Residues in Cdc42 That Specify Binding to Individual CRIB Effector Proteins

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ABSTRACT: Cdc42 is a member of the Rho family of small G proteins. Signal transduction events emanating from Cdc42 lead to cytoskeletal rearrangements, cell proliferation, and cell differentiation. Many effector proteins have been identified for Cdc42; however, it is not clear how certain effectors specifically recognize and bind to Cdc42, as opposed to Rac or Rho, or in many cases, which effector controls what cellular events. Mutations were introduced into Cdc42 at residues: Met1, Val8, Phe28, Tyr32, Val33, Thr35, Val36, Phe37, Asp38, Tyr40, Val42, Met45, Ile46, Glu127, Ala130, Asn132, Gln134, Lys135, and Leu174. Measurements were made of their equilibrium binding constants to the Cdc42 binding domains of the CRIB effectors ACK, PAK, and WASP and to the GTPase-activating protein Rho GAP. Generally, mutations in the effector loop have an equally deleterious effect on binding to all CRIB proteins tested, though the F37A mutation resulted in significant selectivity. Residues outside the effector loop were found to be important for binding of Cdc42 to CRIB containing proteins and also to contribute to selectivity. Mutations such as V42A and L174A resulted in large, selective changes in binding to specific CRIB effectors. Neither mutation resulted in alteration in PAK binding, whereas both severely disrupt binding to ACK and only L174A disrupted binding to WASP. These mutations are interpreted using the structures of the Cdc42/ACK and Cdc42/WASP complexes to give insight into how effectors can specifically recognize Cdc42. Those mutations in Cdc42 that inhibit certain interactions, while retaining others, should aid investigations of the role of specific effectors in Cdc42 signaling in vivo.

Cdc42, Rac, and Rho are members of the Rho family of small GTPases, which regulate a wide variety of cellular functions including cell growth and differentiation, cell motility, and adhesion, and cell cycle progression (*1*−*5*). These small G proteins act as molecular switches, being active in the GTP[1]-bound form and inactive in the GDP-bound form. The interaction of the G protein (in its GTP-bound conformation) with an effector protein triggers a downstream signaling cascade. Many Cdc42 interacting proteins have been identified and proposed to be effector proteins (*6*−*16*). These include: the p21-activated kinases (PAKs, *6*, *7*), the activated Cdc42-associated kinases (ACKs, *8*,*9*), and the Wiskott-Aldrich Syndrome proteins (WASPs, *10*, *11*). Some of these effectors bind to both Cdc42 and Rac, while others are specific for Cdc42. Burbelo et al. (*17*) identified the presence of a conserved motif in these proteins which is part of a minimal Cdc42/Rac binding domain (*18*, *19*). This motif was called the CRIB region, for *C*dc42/Rac interactive binding.

The GTPase binding domains (GBDs) of ACK and WASP, and the Ras binding domains (RBDs) of the Ras specific effectors, Raf-1 and RalGDS, interact in part, by forming an intermolecular β-sheet with strand β2 in their respective G proteins (*20*−*23*). However, the structure of the complex of Cdc42 with the ACK GBD (*22*) shows that it wraps around Cdc42 forming an extensive interface with an average buried surface area of ≈4200 Å^2; in the Rap1a/Raf-1 complex (*20*), for example, the buried surface area is significantly less (≈1200 Å^2). In addition to strand β2, the ACK and WASP GBDs also interact with residues within the C-terminal α-helix and the effector loop of the G-protein (*22*, *23*). We wanted to dissect the interactions between Cdc42 and the CRIB effectors to see which parts of the interface form common interactions and which contribute to specificity between effectors.

GTPase activating proteins, such as Rho GAP, downregulate small G proteins by increasing the GTP hydrolysis rate. Although, like effector proteins, they specifically bind to the GTP form of G proteins, there is little or no direct evidence for an effector function. The structure of Cdc42 complexed with RhoGAP (*24*) shows that the GAP interacts mainly with switch 2 and the P-loop of the G protein, but has very few contacts with the effector loop (switch 1). Formation of the Cdc42/RhoGAP complex buries ≈ 1800 Å of accessible surface.

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[1] Abbreviations: GTP, guanosine-5′-triphosphate; GDP, guanosine-5′-diphosphate; CRIB, Cdc42/Rac interactive binding; ACK, activated Cdc42-associated kinase; PAK, p21-activated kinase; WASP, Wiskott-Aldrich Syndrome protein; GAP, GTPase activating protein; RBD, Ras/Ran binding domain; GBD, GTPase binding domain; wt, wild-type; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; HPLC, high-performance liquid chromatography; SPA, scintillation proximity assay; GMPPNP, guanylyl-5′-imidodiphosphate; ELISA, enzyme-linked immunosorbent assay; GDI, guanine nucleotide dissociation inhibitor.

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Previous studies have identified mutations in both the Ras and Rho family proteins, which interfere with their ability to interact with various effectors. In particular, White et al. (*25*) were the first to show that mutations in the effector loop of Ras could specifically disrupt the interaction with particular effectors. However, there have been no systematic attempts to find mutations in Cdc42 that specifically block one CRIB effector interaction, while leaving others intact. Such discriminatory mutations should allow dissection of signaling cascades in vivo, allowing one to link specific effector proteins to particular downstream effects.

To probe the specificity of Cdc42 for CRIB effector proteins, we have mutated specific residues in Cdc42. These were primarily chosen either as sites previously shown to affect the selectivity of small G proteins for their effector proteins or as novel sites indicated by structural studies. Previous mutational studies of Rho family proteins have not quantified the effects of the mutation on binding affinity. Here, we have used equilibrium binding measurements to accurately define affinities of interactions between wt, Q61L, and mutant forms of Cdc42 and the three CRIB effector proteins, ACK, PAK, and WASP and, for comparison, the GTPase activating protein RhoGAP.

