. The MS/MS data file generated was analysed using the Mascot 2.1 search engine (Matrix Science, London, UK) against the UniRef 100 database, Jan 2008 (5247086 sequences) with no species restriction. The data was searched with tolerances of 0.2 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation selected as a variable modification.

Molecular modelling of the actin and IQGAP1 CHD interface

Models of human IQGAP1 residues 2 - 215 and rabbit skeletal muscle α -actin (which is 100% identical to the human α -actin sequence; UniProt accession numbers: human P46940, rabbit P68135) were constructed using the Phyre2 server in intensive mode [46] and displayed using PyMol (www.pymol.org). These were then minimised using the YASARA force field [47]. Patchdock was utilised in the docking of ADP with actin and Firedock for

refining of these results [48, 49]. The first, most energetically favourable result was taken and minimised using YASARA. Protein flexibility was predicted using FRODAN to generate 500 conformers in the momentum mode [50]. The HADDOCK flexible docking server was used to model potential interactions between actin and CHD1 [51]. The postulated binding regions were used as targets and HADDOCK was set to define its own passive interacting residues. Only the most energetically favourable structure was used. Models were subjected to the structure validation tools of the WHATIF server to check for potential collisions and to validate bond lengths, planarity and dihedral angles of residues [52]. The xWalk algorithm was used to calculate Euclidean distances between the potential cross linked residues in the HADDOCK structure, whilst the ESBRI and PIC solvent accessibility tools allowed calculation of potential salt bridges and solvent accessibility of the residues respectively [53-55]. Residue accessibility was calculated using GETAREA [56].

Multiple sequence alignment, structural alignments, residue conservation and in silico prediction of probable interacting residues

Multiple sequence alignment of CHDs was carried out using Clustal Omega [57, 58]. Sequences were obtained from NCBI using the psiBLAST tool to obtain distant members of the eukaryotic lineage [59]. Sequences that were true IQGAP1 homologues and IQGAP1-like proteins were used as well as the other human and mouse isoforms. The alignment was used along with the Scorecons server to calculate residue conservation at each position based upon the Valdar01 score [60]. The EMBOSScons tool (<http://www.ebi.ac.uk/emboss>) allowed for a consensus sequence of the region to be deduced. Models of the aligned sequences were built in Phyre2 and minimised for use in structural alignments. These were carried out in PyMol using the align function. The WHATIF server was used to calculate residue accessibility in both actin and CHD1 [52]. The programs DisEMBL and DISOclust were employed to

calculate intrinsically disordered domains in the models [61, 62]. The ProFace server was used to analyse the HADDOCK model and to provide the most probable interface residues [63]. NNcon is a program that was used to analyse and provide a readout of intraprotein interactions. Therefore, it was used to identify residues which were not involved in intraprotein contacts and which will, therefore, be more available for protein-protein interactions [64].

Prediction of the biochemical effects of missense mutations on the IQGAP1 CHD

The WHATIF server was used to mutate the potential cross linked residues in the CHD1 to alanines. The resulting model was subject to analysis using the MuPro, SNPeffct and Site directed mutator programs in order to predict potential effects on the protein's functionality [52, 65-67]. The SNPeffct server uses four different programs: TANGO looks at how a given mutation affects the propensity of a protein to aggregate, WALTZ focuses on the effects the mutation may have on the tendency of a protein to form amyloids, LIMBO looks at changes in a proteins ability to bind chaperones and FOLDX analyses the overall effects of the mutation on the proteins stability. The mutated CHD model was also docked to actin using HADDOCK (as described above for the wild type protein) in order to predict changes in predicted energies and root mean square deviations (rmsd).

Results

Actin and the CHD from IQGAP1 can be crosslinked

When actin and CHD1 were mixed in the presence of the crosslinker EDC, an additional band was observed at ~70 kDa (figure 1). This band did not appear when the proteins were mixed in the absence of crosslinker or when each protein alone was exposed to EDC. Therefore, it is likely to correspond to the covalently linked actin-CHD1 complex. The

estimated molecular mass of the complex is consistent with it containing one molecule of actin (42 kDa) and one of CHD1 (26 kDa including the hexahistidine tag).

Bands were excised from these gels and subjected to in-gel digestion with trypsin and the resultant peptides analysed by mass spectrometry. Bands corresponding to CHD1 plus crosslinker and actin plus crosslinker resulted in 22 and 26 peptides with Mascot Ion Scores above the Identity threshold (95% confidence) matching to each sequence respectively. In the band corresponding to the postulated crosslinked complex, peptides from both actin (16 peptides) and CHD1 (7 peptides) could be identified. This confirmed that the additional band did, indeed, correspond to an actin-CHD1 complex. Interestingly, only peptides from α -actin were isolated; therefore bioinformatics and modelling studies concentrated on this isoform.

