The Cdc42 Binding and Scaffolding Activities of the Fission Yeast Adaptor Protein Scd2*

Makoto Endo‡§, Mikako Shirouzu‡§, and Shigeyuki Yokoyama‡§

Received for publication, September 21, 2002, and in revised form, October 24, 2002
Published, JBC Papers in Press, October 29, 2002, DOI 10.1074/jbc.M209714200

The small GTP-binding protein Cdc42, the guanine nucleotide exchange factor Scd1, the p21-activated kinase Shk1, and the adaptor protein Scd2 are involved in the Cdc42-dependent signaling cascade in fission yeast. In the present study, we analyzed the Cdc42 binding and scaffolding activities of Scd2 by co-precipitation assays. We found that two SH3-containing regions, amino acid residues 1–87 (CB1 (Cdc42-binding region 1)) and 110–266 (CB2), of Scd2 can bind to the GTP-bound form of Cdc42. CB2 is cryptic because of the intramolecular binding between the SH3 domain in CB2 (SH3(C)) and the PX domain and binds to Cdc42 only when the Scd2 PB1 domain binds to the PC motif-containing region (residues 760–872) of Scd1. This CB2-Cdc42 association, which would stabilize the open configuration of Scd2, enables the SH3(C) domain to bind to the polyproline motif of Shk1. We also found that the GTP-bound form of Cdc42 binds to the CRIB motif of Shk1 more strongly than to Scd2. Thus, Scd2 functions as a scaffold to form a protein complex, and the GTP-bound Cdc42 might be transferred effectively from the upstream activator Scd1 to the downstream effector Shk1 via Scd2.

The Cdc42/Rac protein is a member of the Rho family of small GTP-binding proteins (1–4). The Cdc42/Rac protein is involved in eukaryotic cellular signaling pathways regulating cell morphology, cell adhesion, actin dynamics, cell cycle progression, and kinase signaling (1–4), as a molecular switch cycling between the GDP-bound, inactive form and the GTP-bound, active form (4–8). Guanine nucleotide exchange factors for Cdc42/Rac, containing Dbl homology domains, promote GDP dissociation and GTP association. The GTP-bound form of Cdc42/Rac interacts with a variety of target proteins to initiate downstream responses (4–8). The Cdc42 protein of the fission yeast Schizosaccharomyces pombe is involved in controlling polarized cell growth (9–11). The disruption of the cdc42 gene is lethal to fission yeast, and the cdc42 mutants exhibit abnormal morphological phenotypes (9). Genetic and molecular studies suggest that a Dbl homology domain-containing Scd1 protein functions as the guanine nucleotide exchange factor for the fission yeast Cdc42 protein and that the GTP-bound Cdc42 activates Ste20 homologous kinase 1 (Shk1) (12–15).

The fission yeast Shk1 belongs to the family of the p21-activated kinase (PAK), which is a highly conserved serine/threonine kinase activated by the Cdc42/Rac protein. PAK consists of an amino-terminal regulatory region and a carboxyl-terminal kinase domain. Most of the PAKs contain a Cdc42 and Rac interactive binding (CRIB) motif in the regulatory region, which binds to the GTP-bound Cdc42/Rac protein (16, 17). Intramolecular binding of the CRIB motif of PAK to the kinase domain inhibits the enzyme activity (18–22). Recent studies indicate that the Cdc42/Rac protein relieves this autoinhibition, thereby allosterically inducing the activation of the kinase activity (18–22). The fission yeast PAK family kinase, Shk1, is positively regulated by the adaptor protein, Scd2 (shape and conjugation deficiency 2, also called Ral3), as well as by Cdc42 (12, 13). Co-expression of Scd2 enhances the binding of Shk1 to Cdc42 in a yeast two-hybrid system (12, 13). Furthermore, the overexpression of Scd2 stimulates the autophosphorylation activity of Shk1 in vivo, whereas the recombinant Scd2 by itself is not able to stimulate it in vitro (12). These results indicate that Scd2 participates in the activation of Shk1 but is not sufficient for it.

