Localization of the PAK1-, WASP-, and IQGAP1-specifying Regions of Cdc42*

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Rong Li‡, Balazs Debreceniš, Baoqing Jia‡, Yuan Gao‡, Gabor Tigyi§, and Yi Zheng¶

From the Departments of ¶Biochemistry and §Physiology and Biophysics, University of Tennessee, Memphis, Tennessee 38163

The Rho family small GTPase Cdc42 transmits divergent intracellular signals through multiple effector proteins to elicit cellular responses such as cytoskeletal reorganization. Potential effectors of Cdc42 implicated in mediating its cytoskeletal effect in mammalian cells include PAK1, WASP, and IQGAP1. To investigate the determinants of Cdc42-effector specificity, we utilized recombinant Cdc42 mutants and chimeras made between Cdc42 and RhoA to map the regions of Cdc42 contributing to specific effector p21-binding domain (PBD) interaction. Site-directed mutants of the switch I domain and neighboring regions of Cdc42 demonstrated differential binding patterns toward the PBDs of PAK1, WASP, and IQGAP1, suggesting that switch I provides essential determinants for the effector binding, but recognition of each effector by Cdc42 involves a distinct mechanism. Differing from Rac1, the switch I domain and the surrounding region (amino acids 29 to 55) of Cdc42 appeared to be sufficient for specific binding to PAK1, whereas determinants outside the switch I domain, residues 157–191 and 84–120 in particular, were necessary and sufficient to confer specificity to WASP and IQGAP1, respectively. In addition, IQGAP1, but not PAK1 nor WASP, required the unique “insert region,” residues 122–134, of Cdc42 to achieve high affinity binding. Microinjection of the constitutively active Cdc42/RhoA chimeras into serum-starved Swiss 3T3 cells showed that although preserving PAK1- and WASP-binding activity could retain the peripheral actin microspike (PAM)-inducing activity of Cdc42, interaction with PAK1 or WASP was not required for this activity. Moreover, IQGAP1-binding alone by Cdc42 was insufficient for PAM-induction. Thus, Cdc42 utilizes multiple distinct structural determinants to specify different effector recognition and to elicit PAM-inducing effect.

The Rho family small GTP-binding proteins RhoA, Rac1, and Cdc42 have been known to regulate a variety of cell biological events involving actin cytoskeletal reorganization (1, 2). Cdc42 was first discovered in Saccharomyces cerevisiae for its role in cell division cycle regulation (3) and has emerged as a key regulator mediating signaling pathways to cell nucleus. The biochemical model that the small GTPase acts as a molecular switch to transduce incoming signals to downstream effector proteins after being activated to the GTP-bound state is well established (16). Based mostly upon the criterion that putative effectors would preferentially recognize the GTP-bound form of the GTPases, a number of candidate effectors of Cdc42 have been unveiled. Initially identified by an overlay assay using the [γ-32P]GTP-bound Cdc42 as a probe (17), a panel of candidate effectors turned out to be a class of novel protein serine/threonine kinases termed p21-activated kinases (PAKs) (18). Subsequent protein data base searches and biochemical characterizations have led to the discovery of a family of proteins sharing a conserved Cdc42/Rac interactive binding (CRIB) motif of PAK (19), many of which have since been shown to belong to the effector family of Cdc42 upon both in vitro and in vivo examinations (16). Two of the CRIB motif-containing putative effectors, PAK1 and Wiskott-Aldrich Syndrome protein (WASP), have been implicated in Cdc42-mediated actin stress fiber dissolution and actin-polymerization process, respectively (20–24, 58). The CRIB motif and the immediate surrounding amino acid residues of these putative effectors constitute the essential p21-binding domains (PBDs) conferring Cdc42 binding activity (52, 56). Another candidate effector, IQGAP1, which contains a RasGAP-homology domain responsible for Cdc42 recognition (25), was identified by Cdc42-GTP affinity binding approach (26) and has been shown to contain an actin-bundling activity (27). IQGAP1 has been suggested recently to play a role in connecting Cdc42-signaling pathway to actin-cytoskeleton structures (28) and in regulating E-cadherin-mediated cell-cell adhesion (29).

