Specificity Determinants on Cdc42 for Binding Its Effector Protein ACK†

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ABSTRACT: Cdc42 and Rac are highly homologous members of the Rho family of small G proteins that interact with several downstream effector proteins thereby causing cytoskeletal rearrangements, cell proliferation, and differentiation. While some effectors, such as the tyrosine kinase, ACK, and the scaffold protein, WASP, are unique to Cdc42, others, such as the serine-threonine kinase, PAK, are shared with Rac. Previous mutagenesis studies identified Val42 and Leu174 as residues that selectively affect binding of Cdc42 to ACK and WASP but not to PAK. However, it is unclear whether these discriminatory residues are sufficient determinants of specificity. In this study we sought to introduce "gain-of function" mutations into Rac to allow it to bind to ACK and WASP, thereby revealing all specificity determinants. Thirteen mutations were made changing Rac residues to those in Cdc42. Equilibrium binding constants of all mutant Rac proteins to ACK, WASP, and PAK were measured. A combination of seven mutations (S41A, A42V, N43T, D47G, N52T, W56F, and R174L) was determined to be necessary to change the binding affinity of Rac for ACK from negligible (K_d < 1 μM) to a comparable affinity to Cdc42 (K_d 25 nM). These mutations are not confined to interface residues. We interpret these data to indicate the importance of the structure of regions of the protein distinct from the contact residues. None of these mutant Rac proteins bound WASP with a similar affinity to Cdc42. Hence, residues as yet unidentified, outside the interface, must be necessary for binding WASP.

The Rho family of small GTP-binding[1] proteins, including Rho, Rac, and Cdc42, play important roles in the control of cell growth, differentiation and migration (*1* and references therein). They act as molecular switches, interconverting between GDP-bound (inactive) and GTP-bound (active) forms. In response to external stimuli, Rho family proteins become GTP-bound and can then interact with a large variety of downstream effector proteins (reviewed in ref *2*). Characterization of these interactions and the solution of many structures of G protein−effector complexes have increased the understanding of these functional relationships. The structures of Cdc42 in complex with the GTPase binding domains (GBDs) of its CRIB (Cdc42/Rac interactive binding) effector proteins, ACK, WASP, and PAK, have been solved (*3*−*5*). PAK, p21-activated kinase, is a serine/threonine kinase that plays a role in regulation of the actin cytoskeleton and JNK signaling (*6*); ACK, activated Cdc42-associated kinase, is a tyrosine kinase implicated in integrin signaling (*7*) and clathrin-mediated endocytosis (*8*); while WASP, Wiskott-Aldrich syndrome protein, is a nonkinase effector involved in regulation of the actin cytoskeleton via the Arp2/3 complex (*9*).

An important question relating to G protein function is how the proteins specifically recognize and bind their effector proteins and which residues contribute to this specificity. This is particularly interesting given the high sequence similarity between Rho family proteins. Cdc42 and Rac are 72% identical, yet some effectors such as PAK are common to both, whereas others e.g. ACK and WASP only bind Cdc42. It is not clear how these Cdc42 effectors specifically recognize and bind to Cdc42 as opposed to Rac. Our previous mutagenesis studies (*3*, *10*) have identified Val42 and Leu174 of Cdc42 as residues that selectively affect binding to ACK and WASP but not to PAK. A study of PAK1, WASP, and IQGAP identified residues 173 (conserved in Rac and Cdc42) and 174 as potential WASP specifying sites (*11*). It is, however, unclear whether the interactions of these residues are sufficient determinants of specificity.

Other studies of small G protein interactions have identified mutations that can disrupt complexes while a few have, more instructively, described mutations which result in a gain of function. Studies of the Ras family G protein−effector interactions have identified residues that have a gain in function effect on interaction with target proteins (*12*, *13*), but these have all involved charge reversal or gain/loss of charge, while Rho family G protein−effector interfaces are largely hydrophobic. For Rho family effector interactions two studies have demonstrated a gain in function following targeted mutagenesis (*14*, *15*). Prieto-Sanchez et al. demon-

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[1] Abbreviations: GBD, G protein binding domain; CRIB, CDC42/Rac interactive binding; ACK, activated Cdc42-associated kinase; PAK, p21 activated kinase; WASP, Wiskott-Aldrich syndrome protein; IPTG, isopropyl-β-D-thiogalactopyranoside; GST, glutathione-S-transferase; GTP, guanosine-5′-triphosphate; GDP, guanosine-5′-diphosphate; DTT, dithiothreitol; SPA, scintillation proximity assay; GMPPNP, guanylyl-5′-imidodiphosphate; GMPPCP, $\beta\gamma$ methylene guanosine-5′-triphosphate; GEF, guanine nucleotide exchange factor.

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strated binding, albeit at a low level, of RhoG to PAK by changing residues 44 and 45 to the equivalent Rac residue while simultaneously substituting the C-terminus for that of Rac (residues 155 onward). More recently Gu et al. achieved wild-type binding of ACK2 to TC10 by substituting residues 36−68 of Cdc42 into TC10 (*15*).

As Rac is so similar to Cdc42 in primary sequence and tertiary fold but does not bind either ACK or WASP, we wished to use it as a nonbinding "scaffold" into which specific mutations could be introduced to enable interaction with these two effector proteins. Our main aim was to design a mutant Rac protein that would be able to bind ACK with an affinity comparable to that of Cdc42, to gain a better understanding of the determinants of G protein recognition. We also examined the effects of the Rac mutations on the interaction with WASP, to identify whether the determinants of recognition were the same for both effectors or whether different sets of residues were involved. As controls, to ensure that mutations did not perturb the structural integrity of the proteins, binding was also measured to PAK and RhoGAP, as these do not discriminate between Rac and Cdc42.

METHODS

Expression Constructs. All the proteins were expressed as glutathione-S-transferase (GST) fusions in the pGEX series of expression vectors (Amersham Biosciences).

Constructs expressing PAK_{75-118} (*16*), $ACK_{504-545}$ (*3*), GST-$WASP_{210-321}$ (*17*), and GST-$RhoGAP_{198-439}$ (*18*) were all kind gifts. C-terminally truncated forms of the small G proteins, Cdc42 Δ7 Q61L (*3*) and Rac Δ7 Q61L (this work), were expressed in pGEX-2T from constructs cloned into the *Bam*H1 and *Eco*R1 sites.

Rac Mutagenesis. Site-directed mutagenesis of the Rac Δ7 Q61L expression construct was performed using the QuikChange multisite directed mutagenesis kit (Stratagene). The mutations were confirmed by sequence analysis on an automated sequencer (Applied Biosystems Inc.) by the Department of Biochemistry DNA Sequencing Facility, Cambridge.

Recombinant Protein Production. GST-fusion proteins were expressed in *Escherichia coli* XL10 Gold (Stratagene) or BL21 (Novagen Inc.). Stationary cultures were diluted 1 in 10, grown at 37 °C to an OD_{600} of 0.8, and induced with 0.1 mM IPTG for 5 h. Proteins were then affinity purified using glutathione agarose beads (Sigma-Aldrich). GST fusions of CRIB domain proteins and RhoGAP were eluted from the glutathione beads and used directly. GST-Cdc42 Q61L Δ7 and GST-Rac Q61L Δ7 variants were cleaved from their GST tag with thrombin (Novagen), while still attached to the beads, prior to use in assays. Protein concentrations for all proteins were evaluated from measurement of their A_{280} using their amino acid composition and the extinction coefficients of tyrosine, phenylalanine, tryptophan, and the guanine nucleotide (for the G proteins) (*19*).

Nucleotide Exchange. Bound nucleotides were replaced with [^{3}H]GTP by nucleotide exchange in the presence of a GTP regeneration system to convert any GDP to GTP. [8,5′-^{3}H]GTP (0.15 mCi) (NEN, Perkin-Elmer) was dried by centrifugal evaporation. To this was added 0.7 mg of G-protein, 15 mM phosphoenol pyruvate, 15 mM KCl, 0.36 M $(NH_4)_2SO_4$, and 6 units of pyruvate kinase (Sigma-Aldrich) in a total volume of 140 μL of buffer: 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT. The mixture was incubated at 37 °C for 2 h, after which 10 mM $MgCl_2$ was added to stop the reaction. One milliliter spin columns containing Sephadex G25 superfine (Amersham Biosciences) equilibrated in buffer with 1 mM $MgCl_2$ added were used to remove unbound nucleotide.