MATERIALS AND METHODS

Expression Constructs. All proteins were expressed as GST fusion proteins in the pGEX series of vectors (Amersham Pharmacia Biotech.). Constructs expressing residues 504−545 of ACK (*22*), 75−132 of PAK (*18*), 210−321 of WASP (*19*), and 198−439 of RhoGAP (*26*) were all kind gifts. Cdc42 wt, Q61L, and all other mutants were expressed in pGEX-2T from constructs encoding residues 1−184 cloned into the *Bam*H1 and *Eco*R1 sites (*24*).

Cdc42 Mutagenesis. Site-directed mutagenesis of the Q61L Cdc42 expression construct encoding residues 1−184 was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The sequence of the Cdc42 coding region of all mutants was verified using an automated DNA sequencer (Applied Biosystems Inc.).

Recombinant Protein Production. GST fusion proteins were expressed in *Escherichia coli* XL1-Blue. Stationary cultures were diluted 1 in 10, grown at 37 °C to an A_{600} of 0.8 and induced with 0.1 mM IPTG for 5 h. Proteins were affinity purified using glutathione-agarose beads (Sigma Aldrich). Fusions of CRIB domain proteins and RhoGAP were eluted from the glutathione-agarose beads and used directly. GST-Cdc42 1−184 variants were cleaved from their GST tag, while attached to the glutathione-agarose beads with thrombin (Novagen) prior to use in assays. Protein concentrations for all proteins were evaluated from measurement of their A_{280} using their amino acid compositions and the extinction coefficients of tyrosine, phenylalanine, tryptophan and the guanine nucleotide (*27*).

Nucleotide Exchange. [^{3}H]GTP complexes of wt and mutant Cdc42 proteins were made by nucleotide exchange in the presence of a GTP regeneration system. [8,5′-3H]GTP (NEN, 0.15 mCi, 26 900 Ci/mL) was taken to dryness by centrifugal evaporation. To this was added Cdc42 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 ammonium sulfate, and 5 μL of pyruvate kinase (Sigma Aldrich, P7768), 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 $MgCl_2$ was added. Unbound nucleotide was then removed on a 1 mL Sephadex G25 (superfine) centrifuge gel filtration column in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl2, and 1 mM dithiothreitol. Wt Cdc42.GMPPNP was prepared as previously described (*18*), and the purity of all nucleotide complexes was checked by HPLC (*18*). All complexes were >99% pure as determined by HPLC analysis.

Scintillation Proximity Assays (SPA). Affinities of Cdc42 proteins for GST−PAK, GST−ACK, GST−WASP, and GST−RhoGAP were measured using Scintillation Proximity Assays (SPAs) in which GST fusion protein was attached to a fluoromicrosphere via an anti-GST antibody in the presence of Q61L Cdc42.[^{3}H]GTP. Binding of Cdc42 to the GST fusion protein brings the labeled nucleotide close enough to the scintillant to obtain a signal. Apparent K_ds for Q61L Cdc42.[^{3}H]GTP itself and proteins incorporating further mutations were measured as described previously (*18*, *28*) by varying the concentration of Cdc42.[^{3}H]GTP at a constant concentration of GST fusion protein. These assays were performed with 30 nM GST−PAK, GST−ACK, GST−WASP, or GST−RhoGAP. For studies of the interaction of GST−WASP with Cdc42 mutants binding with high affinity, the concentration of GST−WASP was decreased to 5 nM. Using this method, the upper and lower limits of the K_ds that can be accurately measured are 1500 and 1 nM, respectively. The affinity of wt Cdc42.GMPPNP for effector proteins and RhoGAP was measured using a competition SPA in which the concentration of wt Cdc42.GMPPNP was varied in the presence of a fixed concentration of Q61L Cdc42.[^{3}H]GTP and GST fusion protein. For each affinity determination, data points were obtained for at least 10 different Cdc42 concentrations. Binding curves were fitted using the appropriate binding isotherms (*18*, *28*) to obtain K_d values and their standard errors.

RESULTS AND DISCUSSION

Measurement of Equilibrium Binding Constants. Scintillation proximity assays (SPAs) have been used successfully to measure protein−protein affinities rapidly under equilibrium conditions. There is no separation step and hence, unlike the commonly used affinity precipitation and ELISA-based techniques, equilibrium binding constants can be measured even when the dissociation rate is very high. This was of particular importance in this study as we wished to determine weak affinities resulting from the effects of mutation. SPAs have been used previously to quantify the interaction between Cdc42 and fragments of PAK (*18*) as well as that of Rho/RhoGAP (*28*) and Ras/Raf (*29*). Here, we have used SPAs to measure the affinity of wt, Q61L and various other mutants of Cdc42 for different effector proteins and for RhoGAP. As full-length, and in the case of Cdc42 naturally processed, proteins are difficult to obtain in sufficient quantity and quality to allow precise binding measurements, minimal domains of all these proteins were used in our experiments. Although these will differ in properties from the in vivo forms of the proteins, we have no reason to believe that the comparative effects of Cdc42 mutations will be qualitatively different.

