Investigation of the Interaction between Cdc42 and Its Effector TOCA1

Handover of Cdc42 to the Actin Regulator N-WASP Is Facilitated by Differential Binding Affinities

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Transducer of Cdc42-dependent actin assembly protein 1 (TOCA1) is an effector of the Rho family small G protein Cdc42. It contains a membrane-deforming F-BAR domain as well as a Src homology 3 (SH3) domain and a G protein-binding homology region 1 (HR1) domain. TOCA1 binding to Cdc42 leads to actin rearrangements, which are thought to be involved in processes such as endocytosis, filopodia formation, and cell migration. We have solved the structure of the HR1 domain of TOCA1, providing the first structural data for this protein. We have found that the TOCA1 HR1, like the closely related CIP4 HR1, has interesting structural features that are not observed in other HR1 domains. We have also investigated the binding of the TOCA HR1 domain to Cdc42 and the potential ternary complex between Cdc42 and the G protein-binding regions of TOCA1 and a member of the Wiskott-Aldrich syndrome protein family, N-WASP. TOCA1 binds Cdc42 with micromolar affinity, in contrast to the nanomolar affinity of the Aldrich syndrome protein family, N-WASP. TOCA1 binds Cdc42 protein-binding regions of TOCA1 and a member of the Wiskott-Aldrich syndrome protein family, N-WASP. TOCA1 binds Cdc42 with micromolar affinity, in contrast to the nanomolar affinity of the N-WASP G protein-binding region for Cdc42. NMR experiments show that the Cdc42-binding domain from N-WASP is able to displace TOCA1 HR1 from Cdc42, whereas the N-WASP domain but not the TOCA1 HR1 domain inhibits actin polymerization. This suggests that TOCA1 binding to Cdc42 is an early step in the Cdc42-dependent pathways that govern actin dynamics, and the differential binding affinities of the effectors facilitate a handover from TOCA1 to N-WASP, which can then drive recruitment of the actin-modifying machinery.

The Ras superfamily of small GTPases comprises over 150 members that regulate a multitude of cellular processes in eukaryotes. The superfamily can be divided into five families based on structural and functional similarities: Ras, Rho, Rab, Arf, and Ran. All members share a well defined core structure of ~20 kDa known as the G domain, which is responsible for guanine nucleotide binding (1). It is this guanine nucleotide binding that underlies their function as molecular switches, controlling a vast array of signaling pathways. These molecular switches cycle between active, GTP-bound, and inactive, GDP-bound, states with the help of auxiliary proteins. The guanine nucleotide exchange factors mediate formation of the active state by promoting the dissociation of GDP, allowing GTP to bind. The GTPase-activating proteins stimulate the rate of intrinsic GTP hydrolysis, mediating the return to the inactive state (reviewed in Ref. 2).

The overall conformation of small G proteins in the active and inactive states is similar, but they differ significantly in two main regions known as switch I and switch II. These regions are responsible for "sensing" the nucleotide state, with the GTP-bound state showing greater rigidity and the GDP-bound state adopting a more relaxed conformation (reviewed in Ref. 3). In the active state, G proteins bind to an array of downstream effectors, through which they exert their extensive roles within the cell. The structures of more than 60 small G protein-effector complexes have been solved, and, not surprisingly, the switch regions have been implicated in a large proportion of the G protein-effector interactions (reviewed in Ref. 4). However, because each of the 150 members of the superfamily interacts with multiple effectors, there are still a huge number of known G protein-effector interactions that have not yet been studied structurally.

The Rho family comprises 20 members, of which three, RhoA, Rac1, and Cdc42, have been relatively well studied. The role of these three proteins in the coordination of the actin cytoskeleton has been examined extensively (5–10). RhoA acts to rearrange existing actin structures to form stress fibers, whereas Rac1 and Cdc42 promote de novo actin polymerization to form lamellipodia and filopodia, respectively (9–12). A number of RhoA and Rac1 effector proteins, including the formins (13) and members of the protein kinase C-related kinase (PRK)6

6 The abbreviations used are: PRK, protein kinase C-related kinase; WASP, Wiskott-Aldrich syndrome protein; TOCA, transducer of Cdc42-dependent actin assembly protein; N-WASP, neural Wiskott-Aldrich syndrome protein; P(II,4,5)P3, phosphatidylinositol 4,5-bisphosphate; HR1, homology region 1; F-BAR, Fez/CIP4 homology BAR; SH3, Src homology 3; CRIB, Cdc42- and Rac-interactive binding; CIP4, Cdc42-interacting protein 4; MBP, maltose-binding protein; GBD, G protein binding domain; SPA, scintillation proximity assay; PAK, p21-activated kinase; ACK, activated Cdc42-associated kinase; HSQC, heteronuclear single quantum correlation; GMP/PNP, guanosine 5′-[(β,γ-imido)triphosphate]; GTPγS, guanosine 5′-3′-O-(thio)triphosphate; GBD, G protein-binding domain; CSP, chemical shift perturbation; PDB, Protein Data Bank.

* The authors declare that they have no conflicts of interest with the contents of this article.

† Author’s Choice—Final version free via Creative Commons CC-BY license. The atomic coordinates and structure factors (code SFRG) have been deposited in the Protein Data Bank (www.pdb.org).

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family (14), along with Cdc42 effectors, including the Wiskott-Aldrich syndrome (WASP) family (15) and the transducer of Cdc42-dependent actin assembly (TOCA) family (16–18), have also been linked to the pathways that govern cytoskeletal dynamics.

Cdc42 effectors, TOCA1 and the ubiquitously expressed member of the WASP family, N-WASP, have been implicated in the regulation of actin polymerization downstream of Cdc42 and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (9, 16, 19–22). N-WASP exists in an autoinhibited conformation, and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (9, 16, 19–22) is released upon PI(4,5)P2 and Cdc42 binding (21, 23) or 19–22. N-WASP exists in an autoinhibited conformation, and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (9, 16, 19–22) is released upon PI(4,5)P2 and Cdc42 binding (21, 23) or 19–22. N-WASP exists in an autoinhibited conformation, and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (9, 16, 19–22) is released upon PI(4,5)P2 and Cdc42 binding (21, 23) or by other factors, such as phosphorylation (24). Following their release, the C-terminal regions of N-WASP are free to interact with G-protein and a known nucleator of actin assembly, the Arp2/3 complex (25). The importance of TOCA1 in actin polymerization has been demonstrated in a range of in vitro and in vivo studies (16, 26–32), but the exact role of TOCA1 in the many pathways involving actin assembly remains unclear. The most widely studied role of TOCA1 is in membrane invagination and endocytosis (28–30, 33, 34), although it has also been implicated in filopodia formation (27), neurite elongation (35), transcriptional reprogramming via nuclear actin (36), and interaction with ZO-1 at tight junctions (37). A role in cell motility and invasion has also been established (38, 39).

