with a Taq Dye-Terminator kit (Perkin-Elmer) and an automated 373A DNA sequencer (Applied Biosystems).

**Isolation of phage and replicative form.** Isolation of phage from *V. cholerae* was done essentially as described. In brief, 1- litre Luria broth cultures were grown overnight at either 30 °C (395) or 37 °C (N19601). Cultures were centrifuged twice at 10,000 g and the supernatant was passed through a 0.45-μm low-protein-binding filter. DNase I and RNase I (Boehringer Mannheim) were added to the filtrate at a final concentration of 1 μg ml−1 and incubated at room temperature for 3 h. NaCl and PEG 8000 were added to a final concentration of 1 M and 10% w/v, respectively, and the mixture was left to precipitate overnight at 4 °C. The supernatant was centrifuged at 11,000 g for 20 min and the pellet resuspended in 4 ml of SM buffer. PEG was removed with an equal volume of chloroform. This supernatant was layered onto a CaCl2 step gradient consisting of 0.25 M of CaCl2 in SM buffer (d = 1.7, 1.5 and 1.45). After centrifugation at 25,000 rpm for 2 h in an SW41 rotor (Beckman), the lower portions (d = 1.45, d = 1.5) were extracted and dialysed against two changes of TM buffer. The phages were further concentrated by the addition of PEG 8000 (10% w/v) and placed on ice for 2 h. The phage preparation was centrifuged at 14,000 g for 20 min and the resulting pellet (containing phage particles) was resuspended in 100 μl of SM buffer. PEG was removed with an equal volume of chloroform. 5 μl of the phage preparation was used for PCR.

The replicative form was isolated from 1 litre of Luria broth culture as described.

Although filamentous phage do not lyse the cell or have a ‘burst size’, we calculated the approximate number of VP1 and CTXφ released per cell and the RF copy number. The number of phage released was calculated by determining the amount of phage DNA (from its absorbance) in 1 litre of overnight culture of DK238 and CVD110 containing ~1012 cells. As strain DK238 is positive for VPI, the difference in amount (in μg) between the two strains should reflect the number of CTXφ genomes (7 kb) and the balance the number of VP1 genomes (40 kb) released per cell. We estimate that 280 and 200 μg of ssDNA is present in a phage preparation from 1-litre cultures of DK238 and CVD110, respectively. If 1 μg of 1-kb ssDNA contains 1.8 × 1012 molecules, then 1 μg of 7-kb ssDNA contains 2.6 × 1011 CTXφ molecules and 80 μg contains 2 × 1013 molecules, so 2 × 1013 molecules/103 cells indicates that an average of 20 CTXφ are made per cell during overnight culture. Likewise, for VP1 we calculate that an average of 9 VP1 are produced per cell. Our calculations assume that no other phage (or RF) is produced and that DNA extraction is 100% efficient, which is unlikely, so values could be underestimated.

We estimated that 57 and 39 μg of ssDNA are present in RF preparations from 1-litre cultures of DK238 and CVD110, respectively, which yields an average CTXφ RF copy number of 2 per cell after overnight culture; the average VP1 RF copy number is similarly estimated as 1 per cell.

**Immunoprecipitation.** DNase- and RNase-treated phage preparations were incubated with rabbit anti-TcpA peptide antibody (1:10,000) at 37 °C and with vigorous shaking for 1 h. Mouse anti-rabbit IgG (whole molecule) agarose beads (Sigma) were added and the reaction was incubated at 4 °C overnight with gentle rotation. After several low-speed centrifugations and washings in deionized water, the pellet was resuspended in deionized water.

**Electron microscopy.** Phage preparations of CVD110 and 395 were placed on a carbon–formvar-coated 300-mesh copper grid (Electron Sciences) for 2 min then negatively stained with 1.5% phosphotungstic acid for 1 min and analysed by electron microscopy. In addition, equal volumes of 395 and N19691 phage preparations were separately incubated with rabbit anti-peptide TcpA antibody (1:10,000) at 37 °C for 20 min, after which this suspension was placed on a grid for 5 min, and 10 μl of 10-nm colloidal gold-conjugated goat anti-rabbit IgG (ICN Biomedicals) was added before negative staining.