Table 1: Affinities of wt and Q61L Cdc42 for Effector Proteins[a]

	apparent K_d(nM)			
	ACK	PAK	WASP	RhoGAP
wt.GMPPNP	770 ± 80	620 ± 138	55 ± 6	6100 ± 1900[b]
Q61L.GTP[c]	30 ± 4	20 ± 4	1[d]	50 ± 6

[a] Equilibrium-binding constants were determined in SPAs as described in the Materials and Methods. K_d values are quoted with the standard errors from curve fitting. [b] K_d obtained from experiments in which the concentration of wt Cdc42.GMPPNP was varied in the presence of Q61L Cdc42.[3H]GTP complex and GST−ACK, −PAK, −WASP, or −RhoGAP. [c] K_d obtained from experiments in which the concentration of Q61L Cdc42.[3H]GTP complex was varied in the presence of either GST−ACK, −PAK, −WASP, or −RhoGAP. [d] The best fits obtained were with K_ds of about 1 nM, but almost identical fits were obtained with K_ds fixed between 0.1 and 1.0 nM.

The intrinsic hydrolysis rate of wt Cdc42−GTP is so fast that it is not possible to use this complex in accurate affinity measurements. Although experiments can be performed with wt Cdc42 complexed with nonhydrolysable nucleotide analogues such as GMPPNP, it was decided to investigate all mutations in a background of Q61L Cdc42, complexed with its natural ligand, GTP. The Q61L mutation sufficiently reduced the rate of GTP hydrolysis such that GTP itself could be used for these binding measurements. Furthermore, Q61L Cdc42 binds with higher affinity than does wt Cdc42 to the CRIB effector proteins and to RhoGAP. This allows more precise data to be obtained for those mutant Cdc42s, which have greatly decreased binding affinities. However, as there have been reports suggesting that there could be differences in effector specificity between wt and Q61L small G proteins (*5*), we also compared the affinities of wt Cdc42.GMPPNP with those of Q61L Cdc42.GTP for the binding proteins under investigation (Table 1).

Using Q61L Cdc42.[3H]GTP a strong SPA signal was obtained with GST fusions of the three CRIB effectors, WASP, ACK, and PAK and also with RhoGAP. The affinities of the four GST fusion proteins were then determined using SPAs in which the concentration of the Cdc42 was varied, while keeping the concentration of GST fusion constant (Figure 1A). The apparent K_d values were 30 nM (ACK), 20 nM (PAK), 1 nM (WASP), and 50 nM (RhoGAP) (Table 1). From at least three independent replicate determinations of K_d, the coefficient of variation was 7−34%. The affinities of wt Cdc42.GMPPNP for these proteins was determined by measuring the effect of varying its concentration on its ability to inhibit binding of Q61L Cdc42.[3H]GTP to its binding partners. The apparent K_d values were 770 nM (ACK), 620 nM (PAK), 55 nM (WASP), and 6100 nM (RhoGAP) (Table 1). Therefore, Q61L Cdc42.GTP binds to the CRIB effector proteins with 30- to 50-fold higher affinity than does wt Cdc42.GMPPNP. However, the Q61L mutation does not result in any significant change in specificity in effector binding for the proteins tested, further justifying our decision to use the Q61L background for the mutagenesis studies described below.

These are the first measurements of the affinity of either Q61L Cdc42.GTP or Cdc42.GMPPNP for ACK. The K_d values obtained for Cdc42 binding to PAK are similar to those previously reported (*18*). The affinity of this WASP fragment for Q61L Cdc42.GTP was not previously reported, but that for Cdc42.GMPPNP found here (K_d = 50 nM) is similar to that obtained using an equilibrium-binding method based on fluorescence quenching (K_d = 77 nM, ref *19*). The affinity of RhoGAP for Q61L Cdc42.GTP is slightly weaker than for Q63L Rho.GTP (16 nM) but stronger than for Q61L Rac.GTP (200 nM) (*28*).

Similar SPA experiments were then performed using a range of Cdc42 mutants in a background of Q61L Cdc42.GTP to investigate their effects on the interaction with the different effector proteins. Experimental data for selected mutants are shown in Figure 1(B−F), and the apparent K_d values obtained for all mutants are summarized in Table 2. Standard errors from curve fitting (shown in Tables 1 and 2) varied from 2.5 to 57.1%, but were typically 15%.

Structural Integrity of Mutant Proteins. A number of criteria were used to assess the structural integrity of the mutants used in these studies. All mutant proteins had expression levels similar to that of Q61L Cdc42 and were soluble. After purification, all showed a similar yield and degree of homogeneity by SDS−PAGE. Successful nucleotide exchange also implies structural integrity of the mutant protein produced. Interestingly, the F28Y mutant, which in Ras is known to decrease the affinity of G proteins for the bound nucleotide, gave reliable data in SPAs. All mutants showed similar binding to RhoGAP, providing a good internal control (although four mutants did show minor impairment).

Mutations in the N-terminal β strand. Mutations were made in the N-terminus of the protein, which interacts with the ACK GBD (*22*). Residues Met1 and Val8 lie at either end of the first strand of the three-stranded β sheet of Cdc42, which is seen to move when ACK binds to Cdc42 *(22)*. When bound to Cdc42, residues 516−520 of ACK adopt an extended conformation, which interacts with and extends this β sheet in the G protein (a mechanism in common with Ras/effector protein binding, see above). Mutation at Met1 had no effect on the binding of ACK, PAK, or WASP, suggesting that its interactions are not critical for effector binding. This is consistent with the Cdc42/ACK structure, which shows that only the backbone of Met1 interacts with both Gln508 and Ile510 at the N-terminus of the ACK GBD. Val8 is involved in the packing of this β sheet to the N-terminus of helix α1 (*22*). However, mutation of Val8 had no effect on the binding of ACK, suggesting that this mutation does not affect the reorientation of the Cdc42 N-terminal sheet. In the Cdc42/WASP structure, no contacts are seen between Met1, Val8, and WASP, explaining the lack of an effect on binding affinities (*23*).