A few major issues related to the mechanism of Cdc42 function concern how the incoming signals would further bifurcate from the activated G-protein to specific effectors, where the specificity of functional coupling between Cdc42 and individual effectors is embedded in the structure of the Rho GTPase, and

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¶ To whom correspondence should be addressed. Tel.: 901-448-5138; Fax: 901-448-7360; E-mail: yzheng@utmem.edu.

† The abbreviations used are: PAM, peripheral actin microspike; GAP, GTPase-activating protein; GST, glutathione S-transferase; PAK, p21-activated kinase; PBD, p21-binding domain; WASP, Wiskott-Aldrich Syndrome protein; CRIB, Cdc42/Rac interactive binding; PKN, protein kinase N; HA, hemagglutinin.
whether the identified putative effectors may truly be involved in mediating a specific cellular function of Cdc42. The switch I region of Rho family GTPases, which includes amino acid residues 32–40 (numbering by the Cdc42 sequences), has been established as a key region required for effector interactions. The switch I region mutants, F37A and Y40C of Rac1 and F39A and Y42C of RhoA, which displayed selective binding patterns toward specific Rac1 or RhoA effectors, have been widely used to rule out potential involvements of given effectors in the respective small G-protein functions (41–43). However, questions have been raised for these approaches by a recent study showing that certain switch I region mutant may have a dominant-negative effect for the cellular functions of the GTPases (49), possibly because of the involvement of the switch I residues in multiple types of interactions with other regulatory proteins including additional effectors, the GTPase-activating proteins, and/or the guanine nucleotide exchange factors. Previous studies of Rac1 interaction with PAK have suggested that, in addition to the switch I region of the molecule, a distant second and possibly a third region may be required to determine the specificity of the functional interaction (30, 31). Recent structural mapping of RhoA interaction with its effectors has also identified multiple regions, the switch I and surrounding residues and amino acids 75–92 of RhoA, Asp-76, Asp-87, and Asp-90 in particular, to be responsible for selective binding to different classes of Rho-binding domains of effectors (32, 33, 53). This “second” or “third” effector-interacting region of Rho GTPases may represent the unique site in the GTPase structures contributing to the specificity of individual effector binding.

To dissect the mechanism of Cdc42-effector interaction and to compare the characteristics of Cdc42-effector coupling with those of Rac1- and RhoA-effector recognition, we have attempted to map the regions of Cdc42 involved in specifying interactions with three effector targets, PAK1, WASP, and IQGAP1, in the current study. Our results obtained by mutational and chimeric approaches identify three distinct regions required for recognition of the three different effector PBDs. Microinjection experiments carried out with constitutively active Cdc42/RhoA chimeras indicated that, although preserving WASP- and PAK1-binding could retain PAM-inducing activity of Cdc42, PAK1 and WASP were not required for the Cdc42-mediated PAM formation in Swiss 3T3 cells. Moreover, IQGAP1-binding alone by Cdc42 was insufficient for this activity. Thus, Cdc42 utilizes multiple distinct structural determinants to specify different effector binding and to elicit PAM-inducing effect.

**EXPERIMENTAL PROCEDURES**

**Construction of Site-directed Mutant and Chimeric cDNAs**—The Cdc42 point mutants were generated by oligonucleotide-directed mutagenesis of human Cdc42 cDNA in pGEX-KG vector by the polymerase chain reaction-based second extension amplification technique using the *Pfu* polymerase (Stratagene), with primers that contained the desired mutations (34). The sequences of mutagenized cDNA inserts were confirmed by manual or automated DNA sequencing. The Cdc42/RhoA chimeric cDNAs were produced by polymerase chain reaction method using the *Pfu* polymerase which generates blunt-ended DNA fragments in PCR reactions. The products amplified from respective cDNAs encoding Cdc42 and RhoA with primers sandwiching the junctional site and the 5'- or 3'-end of the coding sequences containing a BamHI site or EcoRI site were co-inserted into the BamHI and EcoRI sites of the pGEX-KG plasmid as described previously (34, 35). To generate the constitutively active chimeras, the resulting chimeric inserts were re-amplified using a 5'-primer containing a BamHI site, ATG start codon, and the coding sequences including a Gly to Val mutation at the amino acid 12 position (amino acid 14 position for RhoA sequences) and an 3'-primer containing the stop codon and an EcoRI site, and were then reinserted into the pGEX-KG vector. These cDNA constructs were further sequence-confirmed by automated fluorescence sequencing prior to protein expression. All junctions of the chimeras were chosen at the conserved regions between Cdc42 and RhoA (see Fig. 1). The coding region, which is now undergoing recombinant GTPase—Expression and purification of recombinant small GTP-binding proteins and the PBD of effectors from the pGEX-KG vector-transformed *Escherichia coli* were carried out as described previously (36, 37). The PBD of human PAK1 contains amino acid residues 51–135, the PBD of human WASP contains amino acid residues 218–288 of WASP, the PBD of human IQGAP1 contains amino acid residues 864–1657 of the native protein, and the PBD of protein kinase N (PKN) contains residues 1–123 of PKN (gift of Dr. Yoshi Ono). All proteins prepared for measurements were subjected to gel filtration and polyacrylamide gel electrophoresis and Coomasie Blue staining analysis, and the contents of each were judged to be at least 90% pure. Concentrations of the recombinant proteins were determined by using the BCA protein assay reagents from Pierce with bovine serum albumin as a standard or by $^{35}$S/GTP binding for the small GTP-binding proteins (36).