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**Structure of Cdc42 in complex with the GTPase-binding domain of the ‘Wiskott–Aldrich syndrome’ protein**

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The Rho-family GTP-hydrolysing proteins (GTPases), Cdc42, Rac and Rho, act as molecular switches in signalling pathways that regulate cytoskeletal architecture, gene expression and progression of the cell cycle. Cdc42 and Rac transmit many signals through GTP-dependent binding to effector proteins containing a Cdc42/Rac-interactive-binding (CRIB) motif. One such effector, the Wiskott–Aldrich syndrome protein (WASP), is postulated to link activation of Cdc42 directly to the rearrangement of actin.

Human mutations in WASP cause severe defects in haematopoietic cell function, leading to clinical symptoms of thrombocytopenia, immunodeficiency and eczema. Here we report the solution structure of a complex between activated Cdc42 and a minimal GTPase-binding domain (GBD) from WASP. An extended amino-terminal GBD peptide that includes the CRIB motif contacts the switch I, β2 and α5 regions of Cdc42. A carboxy-terminal β-hairpin and α-helix pack against switch II. The Phe-X-His-X2-His portion of the CRIB motif and the extended amino-terminal GBD peptide that includes the CRIB motif contacts the switch I, β2 and α5 regions of Cdc42. Discrimination between the CRIB motif and the α-helix appear to mediate sensitivity to the nucleotide switch through an additional, irregular strand containing a series of conserved β-bulges at WASP residues 240–241, 243–244 and 247–248. Binding of a series of WASP fragments to Cdc42 in complex with the non-hydrolysable GTP analogue GMPPCP and Mg2+, both biochemically and by NMR spectroscopy. All fragments containing the WASP CRIB motif, amino acids 238–251, defined as the minimal conserved sequence in several Cdc42/Rac-binding proteins\(^2\), both biochemically and by NMR spectroscopy. All fragments contained the WASP CRIB motif, amino acids 238–251, defined as the minimal conserved sequence in several Cdc42/Rac-binding proteins\(^2\). As found previously\(^4\), only fragments containing at least residues 230–288 of WASP maintained high affinity for the GTPase (Kd, 20–30 nM). NMR analyses indicated that all unbound peptides were largely disordered in solution. However, in the presence of Cdc42–GMPPCP, they adopted discrete structure, with a minimal binding domain indicated by a conserved group of 44 well dispersed cross-peaks in \(^1\)H/\(^15\)N heteronuclear single-quantum coherence spectra. We carried out structural studies on a fragment of WASP (amino-acids 230–288) bound to Cdc42 (residues 1–179) in complex with GMPPCP and Mg2+. Table 1 shows statistics describing the final set of 20 converged structures. A best-fit superposition of the backbone (N, Cα) atoms of the 20 final NMR structures of the Cdc42 (blue)/WASP (yellow) complex. Residues 278–288 of WASP show no long-range NOEs, appear disordered in solution, and have been removed for clarity. Nucleotide is not shown.