Mutations to the Effector Loop. The effector loop, also referred to as Switch 1, was initially defined for Ras as a region of the protein, which when mutated disrupted its ability to interact with downstream effector proteins and to transform cells (reviewed in *30*). We have made mutations in the effector loop, based largely on mutagenesis studies

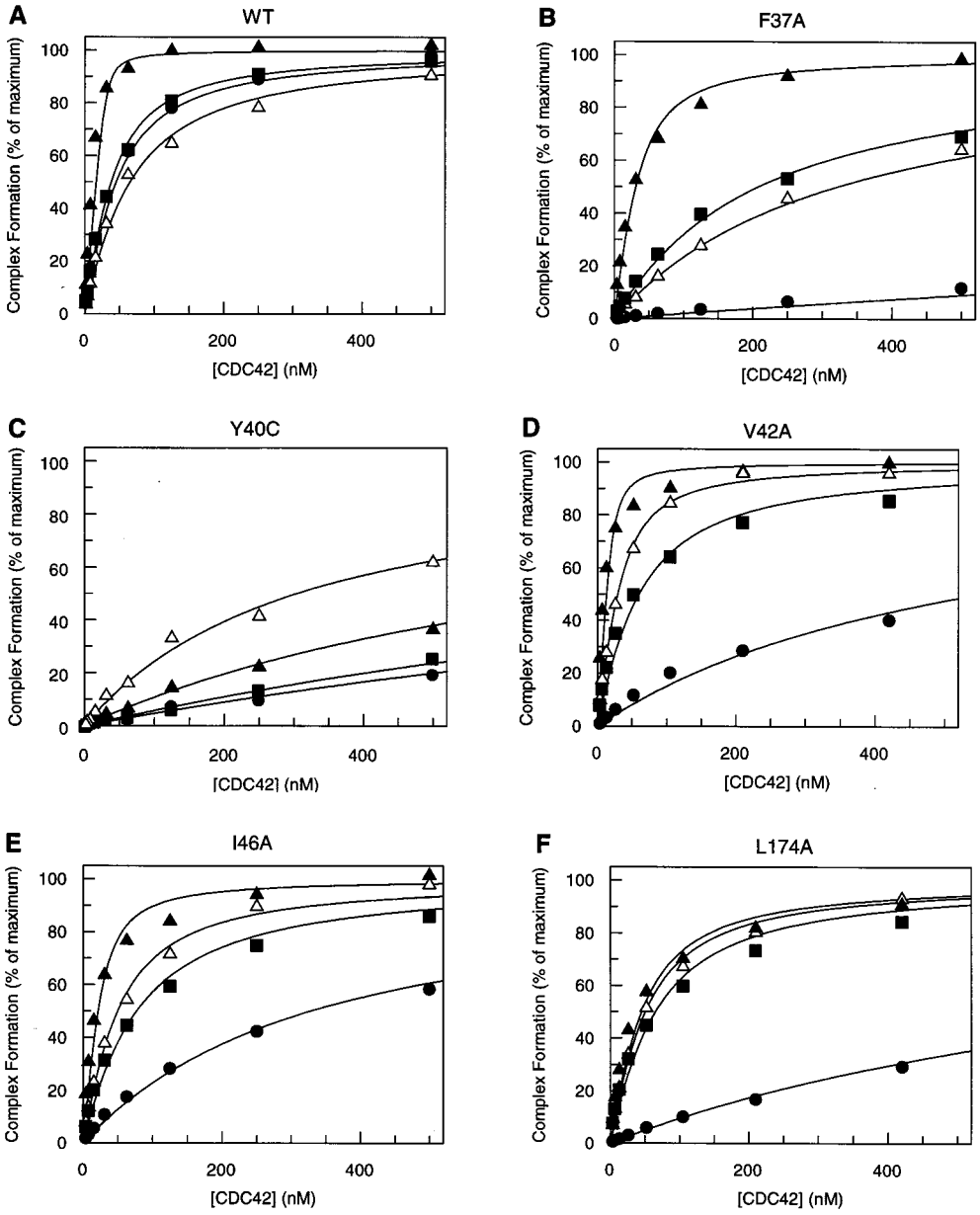

FIGURE 1: Measurement of the affinity of Q61L Cdc42.GTP (A) and selected mutants (B−F) for GST−WASP, GST−ACK, GST−PAK, and GST−RhoGAP (effector protein fragments used are described in Methods and Materials). The indicated concentrations of Q61L Cdc42.[^{3}H]GTP were incubated with 30 nM GST-effector protein in SPAs. The SPA signal was corrected by subtraction of a blank in which the GST-effector protein was omitted. The effect of the [Cdc42] on this corrected SPA cpm signal was fitted to a binding isotherm to give the apparent K_d and the signal at saturating concentration of Cdc42. The data are expressed as a percentage of this maximum signal. The lines shown are the best fits using all data up to 2000 nM. However, for clarity, only datapoints up to 500 nM are shown. The proteins used are GST−WASP (▲), GST−ACK (●), GST−PAK (■), and GST−RhoGAP (△).

