Identification of the Region in Cdc42 That Confers the Binding Specificity to Activated Cdc42-associated Kinase*

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The Rho family small G-protein Cdc42 has been implicated in a diversity of biological functions. Multiple downstream effectors have been identified. How Cdc42 discriminates the interaction with its multiple downstream effectors is not known. Activated Cdc42-associated tyrosine kinase (ACK) is a very specific effector of Cdc42. To delineate the Cdc42 signaling pathway mediated by ACK, we set about to identify the specific ACK-binding region in Cdc42. We utilized TC10, another member of the Rho family of G-proteins that is 66.7% identical to Cdc42, to construct TC10/Cdc42 chimeras for screening the specific ACK-binding region in Cdc42. A region between switch I and switch II has been identified as the specific ACK-binding (AB) region. The replacement of the AB region with the corresponding region in TC10 resulted in the complete loss of ACK-binding ability but did not affect the binding to WASP, suggesting that the AB region confers the binding specificity to ACK. On the other hand, replacement of the corresponding region of TC10 with the AB region enabled TC10 to acquire ACK-binding ability. Eight residues are different between the AB region and the corresponding region of TC10. The mutational analysis indicated that all eight residues contribute to the binding to ACK2. The assays for the Cdc42-mediated activation of ACK2 indicated that the AB region is essential for Cdc42 to activate ACK2 in cells. Thus, our studies have defined a specific ACK-binding region in Cdc42 and have provided a molecular basis for generating ACK-binding-defective mutants of Cdc42 to delineate ACK-mediated signaling pathway.

Cdc42, as a member of the Rho family of small GTPases, plays an important role in cytoskeletal organization, mitogenesis, and membrane trafficking (1–3). Multiple target molecules, such as WASP, IQGAP, PAK, ACK, γ-COP, the partitioning-defective protein 6 (PAR-6), the binder of Rho GTPases (Borg), the myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), and the Cdc42 interactive protein 4 (CIP4), have been identified for mediating these cellular effects (4–17).

Some of the target molecules including WASP, PAK, ACK, and MRCK share a Cdc42interactive motif named “CRIB” domain (18). However, the interactive specificity of Cdc42 with these target molecules is largely unknown. Determination of the specificity becomes critical for us in understanding the mechanisms underlying the multiple cellular function of Cdc42.

ACK, a nonreceptor tyrosine kinase, is a specific downstream target molecule of Cdc42 (19, 20). The ACK family has two members, ACK1 and ACK2, which contain similar functional domains (19). The Cdc42interactive domain (the CRIB domain) in ACK1 and ACK2 are 100% identical (19), indicating that ACK1 and ACK2 have the same biochemical property when interacting with Cdc42. Indeed, both ACK1 and ACK2 interact exclusively with Cdc42 (not Rac or Rho) (19, 20).

Functionally, both ACK1 and ACK2 are activated by cell adhesion through integrin β1 and proteoglycan (21, 22). The cell spreading in HeLa cells is regulated by ACK2 (23). Recent studies have shown that both ACK1 and ACK2 possess a clathrin-binding motif and directly interact with clathrin, a coating protein for receptor endocytotic vesicles (10, 11), suggesting that ACK may play a role in clathrin-mediated receptor endocytosis. Furthermore, ACK2 phosphorylates SH3PX1, a sorting nexin, and facilitates the degradation of the epidermal growth factor receptor (24). In Caenorhabditis elegans, Ark-1 (a homologue of ACK) genetically interacts with UNC101 (the homologue of the mammalian clathrin-associated protein AP47) and SLI-1 (the homologue of mammalian Cbl that is an E3 ubiquitin ligase for the ubiquitination of epidermal growth factor receptor) and negatively regulates epidermal growth factor receptor signaling (25). These data indicate the role of ACK in epidermal growth factor receptor degradation.

