The IQGAP1-Rac1 and IQGAP1-Cdc42 Interactions

INTERFACES DIFFER BETWEEN THE COMPLEXES*

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IQGAP1 contains a domain related to the catalytic portion of the GTPase-activating proteins (GAPs) for the Ras small G proteins, yet it has no RasGAP activity and binds to the Rho family small G proteins Cdc42 and Rac1. It is thought that IQGAP1 is an effector of Rac1 and Cdc42, regulating cell-cell adhesion through the E-cadherin-catenin complex, which controls formation and maintenance of adherens junctions. This study investigates the binding interfaces of the Rac1-IQGAP1 and Cdc42-IQGAP1 complexes. We mutated Rac1 and Cdc42 and measured the effects of mutations on their affinity for IQGAP1. We have identified similarities and differences in the relative importance of residues used by Rac1 and Cdc42 to bind IQGAP1. Furthermore, the residues involved in the complexes formed with IQGAP1 differ from those formed with other effector proteins and GAPs. Relatively few mutations in switch I of Cdc42 or Rac1 affect IQGAP1 binding; only mutations in residues 32 and 36 significantly decrease affinity for IQGAP1. Switch II mutations also affect binding to IQGAP1 although the effects differ between Rac1 and Cdc42; mutation of either Asp-63, Arg-68, or Leu-70 abrogate Rac1 binding, whereas no switch II mutations affect Cdc42 binding to IQGAP1. The Rho family “insert loop” does not contribute to the binding affinity of Rac1/ Cdc42 for IQGAP1. We also present thermodynamic data pertaining to the Rac1/Cdc42-RhoGAP complexes. Switch II contributes a large portion of the total binding energy to these complexes, whereas switch I mutations also affect binding. In addition we identify “cold spots” in the Rac1/Cdc42-RhoGAP/IQGAP1 interfaces. Competition data reveal that the binding sites for IQGAP1 and RhoGAP on the small G proteins overlap only partially. Overall, the data presented here suggest that, despite their 71% identity, Cdc42 and Rac1 appear to have only partially overlapping binding sites on IQGAP1, and each uses different determinants to achieve high affinity binding.

The Rho family of small G proteins are well documented to regulate cell motility through their regulation of the actin cytoskeleton (1). These small G proteins act as molecular switches, being active in the GTP-bound form and inactive in the GDP-bound form. The interaction of the active, GTP-bound, G protein with an effector protein triggers a downstream signaling cascade. Rho family proteins, acting via these effector proteins, have been shown to control the polymerization of actin in the peripheral cortex of the cell. Cdc42 controls filopodia formation; these are actin-rich fingers that establish the direction of motility. Rac1 controls lamellipodia formation; these actin-rich ruffles at the leading edge of the cell initiate motion, whereas Rho controls the establishment of stress fibers whose formation results in the contractile force that moves the body of the cell behind the leading edge (2).

Effector proteins for two members of the Rho family, Cdc42 and Rac1, include the PAK3 family of serine/threonine kinases reviewed in Ref. 3, the ACK tyrosine kinase (4), the Wiskott-Aldrich syndrome proteins (WASPs) (5), and the IQGAP family (6). Many of the effector proteins for Cdc42 and Rac1 are known to be involved in regulation of the cytoskeleton, and at least some of the actin remodeling effects of these small G proteins are mediated through IQGAPs (6).

IQGAP1 is a 190-kDa protein, originally identified serendipitously in a screen for matrix metalloproteases and named for the presence of four calmodulin-binding IQ motifs and a RasGAP-related domain (GRD) (7). Mammalian cells are now known to contain three IQGAP proteins (IQGAP 1–3), which have a well conserved domain structure and show high levels of homology within the conserved domains (8, 9). Although IQGAP1 has a wide tissue distribution, IQGAP2 and -3 are more limited (9). Despite the presence of a domain homologous to the catalytic domain of the RasGAPs, intact IQGAP1 has no GAP activity toward small G proteins (10) but rather has been shown to bind to Rac1 and Cdc42 and act as a downstream effector (10, 11). IQGAP1 inhibits the intrinsic GTPase activity of Cdc42 in vitro, stabilizing Cdc42 in the GTP-bound state (11–14). Consistent with these findings, overexpression of IQGAP1 in mammalian cells substantially increases the pool of GTP-bound Cdc42, stimulating filopodia formation (13), whereas knockdown of endogenous IQGAP1 by RNA interference significantly reduces active Cdc42 (15). Work by several

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3 The abbreviations used are: PAK, p21-activated kinase; GAP, GTPase-activating protein; GRD, GAP-related domain; ACK, activated Cdc42-associated kinase; WASP, Wiskott-Aldrich syndrome protein; MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; isopropyl β-D-thiogalactopyranoside SPA, scintillation proximity assay; PP1, protein kinase C-related kinase 1; GMPPNP, guanosine-5’-(β,γ-imido)triphosphate.
IQGAP1 Binding to Rac1 and Cdc42

Expression Constructs—All proteins were expressed as GST fusion proteins in the pGEX series of vectors (GE Healthcare). Rac1 Q61L, Cdc42 Q61L, and all other mutants were expressed in pGEX-2T from constructs encoding either residues 1–184 Cdc42 or residues 1–192 Rac1, cloned into the BamHI and EcoRI sites. Q61L mutations are present in all small G proteins used in this study. This mutation confers GTPase deficiency, stabilizing the active GTP-bound form and therefore the G protein-effector complexes. Henceforth, Rac1 Q61L and Cdc42 Q61L are referred to as Rac1 and Cdc42, respectively. Other mutations are individually specified. Residues 198–439 of RhoGAP, constituting the GAP domain, were expressed as described previously (33). Residues 864–1657 and 950–1407 of IQGAP1 to β-catenin causes β-catenin to dissociate from α-catenin, weakening the E-cadherin-mediated cell-cell contacts, because of loss of the stabilizing contacts with the actin cytoskeleton. Activation of Cdc42 has been shown to prevent IQGAP1-dependent dissociation of cell aggregates, presumably by sequestering IQGAP1 away from β-catenin (27, 30) and leaving the strongly adhesive cell-cell contacts intact. In a regulatory scenario analogous to the binding of Cdc42 and F-actin, calmodulin has been shown to modulate the binding of IQGAP1 to β-catenin (31). It has also been demonstrated that E-cadherin and calmodulin compete for binding to IQGAP1 (32) and that binding of IQGAP1 to E-cadherin results in the impairment of E-cadherin adhesive functions (32). Thus it seems that IQGAP1 may have access to multiple avenues to disrupt cell-cell contacts and that these activities are collectively modulated by calmodulin.

Information from structural and thermodynamic studies of protein complexes is integral to rational drug design directed toward either the disruption or stabilization of complexes for therapeutic purposes. The absence of any structural data pertaining to IQGAP1 and the complexes that it forms with Cdc42 and Rac1 prompted us to initiate the study presented here, which aims to delineate regions on the small G proteins Cdc42 and Rac1 that are involved in IQGAP1 binding. We have used site-directed mutagenesis to target regions of the small G proteins indicated by previous studies to be involved in binding effector proteins and/or regulatory GAP proteins. We have identified switch I in both Cdc42 and Rac1 as a region of thermodynamic importance in IQGAP1 binding. The switch II region of small G proteins is an important contact surface for GAP proteins and for some effector proteins. We present here the unexpected finding that switch II in Rac1 is involved in IQGAP1 binding, whereas the same region in Cdc42 seems to be energetically unimportant. The “insert loop,” which is specific to the Rho family of small G proteins, is not thermodynamically involved in the interactions with IQGAP1, although our results indicate that it is in close proximity to the IQGAP1 interface. We have also investigated the G protein binding region of IQGAP1 and present data that imply, unexpectedly, that distinct although overlapping interaction surfaces exist for the two highly related G proteins. The mutations described in this study will be useful tools in delineating the regulation of IQGAP1 by both Cdc42 and Rac1 in vivo.

