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Biochemical analysis of the interactions of IQGAP1 C-terminal domain with CDC42

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Author contributions: Elliott SF carried out all protein expression and purification and the crosslinking experiments; Elliott SF and Allen G jointly performed the surface plasmon resonance measurements; Elliott SF, Allen G and Timson DJ analysed these data; Timson DJ carried out the molecular modelling work, was responsible for the overall design of the study, obtained research grants to support the work and wrote the manuscript.

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Abstract

AIM: To understand the interaction of human IQGAP1 and CDC42, especially the effects of phosphorylation and a cancer-associated mutation.

METHODS: Recombinant CDC42 and a novel C-terminal fragment of IQGAP1 were expressed in, and purified from, Escherichia coli. Site directed mutagenesis was used to create coding sequences for three phosphomimicking variants (S1441E, S1443D and S1441E/S1443D) and to recapitulate a cancer-associated mutation (M1231I). These variant proteins were also expressed and purified. Protein-protein crosslinking using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was used to investigate interactions between the C-terminal fragment and CDC42. These interactions were quantified using surface plasmon resonance measurements.

Molecular modelling was employed to make predictions about changes to the structure and flexibility of the protein which occur in the cancer-associated variant.

RESULTS: The novel, C-terminal region of human IQGAP1 (residues 877-1558) is soluble following expression and purification. It is also capable of binding to CDC42, as judged by crosslinking experiments. Interaction appears to be strongest in the presence of added GTP. The three phosphomimicking mutants had different affinities for CDC42. S1441E had an approximately 200-fold reduction in affinity compared to wild type. This was caused largely by a dramatic reduction in the association rate constant. In contrast, both S1443D and the double variant S1441E/S1443D had similar affinities to the wild type. The cancer-associated variant, M1231I, also had a similar affinity to wild type. However, in the case of this variant, both the association and dissociation rate constants were reduced approximately 10-fold. Molecular modelling of the M1231I variant, based on the published crystal structure of part of the C-terminal region, revealed no gross structural changes compared to wild type (root mean square deviation of 0.564 Å over 5556 equivalent atoms). However, predictions of the flexibility of the polypeptide backbone suggested that some regions of the variant protein had greatly increased rigidity compared to wild type. One such region is a loop linking the proposed CDC42 binding site with the helix containing the altered residue. It is suggested that this increase in rigidity is responsible for the observed changes in association and dissociation rate constants.

CONCLUSION: The consequences of introducing negative charge at Ser-1441 or Ser-1443 in IQGAP1 are different. The cancer-associated variant M1231I exerts its effects partly by rigidifying the protein.

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Key words: CDC42; Cytoskeleton; Protein phosphorylation;
Cancer-associated mutation; Protein-protein interaction

**INTRODUCTION**

The IQGAP family of proteins function at the interface between cellular signalling and the cytoskeleton[1-3]. They receive information from a variety of signalling molecules, including kinases, small GTPases, growth factor receptors and calcium sensors[4-21]. This information is relayed directly to the actin cytoskeleton through interaction with filamentous actin (F-actin) which promotes filament bundling and caps the “barbed ends” of the filaments[22-25]. There are also indirect influences on the actin cytoskeleton mediated through the Wiskott-Aldrich Syndrome Protein (WASP) family[26,27] and with microtubules mediated via cytoplasmic linker protein 170 (CLIP-170) and adenomatous polyposis coli (APC) protein[27,28]. The IQGAP proteins are named after two key regions within them—the calmodulin binding IQ-motifs and GTPase activating protein (GAP) related domain (GRD). Although the GRD binds CDC42 in a crosslinking experiment. We then de

also anticipated that there is considerable capacity for conformational change in the molecule in order to receive, integrate, interpret and output signals. The structures of some isolated domains have been determined. The structure of the CHD from human IQGAP1 has been solved by NMR spectroscopy and an x-ray structure of part of the GRD is also available[31,39]. Molecular modelling has predicted largely α-helical structures for the IQ-motifs[22-25].

*In vitro* biochemical studies on IQGAPs have tended to rely on fragments of the protein which are amenable to recombinant expression and purification in bacterial systems. CDC42 and Rac1 interaction with the GRD is promoted by the presence of GTP[4]. Phosphorylation of human IQGAP1 at Ser-1443, however, promotes interaction with CDC42 in the absence of nucleotide[36]. This phosphorylation, along with one at Ser-1441, promotes outgrowth of neurites[37].