**GTPase Activity Assays**—The intrinsic and GAP-stimulated GTPase activities of Cdc42/RhoA chimeras and Cdc42 mutants were measured as described previously (35) by determining the retention of G-protein bound radioactivities on nitrocellulose filters.

**Dot-blot Binding Assay**—The binding interactions between the small G-proteins and effector PBDs were examined by a dot-blot assay as described (19, 35). Briefly, 1–5 μg of GST-fusions of PBDs or GST alone at a 1 μg concentration were spotted onto nitrocellulose filters (BA85, Schleicher & Schuell). The filters were incubated with buffer A containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 1 mM GTP, and 5% dry milk for 1 h at room temperature. 0.2 μg of the active small G-protein mutants, preloaded with $[^{32}P]GTP$, was then added to the buffer mixture and incubated for 5 min at 4 °C under constant agitation. The nitrocellulose filters were washed three times with ice-cold buffer A and were then subjected to autoradiography and radioactive quantification by using an InstantImager (Packard).

**Cell Culture and Western Blot—** COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cDNAs encoding the Cdc42 chimeras were cloned into the pKH3 expression vector, which expresses the respective small G-protein functions (41–43). However, questions have been raised for these approaches by a recent study showing that certain switch I region mutant may have a domain-specific effect for the cellular functions of the GTPases (49), possibly because of the involvement of the switch I residues in multiple types of interactions with other regulatory proteins including additional effectors, the GTPase-activating proteins, and/or the guanine nucleotide exchange factors. Previous studies of Rac1 interaction with PAK have suggested that, in addition to the switch I region of the molecule, a distant second and possibly a third region may be required to determine the specificity of the functional interaction (30, 31).

**In Vivo Analysis**—The effects of the recombinant chimeric GTPases on cell morphology in 2D culture were examined by staining with rhodamine-conjugated phalloidin (Sigma) for filamentous actin and further incubated at 37 °C for 15–20 min followed by fixation and permeabilization into the Locator coverslip. After microinjection cells were observed under an inverted microscope and photographed with a digital camera.

**RESULTS AND DISCUSSION**

Cdc42 is at least 50% identical in amino acid sequences to other Rho family members such as RhoA (Fig. 1) and is about 30% identical to Ras; it shares a similar overall three-dimensional folding with RhoA and Ras (Fig. 1, Ref. 39). It is intriguing that the relative amino acid changes in the primary structure of Cdc42 with other Rho GTPases can result in major quantitative, sometimes qualitative, differences with regard to its ability to recognize a different set of effector targets.

