Crystal Structure of the GTPase-activating Protein-related Domain from IQGAP1

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IQGAP1 is a 190-kDa molecular scaffold containing several domains required for interaction with numerous proteins. One domain is homologous to Ras GTPase-activating protein (GAP) domains. However, instead of accelerating hydrolysis of bound GTP on Ras IQGAP1, using its GAP-related domain (GRD) binds to Cdc42 and Rac1 and stabilizes their GTP-bound states. We report here the crystal structure of the isolated IQGAP1 GRD. Despite low sequence conservation, the overall structure of the GRD is very similar to the GAP domains from p120 RasGAP, neurofibromin, and SynGAP. However, instead of the catalytic “arginine finger” seen in functional Ras GAPs, the GRD has a conserved threonine residue. GRD residues 1099–1129 have no structural equivalent in RasGAP and are seen to form an extension at one end of the molecule. Because the sequence of these residues is highly conserved, this region likely confers a functionality particular to IQGAP family GRDs. We have used isothermal titration calorimetry to demonstrate that the isolated GRD binds to active Cdc42. Assuming a mode of interaction similar to that displayed in the Ras-RasGAP complex, we created an energy-minimized model of Cdc42-GTP bound to the GRD. Residues of the GRD that contact Cdc42 map to the surface of the GRD that displays the highest level of sequence conservation. The model indicates that steric clash between threonine 1046 with the phosphate-binding loop and other subtle changes would likely disrupt the proper geometry required for GTP hydrolysis.

The small GTPase Ras functions as a binary switch in cell signaling processes. When bound to GTP, Ras is able to interact with effector proteins, including Raf kinase, and alter their activities. Ras signaling is terminated when bound GTP is hydrolyzed to GDP and inorganic phosphate. The basal rate of GTP hydrolysis on Ras is quite slow (−1.2 × 10−4 s−1), but this rate of hydrolysis can be enhanced ~10-fold by interaction with a GTPase-activating protein (GAP)2 (1). Several RasGAPs have been identified that bind to activated Ras to effect GTP hydrolysis. However, IQGAP1 expression increases the level of activated Cdc42, initially there was some confusion as to whether the protein might not represent a novel guanine nucleotide exchange factor. However it now appears that IQGAP1 is an effector of Cdc42 and Rac1 and preserves their activated states by tightly binding to the GTPases and stabilizing them in a conformation not conducive to GTP hydrolysis. IQGAP1 appears to be such an important effector for Cdc42 that abrogation of binding to IQGAP1 not only reduces the levels of active Cdc42, it also reduces membrane-localized Cdc42 and the cellular response to bradykinin (12).

A growing body of evidence implicates IQGAP1 in carcinogenesis. Expression of IQGAP1 increases during the transition from a minimally to a highly metastatic form of melanoma, and IQGAP1 has been found to be overexpressed in ovarian, breast, lung, and colorectal cancers (13–17). In vitro, overexpressed IQGAP1 enhances cell motility and invasiveness in a process that requires Cdc42 and Rac (18). β-Catenin is one of the many binding partners of IQGAP1 identified to date.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.
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3 The abbreviations used are: GAP, GTPase-activating protein; NF, neurofibromin; GRD, GAP-related domain; Ex domain, extra domain; GMPPNP, 5’-[(β,γ-imido)triphosphate.© 2009 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
IQGAP1 has been shown to bind to β-catenin and interfere with β-catenin binding to α-catenin, an interaction necessary for stable cell-cell adhesion (19). Another study found that IQGAP2 knock-out mice overexpress IQGAP1 and develop age-dependent liver cancer and apoptosis (20).

To better understand how a protein domain homologous to others that accelerate GTP hydrolysis can function as an effector and preserve the GTP-bound state, we have determined the x-ray structure of the IQGAP1 GRD. Despite low sequence identity, the GRD structure is quite similar to the GAP domains of p120, neurofibromin, and SynGAP; however, unlike those domains, the GRD possesses a conserved threonine in place of the catalytic arginine finger and has a 31-residue insertion that projects from one end of the molecule. Using the coordinates of Ras-GDP-AIF₃, in complex with the GAP domain of p120, we built a model of Cdc42-GTP bound to the GRD. The model indicates that a steric clash between the conserved Thr¹⁰⁴⁶ and the phosphate-binding loop of Cdc42 and other subtle changes within the active site would likely preclude nucleotide hydrolysis. Sequence conservation mapped to the surface of the GRD indicates that the surface with the highest degree of conservation overlaps with the surface that makes contacts to Cdc42 in the model.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Human IQGAP1 GRD**—A cDNA of full-length human IQGAP1 was a kind gift from Dr. Takahiro Nagase of the Kazusa DNA Research Institute. PCR and this cDNA were used to generate two fragments encoding IQGAP1 residues 962–1333 and 962–1345 that were each introduced into pMCSG7 expression vectors using the technique of ligation-independent cloning (21). Our choice of N- and C-terminal limits for the GRD expression constructs was based upon sequence analysis, secondary structure prediction, and the known termini of the NF1 and RasGAP proteins used for their respective structure determinations. Preliminary protein expression experiments indicated that the longer construct (residues 962–1345) yielded greater amounts of soluble protein, so this construct was chosen to produce the GRD protein. The pMCSG7 plasmid encodes an N-terminal tobacco etch protein, so this construct was chosen to produce the GRD protein.