Scintillation Proximity Assays (SPA). Affinities of Cdc42 and Rac proteins for GST-ACK, -PAK, -WASP, and -RhoGAP were measured using SPA. The GST effector proteins, at constant concentrations of 0.05 μM, were immobilized on protein A SPA fluoromicrospheres via anti-GST antibody (Molecular Probes, Invitrogen). The equilibrium binding constants (K_d) of the effector−G protein interaction were determined by monitoring the SPA signal in the presence of varying [^{3}H]GTP·Rac/Cdc42 concentrations as described previously (*16*, *20*). Binding of Cdc42 or Rac to the GST-fusion protein brings the radiolabeled nucleotide close enough to the scintillant to obtain a signal. The highest sample concentration of Cdc42 used was 0.5 μM and of each Rac mutant, 2 μM. In each case a blank experiment was performed in the absence of GST-effector. In the absence of GST-effector, increasing the concentration of [^{3}H]GTP·Rac/Cdc42 resulted in a linear increase in background SPA counts. These background counts were subtracted from the data points obtained in the presence of GST-effector and plotted as a function of increasing G protein concentration. For each affinity determination, data points were obtained for at least 10 different G protein concentrations.

Binding curves were fitted using the appropriate binding isotherms (*16*, *20*) to obtain K_d values and their standard errors for the G protein−effector interactions.

RESULTS

Tools for the Measurement of Equilibrium Binding Constants for Cdc42 and Rac. SPAs were used to measure the affinity of Cdc42, Rac, and various Rac mutants for GST-$ACK_{504-545}$, GST-$WASP_{210-321}$, and the positive controls GST-PAK_{75-118} and GST-$RhoGAP_{198-439}$. These positive controls were required to demonstrate that the mutated proteins were correctly folded and functional. The ACK, PAK, and WASP fragments all bind to the same region of Cdc42, with the N-terminal regions of their GBDs forming an extended intermolecular β-sheet with β2 of Cdc42 (*3*−*5*) (Figure 1). The C-terminal regions of their GBDs, however, interact with Cdc42 in significantly different ways. The C-termini of PAK and WASP form a β-hairpin followed by an α-helix, although these interact in different ways with Cdc42 switch II and have different orientations with respect to each other in the two complexes. The C-terminus of ACK wraps around Cdc42 in an extended conformation making contacts with switch I and II of the G protein. As PAK binds both Cdc42 and Rac and the contact sites on Cdc42 for ACK, WASP, and PAK are generally similar, PAK binding is a useful control for structural changes in the "effector binding surface" of the G protein that could lead to a general disruption of CRIB effector binding after mutation. The GTPase activating protein RhoGAP, like the effector proteins, specifically binds the GTP bound form of G proteins

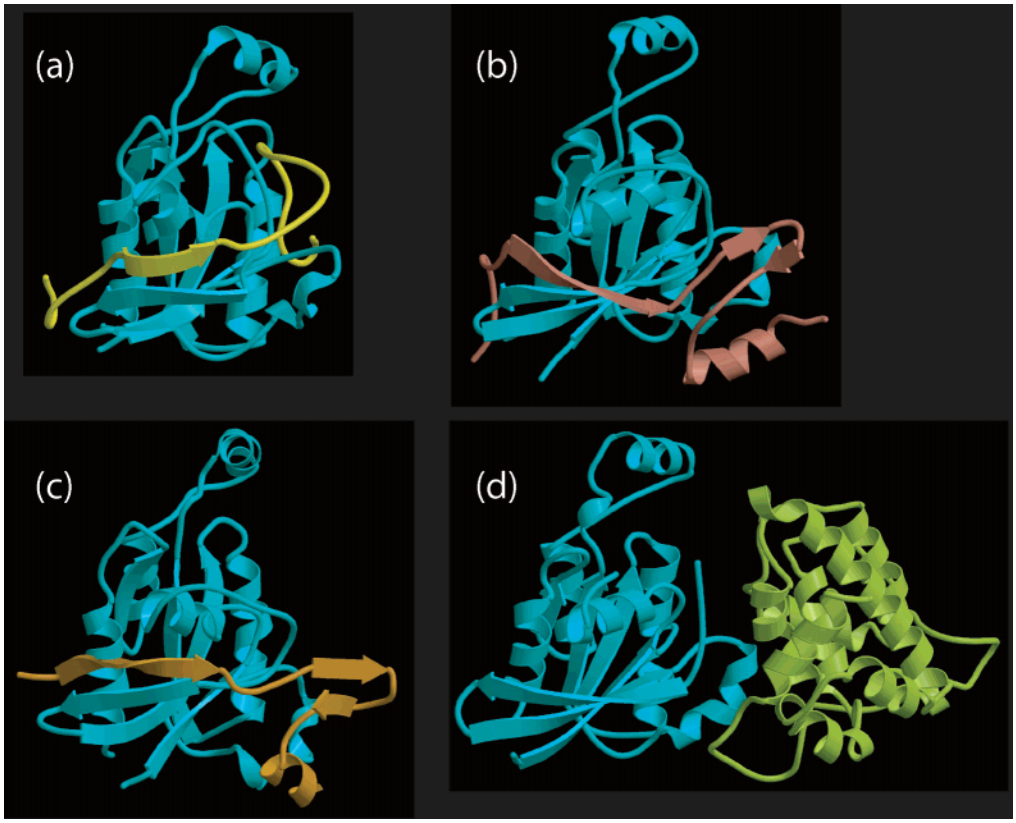

FIGURE 1: Structures of Cdc42 in complex with the three CRIB effectors, ACK, WASP, and PAK, and with the GTPase activating protein, RhoGAP. In each case the G protein is shown in cyan. (a) Cdc42/ACK (*3*). (b) Cdc42/WASP (*4*). (c) Cdc42/PAK (*5*). (d) Cdc42/RhoGAP (*21*).

and is also able to bind both Cdc42 and Rac. However, RhoGAP interacts mainly with switch II and the P-loop of the G protein and has few contacts with switch I (*21*). RhoGAP therefore provides a control for longer distance structural changes within the G protein as a result of mutation as well as being indicative of switch II conformational changes.

The Cdc42 and Rac proteins used were all Q61L mutants that also lacked the C-terminal 7 residues (Δ7). The Q61L mutation produces a constitutively active form of the G protein, with a significantly decreased intrinsic GTPase activity, facilitating the use of G protein complexed to GTP, its natural ligand. Q61L Cdc42·GTP binds with higher affinity than wild-type Cdc42·GMPPNP to the CRIB effector proteins ACK, PAK, and WASP and also RhoGAP but has the same effector specificity as wild-type Cdc42 (*10*). Deletion of the 7 C-terminal amino acids stabilizes the protein in vitro but has no affect on binding to any of the interacting proteins used in this study. Cdc42/Rac Δ7 Q61L is henceforth referred to as wild-type Cdc42/Rac.

Cdc42 binds ACK with an apparent K_d of 25 ± 18 nM, PAK with K_d 23 ± 11 nM, WASP with K_d < 1 nM, and RhoGAP with K_d 32 ± 29 nM (Figure 2A). These values are consistent with the previously published K_ds for the interaction of these effectors with Cdc42 using SPA (*10*).