CHD1 and actin models were docked to predicted interaction sites

Phyre2 modelled both proteins with greater than 90% accuracy and these were virtually indistinguishable from the experimental structures following minimisations and alignment. Both models were validated successfully by the WHATIF server.

The HADDOCK server docked the structure and the first, most favourable result, with a Z-Score of -2.2 was taken and minimised using YASARA. xWalk was then utilised to find the closest point of contact and this was shown to be between Lys-240 of actin and Glu-30 of CHD1 at a Euclidean distance of 5.2 Å. The HADDOCK predicted complex is shown in Figure 2. The top complex highlights residue Lys 240 of actin and residues Glu 29, 30, 33, and Asp 32 of CHD whilst the bottom complex presents the docked structure with a semi-transparent overlay of the surface of the two proteins.. xWalk showed that the solvent accessible surface distance is 4.5 Å; a similar result was calculated by ESBRI (4.45 Å). The

192 PIC solvent accessibility tool validated these residues as being capable of ionic interaction.
 193 While this alone does not prove that these two residues are part of the interaction site it is
 194 consistent with the favourable HADDOCK energies associated with the docking process
 195 (Table 1). Thus the model shown in figure 2 is a feasible representation of part of the
 196 interface between actin and CHD1.
 197
 198 Any interaction site must be accessible in order to take part in a given interaction either
 199 directly or following a conformational change exposing the site. Given this, the relative
 200 accessibility of residues can be a good indicator of whether, or not, they can take part in
 201 initial interactions. The accessibility of the actin and CHD1 residues of interest are given in
 202 Table 2 as side, back and total overall residue accessibility. Of particular interest are charged
 203 residues in the potential binding sites which are potentially crosslinkable by EDC. Residues
 204 Glu-239 and Lys-240 of actin showed good overall accessibility (27.7 and 37.2 Å² total
 205 accessibility respectively) as did the residues Glu-29, Glu-30, Glu-33 and Asp-32 (41.7, 38.0,
 206 13.5 and 44.8 Å² total accessibility respectively) of CHD1. It therefore seems likely that all
 207 these residues are available and are potentially involved in the initial interaction. The Proface
 208 analyser provides a readout of residues involved in the interface of the docked structure. The
 209 results from this prediction are shown in Supplementary Figure S1 and include residues 28,
 210 30, 31, 32, 33, 34 from CHD1 and residues 237, 239 and 240 from the actin. The NNcon
 211 program showed that up to residue 180 in actin there was generally potential for >10
 212 intraprotein contacts between each amino acid and others within the protein. In some cases
 213 there was >40 predicted interactions. After residue 180, the number of potential interactions
 214 per residue fell with some not taking part in any. For the putative actin site, residues 237,
 215 238, 239 and 240 were shown to take part in 0, 4, 4 and 5 intraprotein interactions
 216 respectively. For CHD1, a block of residues from 21-26 and 28-43 inclusive are predicted to

not be involved in any intraprotein interactions. These are the only large blocks of this nature in the whole of the CHD1. Thus, the predicted binding regions in both proteins are free to take part in protein – protein interactions.

The putative binding sites are predicted to be highly flexible

The interaction sites between proteins often involve highly mobile, flexible regions. This is often the case in signalling proteins where high specificity but short-lived interactions are required [68-70]. When interpreting flexibility predictions it is important to look at the flexibility of localised regions as opposed to limiting the analysis to individual residues. FIRST/FRODAN predicted that CHD1 has three highly flexible regions, which are the termini and a zone localised around residues 92-110 (Figure 3a). However, the N-terminus shows extraordinary flexibility and this extends almost as far as residue 40 and peaks at an rmsd of 9.2 Å for Arg-24 (Figure 3b). The results for actin are equally intriguing. It is clear that it is a highly dynamic molecule but two regions stand out, which are those around residue 50 and the larger region spanning residues 224-250. The rmsd peaked at 7.8 Å at Ala-233. The regions correspond to the two lobes in actin with the one around residue 50 being the DNase I binding loop [71]. The predicted interaction regions from both proteins therefore lie within the predicted areas of greatest flexibility. It is possible that, given the high flexibility of the two regions, all residues interact to varying extents by transient hydrogen bonds and other non-covalent interactions. This may therefore be the initial interaction that takes place between CHD1 and actin before a conformational change exposes other actin binding sites.