**Isothermal Titration Calorimetry**—Cdc42(Q61L) was loaded with GTP as described by John et al. (22). Purified IQGAP1 GRD and GTP-bound Cdc42(Q61L) were dialyzed extensively at 4 °C against a buffer consisting of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 100 mM KCl, 5% glycerol, 5 mM MgCl₂, and 2 mM β-mercaptoethanol. After dialysis, protein concentrations were determined using light absorbance at 280 nm and calculated extinction coefficients for the GRD and GTP-bound Cdc42. In this experiment, the GRD (120 μM) was placed into the sample cell of a VP-ITC (MicroCal™), and 680 μM GTP–Cdc42 was injected into the sample cell using 10-μl injections at 210-s intervals. The experiment was conducted at 20 °C.

**Crystallization**—Crystals of Selenomethionine GRD were grown by vapor diffusion in sitting drops at 4 °C. Typically, 4 μl of well solution (500 mM MgCl₂, 20% polyethylene glycol 2000 methyl ether, 100 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.5 using Tris(hydroxymethyl)aminomethane-HCl)) would be added to 2 μl of protein solution. Crystals of Selenomethionine GRD were grown by vapor diffusion in sitting drops at 4 °C. Typically, 4 μl of well solution (500 mM MgCl₂, 20% polyethylene glycol 2000 methyl ether, 100 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.5 using Tris(hydroxymethyl)aminomethane-HCl)) would be added to 2 μl of protein solution. Crystals of Selenomethionine GRD were grown by vapor diffusion in sitting drops at 4 °C. Typically, 4 μl of well solution (500 mM MgCl₂, 20% polyethylene glycol 2000 methyl ether, 100 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.5 using Tris(hydroxymethyl)aminomethane-HCl)) would be added to 2 μl of protein solution. Crystals of Selenomethionine GRD were grown by vapor diffusion in sitting drops at 4 °C. Typically, 4 μl of well solution (500 mM MgCl₂, 20% polyethylene glycol 2000 methyl ether, 100 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.5 using Tris(hydroxymethyl)aminomethane-HCl)) would be added to 2 μl of protein solution. Crystals of Selenomethionine GRD were grown by vapor diffusion in sitting drops at 4 °C. Typically, 4 μl of well solution (500 mM MgCl₂, 20% polyethylene glycol 2000 methyl ether, 100 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.5 using Tris(hydroxymethyl)aminomethane-HCl)) would be added to 2 μl of protein solution. Crystals of Selenomethionine GRD were grown by vapor diffusion in sitting drops at 4 °C. Typically, 4 μl of well solution (500 mM MgCl₂, 20% polyethylene glycol 2000 methyl ether, 100 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.5 using Tris(hydroxymethyl)aminomethane-HCl)) would be added to 2 μl of protein solution. Crystals of Selenomethionine GRD were grown by vapor diffusion in sitting drops at 4 °C. Typically, 4 μl of well solution (500 mM MgCl₂, 20% polyethylene glycol 2000 methyl ether, 100 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.5 using Tris(hydroxymethyl)aminomethane-HCl)) would be added to 2 μl of protein solution. Crystals of Selenomethionine GRD were grown by vapor diffusion in sitting drops at 4 °C. Typically, 4 μl of well solution (500 mM MgCl₂, 20% polyethylene glycol 2000 methyl ether, 100 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.5 using Tris(hydroxymethyl)aminomethane-HCl)) would be added to 2 μl of protein solution.

**X-ray Data Collection**—For data collection at 100 K, the crystals were stepwise transferred to a cryo-solution containing 24% polyethylene glycol 2000 methyl ether, 500 mM MgCl₂, 100 mM Tris, pH 8.5, 50 mM LiCl, 0.05 mM dithiothreitol, and 26% ethylene glycol and then flash-frozen in liquid nitrogen. All of the data were collected on a MARCCD 165 from single crystals (selenomethionine and thimerosal soaked) maintained at 100 K at the Gulf Coast Protein Crystallography Consortium beam line at the Louisiana State University Center for Advanced Microstructures & Devices. Data from the selenomethionine
crystal were collected at wavelengths corresponding to the dispersive minimum (λ₁ = 0.97924Å), the anomalous difference maximum (λ₃ = 0.97900Å), and a remote wavelength to measure the dispersive differences (λ₃ = 0.92523Å). Data for the thimerosal derivative was collected at λ = 1.0063 Å, which maximized the mercury-anomalous signal (Table 1).