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                          +    *    *● ●  ■
ACK(504-545)      GLSAQDISQP LQNSFIHTGH GDSDPRHCWG FPDRIDELYL GN
PAK1(69-108)      EKERPEISLP ..SDFEHTIH VGFDAVTGEF TGMPEQWARL LQ
WASP(233-274)     KISKADIGAP ..SGFKHVSH VGWDPQNGFD VNNLDPDLRS LF

CRIB_Consensus    ......IS.P ....F.H..H VG........ .......... ..
(17)
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FIGURE 2: Sequence alignment of the CRIB motifs from ACK, αPAK, and WASP showing the CRIB consensus (*17*). Some residues in Cdc42 that when mutated can discriminate in binding to CRIB proteins are indicated and the contacts they make to ACK are highlighted: Leu174 (+), Ile46 (★), Met45 (●), and Val42 (■).

available for Ras. Most mutations in the effector loop have a deleterious effect on binding to all three CRIB proteins. From the structures of Cdc42/ACK and Cdc42/WASP, it can be seen that most of the conserved residues in the CRIB region (Figure 2) interact with the effector loop and strand *β*2 (*22*, *23*, and Figure 3A). Thus, the indiscriminate detrimental effects of many of these mutations are not surprising. The same mutations have a much lesser effect

Table 2: Affinities of Q61LCdc42.GTP Mutants for Effector Proteins[a]

mutation	Apparent K_d (nM) ACK	PAK	WASP	RhoGAP	notes
	30 ± 4	20 ± 4	1[b]	50 ± 6	
M1T	30 ± 7	7 ± 4	1[b]	27 ± 4	CDC42/ACK structure[c]
V8A	50 ± 9	14 ± 4	1[b]	24 ± 5	CDC42/ACK structure[c]
F28Y	50 ± 10	15 ± 5	1[b]	29 ± 4	nucleotide binding
Y32F	200 ± 77	90 ± 50	200 ± 35	70 ± 20	effector loop
Y32K	890 ± 170	680 ± 90	90 ± 20	140 ± 10	effector loop
V33N	110 ± 18	28 ± 5	1[b]	11 ± 5	effector loop
T35S	1300 ± 33	520 ± 82	35 ± 5	54 ± 10	effector loop
V36A	520 ± 29	220 ± 13	6 ± 0.6	28 ± 5	effector loop
F37A	>5000	190 ± 23	16 ± 3	310 ± 50	effector loop
D38A	>2000	>2000	50 ± 4	20 ± 4	effector loop
D38E	780 ± 100	550 ± 53	200 ± 20	200 ± 50	effector loop
Y40C	>1000	>1000	830 ± 70	290 ± 35	effector loop
V42A	540 ± 90	40 ± 8	3 ± 0.5	14 ± 4	CDC42/ACK structure[c]
M45T	130 ± 23	30 ± 4	1[b]	50 ± 10	CDC42/ACK structure[c]
I46A	310 ± 23	60 ± 8	9 ± 3	35 ± 4	CDC42/ACK structure[c]
K135Q	50 ± 9	15 ± 5	1[b]	50 ± 4	insert loop
L174A	930 ± 95	50 ± 9	30 ± 7	35 ± 4	CDC42/ACK structure[c]
ΔInsert	30 ± 5	13 ± 4	1[b]	25 ± 4	insert loop

[a] Equilibrium-binding constants were determined in SPAs in which the concentration of the appropriate Cdc42.[^{3}H]GTP complex was varied in the presence of either GST−ACK, −PAK, −WASP, or −RhoGAP as described in the Materials and Methods. K_d values are quoted with the standard errors from curve fitting. [b] The best fits obtained were with K_ds of about 1 nM, but almost identical fits were obtained with K_ds fixed between 0.1 and 1.0 nM. [c] Mutation at these positions were designed based on information gained from the solution structure of Cdc42/ACK GBD (*22*).