Given the protein’s involvement in the transduction of information from signalling pathways to the cytoskeleton, it is not surprising that it has been implicated in various types of cancer[38,39]. However, only one cancer-associated mutation in the coding sequence of the *iqgap1* gene has been identified; this mutation results in the amino acid change M1231I[36]. It is not clear how this change affects the function of IQGAP1, although it does lie in the GRD prompting the hypothesis that it interferes with GTPase binding. However, this has not been tested experimentally.

Here, we identified a novel, biochemically amenable fragment from the C-terminal region of human IQGAP1 and confirmed that it is active, as judged by its ability to bind CDC42 in a crosslinking experiment. We then describe a detailed, quantitative investigation into the affinity of this interaction in the absence of added GTP. To probe the molecular consequences of phosphorylation in this region we used “phosphomimicking” variants in which serine residues are replaced with negatively charged ones. We also recapitulated the cancer-associated variant M1231I in order to investigate its binding properties and carried out molecular modelling studies to provide further understanding the consequences of this alteration.

**MATERIALS AND METHODS**

*Expression and purification of wild type and variant human IQGAP C-terminal region*

The sequence encoding amino acids 877-1558 in human IQGAP1 was amplified by polymerase chain reaction
Expression and purification of human CDC42

The complete coding sequence of human CDC42 was amplified by PCR using IMAGE clone 3626647[41] as a template and inserted into pET-46 EK/LIC. The DNA sequence of the insert was verified. The expression and purification of the protein was carried out using the same protocol as for IQGAP1-CTD.

Crosslinking of CDC42 and IQGAP

GTP bound CDC42 was prepared by incubating a mixture of 6 μmol/L CDC42, 0.9 mmol/mL GTP and 0.9 mmol/L magnesium chloride on ice for 30 min. Nucleotide-depleted (ND) CDC42 was prepared by incubating 6 μmol/L CDC42 with 5 mmol/L EDTA on ice for 30 min. Protein-protein crosslinking was carried out using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Methods were based on those previously used for the detection of interaction between the atrial myosin essential light chain and F-actin[45,46]. Untreated, GTP-loaded or ND CDC42 (3 μmol/L) was mixed with IQGAP(ADR6) (3 μmol/L) and incubated for 30 min at 22°C. EDC was then added to a final concentration of 0.6 mmol/L and the incubation continued for a further 60 min. Products were analysed by SDS-PAGE.

Surface plasmon resonance

Surface plasmon resonance was measured using a BiAcore 3000 instrument (BIAcore, Uppsala, Sweden). Prior to analysis all proteins were dialysed into HBS Buffer (BIAcore; 10 mmol/L Hepes, pH 7.4, 150 mmol/L NaCl). CDC42 was immobilised onto a CM5 sensor chip (BIAcore) using N-hydroxysuccinimide (NHS)/EDC chemistry. The surface was activated with a mixture of 100 mmol/L NHS and 400 mmol/L EDC for 30 min. CDC42 (25 μmol/L) was then flowed over the surface for two 7 min periods and the surface was then blocked and deactivated with 1 M ethanolamine for 30 min. Immobilisation of CDC42 resulted in a change in the response units (RU) of approximately 1400 RU.

Binding was measured by flowing 0.5 μmol/L to 2.5 μmol/L IQGAP(ADR6) over the surface for 300 s (association phase) followed by buffer for 300 s (dissociation phase). In between binding measurements, the surface was regenerated by the injection of sodium hydroxide (5 mmol/L for 220 s). For each binding measurement controls were carried out in parallel in which the protein was flowed over a cell which had been activated with NHS/EDC and blocked with ethanolamine. To determine the response due to interaction between IQGAP(ADR6) and CDC42, the readings for the controls were subtracted from the experimental ones. The association and dissociation rate constants (k0 and k1, respectively) and the dissociation equilibrium constant (K0) were determined by non-linear curve fitting of the data using BIAevaluation software.