The structural studies of the complex of Cdc42 and the CRIB domain of ACK by NMR suggest that Val-42 and Leu-174 of Cdc42 are the residues that interact with the CRIB domain of ACK (26). Further mutational analysis of the interaction of Cdc42 with the CRIB domains derived from ACK, PAK, and WASP identified additional residues, such as Met-45 and Ile-46, for binding to ACK (27). However, the residues Val-42 and Leu-174 are conserved in TC10, the closest member to Cdc42 in the Rho family of small GTPases that does not interact with ACK (28), suggesting that Val-42 and Leu-174 may not be the residues determining the binding specificity of Cdc42 to ACK.

Our initial mutagenesis studies also indicated that Met-45 and Ile-46 alone affected little of the binding of Cdc42 to ACK2. Therefore, there should be additional residues or regions that determine the binding specificity of Cdc42 to ACK.

To define the specific ACK-binding region in Cdc42, we utilized TC10 to construct Cdc42/TC10 chimeras as GST fusion proteins and screen the ACK-binding region in Cdc42. By this approach, we identified a specific ACK-binding (AB) region in Cdc42. This AB region sits between the switch I and switch II regions and possesses a structure containing one U-turn and
ACK-binding Region in Cdc42

**A**

| Cdc42 | TC10 |
|-------|------|
| T10G  | MPASGAGRSBHAGPMALMKCIVVGAVGKTLKCISSLTSNPKFSTVQPYDCATVALGTVMTI | |
| T10S  | T10G | |

**B**

- ACK2
- GST fusion protein

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**EXPERIMENTAL PROCEDURES**

**Construction of Cdc42/TC10 Chimeras and Point Mutants of Cdc42 and TC10—**Wild type pCDNA3-Cdc42 and pCDNA3-TC10 were used as templates to construct chimera and point mutations. The chimera constructs were made by PCR. In the chimera primers, one-half of the primer sequence was from Cdc42 and the other half from TC10. The T7 and SP6 primers were used for the flanking primers. The chimera fragments from PCR were digested by BamHI/EcoRI and cloned into the GST fusion protein vector pGEX-4T3. The point mutations of Cdc42 or TC10 were made using the mutagenesis kit from Stratagene. The chimera or their mutants were washed three times with bacterial lysis buffer (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.5% Triton X-100, 10 μM MgCl₂ and were then ready for the binding assays.

**The Binding Assays with GST Fusion Proteins—**The GTPγS-loaded glutathione-agarose bead-bound GST chimeras Cdc42 and TC10 or their mutants were incubated with HA-tagged ACK2, Myc-tagged ACK1, or Myc-tagged WASP-overexpressed COS-7 cell lysates in the presence of 10 mM MgCl₂ for 1–2 h at 4 °C. The beads were washed three times with the mammalian cell lysis buffer, and the bound proteins were eluted in 2× SDS sample buffer and subjected to 10% SDS-PAGE. The co-precipitated HA-tagged ACK2 or Myc-tagged WASP was detected by immunoblotting with an anti-HA antibody or an anti-Myc antibody. The GST fusion proteins were visualized with Coomassie Blue staining.

**Immunoprecipitation and Immunoblots—**For immunoprecipitation, the pre-cleared cell lysate was incubated with primary antibody on ice for 30 min, protein A beads were added, and the mixture was incubated at 4 °C for 2 h with rotation. The beads were washed with lysis buffer three times, and the immunoprecipitation complexes were either made ready for enzymatic assays or directly dissolved in SDS-PAGE sample buffer for SDS-PAGE. The immunoblots were performed as instructed using ECL immunoblot kits (Amersham Biosciences).

**RESULTS AND DISCUSSION**

**ACK2 Interacts with Cdc42 (Not TC10)—**As shown in Fig. 1A, TC10 is highly homologous to Cdc42, and its primary sequence is 66.7% identical to that of Cdc42. In addition, TC10 shares multiple binding partners with Cdc42, such as PAK, WASP, and the Cdc42 interactive protein CIP4 (28). However, as shown in Fig. 1B, although the GTPγS-loaded Cdc42 strongly interacted with ACK2, the GTPγS-loaded TC10 did not bind to ACK2. Thus, determination of the binding of the two β-sheets (β2 and β3). Our studies provide a molecular basis for generating the ACK binding-defective mutants of Cdc42 to delineate the ACK-mediated signaling pathway.
Cdc42/TC10 chimera proteins to ACK2 will enable us to define the AB region in Cdc42.