Structure Determination and Refinement—The locations of all 10 selenium atoms in the crystal asymmetric unit were determined using SHELDX and data extending to 2.8 Å (23). MLPHARE and DM (24) were used to calculate and improve phases, respectively. Inspection of the density-modified electron density map indicated that the original SHELXD selenium substructure was of the correct hand. Phases were then refined and density-modified using data in the range 25-2.4 Å (Table 1). The resulting experimental electron density map was readily interpretable (see Fig. 1) and was used for the initial model building with the program O (25). The final model is the result of successive rounds of manual model building and automated refinement using CNS (26) and data in the range 25-2.2 Å. The final model contains 3,037 protein atoms, 147 waters, and one Tris molecule (Table 2). The GRD coordinates have been deposited at the RCSB Protein Data Bank (accession number 3FAY).

Sequence Alignment—ClustalX (Version 1.83) (27) using default settings was used to align the IQGAP-related domains of 55 IQGAP1 metazoan homologs. The alignment was then loaded into JalView, which calculates the percentage of identity at each position of an alignment (28). The sequences for the alignment were found using the “Simple Modular Architecture Research Tool.” The sequences used are: Ciona intestinalis, ENSCINP00000001418 and ENSCINP0000001433; Ciona savignyi, ENSCASAVP00000013378, ENSCASAVP00000013379, and ENSCASAVP00000013380; Felis catus, ENSFCAp-0000005901 and ENSFCAp0000006085; Gasterosteus aculeatus, ENSGACp00000004075, ENSGACp00000004080, ENSGACp00000013363, and ENSGACp00000023048; Macaca mulatta, ENSMMP00000015598, ENSMMP00-00000030270, and UP10000D99C7D; Monodelphis domestica, ENSMODP00000013457, ENSMODP00000033667, and UP1-0000F2BE38; Otolemur garnettii, ENSOGAP00000012178; Oryzias latipes, ENSORLPP000000201, ENSORLP-0000002236, and ENSORLP0000002239; Sorex araneus, ENSSARP00000013004; Tupaiya belangeri, ENSTBEP-00000003239; Danio rerio, A2CE93; Bos taurus, A6H7H0 and P10000F31324; Homo sapiens, UP1000020CB2C, P46940, and A6NET5_HUMAN; Tetraodon nigroviridis, Q4SGG0 and Q4SWDS; Oryctolagus cuniculus, Q53J12; Mus musculus, Q6ZQK2, UP100004773A0, and UP10000E86CA8; Xenopus laevis, Q7ZT76 and Q7ZT77; Hydra vulgaris, Q9XZ9E; Rattus norvegicus, UP1000056512 and UP10000DA1D60; Canis lupus familiaris, XP_536318, XP_547533, and UP10000EB44B5; Takifugu rubripes, UP1000065CA9A, UP1000065CDAF, and UP1000065FF85; Xenopus tropicalis, UP100006DA11; Pan troglodytes, UP10000E1ECD8, UP10000E2084B, and UP10000E23-EF0; Gallus gallus, UP1000080D04 and UP10000ECC377; and Equus caballus, UP1000155D8BC, UP1000155F722, and UP1-

TABLE 1
Crystallographic data collection
The figure of merit was <Σ²F₀ - σ²F(A)>, where α is the structure factor phase, and ρ(α) is the phase probability distribution. The overall figure of merit before density modification was 0.535, and that after density modification was 0.777. The experimental phases were calculated using all three SeMet wavelengths (25-2.4 Å) and the thimerosal data in the resolution range 25-3.0 Å.

| Data set | Wavelength (Å) | Resolution (shell) | Observations | Completeness | <I/σI>* | Rcryst (%) |
|----------|----------------|---------------------|--------------|--------------|---------|-----------|
| Native λ₁ | 0.97924 | 25-2.2 (2.28-2.20) | 115,903/20,692 | 86.8 (38.2) | 8.9 (2.1) | 6.2 (24.2) |
| Native λ₂ | 0.97900 | 25-2.2 (2.28-2.20) | 109,111/19,664 | 87.0 (43.5) | 8.9 (2.1) | 7.8 (28.4) |
| Native λ₃ | 0.92523 | 25-2.2 (2.28-2.20) | 104,169/20,052 | 91.0 (57.4) | 8.9 (2.3) | 6.7 (26.3) |
| Thimerosal | 1.0063 | 25-2.8 (2.9-2.8) | 59,073/10,598 | 95.6 (77.0) | 6.0 (2.2) | 8.6 (34.6) |

* <I/σI> = mean signal to noise, where I is the integrated intensity of a measured reflection, and σI is the estimated error in the measurement.

* Rcryst = 100 × Σ|Fobs - Fcalc| / ΣFobs, where Fobs and Fcalc are the observed and calculated structure factor amplitudes for the reflection k. The sumations are over all reflections.

* R factor calculated as for Rcryst except using 1,971 observed reflections that were chosen randomly and were not included in refinement.