TOCA1 comprises an N-terminal F-BAR domain, a central homology region 1 (HR1) domain, and a C-terminal SH3 domain. The F-BAR domain is a known dimerization, membrane-binding, and membrane-deforming module (33, 40, 41) found in a number of cell signaling proteins. The TOCA1 SH3 domain has many known binding partners, including N-WASP (16) and dynamin (40). The HR1 domain has been directly implicated in the interaction between TOCA1 and Cdc42 (16), representing the first Cdc42-HR1 domain interaction to be identified.

Other HR1 domains studied so far, including those from the PRK family, have been found to bind their cognate Rho family G protein-binding partner with high specificity and affinities in the nanomolar range (42–45). The structures of the PRK1 HR1a domain in complex with RhoA (42) and the HR1b domain in complex with Rac1 (46) show that the HR1 domain comprises an anti-parallel coiled-coil that interacts with its G protein binding partner via both helices. Both of the G protein switch regions are involved in the interaction. The coiled-coil fold is shared by the HR1 domain of the TOCA family protein, CIP4 (47), and, based on sequence homology, by TOCA1 itself. These HR1 domains, however, show specificity for Cdc42, rather than RhoA or Rac1 (16, 17). How different HR1 domain proteins distinguish their specific G protein partners remains only partially understood, and structural characterization of a novel G protein-HR1 domain interaction would add to the growing body of information pertaining to these protein complexes. Furthermore, the biological function of the interaction between TOCA1 and Cdc42 remains poorly understood, and so far there has been no biophysical or structural insight.

The interactions of TOCA1 and N-WASP with Cdc42 as well as with each other have raised questions as to whether the two Cdc42 effectors can interact with a single molecule of Cdc42 simultaneously. There is some evidence for a ternary complex between Cdc42, N-WASP, and TOCA1 (30), but there was no direct demonstration of simultaneous contacts between the two effectors and a single molecule of Cdc42. Nonetheless, the substantial difference between the structures of the G protein-binding regions of the two effectors is intriguing and implies that they bind to Cdc42 quite differently, providing motivation for investigating the possibility that Cdc42 can bind both effectors concurrently. WASP interacts with Cdc42 via a conserved, unstructured binding motif known as the Cdc42- and Rac-interactive binding region (CRIB) (48), which forms an intermolecular β-sheet, expanding the anti-parallel β2 and β3 strands of Cdc42 (49). In contrast, the TOCA family proteins are thought to interact via the HR1 domain, which may form a triple coiled-coil with switch II of Rac1, like the HR1b domain of PRK1 (46).

Here, we present the solution NMR structure of the HR1 domain of TOCA1, providing the first structural data for this protein. We also present data pertaining to binding of the TOCA HR1 domain to Cdc42, which is the first biophysical description of an HR1 domain binding this particular Rho family small G protein. Finally, we investigate the potential ternary complex between Cdc42 and the G protein-binding regions of TOCA1 and N-WASP, contributing to our understanding of G protein-effector interactions as well as the roles of Cdc42, N-WASP, and TOCA1 in the pathways that govern actin dynamics.

Experimental Procedures

Expression Constructs—The Xenopus tropicalis TOCA1 HR1 domain (residues 330–426 and N-terminally extended constructs as indicated) were amplified from cDNA (TOCA1 accession number NM_001005148) and cloned into pGEX-6P-1 (GE Healthcare) or pGEX-HisP (44). The HR1 domain of human CIP4 (residues 388–481) was amplified from IMAGE clone 5352036, the Xenopus laevis FBPl7 HR1 domain (residues 385–486) from IMAGE clone 5514481, and the X. tropicalis N-WASP G protein-binding domain (GBD) (residues 197–255) from IMAGE clone 5379332, and all were cloned into pGEX-6P-1. The resulting constructs express the proteins as N-terminal GST fusions with a 3C protease-cleavable tag, with pGEX-HisP expressing an additional C-terminal His6 tag. Human Cdc42ΔQ61L and full-length Cdc42 were cloned into pGEX-2T (GE Healthcare) and pGEX-6P-1, respectively. A C-terminally extended construct of TOCA1 comprising residues 330–545 was cloned into pMAT10-P.7 The resulting construct expresses TOCA1 330–545 as an N-terminal His-MBP fusion protein with a 3C protease-cleavable tag. Full-length X. tropicalis TOCA1, TOCA1 F-BAR (residues 1–287), and TOCA1 ΔSH3 (residues 1–480) were PCR-amplified from a cDNA clone (IMAGE 5157175) and cloned into pET-His6-SNAP using FseI and Ascl sites that had been incorporated into the primers to create His-SNAP-TOCA1 proteins.

Protein Expression—GST fusion proteins (HR1 domains and Cdc42) were expressed in E. coli BL21 cells (Invitrogen). Stationary cultures were diluted 1:10 and grown at 37 °C until an A600 of ~0.8 was reached and then induced with 0.1 mM isopro-

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7 D. Owen, unpublished data.
The "strong" NMR data were processed using NOESY (100-ms mixing time) recorded on an Avance AV600. The residues that had shifted more than the mean chemical shift change across the spectra were classed as significant and were filtered for solvent accessibility using NACCESS (58). Residues with <50% solvent accessibility were considered to be buried and unavailable for binding.