The affinity of Rac for ACK and WASP is too low to measure by SPA (K_d ≫ 1500 nM). Rac binds PAK with a K_d of 56 ± 14 nM and RhoGAP with a K_d of 194 ± 29 nM (Figure 2B). The K_ds of Rac for PAK and RhoGAP are consistent with previously determined values (*16*, *20*). In addition, Thompson et al. (1998) report that PAK fragments

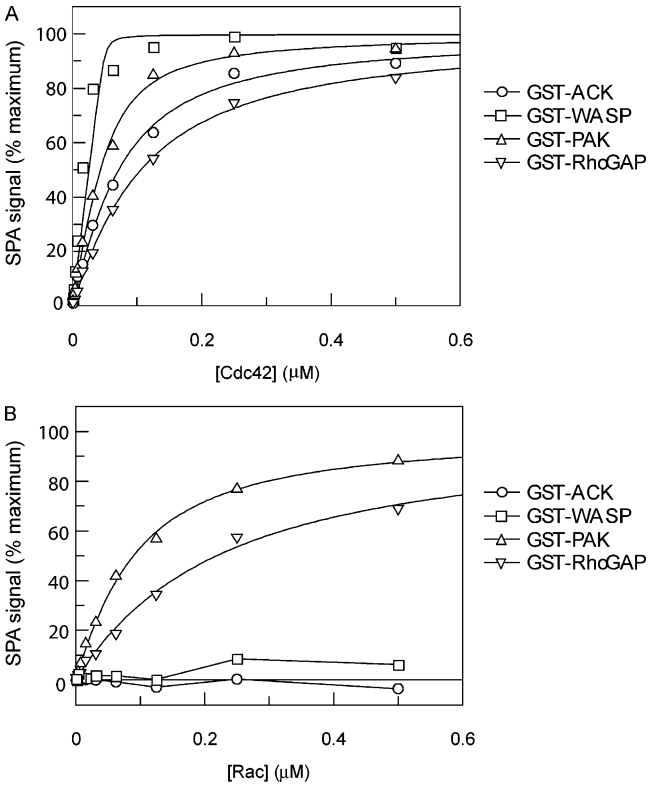

FIGURE 2: (A) Fits of Cdc42·[^{3}H]GTP and (B) Rac·[^{3}H]GTP binding to GST-ACK (○), GST-WASP (□), GST-PAK (△) and GST-RhoGAP (▽).

bind Cdc42 with slightly higher affinity than Rac (3−10-fold), and this is consistent with the 2-fold difference in affinities measured here (*16*).

Table 1: Affinities Measured by SPA Direct Binding Assay of [^{3}H]·GTP Rac, [^{3}H]·GTP Cdc42, and [^{3}H]·GTP Rac Mutants Binding to GST-ACK, GST-PAK, GST-WASP, and GST-RhoGAP[a]

	apparent K_d (nM)			
G protein	GST-ACK	GST-PAK	GST-WASP	GST-RhoGAP
Cdc42	25 ± 18	23 ± 11	<1[b]	32 ± 29
Rac		65 ± 5		194 ± 29
Rac‘8’	850 ± 94	25 ± 2	207 ± 22	38 ± 29
Rac‘8’$^{N52T\ K116Q}$	208 ± 17	10 ± 4		15 ± 4
Rac‘8’$^{N52T\ K116Q\ A27K}$	72 ± 12	5 ± 3		
Rac‘8’$^{V51Y\ W56F}$	25 ± 5	22 ± 5		65 ± 24
Rac‘8’$^{V51Y\ W56F\ A27K}$	20 ± 5	15 ± 4		110 ± 24
Rac‘13’	60 ± 10	45 ± 12	108 ± 20	49 ± 24

[a] Cdc42 and Rac affinities are included for comparison. [b] This K_d is taken from previous work (*10*).

If mutations in Rac do not disrupt the structure of the protein, the K_ds for the PAK and RhoGAP controls should be approximately 50 nM and 200 nM, respectively. Potentially these affinities could increase in some of the mutants, because Cdc42 binds with a 6-fold higher affinity to RhoGAP than Rac and with a 2.5-fold higher affinity to PAK (Table 1).

Design of Mutations. Using the SPA system under the conditions stated in the methods section, precise K_d values can be obtained in the range between 10 and 1500 nM. Thus, any significant gain in affinity of the Rac mutants that reduces the K_d to within these limits should be readily identifiable. The structures of the Cdc42−ACK (*3*) and Cdc42−WASP (*4*) complexes were used to identify residues of Cdc42 that make contacts in the two interfaces. The initial Cdc42 residues selected were those situated in the Cdc42−ACK interface, which had side chains within 4 Å of effector residue side chains and whose NMR resonances shifted on complex formation. As it seemed reasonable that residues specifying ACK or WASP binding to Cdc42 would be residues that differed between Cdc42 and Rac, 8 residues in Cdc42 that are not conserved in Rac were identified as making the major contacts with ACK (i.e. within 4 Å of the effector side chains). These residues were Val33, Ala41, Val42, Thr43, Ile46, Gly47, Leu174, and Glu178 (Figure 3A,B). The corresponding residues in Rac, i.e. Ile33, Ser 41, Ala42, Asn43, Val45, Asp47, Arg174, and Cys178, were, therefore, mutated to those in Cdc42. These residues were later considered in the context of the Cdc42−WASP structure where they also make the major contacts (see below). The mutations identified as making minor contacts (4−5Å from effector side chains) in the Cdc42−ACK and Cdc42−WASP complexes were found by a similar evaluation of the available structures. The group of 8 residues making major contacts included those nonconserved amino acids whose side chains had the largest change in solvent accessibility between free and effector-bound Cdc42. It has been suggested that residues with a large change in solvent accessibility can act as anchor residues in protein−protein interactions (*22*).

Initial Mutations. The mutations I33V, S41A, A42V, N43T, V46I, D47G, R174L, and C178E of Rac were made individually, but none increased the affinity of Rac for ACK to a K_d that could be measured accurately by SPA (Figure 4A). While it seemed possible from a visual inspection of the SPA data that the A42V and N43T mutations slightly increased the affinity (as judged by a small increase in cpm with increasing [Rac]), this effect was very small and the data could not be fitted to give an accurate measure of K_d. If Rac^{A42V} or Rac^{N43T} are binding ACK, they have an affinity weaker than 1500 nM. None of these mutations however changed the affinity of Rac for PAK or RhoGAP, demonstrating that they do not significantly disrupt the structure of the protein (data not shown).

Combination Mutants. As the individual mutations had little measurable effect on the affinity of Rac for ACK, all eight mutations were combined in a single Rac protein Rac$^{I33V\ S41A\ A42V\ N43T\ V46I\ D47G\ R174L\ C178E}$ referred to henceforth as Rac‘8’. In addition, two other combinations of mutations were made. The binding curves for A42V and N43T implied a slight increase in the affinity (Figure 4A and above) suggesting that this region might be a hotspot of interaction. As Ser41 is juxtaposed to Ala42 and Asn43, interactions between the residues could play a role orientating them in the interface and therefore the combination mutant, Rac S41A A42V N43T, was made. The second, Rac$^{I33V\ S41A\ A42V\ N43T\ R174L\ C178E}$, was an intermediate between the other two mutants. Leu174 had been previously implicated in Cdc42−ACK specificity (*10*), and as Glu178 is situated close by, this region possibly constituted a second hotspot. Thus this second intermediate mutant included both potential hotspots.

Rac$^{S41A\ A42V\ N43T}$ exhibited an increased affinity for ACK with a K_d of 1500 nM (Figure 4B). Rac$^{I33V\ S41A\ A42V\ N43T\ R174L\ C178E}$, and Rac‘8’ had similar affinities for ACK, with a K_d of 850nM (Figure 4B), indicating that residues 46 and 47 do not make a significant contribution to the affinity. Again, none of the mutations had any effect on affinity for PAK or RhoGAP, demonstrating that they do not disrupt the structure of the protein.

The affinity of the Rac‘8’ mutant protein for ACK was however, still 30-fold lower than the affinity of Cdc42 for ACK (K_d = 30 nM). It was therefore apparent that other residues that had not been identified in the original structural search must be required for high affinity binding.

Identification of Further Residues for Mutation. From further analysis of the Cdc42-Rac sequence alignment (Figure 3A) and the Cdc42−ACK structure (*3*), two additional residues, Thr52 and Gln116, were identified as potentially making minor contacts with ACK (i.e. 4−5 Å from the effector). In addition, from similar analysis of the Cdc42−WASP structure two residues, Tyr51 and Phe56, were identified in the WASP contact site (*4*). A fifth residue, Lys27, was identified which is involved in the interaction between Cdc42 and PAK (which interacts with both Cdc42 and Rac) (*5*). This residue is located on the edge of the Cdc42−ACK interface and so could potentially contribute to the interaction (Figure 3B).