TABLE 2
Refinement statistics

| Resolution (Å) | No. reflections | Rcryst (%) | Rfree (%) | No. of atoms (protein/solvent) | Root mean square deviation bond length (Å) | Root mean square deviation bond angle (%) | ϕ/ψ (most favored/additional allowed/disallowed, %/%/%) | Rcryst = Σ|Fobs - Fcalc| / ΣFobs |
|----------------|----------------|------------|----------|-------------------------------|------------------------------------------|-----------------------------------------|-------------------------------------------------|------------------|
| 25-2.2 | 17,549 (working)/1,971 (test) | 22.84 | 25.82 | 3037/155 | 0.006 | 1.153 | 90.8/8.6/0.6 |

FIGURE 1. Experimental electron density. Density-modified experimental phases and λ, amplitudes (all |F| > 0) were used to calculate this map in the resolution range of 25-2.4 Å. The map is contoured at 1.4 σ. In the lower right of the picture are Tyr1193 and Arg1194.
The GRD/Cdc42 Model—The coordinates of the GAP domain of p120RasGAP in complex with Ras-GDP-AlF$_3$ (Protein Data Bank code 1WQ1, hereafter referred to as Ras/GAP-334) were positioned onto the GRD using the method of “Combinatorial Extension” to avoid user-introduced bias (29). The program O and explicit least squares superpositioning was used to position the coordinates of Cdc42 (residues 1–177; Protein Data Bank code 1AN0, chain A) onto Ras. The two switch regions of Ras were mutated to the residues in Cdc42 using O, and rotamers with minimal steric clash were chosen for these mutated residues. The coordinates of the GDP and AlF$_3$ from 1WQ1 were used to position a model of GTP. Topology and parameter files for GTP were acquired from the Dundee PRODRG2 server. The Mg$^{2+}$ ion and water 230 from 1WQ1 were included prior to energy minimization. Hydrogens were added to the coordinates, and then the model was subjected to 50 steps of positional minimization followed by one picosecond of molecular dynamics (29) and finally 300 steps of positional minimization using CNS version 1.1 (26). For comparison purposes, the starting coordinates of Cdc42, GTP, Mg$^{2+}$, and water 230 were energy-minimized (in isolation) exactly as described above for the model complex. Figs. 1, 2, 4, and 6 were prepared using PyMol, and GRASP (30) was used to make Fig. 5.

RESULTS

Structure Description—The GRD is an elongated, crescent-shaped, all-helical molecule that can be described as containing two separate domains (Fig. 2). The central domain is comprised of GRD residues 1022–1290 and contains residues whose equivalents in GAP-334 afford 90% of all contacts to Ras as evidenced by the crystal structure of the Ras/GAP-334 complex (31). Residues 962–1021 and 1291–1339 at the termini of the GRD comprise what Scheffzek et al. (2–4, 31) describe as an “extra domain” (Ex domain) in GAP-334, the GAP domain from neurofibromin (NF1–333), and the recently determined structure of the C2-GAP domains of SynGAP. Using the coordinates of the GRD and GAP-334 (Protein Data Bank code 1WER) and the method of “combinatorial extension” (29), a structural superposition was performed. Overall, GAP-334 and the GRD superimpose with a root mean square difference of 3.3 Å for 312 Ca atoms. A sequence alignment based on the structural superposition (Fig. 3) indicates that

FIGURE 2. The IQGAP1 GRD and GAP-334 have very similar tertiary structures. The GRD (left) and GAP-334 (Protein Data Bank code 1WER; right) were superimposed using combinatorial extension (29) to align the coordinates for this figure (root mean square deviation = 3.3 Å for 312 Ca atoms). The cartoon has been colored orange and yellow for the Ex domains of the GRD and GAP-334, respectively. The central domains are colored blue or light blue, and the 31-residue insertion characteristic of IQGAP GRDs is colored green. The helices are shown in dark gray, and a five-residue helix containing the YYR motif in the GRD is shown in dark blue. The IQGAP of GAP-334 are shown as stick representations in magenta.

FIGURE 3. Structure-based sequence alignment of the GRD and GAP-334. The three-dimensional coordinates of the GRD and GAP-334 (Protein Data Bank code 1WER) were superimposed using combinatorial extension (29) to produce this structure-based sequence alignment. Residues at the termini of the GRD and GAP-334 that were disordered or did not have a structural equivalent are shown as blue or gray letters, respectively. Secondary structure was assigned using DSSP (46) and colored as in Fig. 2. Residues that are invariant in an alignment of 55 IQGAP GRD homologs are underlined, and residues that contact Cdc42 in the model or Ras in the Ras/GAP-334 complex (Protein Data Bank code 1WQ1) are colored red. The GRD secondary structure colored green (residues 1099–1129) has no equivalent in GAP-334.
the GRD and GAP-334 share an overall 15.1% sequence identity for the 312 aligned residues, with 18% identity and 7.8% identity between the central and Ex domains, respectively.