### Table 1

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**Figure 1** Sequence alignment and structure overlay of the Cdc42–WASP complex. a, Sequence alignment of WASP (residues 230–277) with similar regions of selected CRIB-containing proteins. Qualitative GTPase binding preferences are listed at right. Residues appearing in four or more sequences are boxed in red. Consensus CRIB motif is indicated below the alignment. Consensus WASP secondary structure\(^29\) observed in the Cdc42 complex is shown above. Residues in the CRIB motif bind Cdc42 in an extended conformation, contacting the five C-terminal residues of switch I, and the entire adjacent β2 strand (residues 41–47; Fig. 2a). This interaction effectively appends the central β-sheet of the GTPase with an additional, irregular strand containing a series of conserved β-bulges at WASP residues 240–241, 243–244 and 247–248. Binding through strand-like contacts is also found in the two reported Ras-family GTPase–effector complexes, Rap1A–Raf (ref. 5) and Ras–RalGDS (ref. 6) (Fig. 2b), indicating that this may be a common feature of target recognition by small GTPases. However, in these cases binding involves an independently folded domain of the effector, and the contacts cover a larger region of switch I without extending across β2. N-terminal to the CRIB region, WASP residues 231–237 form a compact loop that packs against the C-terminal 5 helix of Cdc42. The C-terminal region of the GBD is folded into a two-stranded antiparallel β-hairpin (residues 252–253 and 257–258) and a short α-helix (residues 265–274). Both of these elements are amphipathic and contact a
The hydrophobic patch on the surface of Cdc42 composed of the C-terminal portions of switch I, switch II and strand β3 (Fig. 2a, c). Figure 3 shows intermolecular nuclear Overhauser effects (NOEs) that define packing of the switch-II residues Leu 67 and Leu 70 against aliphatic and aromatic residues of the WASP β-hairpin and helix (Phe 258, Leu 267, Leu 270, Phe 271, Ala 274 and Ile 276). The extensive Cdc42–WASP interface results in burial of over twice as much total surface area as in the Rap1A–Raf (ref. 5) complex (2,930 ± 60 Å² versus 1,270 Å²).

The WASP GBD has about 500-fold greater affinity for Cdc42–GTP than for Cdc42–GDP (ref. 4). Although high-resolution structures of the two Cdc42 states are not available, chemical shift comparisons, as well as analogy to the Ras system, indicate that conformational differences will be localized primarily to switch I and switch II. Thus, contacts to these regions will mediate the sensitivity of WASP binding to the nucleotide switch. Cdc42 residues Phe 37, Asn 39 and Ala 41, near the C terminus of switch I, form main-chain hydrogen bonds to WASP residues Val 250, Val 247/Ser 248 and Lys 245, respectively (Fig. 2c). These interactions position the Asp 38 side chain of Cdc42 near WASP residues His 246 and His 249. There is a hydrogen bond from the Asp 38 carboxylate to either the His 246 or His 249 imidazole ring in nearly all members of the NMR ensemble. Disruption of these contacts through mutation of either the GTPase (for example, Asp 38 to glutamate in Cdc42; ref. 8) or effector (for example, His 208 to aspartate in N-WASP; ref. 9) significantly (>50-fold for Asp 38 to glutamate) decreases the affinity of Cdc42 and Rac for CRIB proteins in vitro and in vivo, explaining the high conservation of the two histidine residues throughout the CRIB effector family. Sheet-like intermolecular hydrogen bonds also position the side chain of conserved WASP residue Phe 244 in a shallow hydrophobic pocket between α1 and β2 of Cdc42 formed by Ile 21, Thr 25 and Tyr 40. The importance of these interactions was shown by mutagenesis of Tyr 40 to cysteine or lysine in Cdc42 or Rac, which substantially decreases their affinity for CRIB-containing targets.

The side chains of switch I residues Val 36 and Phe 37 form one side of an exposed hydrophobic surface of Cdc42, with Phe 56 in β3, and Leu 67 and Leu 70 in switch II. This site interacts with the hydrophobic faces of the GBD C-terminal β-hairpin and helix (Fig. 2c) with numerous intermolecular NOEs from Phe 37 and Phe 56 to WASP residues Val 260, Leu 263 and Leu 267 (see also Fig. 3). These contacts are significant to recognition, as elimination of the C-terminal GBD helix decreases affinity for Cdc42 approximately six-fold. Preferential binding of WASP to Cdc42–GTP thus appears to derive in part from polar and hydrophobic contacts involving highly conserved residues in the CRIB motif.
contacts outside the CRIB motif also appear to be important in response to the nucleotide switch.

CRIB-family proteins bind activated Cdc42 and Rac, but not Rho. Within the family there are variable levels of additional selectivity: proteins such as WASP, ACK and MRC kinase preferentially bind Cdc42 over Rac\(^{3,11,13}\), whereas others such as PAK discriminate little between the two GTPases\(^3\). The Cdc42–WASP structure allows us to understand some aspects of these specificities. The three Rho-family members are identical in the switch I and switch II portions of the WASP-binding site, except at position 38, which is aspartate in Cdc42 and Rac but glutamate in Rho (Fig. 1b). Surprisingly, mutation of Asp 38 in Rac to glutamate\(^3\) decreases the affinity for the PAK GBD by a factor of 50, probably by disrupting contacts to the conserved HXXH motif in the CRIB region, as already described. Thus, this single conservative difference appears to be important for mediating CRIB specificity against Rho.