Results

Cdc42-TOCA1 Binding—TOCA1 was identified in Xenopus extracts as a protein necessary for Cdc42-dependent actin assembly (16) and was shown to bind to Cdc42-GTPγS but not to Cdc42-GDP or to Rac1 and RhoA. Given its homology to other Rho family binding modules, it is likely that the HR1 domain of TOCA1 is sufficient to bind Cdc42. The C. elegans TOCA1 orthologues also bind to Cdc42 via their consensus HR1 domain (34). The HR1 domains from the PRK family bind their G protein partners with a high affinity, exhibiting a range of submicromolar dissociation constants ($K_d$) as low as 26 nM (45). $K_d$ in the nanomolar range was therefore expected for the interaction of the TOCA1 HR1 domain with Cdc42.

We generated an X. tropicalis TOCA1 HR1 domain construct encompassing residues 330–426. This region comprises the complete HR1 domain based on secondary structure predictions and sequence alignments with another TOCA family member, CIP4, whose structure has been determined (47). The interaction between [3H]GTP-Cdc42 and a C-terminally His-

NMR Spectroscopy—The NMR experiments and resonance assignments of the HR1 domain are described (54). The NMR experiments were carried out with 0.9 mM 13C/15N-labeled HR1 domain in 20 mM sodium phosphate, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 5 mM DTT, 10% D2O on a Bruker DRX500. Residues with <50% solvent accessibility were considered to be buried and unavailable for binding.
The TOCA1 HR1-Cdc42 interaction is low affinity. A, curves derived from direct binding assays in which the indicated concentrations of Cdc42 or full-length Cdc42 were titrated into 30 nM GST-ACK and [3H]GTP. The data were fitted to a binding isotherm to give an apparent 

A complex of a GST fusion of the GBD of ACK, which binds with a high affinity to Cdc42 (61), with radio-labeled [3H]GTP-Cdc42 was preformed, and the effect of increasing concentrations of untagged TOCA1 HR1 domain was examined. Competition of GST-ACK GBD bound to [3H]GTP-Cdc42 by free ACK GBD was used as a control and to establish the value of background counts when Cdc42 is fully displaced. The data were fitted to a binding isotherm describing competition (53). Free ACK competed with itself with an affinity of 32 nM, similar to the value obtained by direct binding of 23 nM (61). The TOCA1 HR1 domain also fully competed with the GST-ACK but bound with an affinity of 6 μM (Fig. 1, B and C), in agreement with the low affinity observed in the direct binding experiments.

The Cdc42 construct used in the binding assays has seven residues deleted from the C terminus to facilitate purification. These residues are not generally required for G protein-effector interactions, including the interaction between RhoA and the PRK1 HR1 domain (53). In contrast, the C terminus of Rac1 contains a polybasic sequence, which is crucial for Rac1 binding to the HR1b domain from PRK1 (43, 46). As the observed affinity between TOCA1 HR1 and Cdc42 was much lower than expected, we reasoned that the C terminus of Cdc42 might be necessary for a high affinity interaction. The binding experiments were repeated with full-length [3H]GTP-Cdc42, but the affinity of the HR1 domain for full-length Cdc42 was similar to its affinity for truncated Cdc42 (K_d ~ 5 μM; Fig. 1C). Thus, the C-terminal region of Cdc42 is not required for maximal binding of TOCA1 HR1.

Another possible explanation for the low affinities observed was that the HR1 domain alone is not sufficient for maximal binding of the TOCA proteins to Cdc42 and that the other domains are required. Indeed, GST pull-downs performed with in vitro translated human TOCA1 fragments had suggested that residues N-terminal to the HR1 domain may be required to stabilize the HR1 domain structure (16). Furthermore, both BAR and SH3 domains have been implicated in interactions with small G proteins (e.g., the BAR domain of Arfaptin2 binds to Rac1 and Arl1) (62), while an SH3 domain mediates the interaction between Rac1 and the guanine nucleotide exchange factor, β-PIX (63). TOCA1 dimerizes via its F-BAR domain, which could also affect Cdc42 binding, for example by presenting two HR1 domains for Cdc42 interactions. Various TOCA1 fragments (Fig. 2A) were therefore assessed for binding to full-length Cdc42 by direct SPA. The isolated F-BAR domain showed no binding to full-length Cdc42 (Fig. 2B). Full-length TOCA1 and ΔSH3 TOCA1 bound with micromolar affinity (Fig. 2B), in a similar manner to the isolated HR1 domain (Fig. 1A). The HR1-SH3 protein could not be purified to homogeneity as a fusion protein, so it was assayed in competition assays after cleavage of the His tag. This construct competed with other methods were employed, which utilized untagged proteins. Isothermal titration calorimetry was carried out, but no heat changes were observed at a range of concentrations and temperatures (data not shown), suggesting that the interaction is predominantly entropically driven. Other G protein-HR1 domain interactions have also failed to show heat changes in our hands. Infrared interferometry with immobilized Cdc42 was also attempted but was unsuccessful for both TOCA1 HR1 and for the positive control, ACK.

The affinity was therefore determined using competition SPAs. A complex of a GST fusion of the GBD of ACK, which binds with a high affinity to Cdc42 (61), with radioactive [3H]GTP-Cdc42 was preformed, and the effect of increasing concentrations of untagged TOCA1 HR1 domain was examined. Competition of GST-ACK GBD bound to [3H]GTP-Cdc42 by free ACK GBD was used as a control and to establish the value of background counts when Cdc42 is fully displaced. The data were fitted to a binding isotherm describing competition (53). Free ACK competed with itself with an affinity of 32 nM, similar to the value obtained by direct binding of 23 nM (61). The TOCA1 HR1 domain also fully competed with the GST-ACK but bound with an affinity of 6 μM (Fig. 1, B and C), in agreement with the low affinity observed in the direct binding experiments.

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Investigation of the TOCA1-Cdc42 Interaction

The Cdc42-HR1 interaction is of low affinity in the context of full-length protein and in TOCA1 paralogues. A, diagram illustrating the TOCA1 constructs assayed for Cdc42 binding. Domain boundaries are derived from secondary structure predictions; B, binding curves derived from direct binding assays, in which the indicated concentrations of Cdc42ΔQ61L-[3H]GTP were incubated with 30 nm GST-ACK or His-tagged TOCA1 constructs, as indicated, in SPA. The SPA signal was corrected by subtraction of control data with no fusion protein. The data were fitted to a binding isotherm to give an apparent Kd. C-E, representative examples of competition SPA experiments carried out with the indicated concentrations of the TOCA1 HR1-H3 construct titrated into 30 nm GST-ACK and 30 nm Cdc42ΔQ61L-[3H]GTP (C) or HR1CIP4 (D) or HR1FBP17 (E) titrated into 30 nm GST-ACK and 30 nm Cdc42FLQ61L-[3H]GTP.