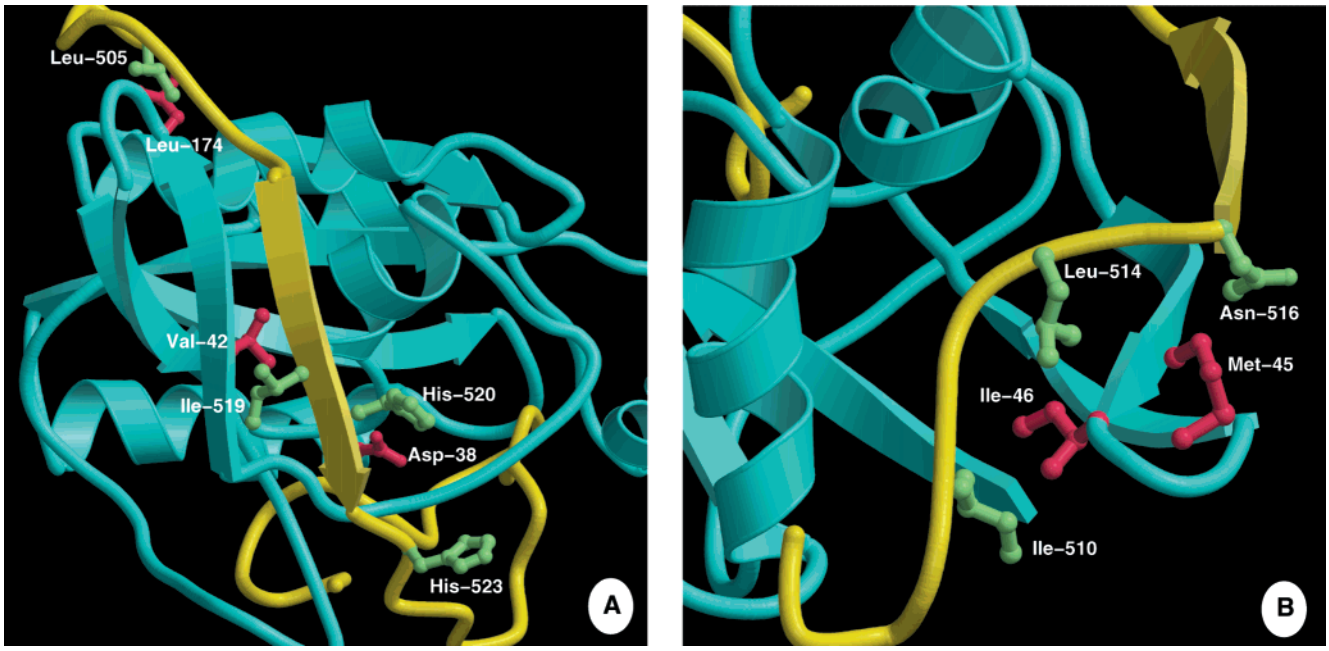

FIGURE 3: (A) Schematic representation of part of the Cdc42/ACK−GBD structure (*22*) showing the contacts made by Asp38 with His520 and 523, Val42 and Leu174 with Leu505. Val42 and Leu174 make a major contribution to the specificity of the interaction between Cdc42 and ACK. Cdc42 is colored blue with side chains shown in red. ACK is colored yellow with side chains in green. (B) Schematic representation of part of the Cdc42/ACK−GBD structure (*22*) showing the contacts made between Met45 with Leu514 and Asn516 and Ile46 with Ile510 and Leu514. Coloring is as in A. This figure was generated using Molscript (*44*) and Raster 3D (*45*).

(or no effect) on RhoGAP binding, consistent with the Cdc42/RhoGAP structure, which shows that the interaction of this loop with RhoGAP is not nearly so extensive (*24*). Some differences can, however, be seen between specific mutants, which are discussed below.

The Y32F and Y32K mutations affect binding of all three CRIB proteins; however, Y32F has a relatively larger effect on binding to WASP than to ACK or PAK (≈10-fold greater effect), whereas Y32K is equally impaired in binding to all three CRIB proteins. The side chain of Tyr32 is poorly defined in both the Cdc42/ACK and Cdc42/WASP structures, so it is not possible to comment on the reasons behind these differences. The V33N and V36A mutations result in relatively minor effects on affinities, but both show some specificity in their effects. V33N only affected binding to ACK. In the Cdc42/ACK structure, Val33 contacts Pro528, while in the Cdc42/WASP complex the residue is not involved in intermolecular contacts. V36A affected binding to all three CRIB effectors, but the affinity for ACK was most reduced. Val36 contacts Gly524 in ACK and Val250 in WASP; however, these interactions cannot contribute significantly to overall binding as shown by the small

effects on affinities. These residues lie in the nonconserved regions of the effector proteins (Figure 2), making it difficult to comment on effects seen with PAK.

The F37A mutation reduces affinity for WASP, PAK, and RhoGAP (16-, 9- and 6-fold, respectively), but binding to ACK is most severely affected with a decrease in affinity of over 160-fold. Initially the F37A substitution was thought not to affect the interaction between Cdc42/Rac and the CRIB proteins (*5*). However, later it was shown that Rac F37A is unable to bind MLK2 and 3 (both CRIB motif proteins) (*31*). We show here that Cdc42 F37A also has a severely impaired affinity for ACK. Therefore, cellular effects seen with this mutation must be considered with care. Unfortunately, we cannot comment on the structural basis for the effects of the F37A mutation on ACK binding, as Phe37 is not well-defined in the Cdc42/ACK structure. In the Cdc42/WASP complex, Phe37 lies within 4 Å of Val250, Leu263, Leu267, and Phe271 (*23*) and would be capable of making many hydrophobic contacts. However these interactions clearly do not contribute greatly to the binding energy of this complex as can be seen by the small change in affinity for WASP. It is interesting to note that the analogous Ras effector loop mutation, E37G, disrupts the interaction with two effector proteins, Raf1 and PI3Kinase, but stills allows the binding of Byr2 (*24*). Sahai et al. (*32*) have also found that the equivalent mutation in RhoA (F39A) blocks the interaction with seven known Rho effectors, while leaving the interaction with mDia2 intact. They also found that specificity could be influenced by replacing F39 with different amino acids; the F39V and F39L mutants each retain the ability to interact with a different subset of the eight different effectors tested.

D38A deleteriously affects binding to all three CRIB proteins to a similar extent (ACK, 67-fold reduction in affinity; PAK, 100-fold; WASP, 50-fold), while the more conservative D38E affects binding to ACK and PAK to a lesser extent (26-fold and 27.5-fold respectively) but the affinity for WASP is more severely affected (200-fold). This result is consistent with the Cdc42/ACK structure (*22*) where Cdc42 Asp38 interacts with His520 in ACK and with His523 to a lesser extent (Figure 3A). Both His are invariant in CRIB domains and it is, therefore, easy to see why mutation of Asp38 to Ala affects binding to all three CRIB proteins. In the Cdc42/WASP complex (*23*), Asp38 also makes contacts with the second invariant His (His249); however, the position of Switch 2 in the two complexes is slightly different, changing the relative orientations of Asp38 and Asp57. In Cdc42/WASP, the two Cdc42 Asp residues are closer in space than is seen in the ACK complex suggesting the possibility that charge repulsion between Glu38 and Asp57 in the D38E Cdc42/WASP could occur. This could alter the position of Glu38 in the complex destabilizing packing with His249 or alternatively restructuring Switch 2 such that it no longer interacts with WASP, so possibly explaining the counter-intuitive effect of D38E on WASP affinity.