EXPERIMENTAL PROCEDURES
IQGAP1 Binding to Rac1 and Cdc42

IQGAP1 were expressed in a modified pGEX-4T vector, which has a tobacco etch virus protease cleavage site inserted between GST and IQGAP1 (34).

Cdc42/Rac1 Mutagenesis—Site-directed mutagenesis of the Cdc42 Q61L and the Rac1 Q61L expression constructs was performed using the QuikChange multisite-directed mutagenesis kit (Stratagene). The sequences of the Cdc42 and Rac1 coding regions of all mutants were verified using an automated DNA sequencer (Applied Biosystems Inc.) by the DNA Sequencing Facility, Department of Biochemistry, University of Cambridge.

Recombinant Protein Production—GST fusion proteins were expressed in Escherichia coli BL21. Stationary cultures were diluted 1 in 10, grown at 37 °C to an A600 of 0.8, and induced with 0.1 mM isopropyl β-d-thiogalactopyranoside for 5 h. Proteins were affinity-purified using glutathione-agarose beads (Sigma). The GST-IQGAP1 and GST-RhoGAP fusion proteins were eluted from the glutathione-agarose beads and used directly. GST-Cdc42 and Rac1 and variants were cleaved from their GST tag while attached to the glutathione-agarose beads with thrombin (Merck), and the cleaved G proteins were finally purified by gel filtration (16/60 S75 column, GE Healthcare). Free RhoGAP was prepared in a similar manner. Protein concentrations for all proteins were evaluated from measurement of their A280 using their amino acid compositions and the extinction coefficients of tyrosine, phenylalanine, tryptophan, and the guanine nucleotide (35).

Nucleotide Exchange—[3H]GTP complexes of Cdc42 and Rac1 proteins were made by nucleotide exchange in the presence of a GTP regeneration system. [8,5-3H]GTP (0.15 mCi, 26,900 Ci/ml, PerkinElmer Life Sciences) was dried by centrifugal evaporation. To this was added Cdc42 or Rac1 protein (0.7 mg in a volume of 0.12 ml), followed by 12 μl of 200 mM phosphoenolpyruvate, 0.5 μl of 1 M KCl, 15 μl of 3 M NH₄(SO₄)₂, and 5 μl of pyruvate kinase (Sigma), making a final volume of 152.5 μl. The mixture was incubated at 37 °C for 2 h, and then 1 μl of 1 M MgCl₂ was added. Unbound nucleotide was removed using a 1 ml of G-25 Sephadex (Superfine, GE Healthcare) centrifuge gel filtration column in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl₂, and 1 mM dithiothreitol.

Scintillation Proximity Assays (SPA), Direct Binding SPAs—For competition assays, free Rac1·GMPPNP, Cdc42·GMPPNP, or RhoGAP was titrated into a mixture of 30 nM [3H]GTP-Cdc42/Rac1 and 30 nM GST-IQGAP1 immobilized on fluoromicrospheres as above. The added Rac1, Cdc42, or RhoGAP competes with the GST-effector-[3H]GTP-Cdc42/Rac1 interaction, abolishing the scintillation signal. The highest sample concentrations of competitor used were 2 μM. In each case, a blank was performed in the absence of GST-effector. For each G protein-effector affinity determination, data points were obtained for at least 10 different competitor concentrations. Kd values and their standard errors were obtained by fitting the dose-response curves to binding isotherms that describe competition between two proteins binding to one site on another protein and account for mutual depletion of the interacting components. The value of Kd for the GST-effector-Cdc42/Rac1 interaction was also required, and this was obtained from direct binding SPAs. The equations used were adapted for SPA from the previously published derivations (38, 39) and have been fully described elsewhere (40). Where the data did not appear consistent with a simple competitive model, data were fitted to a model describing partial competition (Scheme 1). In Scheme 1, ternary complexes consisting of either GST-IQGAP bound to both Rac and Cdc42 or GST-IQGAP bound to both RhoGAP and Rac (or Cdc42) exist. The fits used Equation 1.

\[
\text{SPA signal} = S_{\text{max}} + \frac{((S_{\text{max}} - S_{\text{min}}) y) / (P - \sqrt{Q^2 - 4AQ_sB_o})}{2Q}
\]

(Eq. 1)

where

\[
P = A_o + B_o + K_{d_i}
\]

\[
y = \frac{z - \sqrt{z^2 - 4Q^2A_oB_o}}{2Q}
\]

and

\[
Q = \frac{I_oK_{di} + K_{ci}K_{di}}{K_{di}K_{ci} + I_o} \\
z = QA_o + QB_o + 1
\]

S_{\text{max}} is the observed SPA signal in the absence of inhibitor, and S_{\text{min}} is the signal in the absence of the GST-effector and inhibitor. A_o is the total concentration of A; B_o is the total concentration of B; I_o is the total concentration of inhibitor; K_{d_i} is the equilibrium dissociation constant for binding A to B; K_{ci} is the equilibrium dissociation constant for binding the inhibitor to A; and K_{di} is the equilibrium dissociation constant for binding B.
to Al. For the experiments where Rac1 and Cdc42 compete for binding to GST-IQGAP1, A is GST-IQGAP1; B is the [3H]GTP-Rac1/Cdc42; and I is the unlabeled Rac1/Cdc42. For the experiments where RhoGAP displaces IQGAP1 from binding to Rac1/Cdc42, A is [3H]GTP-Rac1/Cdc42; B is GST-IQGAP1; and I is RhoGAP.

Molecular Modeling—The Rac1-IQGAP1 complex was modeled from the structures of the Ras-RasGAP complex (41) and Rac1 (42) using the program MODELLER (43).

RESULTS

The C-terminal half of IQGAP1 (residues 864–1657) contains the GRD and encompasses the region that binds to G proteins (11). Using SPA, we measured the affinity of this fragment (hereafter IQGAP1) for activated (Q61L) Cdc42 and Rac1. We found that this effector binds Cdc42 and Rac1 with affinities of 24 and 18 nM, respectively (Table 1). The G proteins are 71% identical (Fig. 1), so it might be expected that they would bind IQGAP1 in a similar fashion. We chose to investigate the involvement of several regions of Cdc42 and Rac1 in IQGAP1 binding. This comparison should allow us to determine how similar the modes of interaction of Cdc42 and Rac1 are with this effector.

Mutations in Switch I—Switch I, or the effector loop, is one of the two nucleotide-sensitive regions of small G proteins and is often involved in effector binding. We mutated several residues in switch I of both Cdc42 and Rac1 that are involved in effector binding in other G protein-effector complexes (reviewed in Ref. 44). Switch I is relatively well conserved among small G proteins and is almost identical in Cdc42 and Rac1 (Fig. 1). Several mutations, such as T35S and Y40C, are based on Ras mutants isolated from a random mutagenesis study, which were later characterized to be effector discriminating mutants (45, 46). Most of the other mutants that we have tested, for example F37A, D38A and V42A, are based on our previous work on Cdc42 binding to the CRIB effectors ACK, PAK, and WASP (47). We calculated the affinity of all of these mutant proteins for IQGAP1 using SPA. Results for the switch I mutants are summarized in Table 1 and representative data are shown in Fig. 2. Val-36 is conserved between Cdc42 and Rac1, and mutation of this residue to Ala decreases the affinity for IQGAP1 by ~8- and 19-fold, respectively. The binding energies of the Cdc42-IQGAP1 and Rac1-IQGAP1 complexes are 10.39 and 10.56 kcal/mol, respectively. Val-36 contributes significantly to the binding energy as follows: 1.25 kcal/mol (12.5% of the total) to the Cdc42-IQGAP1 complex and 1.75 kcal/mol (17.5% of the total) to that of Rac1-IQGAP1. Replacement of the hydrophobic Val with the polar Asn had a similar effect (Table 1 and Fig. 2).