The individual mutations of Rac, namely, A27K, V51Y, N52T, W56F, and K116Q, were produced in Rac to confirm their independent contributions to ACK/WASP binding. In addition, in a background of Rac with all eight previous mutations (Rac‘8’), the following mutations were made: Rac‘8’$^{N52T\ K116Q}$, Rac‘8’$^{N52T\ K116Q\ A27K}$, Rac‘8’$^{V51Y\ W56F}$, and Rac‘8’$^{V51Y\ W56F\ A27K}$. Finally, Rac‘13’ with all 13 mutations, Rac$^{A27K\ I33V\ S41A\ A42V\ N43T\ V46I\ D47G\ V51Y\ N52T\ W56F\ K116Q\ R174L\ C178E}$, was made.

None of the individual mutations increased the affinity of Rac for GST-ACK, but neither did they have an effect on

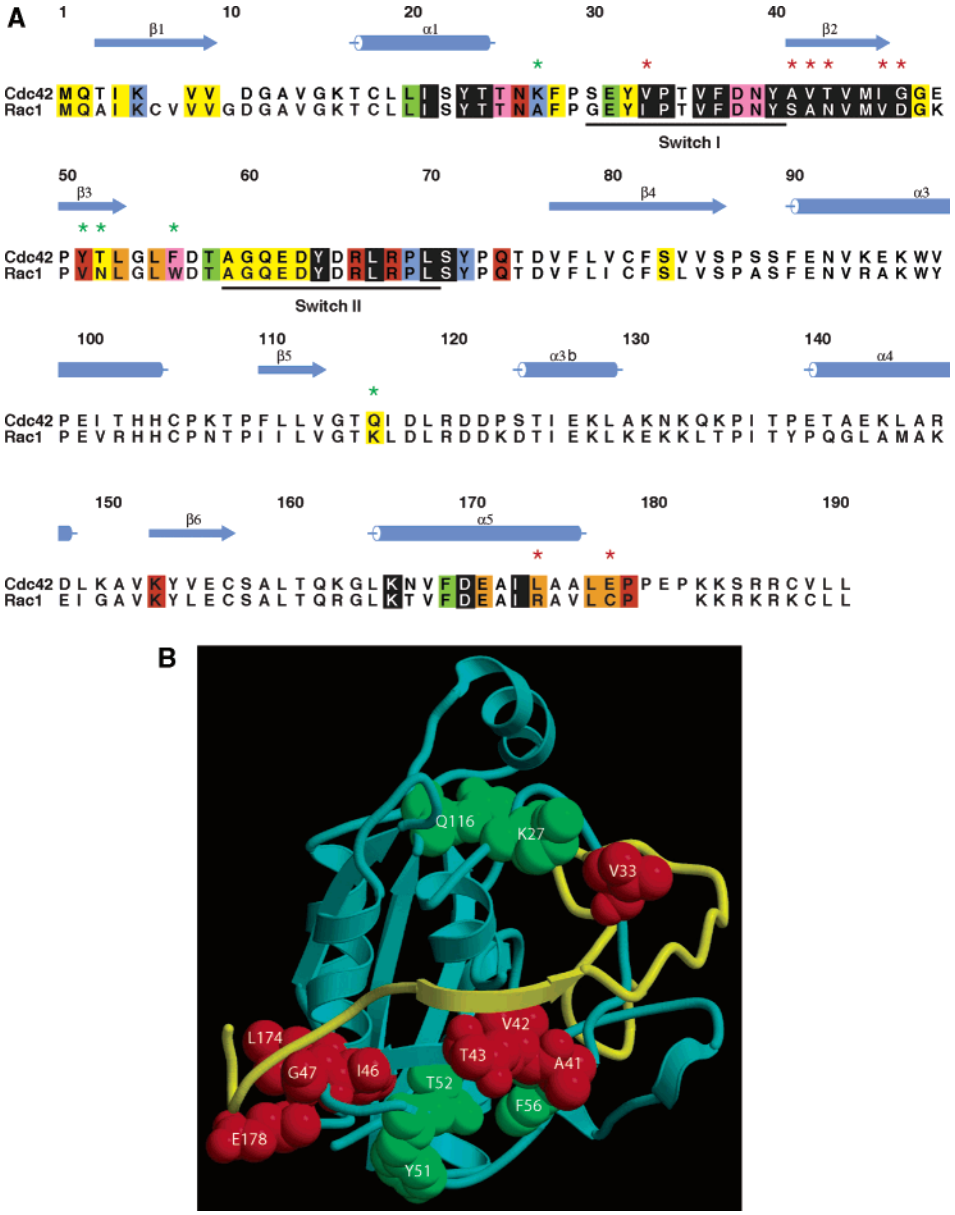

FIGURE 3: Residues that we remutated in this study. (A) Sequence alignment of Cdc42 and Rac. The secondary structure of both proteins is shown in blue above the sequences: α-helices as cylinders, *β*-strands as arrows. The residues that are involved in intermolecular contacts in the CRIB effector/Cdc42 complexes are colored as follows: ACK, yellow; WASP, red; PAK, blue; ACK and WASP, orange; ACK and PAK, green; WASP and PAK, pink; ACK, WASP, and PAK, black. Residues that were mutated are denoted by asterisks above the sequences; red asterisks mark the position of the first 8 mutants, and green asterisks mark the position of the 5 extra mutations. The positions of switch I and switch II, which change conformation on GDP/GTP exchange, are marked below the sequences. (B) The structure of Cdc42/ACK with the thirteen residues mutated in this study shown in a space-filling representation. The first 8 residues mutated are shown in red, and the 5 extra residues mutated are shown in green. Cdc42 is cyan, and the ACK fragment is yellow.

the affinities for GST-PAK or GST-RhoGAP (data not shown). However, all the further mutations when made in the Rac'8' background increased the affinity for GST-ACK (Table 1). Rac'8'$^{N52T\ K116Q}$, the protein with additional mutations designed to increase the affinity for ACK, had an affinity of 208 nM for GST-ACK. This is a 4-fold increase in affinity compared to Rac'8'. However, the incorporation of the additional mutation A27K (Rac'8'$^{N52T\ K116Q\ A27K}$) increases the affinity to a K_d of 72 nM, a further 3-fold increase. This affinity is close to that for Cdc42 binding ACK (30 nM).

The mutations, V51Y and W56F, which were designed to increase affinity with WASP, when incorporated into the Rac'8' background also increased the affinity for ACK (Table 1), even though Tyr51 does not directly contact this effector and the contribution of Phe56 is unclear (see below) (*3*). The Rac'8'$^{V51Y\ W56F}$ and Rac'8'$^{V51Y\ W56F\ A27K}$ mutants increased the affinity for GST-ACK, to ~20 nM, comparable to that of the wild-type Cdc42−ACK interaction. It is noteworthy that the incorporation of the A27K mutation had no effect when combined with Rac'8'$^{V51Y\ W56F}$, whereas when it was introduced into Rac'8'$^{N52T\ K116Q}$ protein it increased the affinity for ACK.