TABLE 1
Experimental restraints and structural statistics

| Experimental restraints | <SA> a | <SA> b |
|-------------------------|--------|--------|
| Coordinate precision for well-ordered regions | | |
| RMSD of backbone atoms (342-379, 386-419) (Å) | 0.67 ± 0.14 | 0.46 |
| RMSD of all heavy atoms (342-379, 386-419) (Å) | 1.10 ± 0.12 | 0.96 |
| Ramachandran analysis for all residues | | |
| Residues in most favoured regions: | 99.4 % | 96.1 % |
| Residues in additionally allowed regions: | 0.6 % | 3.9 % |
| Residues in disallowed regions: | 0 % | 0 % |
| RMS deviations for all residues | | |
| from the experimental restraints: | | |
| NOE distances (Å) | 0.011 ± 0.0027 | 0.011 |
| Dihedral angles (°) | 0.14 ± 0.067 | 0.18 |
| from idealised geometry: | | |
| Bonds (Å) | 0.0027 ± 0.00009 | 0.00269 |
| Angles (°) | 0.434 ± 0.012 | 0.427 |
| Improper (°) | 1.025 ± 0.073 | 1.11 |

* <SA>, the average root mean square deviations for the ensemble ± S.D.
* b <SA>, values for the structure that is closest to the mean.

GST-ACK GBD to give a similar affinity to the HR1 domain alone (Kd = 4.6 ± 4 μM; Fig. 2C). Taken together, these data suggest that the TOCA1 HR1 domain is sufficient for maximal binding and that this binding is of a relatively low affinity compared with many other Cdc42-effector complexes.

The low affinity of the TOCA1 HR1-Cdc42 interaction raised the question of whether the other known Cdc42-binding TOCA family proteins, FBP17 (18) and CIP4 (17), also bind weakly. The HR1 domains from FBP17 and CIP4 were purified and assayed for Cdc42 binding in competition SPAs, analogous to those carried out with the TOCA1 HR1 domain. The affinities of both the FBP17 and CIP4 HR1 domains were also in the low micromolar range (10 and 5 μM, respectively) (Fig. 2, D and E), suggesting that low affinity interactions with Cdc42 are a common feature within the TOCA family.

Structure of the TOCA1 HR1 Domain—Because the TOCA1 HR1 domain was sufficient for maximal Cdc42-binding, we used this construct for structural studies. Initial experiments were performed with TOCA1 residues 324–426, but we observed that the N terminus was cleaved during purification to yield a new N terminus at residue 330 (data not shown). We therefore engineered a construct comprising residues 330–426 to produce the minimal, stable HR1 domain. Backbone and side chain resonances were assigned as described (54). 2,778 non-degenerate NOE restraints were used in initial structure calculations (1,791 unambiguous and 987 ambiguous), derived from three-dimensional 13C-separated NOESY and 13C-separated NOEY experiments. There were 1,845 unambiguous NOEs and 757 ambiguous NOEs after eight iterations. 100 structures were calculated in the final iteration; the 50 lowest energy structures were water-refined; and of these, the 35 lowest energy structures were analyzed. Table 1 indicates that the HR1 domain structure is well defined by the NMR data.

The structure closest to the mean is shown in Fig. 3A. The two α-helices of the HR1 domain interact to form an anti-parallel coiled-coil with a slight left-handed twist, reminiscent of the HR1 domains of CIP4 (47) (PDB code 2KE4) and PRK1 (42, 43) (PDB codes 1CXZ and 1URF). A sequence alignment illustrating the secondary structure elements of the TOCA1 and CIP4 HR1 domains and the HR1a and HR1b domains from PRK1 is shown in Fig. 3B.
In the HR1a domain of PRK1, a region N-terminal to helix 1 forms a short \( \alpha \)-helix, which packs against both helices of the HR1 domain (42). This region of TOCA1 HR1 (residues 334–340) is well defined in the family of structures (Fig. 3A) but does not form an \( \alpha \)-helix. It instead forms a series of turns, defined by NOE restraints observed between residues separated by one (residues 332–334, 333–335, etc.) or two (residues 337–340) residues in the sequence and the \( \phi \) and \( \psi \) angles, assessed using Stride (64). These turns cause the chain to reverse direction, allowing the N-terminal segment (residues 334–340) to contact both helices of the HR1 domain. Long range NOEs were observed linking Leu-334, Glu-335, and Asp-336 with Trp-413 of helix 2, Leu-334 with Lys-409 of helix 2, and Phe-337 and Ser-338 with Arg-345, Arg-348, and Leu-349 of helix 1. These contacts are summarized in Fig. 3C.

The two \( \alpha \)-helices of TOCA1 HR1 are separated by a long loop of 10 residues (residues 380–389) that contains two short \( 3_{10} \) helices (residues 381–383 and 386–389). Interestingly, side chains of residues within the loop region point back toward helix 1; for example, there are numerous distinct NOEs between the side chains of Asn-380 and Met-383 of the loop region and Tyr-377 and Val-376 of helix 1 (Fig. 3D). The backbone NH and CH groups of Gly-384 and Asp-385 also show NOEs with the side chain of Tyr-377.

Mapping the TOCA1 and Cdc42 Binding Interfaces—The HR1\(^{TOCA1}\), Cdc42 interface was investigated using NMR spectroscopy. A series of \( ^{15} \)N HSQC experiments were recorded on \( ^{15} \)N-labeled TOCA1 HR1 with the presence of increasing concentrations of unlabeled Cdc42\(^{7Q61L}\)-GMPPNP to map the Cdc42-binding surface. A comparison of the \( ^{15} \)N HSQC spectra of free HR1 and HR1 in the presence of excess Cdc42 shows that although some peaks were shifted, several were much broader in the complex, and a considerable subset had disappeared (Fig. 4A). This behavior cannot be explained by the increase in molecular mass (from 12 to 33 kDa) when Cdc42 binds and is more likely to be due to conformational exchange. This leads to broadening of the peaks so that they are not detectable. Overall chemical shift perturbations (CSPs) were calculated for each residue, whereas those that had disappeared were assigned a shift change of 0.2 (Fig. 4B). A peak that disappeared or had a CSP above the mean CSP for the spectrum was considered to be significantly affected.