The mostly expected effector-interaction site of Cdc42 is the switch I domain. The switch I and surrounding residues of Rho protein have been established as an important region for inter-
acting with effector molecules to elicit cellular transformation (40). Recent mutagenesis studies of RhoA and Rac1 have also pinpointed this region as an essential effector site mediating their biological activities, which include the RhoA-induced actin-stress fiber formation and integrin complex assembly (41), the RhoA-mediated serum response factor activation and cell transformation (41, 43), the Rac1-induced lamellipodia formation and cell transformation (42, 43), and the Rac1-mediated NADPH oxidase activation (44). Moreover, site-specific mutants of RhoA, Rac1, and Cdc42 at the amino acid 37 and 40 positions (numbered by that of Cdc42) have been in use to delineate the downstream signaling pathways based on their selective binding patterns to certain effectors such as PAK and ROK (41, 43). To examine the requirement of individual residues of the switch I and neighboring regions of Cdc42 for effector recognition in more detail, we have compared the ability of Cdc42 mutants made at five conserved residues in switch I and at seven nonconserved residues in switch I and immediate adjacent regions (Fig. 1) to bind to the PBDs of the implicated effectors, PAK1, WASP, and IQGAP1. As shown in Fig. 2A, four of the five mutants made at the conserved residues of switch I, Y32K, T35A, F37A, and Y40K, demonstrated a selective binding pattern to the three effectors, whereas V33D does not affect binding to any of the three PBDs. Y32K retained the ability of binding to PAK1 and IQGAP1 while impaired the potential to bind to WASP. T35A, which may have an altered Mg\(^{2+}\) binding property because Thr-35 is expected to contribute directly to the Mg\(^{2+}\) coordination, partially retained the WASP binding ability while lost the PAK1- and IQGAP1-binding activities. Consistent with the previous observations (42), F37A was able to bind to PAK1 whereas Y40K was mostly inactive toward PAK1. In addition, these two mutants displayed opposite recognition patterns toward WASP and IQGAP1, i.e., F37A remained capable of binding to WASP but was inactive in binding to IQGAP1 whereas Y40K was active toward IQGAP1 but was inactive for WASP (Fig. 2A). Further examination of the Cdc42-unique residues in the switch I and surrounding regions by mutation of the Cdc42 residues to the corresponding residues of RhoA revealed that Thr-25, Ser-30, Glu-31, and Thr-43, which are located immediately outside switch I, are not essential for the effector-recognition, whereas the Asp-38 and Thr-43 residues constitute the important elements in PAK1-binding (Fig. 2B). These results suggest that switch I, and its C-terminal neighboring region in the case of PAK1, is necessary for the various effector binding interactions of Cdc42. The data also provide further support for the previous observations made of the F37A and Y40K mutants (42, 43), that different effectors utilize distinct residues of switch I to make a contact with Cdc42.

In the best characterized cases of small G-protein-effector interactions, Ras and Rap1 were found to complex with Raf/RalGDS PBDs exclusively through the switch I and the extended region by forming an intermolecular β-sheet at the contact site (45, 62). Chimeras of Rap1 containing the Ras residues of this region became oncogenic like Ras (46), suggesting that the switch I and neighboring regions of Ras are sufficient for specifying effector binding and for its biological function. We therefore examined whether the switch I and surrounding residues of Cdc42 are sufficient for specific binding to the effectors. Chimera A, in which the amino acids 29 to 55 of Cdc42 were replaced by the corresponding residues of RhoA, was mostly inactive toward PAK1 while retaining the Cdc42 property in recognition of WASP and IQGAP1 (Fig. 3). Chimera B, which contains RhoA sequences 23 to 31 adjacent to switch I in the Cdc42 backbone, behaved similarly as wild-type Cdc42 in binding capabilities to all three effectors. On the other hand, chimera C, made by replacing the RhoA residues with the corresponding Cdc42 residues 30 to 54, displayed a reverse binding pattern to the effectors of chimera A, i.e., active toward PAK1 while inactive toward WASP and IQGAP1 (Fig.
Cdc42 does not function as a universal effector binding site. Rather, in addition to its previously implicated role in RhoGDI function (50), this region of Cdc42 contributes in part to high affinity recognition of certain effectors such as IQGAP1, which may be related to the cellular transformation activity of Cdc42 (51). Moreover, Cdc42 apparently does not require its C-terminal residues for efficient PAK1-binding, which differs from the case of Rac1 (31).