The AB Region of Cdc42 Is Located between Residues 36 and 68

To construct the Cdc42/TC10 chimeras, we replaced the portions of TC10 starting from the amino terminus with the corresponding portions of Cdc42 or the portions of Cdc42 starting from the amino terminus with the corresponding portions of TC10 (Fig. 2A). The chimeras were subcloned into a GST fusion protein vector, expressed in bacteria, and purified and immobilized on glutathione-agarose beads.

FIG. 2. The AB region of Cdc42 is located between residues 36 and 68. A, schematic representation of Cdc42/TC10 chimeras. The filled blocks represent the regions of TC10, whereas the empty blocks represent the portions of Cdc42. The chimera names are as follows. T stands for TC10 and C for Cdc42. The numbers following the letter T or C indicate the positions of the amino acid residues in TC10 or Cdc42 at the conjunction boundaries of the chimeras. For example, T49C36 means the chimera has the residues 1–49 of TC10 at the amino terminus followed by the residues 36–191 of Cdc42. The ACK2-binding capacity of each chimera is summarized at the right of the figure. +, binding; −, no binding. B–D, the residues 36–68 of Cdc42 (the AB region) are required for binding to ACK2. The binding was assayed by GST fusion protein pull-down. The glutathione-agarose bead-bound GST chimeras were loaded with GTP-S and subsequently incubated with HA-tagged ACK2- or Myc-tagged WASP-overexpressed COS-7 cell lysates. The co-precipitated ACK2 or WASP was detected by immunoblotting with an anti-HA antibody (top panels in B and C) or an anti-Myc antibody (top panel in D). The GST fusion protein stained by Coomassie Blue is shown in the bottom panels. B, the chimera containing the AB region bind to ACK2 (lanes 1 and 4–8). The chimeras without the AB region do not bind to ACK2 (lanes 2 and 3). C, GST-TC10(C36–68), in which the corresponding region in TC10 was replaced with the AB region, gains the binding to ACK2 (compare lane 2 with lane 1). D, GST-Cdc42(T50–82), the AB region replacement chimera that loses the ACK2-binding capacity, has the full capacity to bind to WASP.

Fig. 3. No single residue in the AB region determines the binding to ACK. A, alignment of the amino acid sequence of the AB region with that of the corresponding region of TC10. The identical residues are linked by vertical lines. The eight residues that are different between the AB region and the corresponding region of TC10 are in bold. B, schematic representation of the point mutants in the AB region. Cdc42[T50–82] is the chimera in which the AB region is replaced with the corresponding region of TC10. In this experiment, we used the GTPase-defective mutant Cdc42Q61L as the template to avoid GTP-S loading. Therefore, all of the point mutants include Q61L mutation. C, the ACK2 pull-down assays of the AB region point mutants. The assays were performed by the same procedures as described in the legend to Fig. 2, except there was no GTP-S loading. The co-precipitated HA-tagged ACK2 was detected by immunoblotting using an anti-HA antibody (top panel), and the GST fusion proteins of the mutants and chimeras were stained by Coomassie Blue (bottom panel). All of the point mutants attained the ACK2-binding capacity.
bilized by glutathione-conjugated agarose beads. The immobilized chimera GTPase proteins were loaded with GTP\(_\gamma\)S and then incubated with HA-tagged ACK2-overexpressed COS-7 lysates. The amount of ACK2 precipitated by the GTP\(_\gamma\)S-loaded Cdc42/TC10 chimeras was detected by immunoblotting with an anti-HA antibody.