\( ^{15} \)N HSQC shift mapping experiments report on changes to amide groups, which are mainly inaccessible because they are buried inside the helices and are involved in hydrogen bonds. Therefore, \( ^{13} \)C HSQC and methyl-selective SOFAST-HMQC (65) experiments were also recorded on \( ^{15} \)N,\(^{13} \)C-labeled TOCA1 HR1 to yield more information on side chain involvement. The affected CH groups underwent significant line broadening, similarly to the NH peaks. Side chains whose CH groups disappeared in the presence of Cdc42 are marked on the graph in Fig. 4B with green asterisks.

TOCA1 residues whose signals were affected by Cdc42 binding were mapped onto the structure of TOCA1 HR1 (Fig. 4C).
The changes were localized to one end of the coiled-coil, and the binding site appeared to include residues from both α-helices and the loop region that joins them. Residues outside of this region were not significantly affected, indicating that there was no widespread conformational change. The residues in the interhelical loop and helix 1 that contact each other (Fig. 3D) show shift changes in their backbone NH and side chains in the presence of Cdc42. For example, the side chain of Asn-380 and the backbones of Val-376 and Tyr-377 were significantly affected but are all buried in the free TOCA1 HR1 structure, indicating that local conformational changes in the loop may facilitate complex formation. The chemical shift mapping data indicate that the G protein-binding region of the TOCA1 HR1 domain is broadly similar to that of the CIP4 and PRK1 HR1 domains (Figs. 3B and 4D).

The corresponding 15N and 13C NMR experiments were also recorded on 15N-Cdc42Δ7Q61L-GMPPNP or 15N/13C-Cdc42Δ7Q61L-GMPPNP in the presence of unlabeled HR1 domain. The overall CSP was calculated for each residue. As was the case when labeled HR1 was observed, several peaks were shifted in the complex, but many disappeared, indicating exchange on an unfavorable, millisecond time scale (Fig. 5A). Detailed side chain data could not be obtained for all residues due to spectral overlap, but constant time 13C HSQC and methyl-selective SOFAST-HMQC experiments provided further information on certain well resolved side chains (marked with green asterisks in Fig. 5B).

As many of the peaks disappeared, the mean chemical shift change was relatively low, so a threshold of the mean plus one
S.D. value was used to define a significant CSP. Residues that disappeared were also classed as significantly affected. Parts of the switch regions (Fig. 5, B and C) are invisible in NMR spectra recorded on free Cdc42 due to conformational exchange. These switch regions become visible in Cdc42 and other small G protein-effector complexes (46, 61, 66) due to a decrease in conformational freedom upon complex formation. The switch regions of Cdc42 did not, however, become visible in the presence of the TOCA1 HR1 domain. Indeed, Ser-30 of switch I and Arg-66, Arg-68, Leu-70, and Ser-71 of switch II are visible in free Cdc42 but disappear in the presence of the HR1 domain. This suggests that the switch regions are not rigidified in the HR1 complex and are still in conformational exchange. Nevertheless, mapping of the affected residues onto the NMR structure of free Cdc42\(\Delta Q61L\)-GMPPNP (Fig. 5C)\(^8\) shows that, although they are relatively widespread compared with changes in the HR1 domain, in general, they are on the face of the protein that includes the switches. Although the binding interface may be overestimated, this suggests that the switch regions are involved in binding to TOCA1.

\(^{8}\) H. R. Mott and D. Owen, unpublished data.

Modeling the Cdc42-TOCA1 HR1 Complex—The Cdc42-HR1\(^{TOCA1}\) complex was not amenable to full structural analysis due to the weak interaction and the extensive exchange broadening seen in the NMR experiments. HADDOCK (67) was therefore used to perform rigid body docking based on the structures of free HR1 domain and Cdc42 and ambiguous interaction restraints derived from the titration experiments described above. Residues with significantly affected resonances and more than 50% solvent accessibility were defined as active. Passive residues were defined automatically as those neighboring active residues.

The orientation of the HR1 domain with respect to Cdc42 cannot be definitively concluded in the absence of unambiguous distance restraints; hence, HADDOCK produced a set of models in which the HR1 domain contacts the same surface on Cdc42 but is in various orientations with respect to Cdc42. The cluster with the lowest root mean square deviation from the lowest energy structure is assumed to be the best model. By these criteria, in the best model, the HR1 domain is in a similar orientation to the HR1a domain of PRK1 bound to RhoA and the HR1b domain bound to Rac1. A representative model from...
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A sequence alignment of RhoA, Cdc42, and Rac1 is shown in Fig. 6C. The RhoA and Rac1 contact residues in the switch regions are invisible in the spectra of Cdc42, but they are generally conserved between all three G proteins. Several Cdc42 residues identified by chemical shift mapping are not in close contact in the Cdc42-TOCA1 model (Fig. 6A). Some of these can be rationalized; for example, Thr-24Cdc42, Leu-160Cdc42, Arg-68Cdc42, Lys-16Cdc42 is unlikely to be a contact residue because it is involved in nucleotide binding, but the others may represent specific Cdc42-TOCA1 contacts. In the model, these side chains are involved in direct contacts (Fig. 6D).

Competition between N-WASP and TOCA1—From the known interactions and effects of the proteins in biological systems, it has been suggested that TOCA1 and N-WASP could bind Cdc42 simultaneously (16). Studies in CHO cells indicated that no longer binds Cdc42. An overlay of the HADDOCK model of the Cdc42-HR1TOCA1 complex and the structure of Cdc42 in complex with the GBD of the N-WASP homologue, WASP (49) (PDB code 1CEE), shows that the HR1 and GBD binding sites only partly overlap, and, therefore, a ternary complex remained possible (Fig. 7A). Interestingly, the presence of the TOCA1 HR1 would not prevent the core CRIB of WASP from binding to Cdc42, although the regions C-terminal to the CRIB that are required for high affinity binding of WASP (68) would interfere sterically with the TOCA1 HR1. A basic region in WASP including three lysines (residues 230–232), N-terminal to the core CRIB, has been implicated in an electrostatic steering mechanism (69), and these residues would be free to bind in the presence of TOCA1 HR1 (Fig. 7A).