Rac'13' bound GST-ACK with an affinity (K_d = 60 nM) comparable to that of Cdc42 (Table 1). Thus the two pairs/trios of additional mutations made in Rac'8' increased the affinity for ACK, but when all five mutations were combined (Rac'13'), no further increase in affinity was observed. These data indicate a potential maximum affinity for these complexes, which corresponds to the wild type interaction

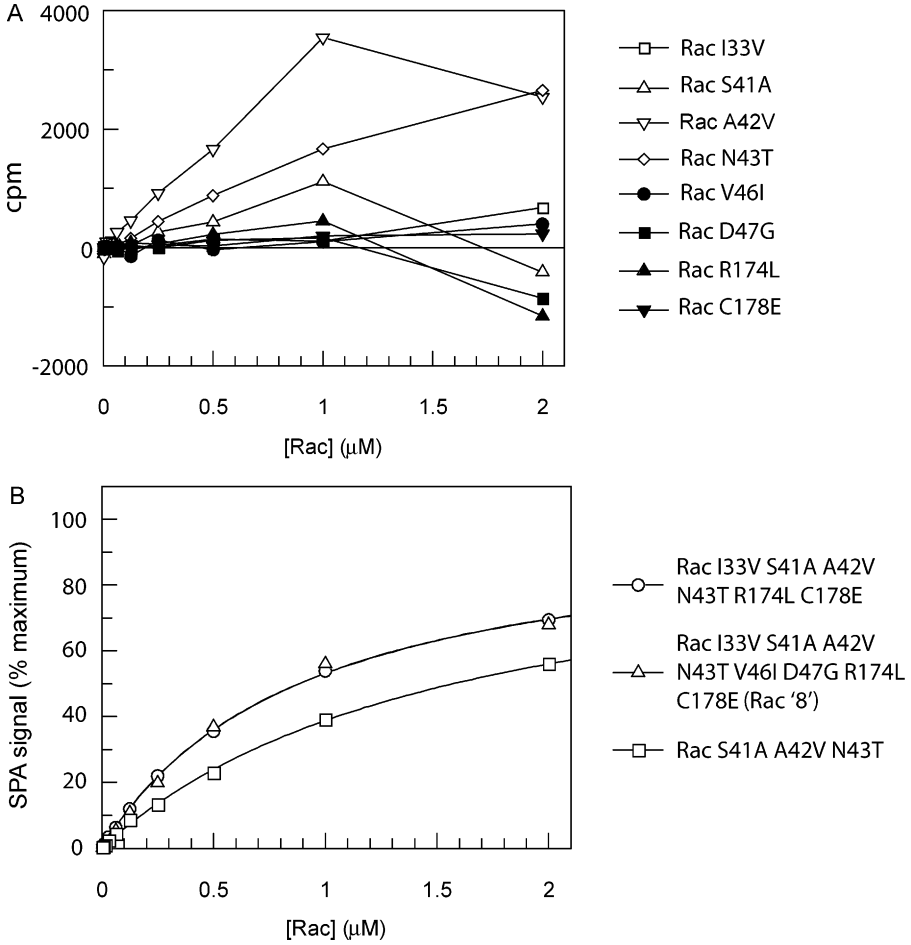

FIGURE 4: (A) SPA cpm of the 8, initial [^{3}H]GTP•Rac mutants binding to GST-ACK: RacI33V (□), RacS41A (△), RacA42V (▽), RacN43T (◇), RacV46I (●), RacD47G (■), RacR174L (▲), and RacC178E (▼). (B) Fits of [^{3}H]GTP•Rac$^{S41A\ A42V\ N43T}$ (□), Rac$^{I33V\ S41A\ A42V\ N43T\ R174L\ C178E}$ (○), and Rac '8' (△) binding to GST-ACK.

between Cdc42 and ACK. This is presumably due to experimental design, as all the mutations investigated change the Rac residue to the corresponding Cdc42 residue.

Analysis of the Importance of Individual Residues. To clarify the relative importance of each of the individual mutations studied, we next removed each mutation from Rac'13' separately, obtaining a series of thirteen mutant Rac proteins, each with twelve residues mutated to the corresponding Cdc42 residues. Mutations K27A, V33I, I46V, Y51V, Q116K, and E178C in Rac'13' had little or no effect on ACK binding, while the other mutations of Rac'13', namely, A41S, V42A, T43N, G47D, T52N, F56W, and L174R, decreased the affinity for ACK between 2.3- and 23-fold (Table 2).

Mutations V42A, T52N, and F56W of Rac'13'. The individual mutations V42A, T52N, and F56W of Rac'13' have the greatest effects on binding to ACK (Table 2). The 23-fold decrease in affinity as a result of the V42A mutation in Rac'13' concurred with our previous results (*3*, *10*). Val42 is located adjacent to switch I, in the interface between Cdc42 and ACK, and was one of the initial residues identified as likely to make important contacts in this interaction. From the structure of Cdc42 in complex with the GBD of ACK, Val42 forms hydrophobic contacts with Ile519 of ACK (*3*). In addition, this residue has already been implicated in having a role in the selectivity of the Cdc42 interaction with ACK (*10*), with the mutation in Cdc42 resulting in a similar (18-fold) decrease in affinity for ACK. In contrast, the 7- and 11-fold effects of the T52N and F56W mutations cannot be explained by direct interactions withACK. Thr52 was identi-

Table 2: Affinities Measured by SPA Direct Binding Assay of [^{3}H]GTP•Rac Δ7 Q61L Proteins with 12 Mutated Residues Binding to GST-ACK, GST-WASP, GST-PAK, and GST-RhoGAP[a]

	apparent K_d (nM)			
G protein	GST-ACK	GST-WASP	GST-PAK	GST-RhoGAP
Cdc42	25 ± 18	<1[b]	23 ± 11	32 ± 29
Rac			65 ± 5	194 ± 29
Rac'13'	60 ± 10	108 ± 20	45 ± 12	49 ± 24
Rac'13'^{K27A}	62 ± 8	131 ± 9	27 ± 5	
Rac'13'^{V33I}	66 ± 11	111 ± 17	29 ± 4	40 ± 8
Rac'13'^{A41S}	137 ± 29	117 ± 15	9 ± 2	46 ± 15
Rac'13'^{V42A}	1350 ± 155	1160 ± 238	93 ± 20	54 ± 14
Rac'13'^{T43N}	196 ± 32	187 ± 31	42 ± 7	19 ± 21
Rac'13'^{I46V}	42 ± 14	75 ± 14	11 ± 3	38 ± 23
Rac'13'^{G47D}	140 ± 22	99 ± 22	5 ± 2	25 ± 17
Rac'13'^{Y51V}	72 ± 10	231 ± 42	35 ± 5	36 ± 23
Rac'13'^{T52N}	389 ± 43	135 ± 70[c]	81 ± 7	
Rac'13'^{F56W}	638 ± 66	68 ± 14	24 ± 10	
Rac'13'^{Q116K}	63 ± 8	86 ± 12	17 ± 12	137 ± 22
Rac'13'^{L174R}	184 ± 29	97 ± 30[c]	32 ± 5	32 ± 9
Rac'13'^{E178C}	21 ± 7	117 ± 18	15 ± 5	33 ± 20

[a] Cdc42 and Rac affinities are included for comparison. Here the mutation in superscript represents the residue of the Rac'13' protein, which is mutated from a Cdc42 residue in Rac'13' back to the corresponding Rac residue. [b] This K_d is taken from previous work (*10*). [c] S_{max} ratio (WASP/PAK) decreased in comparison to other mutants.

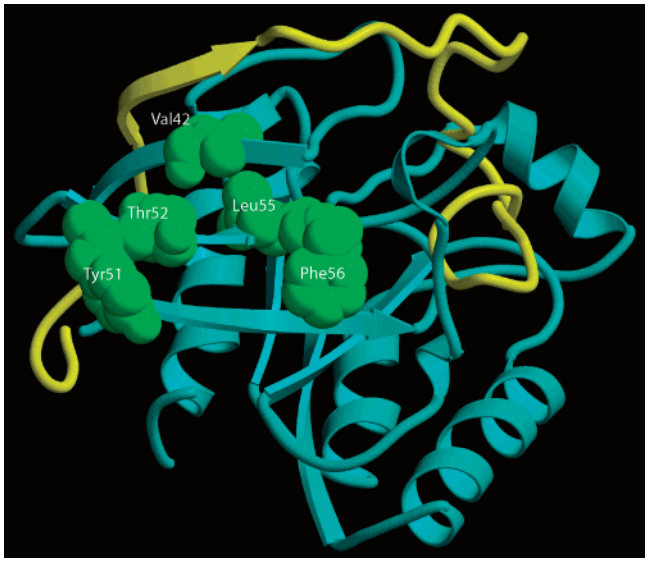

FIGURE 5: Compensating effects of mutation of the residues packing behind Val42. The V42A, T52N, and F56W mutations all decrease the binding of Rac'13' to ACK. Thr52 and Phe56 do not directly contact ACK, but may be involved in maintaining the region around Val42 in the correct conformation for ACK binding. This role for Phe56 is reinforced by the observation that the back mutation Y51V can compensate for F56W. Addition of the bulkier Trp56 at one end of the β-strand behind Val42 can be compensated for by replacement of the Tyr51 ring with a smaller Val side chain.

fied as a residue making minor contacts with ACK in the Cdc42−ACK complex. It seems unlikely that these contacts (4−5 Å distant from Leu514) alone can entirely explain the effect of the mutation. From the structure of the Cdc42−ACK complex it can be seen that this residue packs behind switch I and may therefore have an important structural role holding the switch region in the correct orientation for ACK binding (Figure 5). More specifically, the residue packs directly behind and contacts (within 2 Å) Val42, which, as previously discussed, has been demonstrated to be important in the Cdc42−ACK interaction. We therefore suggest that Thr52 has an important, indirect effect on ACK binding by stabilizing Val42 and switch I in the correct orientation for the interaction.