When the first 49 amino acid residues at the amino terminus of TC10 were replaced by the corresponding portion of Cdc42 (the first 35 amino acid residues at the amino terminus), the resulting chimera C35T50 did not gain any ACK2-binding capacity (Fig. 2B, lane 3), indicating that the first 35 amino acid residues in Cdc42 are not critical for interaction with ACK. When the first 82 amino acid residues of TC10 were replaced, the resulting chimera, C68T83, acquired full ACK2-binding capacity (Fig. 2B, lane 4). Further replacement of the residues toward the carboxyl terminus of TC10 with the corresponding region of Cdc42 yielded the same ACK2-binding capacity as that of C68T83 (Fig. 2B, lanes 5–8). These data suggest that (i) the region between amino acid residues 36 and 68 of Cdc42 is the ACK2-binding domain and (ii) the region after amino acid residue 69 of Cdc42 (i.e. amino acid residues 69–191) is not the determinant region for interaction with ACK2. To confirm these conclusions, we replaced the portions of Cdc42 at the amino terminus with the corresponding regions of TC10. As shown in lanes 1 and 2 of Fig. 2B, replacement of the first 35 amino acid residues of Cdc42 did not change the ACK2-binding capacity (lane 1), whereas replacement of the first 68 amino acid residues of Cdc42 with the corresponding region of TC10 completely lost the binding to ACK2 (lane 2). These data supported the conclusion that the region in Cdc42 con-

**Fig. 4.** All of the eight residues in the AB region contribute to the binding to ACK. A, schematic representation of the point mutations of the eight residues and chimeras in the AB-corresponding region of TC10. The filled blocks represent the regions of TC10, whereas the empty blocks represent the regions of Cdc42. The numbers above the filled blocks indicate the positions of the amino acid residues in TC10, and the numbers above the empty blocks indicate the position of the amino acid residues in Cdc42. B, the ACK2 pull-down assays with GST fusion proteins of point mutants or chimeras in the AB-corresponding region of TC10. The assays were performed as described in the legend to Fig. 2. The co-precipitated HA-tagged ACK2 was detected with immunoblotting with an anti-HA antibody (top panel), and the GST fusion proteins of the mutants and chimeras were stained by Coomassie Blue (bottom panel).
The Binding to ACK by the AB Region Is Co-determined by Multiple Residues—The alignment of the amino acid sequence of the AB region of Cdc42 and the corresponding region of TC10 shows only eight residues that are different between Cdc42 and TC10 (Fig. 3A). These residues should determine the binding to ACK. To identify the residues in the AB region that specifically interact with ACK, we made point mutations for each residue among the eight residues in the AB region and mutated the residue to the corresponding residue in TC10 (Fig. 3B). To avoid GTP\-S loading, we used Cdc42Q61L, a GTPase-defective mutant, as the template for the point mutation. The ACK2 pull-down assays with these point mutants of Cdc42, as shown in Fig. 3C, indicated that no mutation of a single residue among the eight residues significantly affected the binding to ACK2, suggesting that the binding involves multiple residues.

To further identify the residues for binding to ACK2, we replaced the residues in the corresponding region of TC10 with the residues in the AB region of Cdc42 and determined the ACK2-binding capacity of the mutants (Fig. 4A). Replacement of five of the eight residues from either the amino terminus or the carboxyl terminus of the AB-corresponding region of TC10 with the residues in the AB region of Cdc42 partially recovered ACK2-binding ability compared with TC10[C36–68], the AB region substitution (Fig. 4B, lanes 2, 4, and 13). The three-residue substitutions of TC10, such as TC10[C44–49] and TC10[C50–68], also partially recovered ACK2-binding capacity (Fig. 4B, lanes 11 and 12). However, TC10[S57T/T59M/V60I], a three-residue substitution, did not recover any of the binding capacity (Fig. 4B, lane 8). Only a two-residue substitution chimera, TC10[C36–43], partially recovered ACK2-binding capacity (Fig. 4B, lane 3). The other two-residue substitution (TC10[K63E/Q64P]) and all of the single-residue substitutions, such as TC10[S57T], TC10[T59M], TC10[V60I], and TC10[K63E], did not recover any of the ACK2-binding capacity (Fig. 4B, lanes 5–7, 9, and 10). The data suggest that the binding to ACK2 is mediated by all of the eight residues in the AB region. No single residue dominates the binding.