To map the region of Cdc42 responsible for specifying interaction with the effectors, we next examined the binding patterns of a few additional Cdc42/RhoA chimeras to the effector PBDS. As shown in Fig. 5, chimera D, which contains N-terminal 155 residues from Cdc42 and the rest from RhoA, remained active in binding to PAK1 and IQGAP1 but lost most of the WASP binding ability. Chimera E, which had the last 71 residues of Cdc42 replaced by that of RhoA, behaved similarly as chimera D. When only the first 54 amino acids of Cdc42 was preserved in chimera F, the binding activities to both WASP and IQGAP1 were lost, whereas binding to PAK1 remained intact. When the C-terminal residues (amino acids 155 to 191) of Cdc42 were introduced into the corresponding positions of chimera C (to generate chimera G), binding to WASP was restored, whereas the molecule (chimera G) remained active toward PAK1 and inactive toward IQGAP1 like chimera C. Finally, replacement of the corresponding residues of RhoA by residues 83 to 120 of Cdc42 (chimera II) was able to restore most of the binding affinity for IQGAP1 (Fig. 5). Combined with the results of the C-7 and Del mutants, these results indicate that the C-terminal region of Cdc42, residues 156 to 184, contains the structural elements necessary and sufficient for WASP-binding specificity. The region encompassing residues 83 to 120, together with the insert, is important for the high affinity, specific IQGAP1 recognition. The residues of the insert region required for high affinity IQGAP1-binding must be conserved between Cdc42 and RhoA; however, because chimera E which contains the insert region of RhoA remained capable of tight binding to IQGAP1. Moreover, the results obtained with

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**FIG. 3.** The switch I domain and neighboring residues of Cdc42 are sufficient for PAK1-binding specificity but are not essential for specifying WASP or IQGAP1 interaction. ~0.2 μg of [γ-32P]GTP loaded V12Cdc42 or the indicated Cdc42/RhoA chimeras containing the Val12 (or Val14 in case the N terminus was derived from RhoA) mutation were used to probe the GST-PBD (~5 μg) spotted nitrocellulose filters. Bound GTPases were quantified by using an InstantImager. Radioactive counts bound to GST alone were taken as background and were subtracted from each set of G-protein-effector binding data. Data shown are representative of three independent experiments.

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**FIG. 4.** Binding of the C-7 and Del mutants of Cdc42 to the effector PBDS. A, the GTP-binding and GTP-hydrolysis activities of the C-7 and Del mutants compared with wild-type Cdc42. 0.2 μg of wild-type or the mutant of Cdc42 was preloaded with [γ-32P]GTP and subjected to the GTPase activity assay in the presence or absence of 10 ng of p50RhoGAP at 23 °C. B, interaction of wild-type or the mutant Cdc42 proteins with the effector PBDS. Conditions were similar to those in Fig. 2.
Cdc42, RhoA, and the chimeras used in the analysis are drawn on the left and all contain a Gly-12 (or Gly-14, in case the N-terminal end of the molecule was derived from RhoA) to Val mutation. Their interactions with respective effector PBDs were determined similarly as those in Fig. 3 and were quantified by radiolabeled, bound G-proteins. Counts were normalized to 100 according to that of Val12Cdc42 mutant (for PAK1, WASP, and IQGAP1) or V14RhoA mutant (for PKN) after the background counts to GST alone were subtracted. The PAM-inducing activities were summarized on the side. Data are representative of four independent experiments.

chimeras D to H further reinforce our conclusion on PAK1-specifying region of Cdc42, i.e., the switch I and surrounding residues from 30 to 54 contains the determinant(s) that are necessary and sufficient for efficient PAK1 interaction. In each case of the chimera binding assay, the PBD of a RhoA-specific effector, PKN, was used as an additional control for chimera functions. Residues in the region between amino acids 70 and 92 of RhoA have been shown to contain the determinants required for PKR2, a PKN-like molecule, specificity (53).