An N-WASP GBD construct was produced, and its affinity for Cdc42 was measured by competition SPA (Fig. 7B). The $K_d$ that was determined (37 nM) is consistent with the previously reported affinity (69). Unlabeled N-WASP GBD was titrated into $^{15}$N-Cdc42A7Q61L-GMPPNP, and the backbone NH groups were monitored using HSQCs (Fig. 7C). Unlabeled HR1TOCA1 was then added to the Cdc42-N-WASP complex, and no changes were seen, suggesting that the N-WASP GBD was not displaced even in the presence of a 5-fold excess of HR1TOCA1. These experiments were recorded at sufficiently high protein concentrations (145 μM Cdc42, 145 μM N-WASP GBD, 725 μM TOCA1 HR1 domain) to be far in excess of the $K_d$ values of the individual interactions (TOCA1 $K_d \approx 5$ μM, N-WASP $K_d = 37$ nM). A comparison of the HSQC experiments recorded on $^{15}$N-Cdc42 alone, in the presence of TOCA1 HR1, N-WASP GBD, or both, shows that the spectra in
the presence of N-WASP and in the presence of both N-WASP and TOCA1 HR1 are identical (Fig. 7C).

Furthermore, $^{13}$N-TOCA1 HR1 was monitored in the presence of unlabeled Cdc42 $^{7}Q61L$-GMPPNP (1:1) before and after the addition of 0.25 and 1.0 eq of unlabeled N-WASP GBD. The spectrum when N-WASP and TOCA1 were equimolar was identical to that of the free HR1 domain, whereas the spectrum in the presence of 0.25 eq of N-WASP was intermediate between the TOCA1 HR1 free and complex spectra (Fig. 7D). When in fast exchange, the NMR signal represents a population-weighted average between free and bound states, so the intermediate spectrum indicates that the population comprises a mixture of free and bound HR1 domain. Hence, a third, intermediate state that includes all three proteins is unlikely. Again, the experiments were recorded on protein samples far in excess of the individual $K_d$ values (600 $\mu$M each protein). These data indicate that the HR1 domain is displaced from Cdc42 by N-WASP and that a ternary complex comprising TOCA1 HR1, N-WASP GBD, and Cdc42 is not formed. Taken together, the data in Fig. 7, C and D, indicate unidirectional competition for Cdc42 binding in which the N-WASP GBD displaces TOCA1 HR1 domain in complex with Cdc42 in the absence and presence of the N-WASP GBD, showing displacement of Cdc42 from the HR1 domain by N-WASP.

![Figure 7](image-url)

**FIGURE 7. The N-WASP GBD displaces the TOCA1 HR1 domain.** A, the model of the Cdc42-TOCA1 HR1 domain complex overlaid with the Cdc42-WASP structure. Cdc42 is shown in green, and TOCA1 is shown in purple. The core CRIB region of WASP is shown in red, whereas its basic region is shown in orange and the C-terminal region required for maximal affinity is shown in cyan. A semitransparent surface representation of Cdc42 and WASP is shown overlaid with the schematic. B, competition SPA experiments carried out with indicated concentrations of the N-WASP GBD construct titrated into 30 nM GST-ACK or GST-WASP GBD and 30 nM Cdc42$^{7}Q61L$-[3H]GTP. C, Selected regions of the $^{13}$N HSQC of 145 $\mu$M Cdc42$^{7}Q61L$-GMPPNP with the indicated ratios of the TOCA1 HR1 domain, the N-WASP GBD, or both, showing that the TOCA HR1 domain does not displace the N-WASP GBD. D, selected regions of the $^{13}$N HSQC of 600 $\mu$M TOCA1 HR1 domain in complex with Cdc42 in the absence and presence of the N-WASP GBD, showing displacement of Cdc42 from the HR1 domain by N-WASP.
shown to depend on TOCA1 and N-WASP (32). Endogenous N-WASP is present at ~100 nM in Xenopus extracts, whereas TOCA1 is present at a 10-fold lower concentration than N-WASP (16).

The addition of the isolated N-WASP GBD significantly inhibited the polymerization of actin at concentrations as low as 100 nM and completely abolished polymerization at higher concentrations (Fig. 8). The GBD presumably acts as a dominant negative, sequestering endogenous Cdc42 and preventing endogenous full-length N-WASP from binding and becoming activated. The addition of the TOCA1 HR1 domain to 100 μM had no significant effect on the rate of actin polymerization or maximum fluorescence. This is consistent with endogenous N-WASP, activated by other components of the assay, outcompeting the TOCA1 HR1 domain for Cdc42 binding.

**Discussion**

The Cdc42-TOCA1 Interaction—The TOCA1 HR1 domain alone is sufficient for Cdc42 binding *in vitro*, yet the affinity of the TOCA1 HR1 domain for Cdc42 is remarkably low (K₀ ~ 5 μM). This is over 100 times lower than that of the N-WASP GBD (K₀ = 37 nM) and considerably lower than other known G protein-HR1 domain interactions. The polybasic tract within the C-terminal region of Cdc42 does not appear to be required for binding to TOCA1, which is in contrast to the interaction between Rac1 and the HR1b domain of PRK1 but more similar to the PRK1 HR1a-RhoA interaction. A single binding interface on both the HR1 domain and Cdc42 can be concluded from the data presented here. Furthermore, the interfaces are comparable with those of other G protein-HR1 interactions (Fig. 4), and the lowest energy model produced in rigid body docking resembles previously studied G protein-HR1 complexes (Fig. 6). It seems, therefore, that the interaction, despite its relatively low affinity, is specific and sterically similar to other HR1 domain-G protein interactions.