Biochemical evidence and sequence comparisons indicate that there may be additional specificity determinants in the C-terminal α5 helix of the GTPase\(^3\). Hydrophobic interactions anchor WASP residues 232–238 to the Cdc42 β2/β3 hairpin and α5 helix (Fig. 2d). WASP residue Ile 233, which is a hydrophobic amino acid in most CRIB proteins, packs into a hydrophobic pocket formed by Cdc42 residues Ile 46, Tyr 51, Leu 177 and Leu 174, and the highly conserved Ile 238 packs against Cdc42 residues Ile 46 and Ile 173. A β-turn in the GBD, stabilized by backbone and side-chain hydrogen bonds between Asp 237 and Ser 234, then packs the hydrophobic portion of the Lys 235 sidechain of WASP against the Cdc42 Leu 174 sidechain. This arrangement positions the Lys 235 amine close to the carboxylate of Cdc42 Glu 171, facilitating hydrogen-bond formation. In Rac and Rho, Leu 174 is replaced by arginine, and Gly 47 in the β2/β3 hairpin is replaced by aspartate. Hydrogen bonding between these side chains, which is seen in activated Rac\(^14\) and Rho\(^15\), closes off the pocket contacted by Ile 233 of WASP, and would disrupt the hydrophobic contacts to Lys 235. Thus, these differences may enable the ability of WASP to distinguish Cdc42 from Rac and Rho. PAK, which has a similar affinity for Cdc42 and Rac, contains a lysine at the position corresponding to Ile 233 of WASP (Fig. 1a), suggesting that it may interact differently with the GTPase in these regions. Thus, the extended nature of the WASP–GTPase interface may enable binding energy and specificity elements to be distributed in regions of Cdc42 that are modulated by nucleotide exchange and in more static portions of the molecule. In contrast, in the Ras family, nucleotide exchange and selectivity are intimately coupled, with a single residue in switch I at position 31 of Ras and Rap1A largely governing discrimination between Rap and Rap1GDS (ref. 16).

The Cdc42–ACK structure\(^7\) shows similar interactions throughout the CRIB motif to those observed for Cdc42–WASP, with both effectors extending the GTPase β-sheet by an additional strand. However, interactions in regions outside the CRIB motif, particularly in the C termini of the two GBDs, are quite different. Such variability in extra-CRIB interactions may be a common theme in structures throughout the effector family. Sequence conservation outside the CRIB motif is very low (Fig. 1a), and biochemical and structural data indicate that, like ACK, PAK contacts switch I more extensively than we observe for WASP. Thus, whereas PAK binding strongly inhibits nucleotide dissociation from Cdc42 (ref. 8), WASP has only a fourfold effect\(^6\). In addition, covalent modification of the ribose ring of bound nucleotide (proximal to Glu 31/Tyr 32) decreases the affinity of PAK for Cdc42 approximately 30-fold\(^8\), but has no effect on WASP binding\(^4\). Finally, an intermolecular NOE has been reported from the N-terminal region of switch I in Cdc42 to bound PAK\(^3\) that is incompatible with the binding mode in the WASP complex. The functional explanation for these differences may lie in the specific mechanisms of signalling through WASP and PAK. In N-WASP, the GBD binds to and inhibits the actin depolymerization activity of an acidic C-terminal domain, an interaction postulated to involve the highly basic region immediately N-terminal to the CRIB motif\(^6\). This inhibition is relieved through competitive binding of Cdc42 to the GBD. Similarly, in PAK, C-terminal regions of the GBD appear to regulate the serine/threonine kinase domain of the protein negatively, as mutation of