Of the residues in GAP-334 that contact Ras, only four are conserved in the GRD. These are Arg1194, Pro1198, Lys1230, and Gln1233 in the GRD with equivalents in GAP-334 of Arg903, Pro907, Lys935, and Gln938. A hallmark of Ras GAsPs is the conserved sequence Phe-Leu-Arg (FLR). In IQGAsPs, instead of an FLR motif, there is a Tyr-Tyr-Arg motif, and for human IQGAP1 this sequence is 1192YYR1194. These residues are located in a five-residue π-helix similar to the "four residue turn" that contains 901FLR903 in GAP-334 and 579FLR581 in Syn-GAP, and different from the α-helix that contains the 1389FLR1391 signature in NF1-333. In GAP-334, the mutation L902I does not significantly reduce binding to Ras but reduces GTPase activation to ~0.5% of wild type (32, 33). In the Ras/GAP-334 structure, Leu902 is positioned adjacent to Gln61 of Ras; in fact, it appears that the two terminal methyl groups of the leucine side chain are each used to precisely position the amide oxygen and nitrogen atoms of Gln61. In the GRD, Thr1193 occupies the position equivalent to Leu902 in GAP-334, and a tyrosine side chain would be incapable of replicating the interaction seen in the Ras/GAP-334 complex.

Probably the most noticeable single difference between GAP-334 and the GRD is the replacement of the catalytic arginine "finger" (Arg789) of GAP-334 with a threonine in the GRD (Thr1046). Based on the Ras/GAP-334 structure, Arg789 in GAP-334 appears to provide positive charge to stabilize developing negative charge on the nucleotide γ-phosphate in support of an associative mechanism of GTP hydrolysis (31, 34). The conservative mutation of R789K in GAP-334 lowers the rate of GAP-stimulated hydrolysis ~2000-fold without reducing the binding affinity for Ras (35), so it seems likely that substitution of a threonine at this position would completely abolish GAP activity within an otherwise bona fide GAP domain. Thr1046 is highly conserved and is found at the C terminus of helix α2αc, whereas in GAP-334, Arg789 is in a more flexible "finger loop." In GAP-334, Arg903 of the FLR motif is hydrogen-bonded to the backbone oxygen atoms of Phe788, Arg789, and Ala790, thereby stabilizing the finger loop conformation (Fig. 4). Similar intramolecular interactions are seen in the NF1–333 and Syn-GAP structures. In the GRD, the equivalent Arg1194 is hydrogencode to only the backbone oxygen of Ile1044, however, helix α2α is otherwise strongly stabilized by hydrophobic interactions between Ile1044 and Leu1167 and Leu1177 of helix α5. The positioning of the Thr1046 may be further stabilized by hydrogen bonding via its γ-hydroxyl to the backbone oxygen of Gln1042, because the oxygen-to-oxygen distance is 2.9 Å, and there is bridging electron density in the experimental map (not shown). Because the backbone oxygen of Gln1042 is also apparently acting as a hydrogen-bond acceptor to the amide nitrogen of Thr1046, this would be a bifurcated hydrogen bond. Although Thr1046 would apparently be accessible to a kinase, according to the sequence analysis tools available at the Expert Protein Analysis System, the flanking sequences do not appear to specify a phosphorylation site of high probability. Why this threonine is highly conserved in IQGAsPs is at present unknown.

As an aid in our analysis of the GRD, we performed a sequence alignment using 55 IQGAP homologs from metazoa. The alignment indicates that the sequences of IQGAP GRDs are very conserved. In all, 51 of 383 GRD residues are identical in the alignment, and the overall sequence identity (averaged over the 383 positions) is 78%. All homologs feature a threonine at the position equivalent to 1046 in human IQGAP1, and 54 of 55 possess the YYR motif. Identical residues are not evenly distributed throughout the GRD but instead cluster toward the N terminus with 47 completely conserved residues located within the first two-thirds of the GRD (Fig. 3). 16 residues in the Ex domain are identical between the 55 IQGAP1 homologs; however, judging by their surface exposure and their general hydrophobic character, most appear to have structural roles in stabilizing the fold of the Ex domain. Two exceptions are at positions equivalent to Arg906 and Leu969 of the GRD, and these two residues in the structure have relative side chain exposures of 74 and 43%, respectively. The Eukaryotic Linear Motif Resource identifies these two residues as constituting a "destruction box" (RXLR) motif recognized by the anaphase-promoting ubiquitin ligase complex. Although the GRD of the yeast IQGAP homolog Iqg1p does not possess these two conserved residues within its GAP-related domain, Iqg1p has recently been shown to be a substrate for anaphase-promoting ubiquitin ligase complex-mediated degradation (36).