To examine whether the observed in vitro interaction of the chimeras with effector PBDs may occur in vivo, we transiently co-expressed the constitutively active form (containing a Gly to Val mutation at the 12th or 14th position of Cdc42 or RhoA sequences at the N terminus) of chimera A, C, D, E, G, or G with the PBD of PAK1, WASP, or IQGAP1 in COS-7 cells and assayed the binding activity of the chimeras to the respective PBDs by immunoprecipitation and Western blot analysis. The HA-tagged chimeras and the constitutively active Cdc42 mutant, Leu61 (bearing a Gln to Leu mutation at residue 61), were expressed at similar level in each case as judged by anti-HA Western blot of the transfected cell lysates (data not shown). As shown in Fig. 6, the HA-tagged chimeras C, D, E, and G effectively formed a complex with the myc-tagged PAK1 in the cell lysates, chimeras A and G co-precipitated with WASP, and chimeras A, D, and E co-precipitated with IQGAP1. This pattern of association and lack of association of the chimeras with the respective effector PBDs is similar to that of the in vitro binding results (Fig. 5), suggesting that the regions of Cdc42 implicated by the dot-blot assay as important for specifying PAK1-, WASP-, and IQGAP1-interactions are also likely to be important in cellular situations. The Leu61 mutant of Cdc42 appears to bind tighter to the effectors than various chimeras containing the Gly-12 (or Gly-14) to Val mutation, possibly because of an altered conformation and enhanced affinity to the effectors caused by the Gln-61 to Leu mutation which has previously been observed in the case of Rac interaction with effector and GAP (63).

To determine whether any of the three effectors may play roles in Cdc42-mediated actin cytoskeletal effect, we next examined the PAM-inducing activity of the chimeras in Swiss 3T3 fibroblasts. Fifteen minutes after the constitutively active Cdc42 (V12Cdc42) was introduced into the cells by microinjection, we observed a remarkable retraction of cell body accompanying PAM and filopodia protrusion formation (Fig. 7B), similarly as described previously (5, 6). When the constitutively active chimera C containing the switch I and neighboring regions of Cdc42 in the RhoA backbone was microinjected into the cells, little PAM induction activity was detected, whereas potent actin stress fiber induction was apparent (Fig. 7C). In contrast, introduction of the constitutive chimeras A and D, each of which contains a stretch of RhoA residues (residues 30–56 and 157–193, respectively) in the Cdc42 backbone, into the cells readily caused rampage PAM formation like that of V12Cdc42 (Fig. 7, D and E), whereas introduction of chimera H, which is still capable of binding to IQGAP1, led only to actin-stress fiber formation without any visible PAMs (Fig. 7F). Furthermore, injection of chimera G, which is capable of binding to PAK1 and WASP, resulted in PAM induction (Fig. 7G) as in the case of chimera D (Fig. 7E). Combined with the effector binding profiles of the chimeras (Figs. 5 and 6), these results indicate that, although preserving WASP- and PAK1-biding could retain PAM-inducing activity of Cdc42, PAK1 and WASP are not required for the Cdc42-mediated PAM formation. Moreover, binding to IQGAP1, if necessary, is not sufficient for this activity. We have also noticed the subtle differences in morphology aside from PAM-formation in the cells microinjected with different chimera constructs (Fig. 7B, D, and G); these differences raise the possibility that there may exist additional unknown effectors of Cdc42 or RhoA that play roles in controlling other morphological aspects of the cells. The microinjection results also demonstrate that the chimeras examined are biologically active.

The conformational changes of Cdc42 induced by GTP-binding have been primarily localized in the switch I, switch II, and the junctional region of the switches by NMR structural studies (39). The switch I domain of Cdc42, in analogy to that of Ras and other Rho GTPases, is expected to be important for its biological functions. Previous studies from our laboratory have identified residues Tyr-32 and Thr-35 in this region to be critical for interaction with the regulators, Cdc42GAP and the GEFs (34, 35). In this study, by analyzing a panel of mutants made at the conserved as well as the unique residues of the switch I and neighboring regions, we provide direct evidence that the relatively flexible effector loop also constitutes a critical docking site for all three effectors examined. However,
Cdc42 appears to utilize a distinct subset of residues in this region for differential effector binding, implying that significant differences exist between the mechanisms of Cdc42-effector pairs. It is possible that GDP/GTP exchange or GTP hydrolysis leads to a conformational transition in switch I, resulting in the recruitment of the conformational state-specific regulators or effectors through a distinct set of conserved and/or unique residues in this region.