The CHD1 and Actin sites show high levels of intrinsic disorder

Intrinsic disorder was first calculated using DisEMBL which carries out these predictions based upon three definitions of disorder [61]. Both actin and CHD1 disorders were calculated for the loops/coils, hot loops and remark-465 definitions (Supplementary Figure S2). For CHD1 there is extensive disorder for the loops/coils definition, significantly less for the hot loops and none for the remark-465 definition (Supplementary Figure S2a). The remark-465 definition is less relevant in this case as it is generally only compatible with actual X-ray data [61]. The hot loops definition is the most significant as this is a refinement of the first loops/coils data. For CHD1, the fragment LTAEEM is implicated as being disordered. For actin the results are much more profound. Only 13% of actin is disordered by the hot loops definition and this includes the region TAASSSSLEK.

The DISOclust results add weight to the above predictions (Figure 3c,d) because they also implicated the postulated regions. DISOclust scores are only stated to be of significance if they exceed the 50% threshold mark [62]. The CHD1 plot looks not unlike the FRODAN results and it is common for disordered regions to also be highly flexible due to the lack of three-dimensional structure. The postulated CHD1 binding site clearly lies within the disordered region. Apart from the ends, only one other region in actin exceeds the DISOclust threshold, residues 227-244 (Figure 3c).

Taken together, these predictions show that both the actin and CHD1 sites form part of intrinsically disordered domains that are surface exposed. This may also implicate these regions in binding of other partners.

In silico mutagenesis of the CHD1 site is stabilising and preserves overall structure, but is predicted to reduce interaction with actin

The residues Glu 29, 30, 33 and Asp 32 of CHD1 were mutated *in silico* to alanines using the WHATIF mutate a residue tool [52]. MuPro [65] was used to analyse the effect of each of the above mutations individually by predicting the change in the conformational stability of the protein ($\Delta\Delta G$ value). MuPro predicted that each mutation would individually cause an increase in the stability of the protein. The original and WHATIF model were then submitted to the SDM server [67] and this too predicted that the protein would increase in stability but also that it would most likely to be non-functional as a result (data not shown).

The SNPeffect server showed that the mutations have no effect on aggregation tendency, amyloid propensity or the ability of the protein to bind chaperones. The FOLDX component, however, predicted that the protein would increase slightly in stability. The fact that these mutations were predicted to cause an increase in stability was surprising since it is common for amino acid changes to destabilise proteins. However, in this case, it is possible that the mutations cause an increase in stability via an elimination of charge repulsion between the glutamates and aspartate. This, combined with the shorter alanine side chain, may enable the protein to adopt a slightly more compact and stable structure that does not cause malign effects such as aggregation. The loss of these potential interacting residues, the stability changes and likely decrease in flexibility were postulated to diminish interaction with actin.

The mutated structure was taken and docked with wild type actin using HADDOCK using the same docking parameters as wild type. The results were intriguing (Table 1) as there was a substantial drop in electrostatic and Van der Waals energies, with the mutant-actin complex having less than half the electrostatic energy of the complex with wild type. It is clear that docking of the mutant is an unfavourable process compared to the wild type.

291 *Implications for calponin homology domains from IQGAP-like proteins from other species*
292 Sequence alignments, between analogous proteins from different species, often identify the
293 functional, structural and/or evolutionary relationships between them [72]. Actin is one of the
294 most conserved eukaryotic proteins and as such it would be difficult to deduce potential
295 binding sites from structural and sequence information alone. CHD1 is not as strongly
296 conserved and some inferences can be drawn from its alignment. Sequences were compared
297 for the human IQGAP isoforms and lower eukaryotes such as *Trachipleistophora hominis*
298 and *Caenorhabditis elegans* (Supplementary Figure S3). It is be immediately obvious that
299 there is reasonable conservation of the region, especially the aspartate and adjacent
300 glutamate, and also a block of arginine residues following the postulated interaction region.
301 These arginines may also be involved in the interaction. When the EMBOSScons sequence
302 tool was used, it generated VDDERLSAEEMDERRR as the consensus sequence. The
303 Scorecons server shows 0.413, 0.627, 0.685, 0.612, 0.684, 0.921, 1.000, 0.912, 0.611, 1.000
304 and 0.640 as conservation values for the residues LTAEEMDERRR. In many cases, when a
305 residue is not conserved in a sequence it is replaced by an amino acid that could carry out the
306 same function e.g. polar threonine for serine, aspartate instead of glutamate and glutamine or
307 lysine instead of arginine.
308
309 However, when the CHD from the budding yeast (*Saccharomyces cerevisiae*) IQGAP-like
310 protein, Iqg1p, is aligned the following sequence lines up with the postulated actin binding
311 region: KYTVDLNYSKIELRYYEF. This does contain largely polar and charged residues
312 and could be involved in the interaction, but the lack of precise sequence alignment means
313 that there must be differences between this protein and those from higher eukaryotes.
314 However, given that sequence defines structure, structure defines function and that different
315 sequences may produce similar overall structures, the structural alignment of proteins is often