Mutation of Tyr-32 also affected IQGAP1 binding, decreasing the affinity about 6-fold for Rac1 and ~18-fold for Cdc42. For Tyr-32 we had made a conservative change to Phe, indicating that the Tyr hydroxyl group plays a role in IQGAP1 binding. Mutation of residues Phe-37 or Asp-38 in Rac1 had a small effect on the interaction, decreasing the affinity for IQGAP1 by ~3–4-fold. Interestingly, the Asp-38 mutation had the opposite effect with Cdc42, increasing its affinity for IQGAP1 4-fold. When we mutated Val-42 of Cdc42 to the equivalent Rac1 residue (Ala), the affinity of IQGAP1 binding increased 8-fold. Thus, this residue may contribute to the slightly higher affinity between Rac1 and IQGAP1. Therefore, the mutational analysis of switch I shows that there is a "hot spot" for the IQGAP1 interaction within switch I of both Rac1 and Cdc42 (Fig. 3, c and e, respectively).

Mutations in Switch II—Switch II, residues 60–70, is the second nucleotide-sensitive region of small G proteins. It forms a major site of

![Sequence alignment of Cdc42 and Rac1](image-url)

**FIGURE 1.** Sequence alignment of Cdc42 and Rac1. The two switch regions and the insert region are underlined. The secondary structures of the G proteins are shown above the alignment in gray, where cylinders denote α-helices and arrows are β-strands. The boxed residues were mutated in this study. Residues shown as white characters on a black background are those which, when mutated, decrease the affinity of IQGAP1 by more than 5-fold and are therefore thermodynamically important.
interaction between GAPs and small G proteins; as IQGAP1 is homologous to RasGAP in the GRD, it was another potential site of contact. In addition, switch II is involved in effector binding in other Rho family-effector complexes such as ACK, PAK, WASP, and Arfaptin (reviewed in Ref. 48). Thus, switch II participates in both effector binding and GAP binding, and so we expected that mutations in this region would affect IQGAP1 binding. Residues 57–77, which include switch II and its flanking regions, are identical in Cdc42 and Rac1 (Fig. 1). The results from mutations in switch II were thus unexpected (Table 2). We mutated all possible residues of switch II to Ala, except Gly-60, which is necessary for the conformational change on GTP binding (49), and Gln-61, which is involved in the GTPase mechanism and is already mutated to Leu in all proteins used in these experiments to stabilize the GTP-bound form. Residues 62–70 in Cdc42 were mutated to alanine, but none significantly affected IQGAP1 binding (Table 2). When we made the same mutations in Rac1, however, D63A, R68A, and L70A significantly reduced IQGAP1 binding (decreases of ~10-, ~14-, and ~50-fold, respectively) as did D65A to a lesser extent (~4-fold) (Table 2). This observation implies that these residues in switch II are energetically involved in the IQGAP1 interaction with Rac1 but not with Cdc42. The energetic contribution of these residues in the Rac1-IQGAP1 complex is significant; from a total binding energy of 10.44 kcal/mol, Asp-65 0.80 kcal/mol, Arg-68 1.57 kcal/mol, and Leu-70 at least 2.38 kcal/mol (13.3, 7.7, and 15.0%, and at least 22.8% of the total binding energy, respectively). Thus for Rac1 at least, switch II represents an energetic hot spot for the IQGAP1 interaction (Fig. 3c).

**Mutations in the Insert Loop**—The Rho family small G proteins are distinct from the other members of the Ras superfamily due the presence of the insert loop, an extra sequence, residues 125–135 in Cdc42 and Rac1 (Fig. 1), that forms two α-helices (42) (Fig. 3a). The role of the insert loop in binding to regulators and effectors is still not clear, although it has been shown to be necessary for formation of membrane ruffles and lamellipodia (50). It has also been shown previously that removal of residues 122–134 prevents Cdc42 binding to IQGAP1 (51). The insert loop is only partially conserved between Cdc42 and Rac1, but it is highly charged in both. We mutated the insert loop of Cdc42, removing the charges or changing to the equivalent Rac1 residues. The effects of these mutations can be seen in Table 3. The only mutation that significantly decreased IQGAP1 binding to Cdc42 is N132K. However, N132A increased the affinity of Cdc42 for IQGAP1, as did the analogous K132A mutation in Rac1. It is noteworthy that both of these mutations decrease the size of the residue at position 132. Deleting the insert loop entirely (residues 124–135) did not significantly affect the binding of IQGAP1 to Cdc42.

**Mutations That Affect Other Effector Proteins**—As the number of residues in the switches and the insert loop that appeared to be energetically important for the interactions between Cdc42/Rac1 and IQGAP1 was small, we reasoned that other regions of the G proteins must be involved in binding this effector. In our previous work on ACK (a Cdc42 effector) and PRK1 (a Rac1 effector), we designed a number of Cdc42 and Rac1 mutants that abrogated or significantly reduced binding to these effectors, along with others that were in or near the binding interface but were not energetically involved with those particular interactions (47, 52). We therefore examined the binding of IQGAP1 to those mutants of Cdc42 and Rac1 that were designed to lie in the interface with ACK or PRK1, respectively, and did not fall into any of the previous three categories of mutants (Table 4), to determine whether Cdc42 or Rac1 utilizes a similar binding surface to interact with IQGAP1 and the other effectors. These mutations included residues adjacent to switches I and II in the protein sequence and also residues within the C-terminal α-helix (Fig. 1). From Table 4 it can be seen that the only mutation that significantly affects IQGAP1 binding is T17N, which abrogates binding of both Cdc42 and Rac1. The only other mutations that had any affect on IQGAP1 binding were Cdc42 T75K and Rac1 S41A, both of which decreased affinity by ~4-fold. The Ser-41 contribution to the Rac1-IQGAP1 binding energy is therefore calculated to be 0.80 kcal/mol. Thus, only two of the other mutations in residues previously identified as being in the interface between Cdc42-ACK (47) and Rac1-PRK1 (52) showed effects on IQGAP1 binding and these were modest.

**Mutations Based on Available GAP Structures**—Finally, because IQGAP1 is homologous to the RasGAPs, we used the available structures of the catalytic domains of GAP proteins in complex with their cognate G proteins to investigate rationally
whether IQGAP1 contacts the G proteins in a manner similar to that of GAPs. We used three structures to design mutants: Ras-p120 Ras-GAP (41), Rho-RhoGAP (53), and Cdc42-RhoGAP (54). None of these structures are exactly analogous to the Cdc42/Rac1-IQGAP1 complexes. The GRD in IQGAP1 is homologous to RasGAP rather than RhoGAP and Cdc42/Rac1 are Rho family small G proteins rather than belonging to the Ras family. Finally, IQGAP1 is an effector protein rather than a catalytic GAP, so the details of its interaction with the active site of the G protein are likely to be different.