The AB Region Localizes between Switch I and Switch II and Has a Unique Structure—The eight residues in the AB region that determine the binding specificity to ACK start from Asn-39 and end at Phe-56. Residues 39–56 sit between switch I and switch II. Mott et al. (26) have solved the structure of a complex between Cdc42 and the ACK CRIB domain by NMR. Part of the structure is shown in Fig. 5. From the amino acid sequence of the AB region (\textit{NYAVTMIGGEPYLGLF} \textit{\textsuperscript{108}}), we noticed that the eight residues, i.e. Asn-39, Thr-43, Met-45, Ile-46, Glu-49, Pro-50, Thr-52, and Phe-56 (in bold), were distributed symmetrically centered by Gly-47 and Gly-48. From the structure in Fig. 5A, the AB region resembles a U-shaped structure that contains two \(\beta\)-sheets (\(\beta2\) and \(\beta3\)). The Gly-47 and Gly-48 residues constitute the turn structure. The eight residues that involve binding to ACK sit in pairs on the two \(\beta\)-sheets of the U-shaped structure (Fig. 5B). The interactions between the four pairs of residues, Ile-46 with Glu-49, Met-45 with Pro-50, Thr-43 with Thr-52, and Asn-39 with Phe-56, may be necessary to sustain the U-shaped structure of the AB region.
The ACK2 interaction conferred by the AB region is essential for Cdc42 to activate ACK2 in cells. A, the AB region is required for Cdc42 to interact with ACK2 in cells. The HA-tagged ACK2 was co-transfected with vector, Myc-tagged wild type Cdc42, the GTPase-defective mutant Cdc42Q61L, or the GTPase- and AB region-defective mutant Cdc42[T50–82]Q61L into COS-7 cells for 48 h. The overexpressed ACK2 was immunoprecipitated (IP) with an anti-HA antibody. The co-immunoprecipitated Cdc42 and its mutants were detected by immunoblotting with an anti-Myc antibody. The expression level of Cdc42 and its mutants was detected by immunoblotting the cell lysates with an anti-Myc antibody (bottom panel).

B, the AB region is essential for Cdc42 to activate ACK2 in cells. The HA-tagged SH3PX1 was co-transfected with Myc-tagged ACK2 combined with Myc-tagged Cdc42, the GTPase-defective mutant Cdc42Q61L, or the GTPase- and the AB region-defective mutant Cdc42[T50–82]Q61L into COS-7 cells. After 36 h of transfection, the cells were starved in serum-free medium for 12 h. The tyrosine phosphorylation of SH3PX1 was detected by immunoblotting the lysates with an anti-phosphotyrosine antibody (4G10). The amount of HA-tagged SH3PX1, Myc-tagged ACK2, Myc-tagged Cdc42, or Myc-tagged Cdc42 mutants in the cell lysates was detected by immunoblotting with an anti-HA antibody or an anti-Myc antibody. The tyrosine phosphorylation of SH3PX1 was shown in the top panel.

From the structure of the complexes of Cdc42/PAK-CRIB, Cdc42/WASP-CRIB, and Cdc42/PAR-6 semi-CRIB/PDZ determined by either NMR or x-ray diffraction (29–31), we noticed that the β3-sheet in the AB region is the binding surface for interaction with all of the CRIB domains. However, the β3-sheet does not interact with any residue on the CRIB domain. Our data show that the residues on the β3-sheet also determine the binding to ACK, suggesting an important role for the β3-sheet in interaction with effectors.