The TOCA1 HR1 domain is a left-handed coiled-coil comparable with other known HR1 domains (42, 43, 47). A short region N-terminal to the coiled-coil exhibits a series of turns and contacts residues of both helices of the coiled-coil (Fig. 3). The corresponding sequence in CIP4 also includes a series of turns but is flexible, whereas in the HR1a domain of PRK1, the equivalent region adopts an α-helical structure that packs against the coiled-coil. The contacts between the N-terminal region and the coiled-coil are predominantly hydrophobic in both cases, but sequence-specific contacts do not appear to be conserved. This region is distant from the G protein-binding interface of the HR1 domains, so the structural differences may relate to the structure and regulation of these domains rather than their G protein interactions.

The interhelical loops of TOCA1 and CIP4 differ from the same region in the HR1 domains of PRK1 in that they are longer and contain two short stretches of 3₁₀ helix. Many of these residues are significantly affected in the presence of Cdc42, so it is likely that the conformation of this loop is altered in the Cdc42 complex. These observations therefore provide a molecular mechanism whereby mutation of Met₃⁸³-Gly₃⁸⁴-Asp₃⁸⁵ to Ile₃⁸³-Ser₃⁸⁴-Thr₃⁸⁵ abolishes TOCA1 binding to Cdc42 (16).

The lowest energy model produced by HADDOCK using ambiguous interaction restraints from the titration data resembled the NMR structures of RhoA and Rac1 in complex with their HR1 domain partners (42, 46). Some speculative conclusions can be made based on this model. For example, Phe-₅₆Cdc42, which is not visible in free Cdc42 or Cdc42-HR1TOCA1, is close to the TOCA1 HR1 (Fig. 6A). Phe-₅₆Cdc42, which is a Trp in both Rac1 and RhoA (Fig. 6C), is thought to pack behind switch I when Cdc42 interacts with ACK, maintaining the switch in a binding-competent orientation (70). This residue has also been identified as important for Cdc42-WASP binding (71). Phe-₅₆Cdc42 is therefore likely to be involved in the Cdc42-TOCA1 interaction, probably by stabilizing the position of switch I.

Some residues that are affected in the Cdc42-HR1TOCA1 complex but do not correspond to contact residues of RhoA or Rac1 (Fig. 6C) may contact HR1TOCA1 directly (Fig. 6D). Gln-²⁴Cdc42, which has also been identified as a contact residue in the Cdc42-ACK complex (61), contacts Val-₃⁷₆TOCA1 and Asn-₃⁸⁰TOCA1 in the model and disrupts the contacts between the interhelical loop and the first helix of the TOCA1 coiled-coil. Thr-₅₂Cdc42, which has also been identified as making minor contacts with ACK (61, 72), falls near the side chains of HR1TOCA1 helix 1, particularly Lys-₃⁷₂TOCA1, whereas the equivalent position in Rac1 is Asn-₅₂Rac1. N52T is one of a combination of seven residues found to confer ACK binding on Rac1 (72) and so may represent a specific Cdc42-effector contact residue. The position equivalent to Lys-₃⁷₂TOCA1 in PRK1 is Glu-₅₈HR1a or Gln-₁₅₁HR1b. Thr-₅₂Cdc42-Lys-₃⁷₂TOCA1 may therefore represent a specific Cdc42-HR1TOCA1 contact. Arg-
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6S_Cdc42 of switch II is positioned close to Glu-395_TOCA1 (Fig. 6D), suggesting a direct electrostatic contact between switch II of Cdc42 and helix 2 of the HR1 domain. The equivalent Arg in Rac1 and RhoA is pointing away from the HR1 domains of PRK1. The importance of this residue in the Cdc42-TOCA1 interaction remains unclear, although its mutation reduces binding to RhoGAP, suggesting that it can be involved in Cdc42 interactions (53).

The solution structure of the TOCA1 HR1 domain presented here, along with the model of the HR1_TOCA1-Cdc42 complex is consistent with a conserved mode of binding across the known HR1 domain-Rho family interactions, despite their differing affinities. The weak binding prevented detailed structural and thermodynamic studies of the complex. Nonetheless, structural studies of the TOCA1 HR1 domain, combined with chemical shift mapping, have highlighted some potentially interesting differences between Cdc42-HR1_TOCA1 and RhoA/ Rac1-HR1PRK1 binding.

We have previously postulated that the inherent flexibility of HR1 domains contributes to their ability to bind to different Rho family G proteins, with Rho-binding HR1 domains displaying increased flexibility, reflected in their lower melting temperatures \( T_m \) and Rac binders being more rigid (44, 45). The \( T_m \) of the TOCA1 HR1 domain is 61.9 °C (data not shown), which is the highest \( T_m \) that we have measured for an HR1 domain thus far. As such, the ability of the TOCA1 HR1 domain to bind to Cdc42 (a close relative of Rac1 rather than RhoA) fits this trend. An investigation into the local motions, particularly in the G protein-binding regions, may offer further insight into the differential specificities and affinities of G protein-HR1 domain interactions.

Significance of a Weak, Transient Interaction—The low affinity of the Cdc42-HR1_TOCA1 interaction is consistent with a tightly spatially and temporally regulated pathway, requiring combinatorial signals leading to a series of coincident weak interactions that elicit full activation. The HR1 domains from other TOCA family members, CIP4 and FBP17, also bind at low micromolar affinities to Cdc42, so the low affinity interaction appears to be commonplace among this family of HR1 domain proteins, in contrast to the PRK family. Weak, transient protein-protein interactions are functionally significant in several systems (73–75); for example, the binding of adaptor proteins to protein cargo during the formation of clathrin-coated vesicles in endocytosis involves multiple interactions of micromolar affinity (76, 77).

The low affinity of the HR1_TOCA1-Cdc42 interaction in the context of the physiological concentration of TOCA1 in Xenopus extracts (~10 nM) (16) suggests that binding between TOCA1 and Cdc42 is likely to occur in vivo only when TOCA1 is at high local concentrations and membrane-localized and therefore in close proximity to activated Cdc42. Evidence suggests that the TOCA family of proteins are recruited to the membrane via an interaction between their F-BAR domain and specific signaling lipids. For example, electrostatic interactions between the F-BAR domain and the membrane are required for TOCA1 recruitment to membrane vesicles and tubules (27), and TOCA1-dependent actin polymerization is known to depend specifically on \( \Pi(4,5)P_2 \) (32). Furthermore, the isolated F-BAR domain of FBP17 has been shown to induce membrane tubulation of brain liposomes and BAR domain proteins that promote tubulation cluster on membranes at high densities (33). Once at the membrane, high local concentrations of TOCA1 could exceed the \( K_d \) of F-BAR dimerization (likely to be comparable with that of the FCHo2 F-BAR domain (2.5 μM) (41)) and that of the Cdc42-HR1_TOCA1 interaction. Cdc42-HR1_TOCA1 binding would then be favorable, as long as coincident activation of Cdc42 had occurred, leading to stabilization of TOCA1 at the membrane and downstream activation of N-WASP.