Finally, the Y40C mutant, commonly used in G protein function analysis, has a severely deleterious effect on binding of all CRIB effectors tested, confirming other work (*5*, *7*, and *31*). The side chain of Tyr40 makes multiple contacts in both the Cdc42/ACK and Cdc42/WASP structures. In ACK, contacts are made to Phe 518, His520, and Thr521 (*22*), while in WASP, contacts are seen to the equivalent residues Phe244, His246, and Val247 (*23*). These Phe and His residues are invariant across the CRIB effectors explaining the indiscriminate effect of this mutation.

Mutations to Strand β2 . Strand β2 lies adjacent to the effector region and is also contacted by ACK in the Cdc42/ACK complex (*22*). We, therefore, made several mutations to this region. V42A decreases the affinity of the interaction with ACK (18-fold), but has little effect on PAK (2-fold) or WASP binding (3-fold) (Table 2 and Figure 1D). The loss of the hydrophobic interaction between Val42 and Ile519 results in a change in ΔG of 1.7 kcal/mol. These results are consistent with the structures of the two complexes. In Cdc42/ACK, Val42 forms a hydrophobic contact with ACK Ile519 (Figure 3A), while in the WASP complex it instead contacts Pro241 and Phe 244 (*23*). These latter residues are invariant across the CRIB proteins (Figure 2), but as the affinity of Cdc42 V42A is changed very little for WASP, these contacts must not contribute greatly to binding. The equivalent residues to ACK Ile519 in PAK and WASP are Glu82 and Lys245, respectively. In WASP, Lys245 does not contact Val42 (*23*) and Glu82 would be less likely to contribute to hydrophobic interactions than Ile, perhaps suggesting that details of the interaction of Cdc42 with PAK will be more similar to WASP than ACK.

Two further Cdc42 residues were mutated in this strand (Met45 and Ile46). Met45 packs against Leu514 and Asn516 in ACK (Figure 3B). Its mutation, however, has no effect on PAK or WASP binding and only a small effect on ACK binding, suggesting that these interactions are not crucial. In the WASP complex, it is the backbone of Met45, which contacts WASP explaining the lack of effect with this mutation (*23*). The Cdc42 I46A mutation causes a 10-fold decrease in affinity for ACK, a 9-fold decrease for WASP, and a 3-fold decrease for PAK (Table 2 and Figure 2E). Ile46 packs against Ile510 and Leu514 in the ACK complex (Figure 3B). Ile510 is invariant across the CRIB domains (Figure 2), while Leu514 is not conserved. In the WASP complex, Ile46 packs against Ile233 and Ile238 (invariant and equivalent to Ile510 in ACK), explaining the similar effects seen on the affinities for ACK and WASP. It would, therefore, be expected that I46A would have a similar effect on PAK affinity. The PAK construct used in these experiments starts at Ile75 (invariant, equivalent to ACK510 and WASP238), and the preceding conserved Glu74 is replaced with Ser, possibly changing the packing in this region and masking an effect on affinity. However, it has previously reported that removal of residues 70−75 in PAK has little effect on affinity (*18*), leaving the possibility that packing in this region is different in the Cdc42/PAK complex from Cdc42/ACK or Cdc42/WASP.

Mutations to the Insert Loop. The insert region is unique to the Rho-family proteins and is absent in other G protein families. Both the crystal and solution structures of Rac and Cdc42 have shown that the insert region forms a helix, which protrudes from the bulk of the G protein and which might, therefore, mediate protein−protein interactions (*22*, *23*, *24*, *33*, and *34*). Furthermore, it has been implicated in regulating the activity of Rho-Guanine-nucleotide dissociation inhibitor (RhoGDI) on Cdc42 (*35*), IQGAP binding (*15*), and in Rac, $p67^{phox}$-binding/NADPH activation (*36*, *37*). Thus, we made mutations to test for its involvement in CRIB domain protein

binding. The insert loop mutations studied, E127Q, A130K, N132K, Q134L, K135T, and K135Q, all behaved in a very similar manner and had no significant effect on binding to any of the effector proteins tested (see results for K135Q, summarized in Table 2). The entire insert loop was also removed and this too had no effect on CRIB effector protein binding (Table 2). These results confirm the lack of an interaction between CRIB domain effectors and the insert loop observed in the Cdc42/ACK and Cdc42/WASP structures (*22*, *23*).

Mutations to the C-terminal Helix. L174A decreases the affinity of Cdc42 for ACK and WASP by about 30-fold, but has very little effect on PAK binding (2.5-fold) (Table 2 and Figure 1F). Cdc42 Leu174 packs against ACK Leu 505 (Figure 3A) and its removal, therefore, would be expected to disrupt the hydrophobic packing of the complex. The loss of the hydrophobic interaction of Leu174 with either Leu505 of ACK or Ile233 of WASP results in a change in ΔG of 2.0 kcal/mol. The equivalent residue in WASP is Ile233 (Figure 2), which makes similar hydrophobic contacts, explaining the similar effect seen on affinity. However, the relevant residue in PAK, Lys70, is polar, implying that this particular hydrophobic packing is not utilized by Cdc42 when binding to PAK. These results were confirmed using a slightly longer PAK fragment (70−132), which included Lys70 and showed no decrease in affinity with L174A Cdc42 (data not shown). These results are consistent with other reports, which implicate residues 143−175 in binding to some Rho family effectors (*38*). Hence, the C-terminal helix, around Leu174, is seen to strongly contribute to specificity of binding as mutations differentially affect the ability of the G protein to interact with its different effectors.