An investigation of the residues in the small G protein that were in the interface with the GAP molecules but did not fall into one of the categories already investigated (e.g. residues in the switches) revealed several more residues of potential interest. Residues at positions 56, 88, 91, 92, 95, and 96 in Cdc42 and Rac1 were all identified as potentially involved in the GAP interaction in one or more of the structures detailed above. Consequently, these residues were mutated in both Cdc42 and Rac1, and the effects of these mutations on the affinity of the small G proteins for IQGAP1 are shown in Table 5. None of the mutations based on the small G protein-GAP complexes showed any deleterious effect on the IQGAP1 affinity for either Rac1 or Cdc42. On the contrary, many actually displayed increased the affinity for IQGAP1.

### Energetic Analysis of Residues Involved in the Cdc42/Rac1-RhoGAP Complexes

Although there is ample structural information available on the small G protein-GAP complexes (41, 53–56), there is very little thermodynamic information available. We therefore investigated which residues in switch II and the other residues that we had identified in the interfaces in the available GAP complex structures were contributing energetically to the complexes formed by Cdc42 and Rac1 with RhoGAP. These data can be seen in

![Figure 3](https://example.com/figure3.png)

**Figure 3.** The structures of Cdc42 and Rac1 showing the positions on the surface of the mutations made in this study. The G proteins are in the same orientation in each panel. Note that the switch regions, particularly switch I, are in different conformations in the Cdc42 and Rac1 structures, because they are not fixed in a single conformation in the uncomplexed G proteins. c–f, residues that were not mutated are colored blue; residues colored white, when mutated, had no effect on affinities; residues colored red, when mutated, decreased the affinity more than 5-fold and thus represent a hot spot for the interaction. a, ribbon diagram of Rac1. The coordinates are taken from Protein Data Bank entry 1MH1. The “insert region” that defines the Rho family and the switch regions are labeled. b, surface representation of Cdc42 showing the binding surfaces for the CRIB effectors WASP, ACK, and PAK. Residues that interact with all three effectors are shown in dark orange; those that interact with two of the effectors are shown in light orange, and those that interact with only one effector are shown in yellow. The coordinates are from the NMR structure of Cdc42-GMPPNP (H. R. Mott et al., unpublished data). c, surface representation of Rac1. Residues in the hot spots for the IQGAP1 interaction are colored red. d, surface representation of Cdc42. Residues in the hot spots for RhoGAP interaction are colored red. e, surface representation of Cdc42. Residues in the hot spots for RhoGAP identified in this study or in previous work (47) are colored red.

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IQGAP1 Binding to Rac1 and Cdc42

Table 2

Affinities of switch II mutants of Q61L Cdc42-GTP and Q61L Rac1-GTP mutants for IQGAP1-(864–1657) and RhoGAP

| Cdc42     | Rac1     | IQGAP1 | RhoGAP | IQGAP1 | RhoGAP |
|-----------|----------|--------|--------|--------|--------|
| Q61L      | 24 ± 4   | 62 ± 4 | 18 ± 5 | 86 ± 6 |        |
| Q61L,E62A | 5 ± 3    | 85 ± 55| 6 ± 1  | 54 ± 12|        |
| Q61L,D63A | 16 ± 5   | 201 ± 9| 188 ± 32| 731 ± 81|       |
| Q61L,Y64A | <1       | >1 μM  | 21 ± 6 | >1 μM  |        |
| Q61L,D65A | 7 ± 5    | 126 ± 23| 70 ± 13| >1 μM  |        |
| Q61L,R66A | 2 ± 2    | 169 ± 72| 33 ± 4 | >1 μM  |        |
| Q61L,L76A | 14 ± 4   | 467 ± 106| 35 ± 9 | >1 μM  |        |
| Q61L,R86A | 33 ± 5   | >1 μM  | 257 ± 31| 616 ± 51|       |
| Q61L,D96A | <1       | 214 ± 12| 6 ± 3  | 657 ± 92|       |
| Q61L,L70A | 17 ± 5   | 146 ± 3| >1 μM  | 241 ± 46|       |

* Equilibrium binding constants were determined in SPAs as described under "Experimental Procedures." Kᵩ values are quoted with the standard errors from curve fitting.

Table 3

Affinities of insert loop mutants of Q61L Cdc42-GTP and Q61L Rac1-GTP mutants for IQGAP1-(864–1657)

| Cdc42     | Rac1     | IQGAP1 | RhoGAP | IQGAP1 | RhoGAP |
|-----------|----------|--------|--------|--------|--------|
| Q61L      | 24 ± 4   | 62 ± 4 | 18 ± 5 | 86 ± 6 |        |
| Q61L,E127K| 1 ± 1    | 1 ± 1  | 1 ± 1  | 1 ± 1  |        |
| Q61L,A130K| 40 ± 3   | 40 ± 3 | 40 ± 3 | 40 ± 3 |        |
| Q61L,K131E| 10 ± 2   | 10 ± 2 | 10 ± 2 | 10 ± 2 |        |
| Q61L,K131Q| 9 ± 2    | 9 ± 2  | 9 ± 2  | 9 ± 2  |        |
| Q61L,N132A| 1 ± 1    | 1 ± 1  | 1 ± 1  | 1 ± 1  |        |
| Q61L,T132K| >1 μM    | >1 μM  | >1 μM  | >1 μM  |        |
| Q61L,K135Q| <1       | <1     | <1     | <1     |        |
| Q61L,K135Q| 41 ± 7   | 41 ± 7 | 41 ± 7 | 41 ± 7 |        |
| Q61L,K135Q| 19 ± 11  | 19 ± 11| 19 ± 11| 19 ± 11|        |
| Q61L,K135T| 91 ± 8   | 91 ± 8 | 91 ± 8 | 91 ± 8 |        |
| Q61L,Rac1 insert | <1 | Q61L,Cdc42 Insert | 11 ± 3 |       |
| Q61L,ΔInsert| 44 ± 4  | Q61L,ΔInsert | 11 ± 3 |       |

* Equilibrium binding constants were determined in SPAs as described under "Experimental Procedures." Kᵩ values are quoted with the standard errors from curve fitting.

Table 4

Affinities of Q61L Cdc42-GTP and Q61L Rac1-GTP mutants (designed to disrupt other effector interactions) for IQGAP1-(864–1657)

| Cdc42 ACK mutants | Rac1 PRK1 mutants | IQGAP1 | RhoGAP | IQGAP1 | RhoGAP |
|-------------------|-------------------|--------|--------|--------|--------|
| Q61L              | 24 ± 4            | 18 ± 5 |        |
| Q61L,M1T          | 1 ± 0.1           | 1 ± 0.1|        |
| Q61L,T17N         | >1 μM             | >1 μM  |        |
| Q61L,T17N         | >1 μM             | >1 μM  |        |
| Q61L,M174T        | 3 ± 2             | 24 ± 13|        |
| Q61L,H46A         | 8 ± 0.7           | 70 ± 34|        |
| Q61L,T75K         | 95 ± 9            | 8 ± 4  |        |
| Q61L,L174A        | 3 ± 0.9           | 9 ± 3  |        |
| Q61L,L174V        | 12 ± 2            | 10 ± 2 |        |
| Q61L,P50A         | 5 ± 3             | 5 ± 3  |        |
| Q61L,N52T         | 20 ± 3            | 20 ± 3 |        |
| Q61L,Q612A        | 12 ± 1            | 12 ± 1 |        |
| Q61L,K166A        | 9 ± 2             | 9 ± 2  |        |
| Q61L,T167A        | 12 ± 5            | 12 ± 5 |        |

* Equilibrium binding constants were determined in SPAs as described under "Experimental Procedures." Kᵩ values are quoted with the standard errors from curve fitting.