The Scd2 protein has two tandem SH3 domains (SH3(N) and SH3(C)), a PX (phox homologous, also known as PB2) domain, and a PB1 (phox and Bem1p 1) domain (Fig. 1A) (13, 23–27). Most SH3 domains are binding modules for Pro-Xaa-Xaa-Pro or polyproline motifs, and actually the SH3(C) domain of Scd2 binds to a polyproline motif in Shk1 (12). The PX domain has a dual role, serving as an SH3-binding polyproline-containing module as well as a phosphoinositide-binding module, in the cases of proteins other than Scd2 (23, 25, 28–31). The PB1 domain binds to the PC (phox and Cdc42) motif-containing region of Scd1 (Scd1-PC) (13, 24, 26, 27, 32). Because the budding yeast Saccharomyces cerevisiae homologue of Scd2, Bem1p, binds to Cdc42 in a GTP-dependent manner (33), Scd2 is expected to bind to the GTP-bound form of Cdc42. However, neither Bem1p nor Scd2 contains the CRIB motif (13, 33), and their Cdc42-binding regions are unknown.

In the present study on the fission yeast system, we analyzed the interactions of Scd2 with Scd1, Cdc42, and Shk1. We found that the four domains of Scd2 have important roles in the interactions with these proteins. The SH3(N)- and SH3(C)-containing regions (CB1 and CB2 (Cdc42-binding regions 1 and 2)) bind to the switch regions of the GTP-bound form of Cdc42.
The SH3(C) domain intramolecularly binds to the PX domain, which prevents CB2 from binding to Cdc42. CB2 is able to bind to the GTP-bound form of Cdc42 when the PB1 domain of Scd2 is associated with the PC motif-containing region of Scd1 (Scd1-PC). Furthermore, the binding of the polypyrrole-binding site of the SH3(C) domain to Shk1 is enhanced when Scd2 binds to Scd1-PC and the GTP-bound form of Cdc42. Thus, these four proteins can form a quaternary complex that includes two Cdc42-binding proteins. On the other hand, we also found that the CRIB motif of Shk1 binds preferentially to Cdc42, even in the presence of Scd2.

EXPERIMENTAL PROCEDURES

**Plasmid Construction**—The DNA fragments encoding Scd1 and Scd2 were amplified by PCR from the *scd1* and *scd2* genes, which were kindly provided by Dr. M. Yamamoto (University of Tokyo). The DNA fragments encoding Cdc42 and Shk1 were amplified by reverse transcription-PCR from mRNA extracted from fission yeast cells. We also used PCR to amplify the *BomHI*/EcoRI fragments encoding the Scd2 and Cdc42 proteins and the *BomHI*/EcoRI fragments encoding the Shk1 proteins. To obtain the Xhol/EcoRI fragment encoding Scd1(760–872), a *SalI* site was introduced after the stop codon. Point mutations of the Scd2 proteins were introduced into the DNA fragments using two subsequent PCRs. Point mutations of the Cdc42 proteins were introduced by site-directed mutagenesis using two subsequent PCRs. Point mutations of the Shk1 proteins were prepared by subcloning into pRS5 A (Invitrogen). The integrity of all of the PCR products was confirmed by sequencing.

**Protein Preparation**—GST fusion and His-tagged proteins were expressed in *Escherichia coli* at 30 °C and were purified using glutathione-Sepharose 4B (Amersham Biosciences) and Talon resins (Clontech), respectively, according to the manufacturer’s instructions. The His-tagged Scd1(1–658) protein was purified as described previously (12).