Phe56 was identified as a residue that makes contacts with WASP in the Cdc42−WASP complex. The importance of this residue in the ACK interaction is however unclear, as the Phe56 side chain is disordered in the Cdc42−ACK complex (*3*). However, from the position of the backbone in the structure, Phe56 must also pack behind switch I and may therefore have a role in maintaining the switch region in the correct conformation for ACK binding **(**Figure 5). The F56W mutation significantly increases the size of the side chain at this position while retaining its rigid, aromatic nature. This size increase could push switch I outward in Rac. Phe56 packs behind conserved residues 37−39, a region of switch I important for effector interaction. Point mutations of Cdc42 residues Phe37 and Asp38 have been demonstrated to severely disrupt the interaction with ACK, decreasing the affinity 167- and 67-fold, respectively (*10*). Structural changes resulting from the F56W mutation in this conserved region of the Rac'13' protein can be considered to be "secondary switching" between two slightly different conformations of active switch I, the Cdc42- and Rac-like conformations. It is therefore possible that ACK is more sensitive than PAK and RhoGAP to slight alterations in the orientation of residues in this region of switch I as it cannot recognize the "Rac conformation". Phe56 is also juxtaposed to, and therefore likely to affect, the conserved residue Leu55, which in turn contacts Val42. It is therefore possible that Phe56 could also be exerting further effects on ACK binding via Val42. As Val42 is emerging as a crucial residue for specificity, these secondary effects of Phe56 are likely to be important.

Interestingly, the Rac'13'^{V42A} and Rac'13'^{T52N} mutants also have a decreased affinity for PAK relative to that of wild-type Rac. This may also be indicative of secondary switching: differences between the switch surfaces in this region of the G protein that determine the slight differences in affinity of Rac and Cdc42 for their shared effector, PAK.

Compensation of Y51V for F56W. Rac'8'$^{N52T\ K116Q\ A27K}$ is equivalent to Rac'13' with back mutations Y51V and F56W. Both Rac'13' and Rac'8'$^{N52T\ K116Q\ A27K}$ bind ACK with similar affinity. As Phe56 has been identified as important to the Cdc42−ACK interaction, it appears that the Y51V mutation can compensate for the effect of the F56W mutation. The F56W mutation introduces a larger, rigid side chain, while the Y51V mutation replaces an aromatic side chain for an aliphatic, which is less rigid and smaller. If the F56W mutation acts through contacts with Leu55 to push the Val42 outward, disrupting the interaction with ACK, the reduction in residue size resulting from the Y51V mutation could allow Val42 to move back inward, via interactions through Thr52 (see Figure 5). As only 4 residues separate residues 51 and 56 in primary sequence, the compensatory change in structure could be transmitted along the backbone of the protein. Alternatively, a similar compensatory arrangement may be taking place in the switch I region (residues 37−39) that we have previously shown to be important for ACK binding (*3*, *10*)

Compensation of Q116K for T52N. Rac'8'$^{V51Y\ W56F\ A27K}$ is Rac'13' with back mutations T52N and Q116K yet binds ACK with an affinity comparable to that of Cdc42. In this case the effect of the T52N mutation therefore seems to be compensated for by Q116K. Gln116 of Cdc42 plays a role in nucleotide binding, contacting the guanidine base and ribose moiety in the nucleotide-binding pocket. The lysine at position 116 of Rac is conserved in H-Ras and RhoA. The fact that this lysine is conserved in Ras, Rac, and Rho but is a glutamine in Cdc42 may reflect slight differences in nucleotide recognition between the two proteins. The compensatory effect of this residue could therefore result from a reorganization of the switch region of the protein via the nucleotide.

Mutations A41S, T43N, G47D, and L174R of Rac'13'. Mutations A41S, T43N, G47D, and L174R of Rac'13' decrease the affinity for ACK by 2.3- to 3.3-fold. The mutation A41S replaces a neutral residue with a polar one, while not greatly altering the size of the side chain. Mutation of Thr43 to Asn is a relatively conservative change; both are hydrophilic, neutral residues although Asn has a different geometry and more polar groups. As well as being identified as making contacts across the Cdc42−ACK interface (with Thr521 and Ser517 respectively), residues Ala41 and Thr43 of Cdc42 are positioned on either side of Val42 and may have an additional effect on ACK binding by maintaining Val42 in the correct orientation for interaction. In particular,

they are situated in a β-sheet and may therefore be important for maintaining its packing and structure. The Rac$^{S41A\ A42V\ N43T}$ mutant had an affinity for ACK of around 1.5 μM while none of the individual mutations in Rac significantly improved the affinity (Figure 4A,B), implying that interactions between these residues may play a combined role in orientating them in the ACK interface.

The G47D mutation replaces a Gly with a much larger, acidic residue. Gly47 contacts Ile510 of ACK, and the presence of a charged residue in the place of Gly would disrupt this interaction. It appeared from the initial combination mutants that residues 46 and 47 were having little or no significant effect on the affinity for ACK, as Rac'8' and Rac$^{I33V\ S41A\ A42V\ N43T\ R174L\ C178E}$ both had a K_d of 850 nM. Rac'8' has both V46I and D47G mutations, so we surmise that the combination of these two mutations confers no further increase in affinity on Rac$^{I33V\ S41A\ A42V\ N43T\ R174L\ C178E}$ while Gly47 alone, when removed from Rac'13', is deleterious to binding. Presumably, replacement of the Gly with the larger Asp side chain can be compensated for by the replacement of Ile with the smaller Val. This compensation would mean that Ile510 can rearrange to contact Val46 only, rather than both Ile46 and Gly47 (as in Cdc42).

Leu174 is located in the C-terminal helix of Cdc42 and has previously been implicated in having a role in the selectivity of the Cdc42 interaction with ACK. Specifically, Leu174 packs against Leu505 of ACK and removal of this side chain would be expected to disrupt the hydrophobic packing of the complex. The mutation L174A in Cdc42 results in a 30-fold decrease in affinity for ACK (*10*) whereas the mutation L174R reduced the affinity of Rac'13' for ACK 3.1-fold. Replacement of Leu174 with the basic Arg therefore has a lesser effect than the mutation to Ala. This may be because Arg, with a relatively long carbon side chain, can form some of the same hydrophobic packing as the Leu. The charged end of the Arg could then protrude from the protein contacting the surrounding solvent. It is however notable that the two mutations L174A and L174R have been made in the context of two different proteins, Cdc42 and Rac'13', respectively, making a direct comparison difficult.

Mutations K27A, V33I, I46V, Y51V, Q116K, and E178C of Rac'13'. The mutations K27A, V33I, I46V, Y51V, Q116K, and E178C had little or no effect on ACK binding, implying that these residues are not significantly involved in the specificity of the interaction of Rac'13' with ACK.

The effect of K27A is complicated by its effect on Rac'8'$^{N52T\ K116Q}$, where incorporation of the mutation A27K in Rac'8'$^{N52T\ K116Q\ A27K}$ increased the affinity for ACK 3-fold, while in Rac'8'$^{V51Y\ W56F}$, addition of A27K (Rac'8'$^{V51Y\ W56F\ A27K}$) has no effect on affinity. This implies that Lys27 has a role in ACK binding as it can rearrange the structure that results from the F56W/Y51V mutations of Rac'13' (since Rac'8'$^{N52T\ K116Q}$ is actually Rac'13'$^{K27A\ Y51V\ F56W}$ and Rac'8'$^{N52T\ K116Q\ A27K}$ is Rac'13'$^{Y51V\ F56W}$). Lys27 is positioned behind switch I and near Tyr32, another residue previously shown to be important for effector interaction (*10*). The effect of the K27A mutation may therefore be a result of structural rearrangements within the switch region, in which Lys27 plays a role in stabilizing Rac'13'$^{Y51V\ F56W}$ but not in stabilizing the more Cdc42-like Rac'13' conformation of the protein. Compensation effects by Q116K have already been discussed.