In the GRD, the N terminus of helix α4αc is structurally equivalent to helix 3αc of GAP-334. However, the coordinates of residues within the C terminus of helix α4αc as well as those in α4bαc, the amino terminus of α4c, and the intervening connecting loops, do not have an equivalent in GAP-334 and appear to be a 31-residue insertion within the central domain (Figs. 2 and 3). These residues form an extension at one end of the molecule, and many have a high degree of solvent exposure. The 31-residue insertion is conserved among IQGAP
IQGAP1 GAP-related Domain

FIGURE 5. Surface representation of sequence conservation and model contacts. Left panel, a surface representation of the GRD, oriented approximately the same as in the lower left of Fig. 2, has been colored to indicate residues that are at least 90% (red) or 100% (magenta) identical among 55 metazoan IQGAP GRDs. Right panel, residues that lose >10 Å² solvent-accessible surface area in the Cdc42/GRD model have been colored blue. In general, the highest degree of sequence conservation includes residues that contact Cdc42. Note the high degree of sequence conservation in the 31 residue insertion (arrow).

homologs, and overall, the average percentage of identity of the residues in the insertion is higher than that for the entire GRD (86% versus 78%) with three residues (Glu¹¹⁰³, Gly¹¹⁰⁷, and Tyr¹¹¹⁴) absolutely conserved (Fig. 3). Only one residue in the insertion appears to make contact with Cdc42 in our model (see below), and this is Asp¹¹¹⁵. This residue is an aspartic acid in 41 of 55 aligned IQGAP homologs with 12 other homologs possessing a glutamic acid at this position. In the model, Asp¹¹¹⁵ is in van der Waals’ contact with the Cdc42 Met¹ side chain. However, a small intermolecular adjustment would bring the positively charged amino terminus of the GTPase within salt-bridging distance of the negatively charged carboxylate of the aspartate (or glutamate).

Although the IQGAP insert sequence contains several potential Ser/Thr phosphorylation and protein-protein interaction motifs that can now be assessed in a structural context, we do not know of any evidence that the GRD is phosphorylated or that these residues are important for mediating binding to known target proteins. However, given that the level of sequence conservation in the insert is higher than that of the entire domain, the insert is clearly important for IQGAP1 activity in some capacity.

Cdc42/GRD Model—Cdc42 and Ras share >30% sequence identity, and their structures are quite similar, as are the structures of GAP-334 and the GRD. Because of these considerations, we undertook to model Cdc42-GTP bound to the GRD using the coordinates of the Ras/GAP-334 structure as a guide (see “Experimental Procedures”). The model indicates that >3000 Å² of mostly hydrophobic surface area would be buried upon complex formation, and GRD residues at the interface are among the most conserved of IQGAP homologs (Fig. 5). Residues from the two “switch” regions (residues 25–40 and 59–75) provide the bulk of the contacts from Cdc42. In addition to the switch regions, residues within helices a3a and a3b and the helical insert characteristic of Rho GTPases (residues 123–134) are also involved at the interface. In the model, the side chain of Thr¹⁰⁴⁶ of the GRD is in van der Waals distance of the nucleotide sugar and the backbone oxygen of Ala¹¹⁰⁶ of Cdc42, whereas the Thr¹⁰⁴⁶ backbone oxygen is 2.7 Å from the backbone oxygen of Gly¹². Intrusion of Thr¹⁰⁴⁶ on the phosphate-binding loop has the effect of slightly distorting the loop and shifting the bound nucleotide away from this contact point (Fig. 6).

The absolutely conserved Tyr¹¹⁹³ and Arg¹¹⁹⁴ (of 1192YYR¹¹⁹⁴) bracket Gln⁶¹ of Cdc42 (Fig. 6). Gln⁶¹ is thought to be important for positioning a nucleophilic water during GTP hydrolysis. Mutations of Gln⁶¹ in Ras decrease the intrinsic rate of hydrolysis significantly and can reduce the rate of GAP-stimulated hydrolysis by up to 10⁶-fold (1, 37). In the model, Tyr¹¹⁹³ loses 94 Å² of solvent-accessible surface area upon interaction with Cdc42. The hydroxyl oxygen of Tyr¹¹⁹³ is located 2.8 Å from the oxygen atom of the nucleophilic water (WAT²³⁰) and also 2.8 Å from the backbone oxygen of Thr³⁵ of Cdc42. This would make the Tyr¹¹⁹³ hydroxyl a hydrogen bond donor to WAT²³⁰. Tyr¹¹⁹³ makes several other contacts to Cdc42 residues including one to the side chain oxygen of Gln⁶¹ (CE2 to OE1 distance of 3.7 Å) and hydrophobic contacts to the Pro⁳⁴ and Val⁴⁵ side chains. In addition, the aromatic ring of Tyr¹¹⁹³ appears to be functioning as a hydrogen bond acceptor from the hydroxyl of Tyr⁶⁴ of Cdc42 (38). Reorientation of the bound nucleotide with respect to Gln⁶¹ of Cdc42 would presumably be sufficient to greatly reduce GTP hydrolysis (Fig. 6).

In this modeling study, we have assumed that the ground state interaction closely resembles the transition state as reflected in the Ras/GAP-334 structure. However, the ground state interaction could be significantly different from the transition state complex as evidenced by a comparison of the structures of Cdc42-GMPPNP and RhoA-GDP-ALF₇ in complex with p50 RhoGAP where a ~20° rotation of the GTPase appears to accompany the conversion from ground state to transition state (39, 40). As mentioned earlier, it should be noted that GAP-334, NF1–333, SynGAP, and the GRD do possess a degree
of structural similarity to the RhoGAPs (7). Ultimately, understanding of how IQGAP1 engages the Rho GTPases awaits the elucidation of a co-crystal structure.