a much better indicator of deducing functionally equivalent sites. Therefore, Phyre2 was used to construct models of CHDs from a variety of species and, following minimisation, these were aligned to the human structure using PyMol. The predicted structures of human CHD1 aligned with the equivalent regions from IQGAP-like proteins from *C. elegans*, *Hydra vulgaris*, and *S. cerevisiae* are shown in Figure 4a,b,c. Although the *S. cerevisiae* sequence aligns only poorly with the human one, it is clear that the structures do align and, interestingly, Asp-93 aligns with the postulated actin-binding domain in human CHD1 (Figure 4c). In all three cases the N-termini of the CHDs are the unstructured, intrinsically disordered regions of the proteins; this was supported by DISOclust and DisEMBL predictions (data not shown).

As a further test of this hypothesis, the IQGAP-like protein from the soil amoeba *Dictyostelium discoideum* was investigated (gapA; Uniprot O00899) [73, 74]. The significant feature of this protein is that it does not have any regions that are recognised as CHDs. Therefore, residues 1-216 were taken and a Phyre2 model produced and subsequently aligned. The resulting model (Figure 4d) is clearly different from others, but alignment with human CHD1 showed that the N-terminus of *D. discoideum* gapA aligned reasonably well with the postulated binding site in the human protein; coincidentally this is also a negatively charged region with an abundance of polar residues (Figure 4d). So, although *D. discoideum* has no recognised CHD, it shows some structural similarity and, therefore, may bind actin through a novel, functionally equivalent domain.

Discussion

It has been proposed that there are two actin binding domains used by some CHD-containing proteins [75-78]. However, in IQGAP1, one CHD is sufficient for actin binding [39]. The

experimental work presented here demonstrated that the stoichiometry of the interaction is 1:1. Here we propose that the regions identified in this work (and the polar, intrinsically disordered analogous regions in other IQGAP-like proteins) form at least part of the initial interface between the proteins. We believe this could be a lower affinity interface associated with transient hydrogen bonds and electrostatic interactions that precedes conformational change and tighter binding at other sites. We further note that calmodulin has been previously predicted to interact with the same part of the CHD and speculate that direct competition for the binding site may be a regulatory mechanism [44]. Further analyses are necessary to fully characterise the interactions between these proteins.

Acknowledgements

DM thanks the School of Biological Sciences, Queen's University, Belfast for a summer studentship and EH thanks the Department of Employment and Learning, Northern Ireland for a postgraduate studentship. The work was funded in part by grants from the BBSRC (BB/D000394/1 To DJT) and by the Wellcome Trust [grant number GR06281AIA] which funded the purchase of the QStar XL mass spectrometer at the BBSRC Mass Spectrometry and Proteomics Facility, University of St Andrews and funded SLS.

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Figure legends

Figure 1: Crosslinking of the CHD of human IQGAP1 (CHD1, 27 μ M) and actin (23 μ M) by EDC. The products of the reaction were resolved by 12.5% SDS-PAGE and visualised by staining with Coomassie blue. M, molecular mass markers (masses are shown to the left of the gel in kDa); A, actin; C, CHD1; AC actin and CHD. In the control lanes, CHD1 (27 μ M) and actin (23 μ M) were incubated with crosslinker under identical conditions. The additional bands in the actin lane most likely represent actin dimers and oligomers. Note that, due to the equilibrium nature of the interaction and the short half-life of EDC, the crosslinking reaction does not go to completion. Therefore, some non-crosslinked product remains visible in lane AC.

Figure 2: Predicted structure of the CHD1/ α -actin complex. α -Actin is shown on the left of both space-filling (top) and cartoon (bottom left) models in green. An enlarged image of the potential interaction site and key residues is also shown (bottom right). α -Actin residue labels are in green whilst those belonging to CHD1 are in blue.

Figure 3: FIRST/FRODAN flexibility predictions for (a) CHD1 and (b) α -actin. DISOclust predicted disordered regions in (c) CHD1 and (d) α -actin

Figure 4: Alignments of the predicted structures of the CHD from the IQGAP-like proteins from (a) *C. elegans*, (b) *H. vulgaris* and (c) *S. cerevisiae* with human CHD1 (green in all three alignments). (d) *D. discoideum* IQGAP-like protein gapA has an atypical CHD-like structure at the N-terminus. Structural alignment of this domain with human CHD1 showing coincident polar, charged residues (human CHD1 in green).

584 Supplementary Figure S1: Results from the Proface server revealed surface-exposed,
585 potentially interacting residues.

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587 Supplementary Figure S2: Results from DisEMBL reveal potentially flexible residues
588 (highlighted in yellow).

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590 Supplementary Figure S3: Protein sequence alignments of the N-termini of IQGAP-like
591 proteins from a variety of species.

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