Our data also suggest that the eight residues identified in the AB region are specific for the binding to ACK. Replacement of these residues with corresponding residues of TC10 did not change the binding affinity to WASP. The residue Leu-174 on the α5 helix of Cdc42 has been proposed as a key residue for binding to ACK and WASP. Our data suggest that Leu-174 alone is not sufficient to bind to ACK, because replacement of the amino acid terminus of Cdc42, which contains the residues 1–68, with the corresponding region of TC10 diminished the binding to ACK2 (Fig. 2B). In addition, the corresponding residue of TC10 to the residue Leu-174 is Ile-188. This change does not affect the binding to ACK, as the replacement of residues 1–82 of TC10 with the corresponding residues of Cdc42 gained the full binding to ACK2 (Fig. 2B).

The AB Region of Cdc42 Determines the Activation of ACK2 in Cells—To examine whether the AB region determines the interaction of Cdc42 with ACK2 in cells, we co-transfected HA-tagged ACK2 with Myc-tagged Cdc42 wild type, the GTPase-defective mutant Q61L, and the GTPase- and AB region-defective mutant Cdc42[T50–82]Q61L into COS-7 cells. We immunoprecipitated HA-ACK2 with an anti-HA antibody and detected the co-precipitated Cdc42 by immunoblotting with an anti-Myc antibody. As shown in Fig. 6A, although the GTPase-defective mutant Cdc42Q61L was co-immunoprecipitated with ACK2 (lane 5), the AB region-defective mutant Cdc42[T50–82]Q61L was not (lane 4), indicating that the AB region determines the binding of Cdc42 to ACK2 in cells.

To examine whether the AB region-conferred interaction has physiological function, we determined the effects of Cdc42Q61L and Cdc42[T50–82]Q61L on the activation of ACK2 in COS-7 cells. Our previous studies have shown that SH3PX1 (sorting nexin 9) is the phosphorylation substrate of ACK2 in cells (24). Therefore, in the activation assays, we used the tyrosine phosphorylation of SH3PX1 as a read-out. As shown in Fig. 6B, co-transfection of SH3PX1 with Cdc42Q61L and ACK2 yielded much stronger tyrosine phosphorylation of SH3PX1 (top panel, lane 6) than the controls (co-transfection with Cdc42 WT and ACK2 (top panel, lane 4)) or ACK2 alone (top panel, lane 3), indicating that ACK2 kinase activity is stimulated by Cdc42Q61L in the cells. However, co-transfection of SH3PX1 with Cdc42[T50–82]Q61L and ACK2 yielded much less tyrosine phosphorylation of SH3PX1 (Fig. 6B, top panel, lane 5) than the controls (top panel, lanes 3 and 4), suggesting that the AB region-defective mutant Cdc42[T50–82]Q61L has a dominant negative effect on the tyrosine kinase activity of ACK2. These data conclude that the interaction of the AB region of Cdc42 with ACK2 plays an essential role in ACK2 activation in cells, and the disruption of the function of the AB region results in dysfunction of ACK2-mediated signaling.

Conclusions—Cdc42 has multiple downstream effectors. Determination of the binding specificity to these effectors becomes a challenge in the dissection of Cdc42-mediated signal pathways. Here we show that the AB region, between amino acid residues 36 and 68 in Cdc42, confers the binding specificity to ACK. The eight residues in the AB region that sit in pairs on the β2 and β3-sheets may be necessary for the structure of the ACK-binding surface. No single residue in the AB region determines the binding to ACK. Our data have proposed a model that shows that the binding specificity of Cdc42 to its downstream effectors is conferred by a set of residues, not by a single residue.

Our studies have provided a molecular basis for the delineation of the ACK2-mediated signaling pathway. We have proven that overexpression of chimera Cdc42[T50–82] disrupts the biological function of ACK2 in cells. Next we will determine the biological effects of Cdc42[T50–82] on cytoskeletal organization, cell migration, cellular transformation, and epidermal growth factor receptor degradation. The outcome of the studies will help us to define the ACK-mediated intracellular signaling pathway.
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