**Mutations in the GRD**—Several mutations within the GRD have been introduced to elucidate IQGAP1 function. Expression of IQGAP1 increases levels of active Cdc42 in cells and confers a migratory phenotype, whereas expression of IQGAP1ΔGRD, lacking residues 1122–1324 of the GRD, has been shown to reduce the migratory and invasive phenotype of cells and reduce the levels of activated Cdc42 (18). Interestingly, this mutation appears to reduce the levels of active Cdc42 by directly stimulating GTP hydrolysis. This was determined because measurable binding was only seen when Cdc42 was loaded with the nonhydrolyzable GTP analog GMPPNP and not the slowly hydrolyzed analog GTPγS (12). It was later determined that IQGAP1ΔGRD does bind to Cdc42-GDP (41). Removal of residues 994–1248 from full-length IQGAP1 (IQGAP1ΔGRDF) abolishes Cdc42 binding regardless of activation state (41). Additional deletion analysis was used to determine that residues 1054–1077 (IQGAP1ΔMK24) are necessary but not sufficient for Cdc42 binding (41). The analogous residues in GAP-334 are located near the center of the Ras-binding site. Another designed mutation in the GRD (IQGAP1(T1050AX2)) that introduces a 17-residue insertion after Gly1047 and converts native residues Thr1050, Val1051, and Ile1052 to alanines appears to function as a constitutively active mutant in that the requirement for Cdc42/Rac1 interaction to enhance binding to CLIP-170 has been eliminated (42). Like IQGAP1ΔMK24, the location of the T1050AX2 mutation in GAP-334 would map to the approximate center of binding surface for Ras (31). If Cdc42/Rac1 binding to IQGAP1 induces a conformational change by disrupting IQGAP1 intramolecular interactions, perhaps the 1050AX2 is bypassing the need for GTPase binding through essentially the same mechanism, that is, significantly altering or blocking access to an important intramolecular binding surface.

From a structure-based point of view, it is difficult to interpret the deletion mutants. For instance, in IQGAP1ΔMK24 the smallest of the GRD deletions, residues ~30 Å apart in the native structure would be adjacent in the deletion mutant. It is very likely that this mutant and the other deletion mutations disrupt the tertiary structure of the GRD and perhaps the fold of portions of the molecule outside the GRD. However, the fact that IQGAP1ΔGRD still binds to Cdc42 to some degree, whereas IQGAP1(AGRDF) shows no binding to Cdc42 regardless of the activity state argues that a measure of the native fold persists in the deletion mutant.

Although a number of studies implicate IQGAP1 in carcinogenesis, there is only one report of a mutant variant within the IQGAP1 GRD. A recent study found that 2 of 33 diffuse type gastric cancer samples contained a mutation in codon 1231 of the GRD (43). This mutation converts the native methionine to an isoleucine. Met1231 resides in α7, of the central domain, and the side chain is directed toward the interior of the protein, where it is packed against Met1138 and is involved in hydrophobic interactions with residues from helices α4C and α8α, that are presumably important for stabilizing the central domain. Introduction of the β-branched isoleucine at this position would likely cause a small, local disruption of the central domain. Because α7, provides three residues that contact Cdc42 in the model, namely Arg1222, Lys1230, and Gln1233, a local disruption to the fold might significantly reduce binding to Cdc42 or Rac1.

**Binding Studies**—The GRD is considered to be necessary but insufficient for binding to Cdc42 and Rac1. We decided to test both active and GDP-loaded Cdc42 for binding to the isolated GRD using isothermal titration calorimetry. For this purpose, the constitutively active Cdc42 (Q61L) was loaded with GTP using an established protocol (22), whereas wild type Cdc42, purified over the course of 3 days without added nucleotides, was considered to be GDP-loaded. We found that GTP-bound Cdc42 displays significant binding activity toward the isolated GRD, with an estimated affinity (Kd) of ~1.3 μM (Fig. 7). Under the conditions used in this experiment, we did not detect a significant interaction between GDP-Cdc42 and the GRD (data not shown).
**IQGAP1 GAP-related Domain**

**DISCUSSION**

After GAP-334, NF1–333, and SynGAP, the IQGAP1 GRD is the fourth GAP-related domain structure determined. Despite low sequence conservation, all four of these structures feature a similar fold of their Ex domains. The minimal fragment of NF1 that can accelerate hydrolysis of GTP on Ras consists of essentially the central domain of NF1–333 alone, whereas that for p120RasGAP is a fragment spanning the first 273 amino acids of GAP-334 and ending at the C terminus of the central domain, in other words, missing approximately half of the Ex domain contributed by C-terminal sequences (44). Attempts to express the isolated central domain of GAP-334 in bacteria were not successful (44); however, considering the structural similarity of NF1–333 and GAP-334, and the fact that the minimal functional RasGAP fragment clearly does not possess an intact Ex domain, it seems fairly likely that the Ex domain does not, in general, perform an essential structural role in stabilizing the central domain in these Ras GAPS. If the structurally conserved Ex domain is not required for stabilizing the central domain, it is possible that it has a distinct, novel function.