Molecular modelling

The structure of human IQGAP1, residues 962-1345 (PDB 3FAY[34]) was taken as a starting point for molecular modelling studies. This structure file describes one, unbroken polypeptide chain. The selenomethionine residues in this structure were altered to methionine using PyMol (www.pymol.org) and the resulting structure energy minimised using YASARA[47]. Residue 1231 in this minimised structure was altered to isoleucine, and the mutated structure
Identification of a biochemically tractable C-terminal fragment of human IQGAP1

Previous reports demonstrated that a fragment beginning at residue 864 and continuing through to the C-terminus of the protein (residue 1657) can be expressed in, and purified from, E. coli, albeit at relatively low levels\textsuperscript{[48,49]}. We noted that the structure of the Ras-GAP C-terminal domain (RGD), a 112 amino acid residue region at the extreme C-terminus of the protein has been deposited in the Protein Data Bank (PDB ID: 1X0H). From this we reasoned that there must be a domain boundary in the region of residue 1545. Therefore, a region beginning at residue 877 and finishing at 1558 was expressed. This fragment, which we named IQGAP1(DR6), can be purified with good yield, typically 1-2 mg per litre of E. coli culture (Figure 2A). Similar purities and yields were achieved with the various variant proteins also described in this work (data not shown). Full length, recombinant, human CDC42 could also be purified in good yield (Figure 2B).

Interactions between CDC42 and IQGAP1(DR6)

Recombinant, human CDC42 was shown to interact with the C-terminal domain fragment. The two proteins could be cross-linked using the reagent EDC which is specific to carboxylate and amino groups (Figure 3). This demonstrates that the recombinant C-terminal fragment is likely to be folded and is functional. The amount of crosslinked product was greatest in the presence of GTP (Figure 3).

Effects of phosphomimicking mutations

To investigate the effects of phosphorylation at serines 1441 and 1443, the phosphomimetic variants S1441E, S1443D and S1441E/S1443D were constructed. These amino acid changes insert negative charges into the structure at the sites which can be phosphorylated. Similar mutants have been shown to recapitulate the effects of phosphorylation in an in vitro cell model\textsuperscript{[37]}. Since it has been hypothesised that phosphorylation increases the affinity for CDC42 in the absence of GTP\textsuperscript{[38]}, this interaction was investigated by surface plasmon resonance. Interaction between the wild type and immobilised CDC42 could be detected by surface plasmon resonance in the absence of added GTP (Figure 4). Fitting of these data resulted in rate constants for the association and dissociation phases of the reactions ($k_a$ and $k_d$) and, consequently, a value for the dissociation constant ($K_D$) (Table 1). It was noted that these fits were not perfect with some non-random residuals (not shown). This may indicate that there is heterogeneity in the preparations and/or that the binding event is more complex. However, for the purposes of comparison, the simple bimolecular interaction model was used.

RESULTS

Identification of a biochemically tractable C-terminal fragment of human IQGAP1

Energy cut off of -1 kcal/mol

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Figure 2 Proteins used in this study. The recombinant expression and purification of (A) IQGAP1(DR6) and (B) CDC42 monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In both (A) and (B), U, extract from cells prior to induction; I, extract from cells 2-3 h after induction; S, soluble material remaining after sonication; F, material which flowed through the column without binding; W, material removed in the washing steps; E, elutions; M, molecular mass markers (with their masses shown to the side of the gel in kDa). In the case of CDC42, F and W were combined into a single sample.

re-minimised using YASARA. Polypeptide flexibility was estimated by generating 500 conformers in the momentum motion type mode of FIRST/FRODAN with an energy cut off of -1 kcal/mol\textsuperscript{[48,49]}.

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Figure 3 Interaction between IQGAP1 and CDC42. An interaction between IQGAP1(DR6) and CDC42 can be detected by EDC crosslinking in the presence of GTP. In the absence of EDC (lanes headed -EDC), a mixture of IQGAP(DR6) and CDC42 (lane C) behaves the same on SDS-PAGE as IQGAP1(DR6) and nucleotide-depleted CDC42 (lane ND) and as IQGAP1(DR6) and GTP-loaded CDC42 (lane GTP). In the presence of the crosslinker (lanes headed +EDC), crosslinking was not observed between IQGAP1(DR6) and CDC42 (C) or IQGAP1(DR6) and nucleotide-depleted CDC42 (ND). However an additional band, corresponding to approximately the combined molecular masses of IQGAP(DR6) and CDC42 is seen with GTP-loaded CDC42 (GTP).
All three phosphomimic variants also bound to CDC42 in the absence of additional nucleotide. However, in the case of S1441E, the affinity was reduced by two orders of magnitude. This arises mainly because of a reduction in the association rate constant. It should be noted that this reduced value (1.2 \text{L.mol}^{-1}\text{s}^{-1}) is very low and, therefore, may be subject to greater error than the other values. Interestingly, the double mutant (S1441E/S1443D) binds with a similar affinity to the wild type (Table 1).