Tables 2 and 5. In fact the Cdc42/Rac1 residues defined in the Cdc42-RhoGAP structure as being ±5 Å from RhoGAP had very little effect on the binding of RhoGAP when mutated. A modest (2-fold) effect is seen with Cdc42 E95A and Rac1 W56A, E91A, and N92A (Table 5). Switch II mutations, however, did show marked reductions in the RhoGAP interaction (Table 2). With the exception of E62A, mutation of all the residues in switch II of Rac1 had a profound effect on the ability of the small G proteins to bind RhoGAP (Table 2 and Fig. 3d), although L70A only decreased the affinity 2.5–4-fold. This suggests that in Cdc42, at least, switch I is also important for RhoGAP binding (Fig. 3f). A similar pattern of results was also obtained for mutation of switch II residues in Cdc42, although the observed decreases in affinity were less pronounced (Fig. 3f), and only mutations in Tyr-64, Leu-67, and Arg-68 had significant effects (i.e. more than 5-fold) on the affinity. The energetic contributions of the residues of switch II in Cdc42 in the RhoGAP complex varies between 0.76 and 2.21 kcal/mol, and in total they combine to equal approximately the binding energy of complex formation. For Rac1 the energetic contributions to binding are larger, varying between 1.54 and 2.38 kcal/mol and the summed contributions of the individual switch II residues far exceeds the total binding energy of the complex. Thus for the RhoGAP complexes switch II may well contribute most of the energy for complex formation. For Cdc42, we found previously that F37A and Y40C mutations in switch I abrogated the binding of RhoGAP (47), reducing the affinity 5–6-fold, whereas Y32K and D38E mutations had a modest effect on the affinity, reducing it 2.5–4-fold. This suggests that in Cdc42, at least, switch I is also important for RhoGAP binding (Fig. 3f).

Similarity of the IQGAP1- and RhoGAP-binding Sites on Cdc42 and Rac1—We also sought to investigate the extent of the overlap of the RhoGAP- and IQGAP1-binding surfaces on Cdc42 and Rac1 by performing competition SPAs. Thus, free RhoGAP was titrated into GST-IQGAP1 that was pre-bound to [3H]GTP-Cdc42 or [3H]GTP-Rac1 (Fig. 4). Initially, the data were fitted to an equation describing pure competition between RhoGAP and IQGAP1. However, at saturating concentrations of RhoGAP, the signal did not return to the experimentally determined zero (i.e. the signal obtained in the absence of GST-effector), which would be expected for a full competitor (Fig. 4). Dependent upon whether or not the signal at saturating RhoGAP was constrained, the resulting fits were either poor or gave much weaker affinities for the RhoGAP-Cdc42/Rac1 complexes than had been previously measured in direct SPAs. This prompted us to refit the data using an equation describing partial competition, in which the competitor modulates the affinity of the monitored reaction but does not abolish it completely. In this scenario, at saturating [RhoGAP], the residual GST-
sections were constrained to start at the SPA signal in the absence of RhoGAP and to end at the SPA signal in the absence of GST-IQGAP1, respectively. The $K_d$ values for IQGAP1 binding to Cdc42 were fixed at 300 and 86 nM, respectively. The effect of altering $K_d$ values for IQGAP1 binding to Cdc42 or Rac1 in the range 80–400 nm on either the quality or the values of the remaining $K_d$s was rather small. Simulations showing a pure competition model with the same fixed $K_d$ values for IQGAP1-Cdc42 or Rac1 binding to GST-IQGAP1 were fixed at 24 and 18 nm, respectively. The $K_d$ values were fixed. The $K_d$ values for IQGAP1 binding to the Cdc42-RhoGAP or Rac-RhoGAP complexes, derived from the fits, were 86 and 37 nm, respectively. This corresponds to $K_d$ values ($K_{iu}$) for IQGAP1 binding to the Cdc42-IQGAP1 or Rac1-IQGAP1 complexes of 1100 or 2100 nm, respectively.

IQGAP1-Rac1/Cdc42 complex would give rise to an SPA signal leading to the observed displacement curves. The fits using partial competition were of good quality, with errors within the expected range. To reduce the number of inter-dependent parameters, the fits shown in Fig. 4 were obtained by fixing the $K_d$ values for the interaction between GST-IQGAP1 and Rac1/Cdc42 and of RhoGAP and Rac1/Cdc42. For comparison, Fig. 4 also shows a simulation of pure competition with the same $K_d$ values, demonstrating the severe deviations from the experimental data points. The $K_d$ values for IQGAP1 binding to the Cdc42-RhoGAP or Rac1-RhoGAP complexes, derived from the fits to the partial competition model, were 86 and 37 nm, respectively. The effect of RhoGAP can be obtained by comparing these with the $K_d$ values for IQGAP1 binding to Cdc42 or Rac1, which were 24 and 18 nm, respectively. These fits are consistent with saturating RhoGAP only causing a 2–3-fold decrease in affinity of the interaction between IQGAP1 and G protein. A full competitor would have caused an infinite decrease in affinity.

The Binding Determinants on IQGAP1—The work described thus far was carried out with a large C-terminal fragment of IQGAP1, which encompassed residues 864–1657. To delineate the minimal binding domain on IQGAP1 for the small G proteins, we have produced multiple GST fusion constructs expressing various residues of IQGAP1 and have tested some for their ability to bind to Cdc42 and Rac1. One such construct, comprising residues 950–1407 IQGAP1, constitutes the smallest region of IQGAP1 that we have produced that retains the ability to bind Cdc42. IQGAP1-(950–1407) bound to Cdc42 with an affinity of 140 nm but failed to interact with Rac1 in a direct SPA (data not shown). This unexpected difference between the apparent binding interfaces for the two GTPases on IQGAP1 led us to investigate whether separable binding sites exist on IQGAP1 for the small G proteins. We performed competition SPAs, where we pre-bound GST-IQGAP1 to either radiolabeled $[^3H]GTP$-Cdc42 or $[^3H]GTP$-Rac1 and then examined the effect of increasing concentrations of unlabeled Rac1-GMPPNP or Cdc42-GMPPNP. Self-competition, e.g. competition of GST-IQGAP1 bound to $[^3H]GTP$-Cdc42 by unlabeled Cdc42-GMPPNP, was used as a control (Fig. 5a).

When the control self-competition data were fitted to a binding isotherm describing pure competition, as expected the data fitted well; with saturating concentrations of Rac1 or Cdc42, the signal returned to the experimentally determined zero (i.e. that in the absence of GST-effector) (Fig. 5a). The fits and deduced parameters using the partial competition model were essentially identical. However, with combinations of Cdc42 and Rac1, the best fits did not precisely return to the experimental zero, and there were small but significant deviations from the data (Fig. 5b). Once again, when refitting to a model describing partial competition, better quality fits for Cdc42 versus Rac1 and Rac1 versus Cdc42 were obtained which decreased to the experimentally determined zero. In this case, however, the competitive element was much lower. Thus, saturating Rac-GMPPNP reduced the affinity of IQGAP1 for Cdc42-GTP by 9-fold and saturating Cdc42-GMPPNP reduced the affinity of IQGAP1 for Rac-GTP by 10-fold. Therefore it seems that Rac1 (or Cdc42) has some affinity for the Cdc42 (or Rac1)-IQGAP1 complex, suggesting that the binding sites for Rac1 and Cdc42 on IQGAP1 do not completely overlap. This is consistent with the data that we obtained for IQGAP1-(950–1407) showing difference binding to Cdc42 and Rac1.