To date, the minimal IQGAP1 fragment identified that binds with high affinity to activated Rac1 and Cdc42 contains ~100 residues N-terminal to the GRD and extends to the C terminus (IQGAP1 residues 864–1657). This IQGAP1 fragment binds to activated Cdc42 and Rac1 with affinities ($K_d$) of 24 and 18 nM, respectively (45). This is ~50-fold tighter binding than we measured for activated Cdc42 binding to the isolated GRD, implying that residues outside the GRD contribute significantly to binding. In p120RasGAP, the C terminus of GAP-334 is the C terminus of the full-length protein, whereas NF1, SynGAP, and IQGAP1 have C termini located hundreds of residues beyond the GAP-related domains. If the IQGAP1 Ex domain per se provides crucial contacts necessary for high affinity binding to Cdc42 and Rac1, then our GRD construct may possess all necessary C-terminal Ex domain residues, but it is missing N-terminal residues that will complete the Ex domain fold and provide additional contacts to the GTPase.

Recently, Owen et al. (45) undertook a thorough study of Cdc42 and Rac1 interactions using IQGAP1(864–1657), site-directed mutagenesis of the GTPases, and a scintillation proximity assay to estimate binding affinity. In general, our model reflects the importance of switch I residues (residues 25–40) for binding to IQGAP1. For instance, mutation of Tyr$^{32}$ to phenylalanine significantly reduces the affinity of Cdc42 and Rac1 for IQGAP1, and this would be explained in our model by a loss of a hydrogen bond by the tyrosine hydroxyl to the backbone oxygen of Lys$^{185}$. Likewise, mutation of Val$^{36}$ to either Asn or Ala would apparently disrupt hydrophobic interactions with Tyr$^{1193}$ and Gin$^{1235}$ of IQGAP1 and Tyr$^{64}$ of Cdc42. The results of mutations made in switch 2 residues are more difficult to understand in light of the model. Although no switch 2 mutation significantly decreased the affinity of Cdc42 for IQGAP1, two mutations, Y64A and P69A, slightly increased affinity. Although Pro$^{69}$ is not at the intermolecular interface in the model, Tyr$^{64}$ appears to be productively interacting with Tyr$^{1193}$ of the GRD; however, the phenolic ring of Tyr$^{64}$ is ~3.5 Å from the amide oxygen of the Asn$^{1197}$, so mutation to alanine may provide space for this polar side chain. Surprisingly, three switch 2 mutations in Rac1 significantly reduced binding affinity for IQGAP1. For instance, mutation of Asp$^{63}$ in switch 2 to alanine, which slightly increases the affinity of Cdc42 for IQGAP1, reduces the affinity of Rac1 for IQGAP1 by ~10-fold. In the model, Asp$^{63}$ is apparently hydrogen bonding with one side chain oxygen to Arg$^{1060}$ but is otherwise in a hydrophobic environment. The environment for Asp$^{63}$ of Rac1 would be expected to be identical because all neighboring Rac1 residues are identical to those in Cdc42. Because Rac1 and Cdc42 are ~70% identical in sequence and have highly similar structures, these results and the results of other experiments led these investigators to conclude that the two GTPases engage IQGAP1 in similar but not identical ways.

We have found that the isolated GRD (residues 962–1345) binds to active Cdc42 with moderate affinity and that GDP-bound Cdc42 does not appear to display significant binding activity toward the GRD under the conditions used in our experiment. The results of our calorimetry experiments are consistent with a previous report that used pulldown assays to show that full-length IQGAP1 binds to both forms of Cdc42 with apparently much higher affinity for the active form of the GTase (9). Presumably, increased affinity toward GDP-bound Cdc42 by full-length IQGAP1 (versus the isolated GRD) is afforded by sequences outside the GRD that also increase affinity toward active Cdc42. This was observed for the larger IQGAP1 fragment (residues 864–1657), which binds active Cdc42 with ~50-fold higher affinity than the isolated GRD (45).

In summary, we have determined the three-dimensional structure of the GAP-related domain from human IQGAP1. The structural similarities between the GRD and GAP-334 and those between Ras and Cdc42 inspired the modeling studies wherein we see that a steric clash between Thr$^{1046}$ and the P-loop shifts the loop and bound nucleotide in Cdc42 and that Tyr$^{1193}$ competes with Gin$^{64}$ for hydrogen bonding to the nucleophilic water. If this mode of binding is correct, these effects on the active site would likely explain the stabilization of active Cdc42 observed for IQGAP1. The model may differ significantly from reality in the details but probably not in essence. The highest degree of sequence conservation roughly coincides with the surface of the GRD equivalent to that on GAP-334 bound by Ras (Fig. 5), and it would seem less than economical for nature to use a GAP domain as an effector module if the mode of binding to that module is distinct from other GTPase/GAP interactions.

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