![Figure 3](image-url)
Leu 107, or proteolytic removal of N-terminal regulatory sequences, including the GBD, lead to constitutive activation 19,20. Thus, sequence divergence in the extra-CRB regions, and the resulting variation in GTPase contacts, may reflect additional regulatory interactions with other enzymatic/functional domains in the effectors. Structural overlap between the intramolecular (GTPase) and intramolecular (enzymatic domain) binding sites of the GBD, and its thermodynamic consequences, could be subtle, and themselves subject to additional regulation. In both WASP and PAK, the GTPase-binding and enzymatic domains are separated by proline-rich sites that bind SH3 domains 21,22. Ligation of these sites could modulate the thermodynamic balance in favour of activation or inhibition. Additional structures of CRIB proteins, both free and in complex with GTPases, will be needed to understand these thermodynamic and structural aspects of recognition and signalling through the Rho-family GTPases. The structure of a Cdc42–WASP-GBD complex represents a first step in this direction.

Methods
Sample preparation. WASP and Cdc42 were overexpressed from vector PET11a in Escherichia coli strain BL21(DE3), and purified from bacterial lysate chromatographically. Uniform 15N and 13C labelling was achieved by growing bacteria in minimal medium supplemented with 15NH4Cl and/or 13C6-glucose as the sole nitrogen and carbon sources. Deuteration was achieved by growth in 50–99.9% D2O with protonated (50–70% 2H) samples or deuterrated (99.9% 2H) samples 15–21. Selective methyl protonation of leucine, valine and isoleucine (51) in a deuterated background was obtained as described 22.

NMR spectroscopy. Spectra were recorded at 25°C on Varian Inova 600 or 800 MHz spectrometers. Sequential assignments were obtained through the following 2H-decoupled, triple-resonance spectra recorded on complexes with 800 MHz spectrometers. Sequential assignments were obtained through the Rho-family GTPases. The structure of a Cdc42–WASP-GBD complex represents a first step in this direction.

Structure determination. For initial calculations, we used a torsion angle dynamics simulated annealing protocol implemented in CNS 28 to dock the GBD onto coordinates of Cdc42–GMPPNP derived from the crystal structure of the GTPase in complex with GMPPNP–Mg2+ and rhoGAP (unpublished coordinates provided by N. Nassar and R. Cerione) through 382 intra-WASP and 170 intermolecular NOEs. These Cdc42 coordinates are similar to those of Rac–GMPPNP–Mg2+ and rhoGTP–S–Mg2+ (backbone r.m.s.d. of 0.88 Å and 1.01 Å, respectively) 29. Structures from these calculations formed the basis for automated, iterative assignment of intra-Cdc42 and remaining intra-WASP NOEs using ARIA 29. In addition to NOE data, calculations used 128 restraints for 64 intra-Cdc42 hydrogen bonds found in regular secondary structure elements in both the Cdc42–GMPPNP–Mg2+–rhoGAP and Rac–GMPPNP–Mg2+ structures 11. Internal GTPase side chains in 55 residues, whose amide chemical shifts were not perturbed by WASP binding, were restrained to within ±20–60° of their rotamers observed in the Cdc42–GMPPNP–rhoGAP crystal structure. Nucleotide and Mg2+ coordinates were fixed in the calculations, and restrained to Cdc42 by hydrogen bonds conserved in Ras-like GTPase structures. The χ1 torsions of nine WASP and four Cdc42 side chains at the molecular interface were restrained to ±20–40° based on patterns of local NOEs. A total of 16 WASP ϕ angles were restrained to ±40° based on qualitative analysis of an HNHa-J experiment. We performed six rounds of calculations with ambiguous cut-off parameter, p (ref. 27), decreasing from 0.96 to 0.7. This was repeated several times, as intermolecular NOEs were added manually. Late in the refinement, we included 97/16 (Cdc42/WASP) ϕ and 97/26 (Cdc42/WASP) ψ restraints, based on analysis of chemical shifts in the program TALOS 30. Dihedrals were restrained to the maximum of 30° or twice the standard deviation observed in the TALOS database matches. We assessed structure quality with PROCHECK-NMR 29.

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