Effects of the cancer-associated mutation, M1231I

The ability of the disease-associated variant to interact with CDC42 was tested by surface plasmon resonance. These experiments suggest that it is able to do so with similar affinity to the wild type protein. However, both the association and dissociation rate constants are reduced compared to wild type (Table 1).

To help understand the biochemistry of the M1231I variant protein, a molecular model was constructed based on the crystal structure of the GRD. This suggested that the overall fold is not greatly changed by the substitution of this methionine for isoleucine (rmsd between the wild type and variant protein 0.564 Å over 5556 equivalent atoms). The residue lies towards the surface of the protein, away from the predicted GTPase binding site. In addition to gross structural changes, the functions of proteins can be affected by the flexibility of the molecule [50]. Computational estimation of the backbone flexibility of the molecule suggested that the M1231I variation results in changes in flexibility at a number of sites within the protein (Figure 5A). The site with the greatest loss of flexibility is a loop (Ser-1212 to Leu-1217) which links the \(\alpha\)-helix containing residue 1231 with residues predicted to play a key role in the CDC42 binding site (Tyr-1192 to Arg-1194; Figure 5B). This loss of flexibility may affect the dynamics of small GTPase interaction.

**DISCUSSION**

These experiments establish a new fragment from the C-terminal region of human IQGAP1 which is amenable to biochemical analysis. The fragment interacts with CDC42, and the strength of interaction is increased throughout.

All three phosphomimic variants also bound to CDC42 in the absence of additional nucleotide. However, in the case of S1441E, the affinity was reduced by two orders of magnitude. This arises mainly because of a reduction in the association rate constant. It should be noted that this reduced value (12 \text{L.mol}^{-1}\text{s}^{-1}) is very low and, therefore, may be subject to greater error than the other values. Interestingly, the double mutant (S1441E/S1443D) binds with a similar affinity to the wild type (Table 1).

<table>
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<tr>
<th>IQGAP1(DR6) variant</th>
<th>(k_a/\text{l.mol}^{-1}\text{s}^{-1})</th>
<th>(k_d/\text{s}^{-1})</th>
<th>(K_D/\mu\text{mol/L})</th>
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<td>Wild type</td>
<td>4900 ± 100</td>
<td>(6.5 ± 0.5) \times 10^3</td>
<td>1.3 ± 0.13</td>
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<td>S1441E</td>
<td>12 ± 1</td>
<td>(2.7 ± 0.03) \times 10^3</td>
<td>220 ± 21</td>
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<tr>
<td>S1443D</td>
<td>5800 ± 100</td>
<td>(4.7 ± 0.2) \times 10^3</td>
<td>0.81 ± 0.048</td>
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<tr>
<td>S1441E/S1443D</td>
<td>3600 ± 100</td>
<td>(4.0 ± 0.2) \times 10^3</td>
<td>1.1 ± 0.086</td>
</tr>
<tr>
<td>M1231I</td>
<td>1800 ± 100</td>
<td>(1.7 ± 0.2) \times 10^3</td>
<td>0.90 ± 0.16</td>
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</table>

These were determined by surface plasmon resonance. The values are reported ± their standard errors as determined by the BIAevaluation fitting programme (see Materials and Methods).
the presence of GTP. Interestingly, previous work has demonstrated, using isothermal titration calorimetry, an interaction between a C-terminal fragment of IQGAP (residues 962-1345) and GTP-loaded CDC42, but not with CDC42 purified in the absence of added nucleotides (assumed to be GDP-loaded)\. This may indicate that the additional residues present in the IQGAP1(DR6) fragment are important in CDC42 interaction in the absence of GTP. The phosphomimicking variants suggest that phosphorylation of the two serine residues has different effects. While the S1443D variant has slightly increased affinity for CDC42, the affinity of S1441E is decreased and introduction of a negative charge at both sites restores the affinity to essentially wild type levels. This suggests that there may be crosstalk between the two serines within the C-terminal domain. The results from the cancer-associated variant emphasise the importance of considering changes in protein flexibility, as well as changes to overall structure.

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