**DISCUSSION**

Initial reports identified IQGAP1 as a putative effector protein for both Cdc42 and Rac1 (10, 11). Subsequent studies have validated that the interaction between IQGAP1 and the GTPases has biological significance, with many functions of the small G proteins mediated through IQGAP1. For example, overexpression of IQGAP1 increases active Cdc42 in cells, leading to the formation of filopodia (13). In addition, IQGAP1 appears to be necessary for Cdc42 to localize to the plasma membrane (13), implying that IQGAP1 has a role in Cdc42 function. IQGAP1 promotes cell motility at least in part by binding to Cdc42 and Rac1 (15, 57). Interestingly, IQGAP1 also appears necessary for Cdc42 to increase motility of epithelial cells (15), implying bidirectional communication between IQGAP1 and Cdc42. IQGAP1 also has a role in cell polarity as it captures growing microtubule ends by binding CLIP-170 in a process regulated by Cdc42 and Rac1 (24).

Although IQGAP1 was originally identified as an effector protein for both Cdc42 and Rac1, later data showed that the in vitro affinity of Cdc42 for IQGAP1 was considerably higher than that of Rac1 for IQGAP1 (58, 59). Here we report that in vitro IQGAP1 has similar affinities for both Cdc42 and Rac1. The affinities that we have measured of ~20 nM are also very similar to those that we have previously reported between...
IQGAP1 binding to Rac1 and Cdc42

![Image of figure 5](image_url)

FIGURE 5. a, displacement of [3H]GTP-Cdc42 from GST-IQGAP1 by Cdc42-GMPPNP and [3H]GTP-Rac1 from GST-IQGAP1 by Rac1-GMPPNP. Increasing concentrations of unlabeled Rac1-GMPPNP (or Cdc42-GMPPNP) were titrated into fixed concentrations of GST-IQGAP1 and [3H]GTP-Cdc42 (or Rac1) in competition SPAs. Data were fitted to complete and partial competition models. The fits were constrained to start at the SPA signal in the absence of RhoGAP and to end at the SPA signal in the absence of GST-IQGAP1, i.e., 10 and 80 cpm with Cdc42 and Rac1, respectively, and the K_i values for Cdc42 or Rac1 binding to GST-IQGAP1 was fixed to the values obtained for these K_i values from direct SPAs, i.e., 24 and 18 nm, respectively. Fits of the data to a pure competition model are shown as dashed lines, whereas the fits of the data to a partial competition model are shown as solid lines. b, Cdc42-GMPPNP titration into [3H]GTP-Cdc42 + IQGAP1; Rac1-GMPPNP titrated into [3H]GTP-Rac1 + IQGAP1. The K_i values for IQGAP1 binding Cdc42-GMPPNP and Rac1-GMPPNP derived from the pure competition model were 440 ± 20 and 130 ± 20 nm, respectively. The derived parameters from the partial competition model were not significantly different. The deviation of these K_i values from those derived from direct SPAs may be due to the use of GMPPNP-loaded Rac1 and Cdc42 as the competitor in these experiments, whereas the direct SPAs measure the affinity of GTP-loaded G protein for IQGAP1. b, displacement of [3H]GTP-Cdc42 from GST-IQGAP1 by Rac1-GMPPNP and of [3H]GTP-Rac1 from GST-IQGAP1 by Cdc42-GMPPNP. Increasing concentrations of unlabeled Rac1-GMPPNP (or Cdc42-GMPPNP) were titrated into fixed concentrations of GST-IQGAP1 and [3H]GTP-Cdc42 (or Rac1) in competition SPAs. Data were fitted with the same constraints as in a. The best fits of the data to a partial competition model are shown as solid lines and those to a pure competition model as dashed lines. c, Rac-GMPPNP titration into [3H]GTP-Cdc42 + IQGAP1; Cdc42-GMPPNP titrated into [3H]GTP-Rac1 + IQGAP1. The K_i values for IQGAP1 binding Cdc42-GMPPNP and Rac1-GMPPNP derived from the pure competition model were 290 ± 20 and 320 ± 30 nm, respectively. Using the partial competition model, the K_i (K_i) values for IQGAP1 binding Cdc42-GMPPNP and Rac1-GMPPNP were both 200 ± 20 nm, and the K_i values for Cdc42-GTP binding to Rac-IQGAP1 complex was 220 ± 30 nm and for Rac1-GTP binding to Cdc42-IQGAP complex was 230 ± 50 nm. These corresponds to K_i values (K_i) for Rac1 binding to Cdc42-IQGAP1 complex or Cdc42 binding to Rac1-IQGAP1 complex of 1800 or 2600 nm, respectively.

Cdc42/Rac1 and members of the CRIB family of effectors and the regulatory protein RhoGAP (36, 47). Previous determinations of the comparative affinities of Rac1 and Cdc42 for IQGAP1 have mainly relied on nonquantitative (affinity precipitation) methods (59) or indirect quantitative assessments (inhibition of GTP hydrolysis) (58), and these experimental differences probably underlie the discrepancies. Previous work also found that the affinity of Cdc42 for IQGAP1 was substantially higher than that for the CRIB effectors, PAK1 and WASP (12); however, these data again were derived from an indirect assay (inhibition of GTP hydrolysis), which may explain deviation from data presented here. We believe that the direct analysis we use (36, 47, 52, 60) and present here provides the most accurate estimation to date of the relative binding affinities.

Switch I or the “effector loop” is a region on the surface of small G proteins that is usually involved in binding target proteins. Our results indicate that this region is also involved in IQGAP1 binding. However, despite the fact that mutation of some residues in switch I, in both Cdc42 and Rac1, affect IQGAP1 binding, there is a striking lack of effect with many classical switch I mutations. This is in distinct contrast to other Cdc42/Rac1 effectors, e.g., the CRIB effectors PAK, ACK, and WASP, where switch I contacts the effector in all three cases (Fig. 3b), and most mutations in the effector loop (at least in Cdc42) have a deleterious effect on affinities (47). Effector loop mutations in Cdc42 also decrease binding of RhoGAP, although far fewer residues appear to be energetically integral to the RhoGAP interface than to the CRIB interfaces (Fig. 3f). Specifically, residues Tyr-32, Phe-37, Asp-38, and Tyr-40 are the only switch I mutations that affect the binding of Cdc42 to RhoGAP (47). Thus, the effects of switch I mutations on IQGAP1 binding to Cdc42 (Tyr-32 and Val-36) are a closer mimic of the effects seen with RhoGAP than with the CRIB effectors. The IQGAP1-Rac1 interaction is also less dependent on switch I, although Phe-37 and Asp-38 mutants also have a small effect on this interaction.

In the CRIB effector complexes (61–63), switch I of Cdc42 is in close contact with most of the conserved residues of the CRIB motif (Fig. 3b), whereas in the complex with RhoGAP (54), switch I interactions are not nearly as extensive. Thus, our mutational analysis of the involvement of switch I in IQGAP1 binding indicates that the interaction is more similar to a GAP interaction than an effector protein interaction.

The effects of some switch I mutations in Cdc42 on IQGAP1 binding have been reported previously (51, 59). Y32K has been demonstrated to decrease binding to IQGAP1, which agrees with our finding that Y32F decreases the affinity of Cdc42 and Rac1 for IQGAP1. In fact mutation of Tyr-32 (or its equivalent) has been shown to be energetically deleterious to various other small G protein–effector complexes (47, 64), and importantly, it is a residue whose orientation is critical for the ability of the G protein to bind effectors (65–67). T35A, however, was reported to abrogate binding to IQGAP1, whereas we see no changes in affinity with a T35S mutation in either Cdc42 or Rac1. This is
probably because the –OH group of Thr-35, which is involved in coordinating the Mg$^{2+}$ cofactor, is retained in Ser. The T35A mutation is likely to destabilize the Mg$^{2+}$ in the nucleotide-binding site, and therefore the orientation of the switches, rather than directly contact the IQGAP1 itself. F37A Cdc42 has been reported by others to have a significant effect on IQGAP1 binding (51), but we see no effect with this mutation in Cdc42 and only a modest decrease in affinity for Rac1. Finally, the conservative mutation D38E was demonstrated to retain binding of Cdc42 to IQGAP1 and IQGAP2 (59), and this is in agreement with our results for D38A in Cdc42 although we see a small effect with this mutation in Rac1.