Table 3: Affinities Measured by SPA Direct Binding Assay of [^{3}H]GTP·Rac Δ7 Q61L Combination Mutants of Residues Appearing Most Important for Interaction with ACK Binding to GST-ACK, GST-PAK, and GST-RhoGAP[a]

G protein	apparent K_d (nM) GST-ACK	GST-PAK	GST-RhoGAP
Cdc42	25 ± 18	23 ± 11	32 ± 29
Rac		65 ± 5	194 ± 29
Rac$^{A42V\ N52T\ W56F}$	1450	44 ± 7	253 ± 23
Rac$^{S41A\ A42V\ N43T\ D47G\ N52T\ W56F\ R174L}$	25 ± 6	38 ± 7	223 ± 80

[a] Cdc42 and Rac affinities are included for comparison.

Combination Mutants of Residues Important for the Interaction with ACK. The three mutations which appeared to have the greatest effect on the affinity of Rac'13' for ACK were V42A, T52N, and F56W, while the mutations A41S, T43N, G47D, and L174R decreased the affinity for ACK to a lesser extent. To clarify the role of these residues in light of compensatory or contradictory effects of some residues in different backgrounds, two final combinations of mutations were made in Rac. These were Rac$^{A42V\ N52T\ W56F}$ (wt Rac with the three residues that appeared most important for the ACK interaction with Rac'13' mutated) and Rac$^{S41A\ A42V\ N43T\ D47G\ N52T\ W56F\ R174L}$ (Rac with all seven residues which appeared to have an effect on the affinity of Rac'13' mutated).

Rac$^{A42V\ N52T\ W56F}$ bound ACK with a K_d of 1450 nM (Table 3). This is 50-fold weaker than the wild-type affinity of Cdc42 for ACK. It is clear therefore that the other residues, which appear less important for the affinity of Rac'13', are nevertheless important for ACK binding.

Rac$^{S41A\ A42V\ N43T\ D47G\ N52T\ W56F\ R174L}$ bound ACK with an affinity of 25 nM, which is comparable to the affinity of the Cdc42−ACK interaction (Table 3). This therefore confirms the data from the Rac mutants with 12 mutated residues (Table 2) that residues Lys27, Val33, Ile46, Tyr51, Gln116, and Glu178 are not important for the specificity of ACK binding Cdc42 versus Rac, while also demonstrating that all of the other seven residues identified are required in combination to achieve a Cdc42-like affinity for ACK.

Effect of Mutations on the Interaction of Rac with WASP. All the Rac mutants described were also used in SPAs with GST-WASP. The K_d of the wt Cdc42−WASP interaction is <1 nM (*10*), while the K_d of wt Rac for WASP (like that for ACK) is too high to be measured by SPA (Table 1).

The mutation V46I of Rac had no effect on the interaction with WASP. The other 12 mutations generally appeared to increase very slightly the affinity of Rac for WASP (by visual inspection of the binding curves), but similarly to the situation with ACK, these increases were insufficient to be able to determine an accurate K_d value (data not shown). Indeed, mutation of all of the eight residues initially identified and predicted to make the major contacts across the interface between Cdc42 and WASP (Rac'8') only increased the affinity of Rac for WASP to a K_d of 207 nM (Table 1).

When the five additional mutations, A27K, V51Y, N52T, W56F, and K116Q, were made to produce Rac'13', the affinity for WASP increased to a K_d of 108 nM (Tables 1 and 2). Although this was a considerably higher affinity than the wild-type Rac−WASP interaction, it was still more than 100-fold lower affinity than the affinity of Cdc42 for WASP.

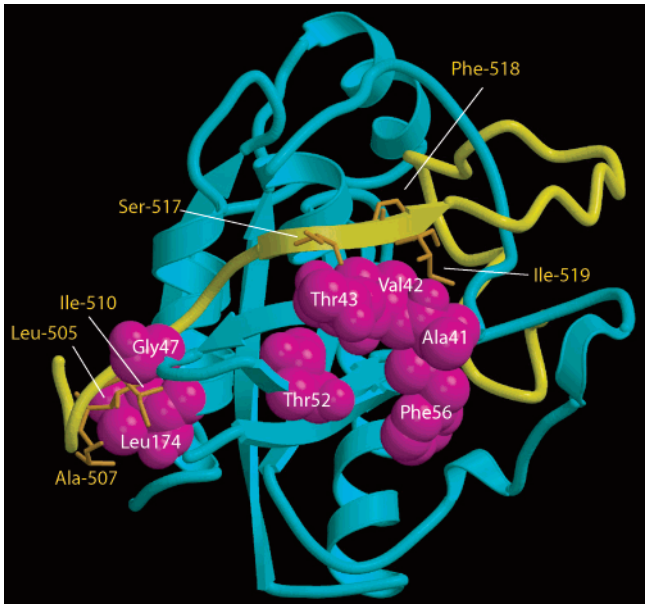

FIGURE 6: The residues highlighted in pink are the "hotspots" for the specificity of the interaction between Cdc42 and ACK. Mutation of just these 7 residues in Rac to the Cdc42 equivalent is sufficient to make Rac bind to ACK with the same affinity as does Cdc42.

We were therefore unable to increase the affinity for WASP to that of the Cdc42−WASP interaction by mutating all the residues in the interface that differ between Cdc42 and Rac and also mutating some residues that clearly, from the data with GST-ACK, have a structural effect on the interface.

Only two of the Rac'13' residues, V42A and Y51V, significantly decreased the affinity for WASP when mutated back to the corresponding Rac residue (Table 2). These data demonstrate distinct differences between the interaction of Cdc42 with ACK and WASP.

DISCUSSION

Our initial aim was to design gain of function mutants of Rac to study the selectivity of Cdc42−effector binding. Using Rac as a "scaffold" protein that does not bind ACK or WASP, we designed mutations that would allow Rac to bind ACK with an affinity comparable to that of the Cdc42 interaction (Figure 6). In addition we examined the effect of these and other mutations (designed from the Cdc42−WASP structure) on the interaction with WASP, and thereby identified whether the determinants of specificity are the same for both effectors.

In the case of the interaction with ACK, we have successfully identified seven residues, Ala41, Val42, Thr43, Gly47, Thr52, Phe56, and Leu174, which together comprise only 5.5% of the total buried surface area, as the determinants of binding specificity of the Cdc42−ACK interaction. Some of these residues are situated in the interface between the two proteins and were therefore relatively easy to identify from the structure of the complex, but others have a more subtle conformational influence on the interface. Thus, while Cdc42 and Rac are very similar in primary structure and tertiary fold, there are minor structural differences which are important for recognition of specific effectors and the interfaces cannot be considered as rigid structures into which different residues can be inserted to alter the binding specificity. The seven residues identified in Cdc42 all make contacts with the N terminal half of the ACK peptide (Figure 6). This is the most conserved part of the ACK, PAK, and WASP GBD sequences and structures (Figure 1). This work implies, however, that it is this N-terminal half of the GBDs that is responsible for discrimination. Thus these seven mutations are enough to alter Rac such that it resembles Cdc42 and can be recognized and bound by ACK. In contrast, $Rac^{S41A\ A42V\ N43T\ D47G\ N52T\ W56F\ R174L}$ binds RhoGAP with an affinity comparable to that of the Rac−RhoGAP interaction (Table 3) rather than the higher affinity Cdc42−Rho GAP interaction. This implies that the residues in Cdc42 that specify high-affinity Rho GAP binding are also different from the residues that are important for ACK binding.