It has been postulated that WASP and N-WASP exist in equilibrium between folded (inactive) and unfolded (active) forms, and the affinity of Cdc42 for the unfolded WASP proteins is significantly enhanced (78). The unfolded, high affinity state of WASP is represented by a short peptide, the GBD, which binds with a low nanomolar affinity to Cdc42 (49). In contrast, the best estimate of the affinity of full-length WASP for Cdc42 is low micromolar (79). In the inactive state of WASP, the actin- and Arp2/3-binding VCA domain contacts the GBD (21, 23, 80), competing for Cdc42 binding. The high affinity of Cdc42 for the unfolded, active form pushes the equilibrium in favor of (N-)WASP activation. Binding of \( \Pi(4,5)P_2 \) to the basic region just N-terminal to the GBD further favors the active conformation (21). A substantial body of data has illuminated the complex regulation of WASP/N-WASP proteins, and current evidence suggests that these allosteric activation mechanisms and oligomerization combine to regulate WASP activity, allowing the synchronization and integration of multiple potential activation signals (reviewed in Ref. 24). Our data are easily reconciled with this model.

We envisage that TOCA1 is first recruited to the appropriate membrane in response to \( \Pi(4,5)P_2 \) via its F-BAR domain, where the local increase in concentration favors F-BAR-mediated dimerization of TOCA1. Cdc42 is activated in response to co-incident signals and can then bind to TOCA1, further stabilizing TOCA1 at the membrane. TOCA1 can then recruit N-WASP (26) via an interaction between its SH3 domain and the N-WASP proline-rich region (16). The recruitment of N-WASP alone and of the N-WASP-WIP complex by TOCA1 and FBP17 has been demonstrated (26). WIP inhibits the activation of N-WASP by Cdc42, an effect that is reversed by TOCA1 (16). It may therefore be envisaged that WIP and TOCA1 exert opposing allosteric effects on N-WASP, with TOCA1 favoring the unfolded, active conformation of N-WASP and increasing its affinity for Cdc42. TOCA1 may also activate N-WASP by effective oligomerization because clustering of TOCA1 at the membrane following coincident interactions with \( \Pi(4,5)P_2 \) and Cdc42 would in turn lead to clustering of N-WASP, in addition to pushing the equilibrium toward the unfolded, active state.

In a cellular context, full-length TOCA1 and N-WASP are likely to have similar affinities for active Cdc42, but in the unfolded, active conformation, the affinity of N-WASP for Cdc42 dramatically increases. Our binding data suggest that TOCA1 HR1 binding is not allosterically regulated, and our NMR data, along with the high stability of TOCA1 HR1, suggest that there is no widespread conformational change in the pres-
domains are poised for membrane distortion. Hence, actin polymerization cannot occur until F-BAR only be robustly recruited when F-BAR domains are already advantage to such an effector handover, in that N-WASP would N-WASP binding. Step 3, electrostatic interactions between Cdc42 and the basic region upstream of the CRIB initiate Cdc42-N-WASP binding. Step 4, the core CRIB binds with high affinity while the region C-terminal to the CRIB displaces the TOCA1 HR1 domain and increases the affinity of the N-WASP-Cdc42 interaction further. The VCA domain is released for downstream interactions, and actin polymerization proceeds.

In conclusion, the data presented here show that the TOCA1 HR1 domain is sufficient for Cdc42 binding in vitro and that the interaction is of micromolar affinity, lower than that of other G-protein-HR1 domain interactions. The analogous HR1 domains from other TOCA1 family members, FBPI7 and CIP4, also exhibit micromolar affinity for Cdc42. A role for the TOCA1-, FBPI7-, and CIP4-Cdc42 interactions in the recruitment of these proteins to the membrane therefore appears unlikely. Instead, our findings agree with earlier suggestions that the F-BAR domain is responsible for membrane recruitment (27, 33). The role of the Cdc42-TOCA1 interaction remains somewhat elusive, but it may serve to position activated Cdc42 and N-WASP to allow full activation of N-WASP and as such serve to couple F-BAR-mediated membrane deformation with N-WASP activation. We envisage a complex interplay of equilibria between free and bound, active and inactive Cdc42, TOCA family, and WASP family proteins, facilitating a tightly spatially and temporally regulated pathway requiring numerous simultaneous events in order to achieve appropriate and robust activation of the downstream pathway. Our data are therefore easily reconciled with the dynamic instability models described in relation to the formation of endocytic vesicles (75) and with the current data pertaining to the complex activation of WASP/N-WASP pathways by allosteric and oligomeric effects (24).

It is clear from the data presented here that TOCA1 and N-WASP do not bind Cdc42 simultaneously and that N-WASP
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is likely to outcompete TOCA1 for Cdc42 binding. We therefore postulate an effector handover mechanism based on current evidence surrounding WASP/N-WASP activation and our model of the Cdc42-HR1TOCA1 complex. The displacement of the TOCA1 HR1 domain from Cdc42 by N-WASP may represent a unidirectional step in the pathway of Cdc42-N-WASP-TOCA1-dependent actin assembly.

Author Contributions—J. R. W. generated constructs and proteins, set up NMR experiments, analyzed NMR data, and performed binding experiments; D. N. set up NMR experiments; H. M. F. generated longer TOCA clones and proteins; J. L. G. supervised the pyrene experiments; D. N. set up NMR experiments; H. M. F. generated the Cdc42 model of the Cdc42 HR1TOCA1 complex. The displacement of the TOCA1 HR1 domain from Cdc42 by N-WASP may represent a unidirectional step in the pathway of Cdc42-N-WASP-TOCA1-dependent actin assembly.

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