The idea that IQGAP1 binding may share some determinants with RhoGAP binding to Cdc42 and Rac1 was tested by making mutations in switch II. This region is often, although not always, involved in contacts between the Rho family proteins and their effectors and, importantly for this study, is seen to make close contacts with GAP proteins. Despite the fact that we mutated every residue in switch II of Cdc42, we found no residues that contributed thermodynamically to the interaction with IQGAP1 (Table 2 and Fig. 5e). In the light of the results with Cdc42, the effects of mutations in switch II of Rac1 are unexpected. Although mutations in some residues of switch II did not affect the affinity, mutations at Asp-63, Arg-68, Leu-70, and to a lesser extent Asp65 had a deleterious effect on IQGAP1 binding. Thus, it seems that switch II is thermodynamically active in the Rac1-IQGAP1 interface, contributing significantly to the binding energy of the complex. This suggests that the binding surfaces for IQGAP1 and RhoGAP on Rac1 must at least partially overlap (Fig. 3, c and d). Past observations suggested that RhoGAP does not effectively compete for IQGAP1 binding to Cdc42, although the data in that study were not quantitative (59). However, the structure of Cdc42 complexed with RhoGAP (54) shows that there are significant contacts between Cdc42 switch II and RhoGAP. If switch II were at least in the interface with IQGAP1, RhoGAP might be expected to compete with IQGAP1 for Cdc42 to some extent, although switch II exhibits conformational flexibility in active Cdc42 (68). This flexibility could allow switch II to bind to RhoGAP, whereas switch I is engaged with IQGAP1. Our competition SPAs demonstrate that RhoGAP only competes partially with IQGAP1 for binding to Cdc42 or Rac1. This suggests that there is partial overlap between the RhoGAP- and IQGAP1-binding interfaces on Rac1 and Cdc42, even though switch II in Cdc42 does not contribute energetically to the interface. These data imply that there are significant differences between the modes of interaction of IQGAP1 and RhoGAP with Cdc42 or Rac1. We were prompted by the differences observed in the contribution of switch II to IQGAP1 binding to Rac1 or Cdc42 to investigate the thermodynamic contribution of this region to RhoGAP binding. Here a large contribution by this region to complex formation was identified for both small G proteins. However as was seen with IQGAP1, switch II seems to contribute more to the interface for Rac1 than for Cdc42. The molecular basis underpinning these results awaits the determination of the structures of both Cdc42-IQGAP1 and Rac1-IQGAP1 complexes. Nevertheless, it appears that the complexes made between the two G proteins and IQGAP1 display significant differences to each other and share limited similarities with the Cdc42-RhoGAP complex. The energetic similarities of the RhoGAP and IQGAP1 interactions with Rac1 suggested that the Ras-RasGAP (41) structure could be used to construct an informative model of the complex formed between Rac1 and IQGAP1. Our model of Rac1-IQGAP1 is based only on homology and not on the mutational data presented here, and it shows that the residues that we have identified as being thermodynamically important would indeed be close to the IQGAP1 if it bound to Cdc42 and Rac1 using the same interface as Ras uses to contact RasGAP (Fig. 6).

The structures of Cdc42 and Rac1 show that the insert region forms a helix that protrudes from the body of the protein (42, 69), making it a potential surface to mediate protein-protein interactions. The insert region has been implicated in regulating the activity of RhoGDI toward Cdc42 (70), for Rac1 in p67phox binding/NADPH activation (71, 72), and also as being involved in IQGAP1 binding (59). Despite this, none of the structures solved to date have shown any interactions between the insert loop and any regulator or effector (reviewed in Ref. 73). To analyze the contribution of the insert loop residues to IQGAP1 binding, we therefore created several mutated versions of the Cdc42 insert loop. The only mutation that decreased the affinity of Cdc42 for IQGAP1 was N132K. Even when the whole insert loop was removed, Cdc42 was able to bind IQGAP1 with the same affinity as wild-type Cdc42. The effect of the N132K mutation can be explained in light of the N132A mutation in Cdc42 and K132A in Rac1. Both of these mutations increased the affinity of the GTPases for IQGAP1. The RhoA-AlF3-RhoGAP structure shows that Met-134 in RhoA (equivalent to 132 of Cdc42/Rac1) contacts RhoGAP (53). In the homology model of the Rac1-IQGAP1 complex that
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we constructed, the insert region is close to the GAP, and indeed Lys-132 is the closest point of contact between the two proteins (Fig. 6). This implies that the interface used by Cdc42 to contact IQGAP1 has at least some residues in common with that used to contact RhoGAP. The model was constructed using the structure of Ras-GDP-AlF₃-GDPGAP and represents the transition state of the hydrolysis reaction. There is no structure available for the ground state for the reaction (i.e. Ras-GMPPPNP-RasGAP), but the structures of the Cdc42-GMPPNP-RhoGAP (53) and Cdc42-GDP-AlF₃-RhoGAP (74) allow the ground state and the transition state of the hydrolysis reaction to be compared for Cdc42. This comparison shows that there is a change in the intermolecular interface when the transition state is formed and furthermore that Asn-132 is in close proximity to RhoGAP only in the transition state. Mutation of the other residues that are close to RhoGAP only in the transition state (Glu-91, Asn-92, and Lys-96) conversely do not affect the binding. This suggests that the interface in the Cdc42-GTP-IQGAP1 complex has at least some features in common with the transition state complex, although it is catalytically unproductive. In this study, we measured the affinities using the Q61L variant of Cdc42-GTP, which prevents GTP hydrolysis (both intrinsic and GAP-assisted), so direct comparison between this and the transition state is limited.

Taken together, we interpret these data as showing that the insert loop itself is not positively contributing to the IQGAP1 interaction but is in the vicinity of the IQGAP1 interface. When the Asn or Lys residue at position 132 in the GTPases is replaced by the smaller Ala side chain, steric hindrance by the larger side chains is relieved. The effects of the N132K mutant in Cdc42 are either due to the introduction of a bulky, positively charged residue or because the mutation results in three consecutive Lys residues, which destabilizes the C terminus of the α-helix, creating a structure that prevents the IQGAP1 interaction. Interestingly, when we replaced the insert region of Cdc42 with that from Rac1, the affinity for IQGAP1 was increased at least 38-fold. This finding suggests that, notwithstanding the negative effects of a bulky group at Lys-132, the Rac1 insert loop in the context of Cdc42 is more favorable for the IQGAP1 interaction. This may be due to the distribution of the charged residues or slight differences in the structures of the two insert regions. Substitution of the Cdc42 insert loop into Rac1, in contrast, has very little effect on the IQGAP1 affinity.