While seven mutations alone were sufficient to increase the affinity of Rac for ACK from immeasurably low to that of Cdc42−ACK binding, none of the mutations or combinations of mutations increased the affinity of Rac for WASP to a value comparable to that of the Cdc42 interaction. The determinants of effector recognition on Cdc42 are therefore significantly different for ACK and WASP with only Val42 implicated in both interactions. Since the mutations we designed did not increase the affinity of Rac for WASP to that of Cdc42, either we have not identified the interface residues important for this interaction or additional subtle structural changes distant from the interface are required for a wild-type Cdc42 interaction with WASP. As we have mutated all the interface residues in the Cdc42−WASP complex, other structural contributions are likely to be required for a wild-type affinity interaction. Indeed, in the case of the Cdc42−ACK interaction, some residues important for the interaction, for example Phe56, make a structural contribution rather than direct interactions across the interface, and we were fortunate in identifying them as making minor interactions with WASP.

Recently Gu et al. used chimeras to create a mutated version of TC10 with the ability to bind ACK2 (*15*). TC10 is another member of the Rho family of small G proteins with 67% identity to Cdc42 and like Rac is unable to bind ACK. In their study residues 36−68 of Cdc42 were identified as the specificity determinants for ACK2 binding. Eight residues in this region, that differ between Cdc42 and TC10, when mutated either individually or in various combinations, did not affect the binding ability of the TC10/Cdc42 chimera, and it was concluded that all 8 residues are required for ACK binding. These results are largely in agreement with those presented in this study and are further evidence that multiple residues are required to confer effector binding specificity in the Rho family of small G proteins.

All the specificity determining residues identified in the TC10 study are located in switch 1, strand β2 and strand β3 of Cdc42. Strand β2 of Cdc42 contacts ACK directly, forming the canonical intermolecular β-sheet seen in G protein−effector complexes, while β3 contacts β2, constituting the juxtaposed strand in the sheet. The TC10 data are consistent with ours, indicating that residues important in stabilizing the conformation of the Cdc42 interface with ACK are as important as residues that are in direct contact with the effector.

Gu et al. identify residues that differ between Cdc42 and TC10, while this study sought to identify specificity determinants between Cdc42 and Rac. The residues highlighted as important by both studies, Thr43, Thr52, and Phe56, are

the only residues in this region that differ between all three G proteins. Gu et al. also conclude that Leu174 is not important to the specificity of ACK binding. This appears to be the case for ACK discrimination between TC10 and Cdc42. However, the data presented here and in our previous studies clearly indicate that Leu174 is an important residue for discrimination between Rac and Cdc42. It is likely that Leu174 is less important for the discrimination between TC10 and Cdc42, since TC10 has an Ile at this position, which is likely to be able to make similar interactions to Leu174 in Cdc42. The advantage of directly measuring K_ds is also apparent in this instance, as it is possible to quantify important but relatively small changes, which are not identifiable by more qualitative methods. These studies taken together also suggest that specificity determinants for the same effector by different G proteins will be distinct.

Another study investigating gain of function within small G proteins was published recently by Heo and Meyer (*23*). In this work the authors showed that expression of one hundred small G proteins induced cell morphologies of nine types. They then sought mutations sufficient to switch the small G proteins between these morphological classes, using Cdc42 and Rac as one of their test cases. No single point mutation was sufficient for a change in morphology. However, a combination of five amino acid changes showed a significant shift and a further four mutations strengthened the switch in morphological class. The five residues that they identified were 47, 95, 116, 144, and 174 while the extended group included 56, 33, 41, and 42. Of this group of nine residues, five are identified in our study as being crucial for ACK binding. As many of the residues that Heo and Meyer identify are outside the interfaces of the known effector complexes, they surmise that these residues may have long distant allosteric effects, a conclusion that fits well with the data that we present here. The phenotypes that Heo and Meyer monitor are obviously the outcome of a combination of interactions the small G proteins participate in with multiple regulatory, as well as effector proteins, however all data indicate that multiple sites on Rho family small G proteins are required to control specificity of interactions.

This idea is further supported by a recent study of the specificity of the PAK−Rac1 versus RhoG interaction (*14*). This work identified two residues of RhoG which, when mutated to the corresponding Rac1 residue, resulted in a slight increase in the interaction of RhoG with PAK. However affinity of this RhoG mutant for PAK was not comparable to that of Rac1−PAK, indicating that other residues (from residue 155 to the C-terminus) are also required for a specific interaction.

In contrast to the work presented here, studies of Rap/Ras−effector and Ral−effector interactions identified only one or two residues that are the major determinants of affinity (*12*). In G protein−GEF interactions a single residue change has also been shown to be sufficient to alter specificity, although these studies monitored GEF activity rather than the actual affinity of interaction. The Ras family interactions involved alteration of electrostatic interactions, while the Cdc42−effector interfaces are largely hydrophobic (Figure 6). Of the seven residues identified as being the determinants of binding specificity of ACK, only two residues (Thr43 and Thr52) are hydrophilic in Cdc42, and both of these are uncharged. In addition, two mutations (D47G and R174L) involve replacement of charged residues of Rac with neutral or hydrophobic Cdc42 residues, and one (S41A) involves replacing a polar residue with a hydrophobic one. It is therefore possible that the involvement of several residues in determining specificity is a feature of hydrophobic interfaces where, for example, charge reversal effects are rarely possible. The apparent structural effects seen with certain mutations in this study suggest that the Cdc42−effector protein interface cannot be considered a rigid structure into which specificity determining residues can be inserted. This is consistent with the flexibility of G proteins, particularly in their switch regions, and the unstructured nature of the free effector fragments.

^{31}P NMR studies of Ras bound to a GTP analogue have suggested that a ground-state equilibrium exists between at least two conformations of switch I (*24*). One of these conformations is stabilized by interaction with the effectors Raf (*24*) and AF6 (*25*). The other is proposed to represent a highly flexible conformation (*26*). It has been shown that mutations of Thr35 in switch 1 can perturb this conformational equilibrium thereby decreasing the affinity of interaction with effectors (*26*). The T35S mutation has been shown to affect specifically the interaction of certain Ras effectors (*27*) indicating that different effectors may recognize slightly different conformational arrangements of switch I. Backbone dynamics studies of Cdc42 have demonstrated that the most flexible parts of the molecule are the switch I and II regions (*28*). It is therefore likely that switch I of both Cdc42 and Rac may be in a conformational equilibrium similar to that seen in Ras. Thus, it is possible that some of the mutations of Rac could affect the dynamic behavior of switch I, perturbing the ground state of the protein such that the conformation recognized by ACK is stabilized relative to other conformations. This would reduce the energy difference between free Cdc42 and the complex, thus increasing the affinity. In addition, a recent study of Cdc42 side chain methyl group dynamics has reported increased mobility, and thus entropy, of hydrophobic residues in regions that are distant from the binding interface when PAK binds (*29*). This increase in disorder represents a favorable contribution to effector binding, offsetting to some extent the loss of entropy that occurs as a result of motional restriction at the protein−protein interface. Thus mutations that affect protein dynamics quite distant from the Cdc42/Rac−effector interface could also have a significant effect on the affinity of interaction.

An added level of complexity in analyzing these interactions comes from the fact that the effector fragments themselves are also flexible, being largely unstructured in their free form. It is likely that it is both G protein and effector flexibility that determine affinity and hence specificity. The possibility exists that the unbound effector proteins sample a series of conformations, only one of which is an active conformer capable of binding the G protein. The presence of a limited amount of secondary structures in the CRIB regions of these effectors has been demonstrated for the free forms of PAK (*5*) and WASP (*17*), while none was observed for ACK (*3*). Alternatively, binding could be achieved via anchor residues, with binding following an induced fit scenario where the G protein and the effectors only adopt their final, bound conformation upon interaction. Mutations introduced into the G protein may therefore be compensated for by rearrangements in the bound conforma-

tion of the effector fragment. This makes prediction of the effects of mutations more problematic and would explain why not all observed changes can be easily predicted from the structures of Cdc42−ACK/WASP. The importance of dynamic rearrangements to these complexes may also explain why the mutations examined have little effect on RhoGAP affinity. The Cdc42/Rac binding domain of RhoGAP is a preformed structured domain that does not undergo dramatic structural rearrangements on binding to the G proteins (*30*). It seems that conformational freedom is likely to be an important requirement for the observed complex combination of selectivity and promiscuity seen in these Rho family/effector interactions.

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