By swapping the residues of Cdc42 with the corresponding amino acids of RhoA, we have found that the switch I and the immediate neighboring region of Cdc42 contain all the necessary determinants for PAK1 specificity, whereas in the cases of WASP and IQGAP1, this region is neither necessary nor sufficient for specificity. In fact, two distantly localized regions of Cdc42, residues 155 to 184 and residues 83–120, constitute the WASP- and IQGAP1-specifying regions, respectively (Fig. 8). The C-terminal polybasic domain is found nonessential for binding to all three effectors, and the Rho family-unique “insert region” of Cdc42 is dispensable for PAK1 and WASP binding but is required for high affinity binding by IQGAP1. These observations bring about a few interesting comparisons with other Rho family members, and with other Cdc42-regulator interactions as follows. (a) PAK interaction with Rac1 is mediated by a second effector site between residues 143 and 175 and a third effector site at the carboxyl polybasic domain of Rac1 in addition to the N-terminal site including switch I (30, 31), which is clearly different from the case of Cdc42. (b) Interaction of two classes of effectors of RhoA, represented by rhophilin and ROCK, appears to involve at least two regions of RhoA for specificity, the switch I domain and a second site between residues 75 and 92 and residues 75 and 119 (32), particularly Asp-87 and Asp-90, respectively (54), whereas the switch I region and the surrounding residues are sufficient for specific binding to the class III effector, citron (32). This, in principle, is similar to the case of Cdc42. (c) The Rho family GTPase regulators, GAPs, GEFs, and RhoGDI, all appear to require multiple regions of the GTPases for functional coupling. For example, p190, the Rho-specific GAP, utilizes both the switch I and loop 6 regions of RhoA for efficient catalysis (35); Lbc, the Rho-specific GEF, requires the switch I and loop 5 regions of RhoA for the GDP/GTP exchange reaction (34); and RhoGDI needs the insert region and at least one other region of Cdc42 (most likely switch I) for functional interaction (50). Therefore, it appears that binding specificity of many Cdc42- and other Rho family GTPase-interacting proteins, including the effectors, may be determined by a complementary second binding site that is subjected to the control of a conformational change conferred by the switch I domain.

Over ten putative effector molecules for Cdc42 have been identified to date (16). Although a number of them, including the three effector molecules examined in this study, have been shown to cooperate with Cdc42 to mediate actin cytoskeletal changes, it remains unclear which effector(s) is truly involved in the induction of filopodium formation by Cdc42. As shown by the effector-specifying Cdc42/RhoA chimeras, although preserving PAK1- and WASP-binding retained the PAM-inducing activity of Cdc42, interaction with PAK1 or WASP is dispensable for this activity; IQGAP1 itself is not sufficient, if necessary, for mediating this activity. Three recently identified Cdc42 effectors, CIP-4, N-WASP, and PAK4 (54, 55, 57, 59), represent new candidates that may contribute solely or cooperatively to the PAM-induction activity in certain cell types. It is possible that a complex mode of Cdc42 interaction with multiple effectors can lead to the induction of PAM formation. It will be of particular interest to determine the effector-specifying regions for the new effector candidates and to rule in or rule out their potential roles in various Cdc42 signaling pathways.

Many of the putative effectors share a homologous PBD which is responsible for binding to the activated Cdc42, yet each may employ a distinct mechanism in coupling to Cdc42 as we have shown in the current study for the two CRIB motifs of PAK1 and WASP. Recently, it has been realized that the switch...
I mutants such as F37A, which selectively recognizes a subset of effectors and has been widely used in delineating effector pathways of Rho proteins, may cause complicated effects in cells, i.e. a constitutively active switch I mutant may simultaneously send out dominant positive and dominant negative signals to the related pathways (49), possibly because of the extensive involvement of this region of Rho GTPases in interaction with multiple types of regulators and effectors. Therefore, it will be of importance to generate the second effector-site mutants which may dictate the effector specificity without interfering with other regulatory functions. When this manuscript was under preparation, Guo et al. (48) reported an intramolecular β-sheet formed along β2 of Cdc42, encompassing residues from 37 to 47, may constitute the PAK3-contact site. Combined with our effector mapping results, this raised the possibility that Asp-38 and Thr-43 in this region served as the major discriminating determinant for PAK-recognition. Characterization of additional point mutants in the WASP-specifying region of Cdc42 has led us to the identification of residues 173 and 174 as the potential WASP-specifying sites. The most recent NMR structural model of Cdc42 in complex with WASP PBD (60) is consistent with our findings and supports the possibility that the Cdc42-unique residues at the distant C-terminal region serve to specify the effector recognition by providing an additional docking site away from the conventional switch domains of the small GTPase.

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