Work with insert loop mutants of Cdc42 and IQGAP1 has also been performed previously. Replacement of the insert loop of Cdc42 with the analogous region of Ras resulted in a protein that was ~5-fold less effective in binding IQGAP1 (59). The discrepancy with our results might be explained by differences between the mutants examined. Our ΔIns mutant has the insert loop deleted (residues 124–135 inclusive), whereas McCallum et al. (59) replaced the insert loop of Cdc42 (residues 120–139) with those from Ras (residues 120–126). It is possible that the Ras sequence affects the IQGAP1 binding in a similar manner to the N132K mutation, as it is positively charged (ARTVESR) compared with our ΔIns mutant (RDDPITP). Alternatively, the Ras substitution has removed residues outside 124–135 that are important for binding. Li et al. (51) have also reported a decrease in IQGAP1 binding with Cdc42 lacking the insert region. Again this deletion (residues 122–134) is slightly different from the one we describe here (residues 124–135). The 122–134-residue deletion retains a loop with overall positive charge (RDKPITP), whereas our 124–135-residue deletion has overall negative charge (RDDPITP), which could explain the differences observed. Other investigations using the same mutation as our ΔIns mutant in Rac1 have shown that although the insert loop is not involved in cytoskeletal control or activation of the JNK cascade, it is critical for mitogenesis, because its deletion abrogates stimulation of DNA synthesis and superoxide production (72). As IQGAP1 is implicated as a downstream effector in small G protein control of the cytoskeleton, it follows that mutations in the insert loop would not be expected to affect IQGAP1 binding.

We also investigated whether Cdc42 and Rac1 employ similar binding interfaces with IQGAP1 and two other effector proteins, ACK and PRK1. No mutations (Table 4) examined showed large changes in affinity for IQGAP1 except T17N. The T17N mutation in both Cdc42 and Rac1 is frequently used as a dominant negative mutant. This mutation is thought to act in cells by sequestering the G proteins in nonproductive interactions with their upstream activator proteins, the GEFs. Thr-17 coordinates the Mg²⁺ cofactor, so its mutation would potentially destabilize the interaction with GTP and therefore the orientation of the switches, rather than being in direct contact with IQGAP1. The T75K mutation in Cdc42 showed a modest decrease in affinity for IQGAP1. Interestingly, Thr-75 was identified in the Cdc42-ACK interface but subsequently determined to play no thermodynamic role in that interaction (47, 61). It seems however that it might make a small contribution to the IQGAP1 interaction. Our previous analyses of the Cdc42-CRIB effector (47, 61) and Rac1-PRK1 (52) complexes had identified various residues as energetically important in these interactions. None of these key residues, however, were identified as important for the Cdc42/Rac1-IQGAP1 interactions with the exception of Rac1 Asp-63 (switch II) and Cdc42 Tyr-32 and Val-36 (switch I). From these data it is apparent that Cdc42 and Rac1 use a binding interface (at least thermodynamically) with IQGAP1 that is significantly different from that with at least two representative effector proteins.

Finally we analyzed available structures of three small G protein-GAP complexes to identify any other regions of the small G proteins that might contribute energetically to IQGAP1 binding (Table 5). However, mutation at these positions did not decrease the affinity for IQGAP1, and in fact in most cases an increase in affinity was observed. These data would imply that, in these regions at least, the interface for IQGAP1 on Rac1 and Cdc42 is dissimilar to that utilized by genuine Rho family GAP proteins. However, when we investigated the contribution of these residues to RhoGAP binding we also found little involvement. Thus, these residues represent a “cold spot” on the RhoGAP interface and could be the same in the IQGAP1 interface.

Our competition studies between IQGAP1 and RhoGAP for Cdc42 or Rac1 showed that RhoGAP only reduced the affinity of the interaction between IQGAP1 and Cdc42 or Rac1 by 2–3-fold, whereas a true competitor would have completely abrogated binding of IQGAP1 to the G proteins. Therefore,
IQGAP1 has significant affinity for the RhoGAP complexes with either Cdc42 or Rac1. This suggests that the binding sites on both Cdc42 and Rac1 for the two interacting proteins only partially overlap. Indeed, the ternary IQGAP1-Cdc42 (or Rac1)-RhoGAP has comparable stability to the binary effector-G protein complexes.

All of our mutagenesis studies targeting the small G protein side of the small G protein-IQGAP1 complex suggest that there are differences in the Cdc42 and Rac1 binding interfaces for IQGAP1. However the limited data that we have regarding IQGAP1 suggests that the binding sites on IQGAP1 for Cdc42 and Rac1 also differ. IQGAP1-(950–1407) contains most of the binding determinants for Cdc42 but conversely shows no interaction with Rac1 that could be detected by SPA, suggesting that critical determinants for Rac1 binding lie outside this region. The displacement data (Fig. 5) also demonstrate that Rac1 and Cdc42 do not fully compete with each other for binding to IQGAP1 and that a ternary, albeit weak, Rac1-IQGAP1-Cdc42 complex can be formed. In contrast to the experiments with RhoGAP (Fig. 4), the difference between pure and partial competition fits was much lower (Fig. 5b). This is supported by the observation that saturating Cdc42-GMPPNP decreases the affinity of [3H]GTP-Rac1 for IQGAP1 by about 10-fold, and vice versa. Hence, the interactions are predominantly competitive, and the affinity of the ternary Rac1 for the IQGAP1-Cdc42 complex (and Cdc42 for the IQGAP1-Rac1 complex) is much weaker than the affinity of the IQGAP1-Rac1 or IQGAP1-Cdc42 binary complex. These data are consistent with the two interfaces for the G proteins on IQGAP1 being largely shared, but with Rac1 having additional interactions in the region deleted from IQGAP1-(950–1407). We cannot exclude the possibility, however, that Rac1 and Cdc42 have distinct binding sites on IQGAP1 and that binding of one results in conformational changes in IQGAP1, which reduce the binding affinity at the second site.

This study of the interactions between IQGAP1, Cdc42, and Rac1 has revealed some unexpected results. IQGAP1 contacts switch I of the small G proteins but in a manner closer to that of GAP proteins than that of effector proteins. It makes significant contacts to switch II in Rac1, as would be predicted for a GAP-like domain, but switch II in Cdc42 does not contribute energetically to the interface at all, despite the fact that the sequences of switch II in Cdc42 and Rac1 are identical. This correlates with the effects of the mutants on RhoGAP binding; switch II mutants in Cdc42 have a much smaller effect than the same mutants in Rac1. Therefore, our model is that the molecular topography of the switch II loops in Cdc42 and Rac1 must be significantly different and that the conformation adopted by switch II in Rac1 leads to the energetic contribution in binding IQGAP1 and RhoGAP. We have previously found evidence indicating the importance of surface conformation, as opposed to simply surface residues, for Rac1 and Cdc42 in binding to their effectors (75). As RhoGAP can partially compete with IQGAP1 for binding to both Cdc42 and Rac1, we expect that the binding interface utilized by IQGAP1 is similar in at least some respects to that which RhoGAP forms with Cdc42. The insert loop together with regions utilized by other effector proteins and other GAP proteins as part of their binding interface are also not utilized energetically by IQGAP1. As IQGAP1 binds with a high affinity to both Rac1 and Cdc42, it might be assumed that other regions of the small G proteins will be involved in binding. However, it is also possible that the binding surface for IQGAP1 on both Rac1 and Cdc42 is relatively large but devoid of energetic hot spots displaying a more homogeneous energy landscape. This type of interface would be more difficult to probe by single site mutagenesis but could be amenable to studies based on multiple mutations.

In conclusion, our extensive study of Cdc42 and Rac1 in complex with IQGAP1 has revealed unexpected differences in the details of the individual interactions. To our knowledge this is the first demonstration of a shared Rho family effector that interacts in a significantly different manner with two such highly related small G proteins. Our data provide insight into the mechanisms by which Cdc42 and Rac1 interact with binding partners and the mechanism by which IQGAP1 binds the Rho GTPases. As the interaction between the Rho GTPases and IQGAP1 regulates cell-cell adhesion by stabilizing E-cadherin/β-catenin/α-catenin contacts and also promotes cell motility, the ability to dissect the regulation of IQGAP1 by Cdc42 and Rac1 may lead to tools to study cellular adhesion and open up new anti-metastasis therapeutic avenues.

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