Implications for Tight Binding and Specificity of Cdc42/Effector Interactions. The results presented here show that the Rho-family proteins utilize regions outside the relatively well-conserved effector loop to obtain tight binding and specificity. In particular, the C-terminal α-helix and the second β-strand of Cdc42 and Rac appear to be important specificity determinants. It is possible that these Rho-family G proteins have had to utilize regions other than the conventional effector loop to obtain sufficient specificity when binding to their large number of effectors. So far, structures of G protein/effector complexes are available for: Ras(Rap)/ubiquitin fold effectors, (Raf, *20* and RalGDS, *21*), Cdc42/CRIB effectors (ACK, *22*, and WASP, *23*), Rab/Rabphilin-3A GBD (*39*), and Ran/RanBP2 RBD (*40*). Only the ubiquitin fold family of Ras effectors show binding primarily to the effector loop. In the Rab3A/Rabphilin3A interaction, a second interface exists, which augments effector binding to switches 1 and 2 and appears to be important for specific interactions between each Rab protein and its effectors (*39*). This second interface involves the extreme N-terminus of the protein, the C-terminal end of Switch 2 and the C-terminal end of helix α5. The Ran/RanBP2 complex shows the largest buried surface area seen in any of these G protein/effector complexes (5640 Å^2) and again, other regions outside the effector loop contacting the effector (*40*). These include the extreme C-terminus of Ran (including its C-terminal helix), the β2-β3 loop, residues 116 (in β5) and 168 (in α5) and residues 154/158 (involved in nucleotide binding). In part, the larger protein−protein interface employed in the Cdc42/ACK interaction as compared to Ras/Raf, might also be required to compensate for the loss in entropy when the CRIB effectors become structured on interaction with the G protein. The ACK GBD, for example, is unstructured in solution and the uncomplexed WASP GBD is only partially structured (*19*), while for Ras/Raf, the interacting surface in Raf (which is part of a structured domain) already has the correct conformation to interact with Ras (*41*).

Our results contribute to the accumulating evidence that over a large protein−protein interaction surface the binding energy is not uniformly distributed, but rather is concentrated in small regions (*42*, *43*). It is particularly noteworthy that despite the very large surface area of the Cdc42/ACK or Cdc42/WASP interfaces, single point mutations can result in losses of binding energy of as much as 2 kcal/mol (e.g., L174A), representing a significant proportion of the total binding energy (10 kcal/mol). Interestingly, we found that both affinity and specificity was defined by several hydrophobic interactions (e.g., Val 42−ACK Ile519; Leu 174−ACK Leu505; Leu174−WASP Ile233), each of which contributed binding energies of 1.7−2 kcal/mol. The data presented here suggest which regions of these interfaces contribute more to the affinity of binding. The results point to the very subtle changes that are required to discriminate between binding by different CRIB effectors. This reflects the complexity of the problem faced by the Rho family proteins in obtaining specific binding to particular effectors. Understanding which residues contribute most to the binding energy will also aid in the rational design of small molecules that might be used to inhibit such interactions, e.g., for use in anti-cancer therapies. The results presented here indicate which region(s) of the extensive G protein CRIB effector interface might be most usefully targeted.

Implications for in Vivo Interactions. A primary objective in this study was to find mutations in Cdc42, which would selectively abolish binding to one of the many effector proteins that have been identified for this Rho family GTPase. Our initial aim, therefore, has been to determine the dissociation constants for binding to the different Cdc42 mutants. The combination of mutagenesis and SPAs presented here provides the first quantitative assessment of the effects of Cdc42 mutations on the equilibrium-binding constants to CRIB effector proteins. Previous studies have utilized the yeast two hybrid system, overlay blots, and affinity precipitation to demonstrate the ability (or inability) of mutant G proteins to bind interacting proteins. While these techniques have other advantages, they do not provide a quantitative assessment of binding ability.

Many factors should be considered when trying to extrapolate between in vitro affinities of mutants for effectors and their possible action in vivo. In these studies (and many others), fragments of the proteins have been used rather than the intact proteins. All the Cdc42 variants used in these studies are Q61L, as discussed above. The small G proteins used in these and other in vitro assays are unprocessed and obviously not membrane localized as they would be in vivo. Another crucial problem in interpreting in vitro affinity data is the lack of information available on the concentrations of the different proteins in vivo. It is the concentrations of the proteins relative to the K_ds that will determine the affect of a mutation in vivo. However, despite all these potential problems in the only study that we are aware of where an

attempt was made to compare in vitro affinity data of an effector binding to a small G protein to in vivo signaling activity, a very good correlation was found between the two (*42*). This was obtained even though in that study, in vitro binding data was obtained using the isolated Ras-binding domain of Raf and truncated unprocessed Ha-Ras complexed with a GTP analogue, mant-GMPPNP. We expect, therefore, that the differences in dissociation constants measured here will reflect the degrees of activation of the different signaling pathways controlled by Cdc42 in vivo.

The availability of a range of mutants affecting a particular interaction to various degrees, as we have provided here, could also allow one to demonstrate whether the in vivo effects on a particular signaling pathway are due to a particular G protein/effector interaction. They also potentially allow one to show whether signaling through a particular pathway is only dependent on formation of the initial complex or if subsequent activation of the effector is required (*42*).

Concluding Remarks. Cdc42 is now known to be active in the control of many different pathways in mammalian cells. It exerts this control via interactions with a wide range of effector proteins. However, it is still not clear how Cdc42 is able to interact with all of these effector proteins specifically and indeed, in some cases, how activation of particular effector proteins goes on to influence different downstream pathways. The ability to knock out the interaction between Cdc42 and its effector proteins individually, while leaving the other interactions intact, will provide a powerful tool that can be used in delineating these Cdc42-controlled signaling pathways in vivo.

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