**RESULTS AND DISCUSSION**

**GTP Dependence of the Scd2(1–536) Binding to Cdc42**—Chang et al. (13) used a yeast two-hybrid system to show that Scd2 binds to the fission yeast Cdc42 protein, but it is not known whether this binding is GTP-dependent. The budding
yeast homologue of Scd2, Bem1p, was shown to bind to the budding yeast Cdc42 protein (Cdc42p) in a GTP-dependent manner (33). To determine the GTP dependence of the Scd2 binding to the fission yeast Cdc42 protein, we performed a co-precipitation assay using recombinant GST fusion and His6-tagged proteins. We incubated the GST fusion form of the full-length Scd2 (GST-Scd2 (1–536)) with the GDP-bound or GTPyS-bound His6-tagged form of the fission yeast Cdc42 protein (His6-Cdc42). The protein complexes bound to the glutathione-Sepharose 4B beads were subjected to SDS-PAGE. The co-precipitating His6-Cdc42 proteins were detected by Western blotting and development using the ECL immunodetection system (Amersham Biosciences), as described under "Experimental Procedures." D and T denote the GDP- and GTPyS-bound His6-Cdc42, respectively.

**Binding Activities of the Scd2 Fragments to Cdc42**—Bose et al. (33) showed that the deletion of the amino-terminal 140 amino acid residues of Bem1p decreases the binding to the GTP-bound form of Cdc42p. Furthermore, their data showed that the deletion of either the amino-terminal 234 or the carboxyl-terminal 35 amino acid residues of Bem1p impairs the binding to the GTP-form of Cdc42p (33). In Bem1p, the amino-terminal 234-residue region contains two SH3 domains (residues 79–127 and 160–212), and the carboxyl-terminal 35 amino acid residues are included in the PB1 domain (residues 472–551) (24, 33).

Considering these observations, we prepared seven fragments containing various regions of Scd2 as GST fusion proteins (Fig. 1, A and B). The amino-terminal half, Scd2(1–266), contains both of the two SH3 domains, whereas the carboxyl-terminal half, Scd2(267–536), contains the PX and PB1 domains. Scd2(1–109), Scd2(1–87), and Scd2(25–87) contain the SH3(N) domain. We expressed GST-Scd2(10–185), corresponding to the SH3(C) domain, but this protein was insoluble (data not shown). Therefore, we constructed two fragments, Scd2(10–536), which contains the SH3(C) domain, and Scd2(186–536), which lacks that domain. GST-Scd2(1–450), lacking the PB1 domain, was insoluble (data not shown). The expression level of each soluble fragment was as high as that of the full-length GST-Scd2(1–536) (data not shown).

To determine the Cdc42-binding region(s) of Scd2, we first investigated the Cdc42 binding activities of these fragments. We found that the GTPyS-bound His6-Cdc42 co-precipitated with GST-Scd2(1–266) (Fig. 2, lanes 3 and 4). Among the shorter mutants containing SH3(N) but not SH3(C), GST-Scd2(1–109) and GST-Scd2(1–87) bind to His6-Cdc42 in a GTPyS-dependent manner (lanes 5–8). In contrast, GST-Scd2(25–87), lacking the amino-terminal 24 amino acid residues, did not bind to His6-Cdc42 (lanes 9 and 10). On the other hand, GST-Scd2(110–536), which contains the SH3(C) domain, did not bind to His6-Cdc42 (lanes 11 and 12). However, we found that GST-Scd2(186–536), which lacks the SH3(C) domain, associated with His6-Cdc42 in both the GTPyS-bound and GDP-bound forms (lanes 13 and 14). Furthermore, GST-Scd2(267–536) did not bind to His6-Cdc42 (lanes 15 and 16).

In our results, the amino-terminal half of Scd2, Scd2(1–266), was important to bind to the GTP-bound form of Cdc42, but Scd2(267–536) was not. Furthermore, the regions located adjacent to the SH3 domains, Scd2(1–24) and Scd2(186–266), were necessary for the binding to Cdc42. These regions have no known binding motifs and do not have significant homology with each other. However, using homology search algorithms, we found that Scd2(1–24) and Scd2(181–228) have low sequence homology to the corresponding regions of Bem1p, within amino acid residues 52–78 and 213–260, respectively.
The co-precipitating Scd2(1–266) was tested in co-precipitation experiments with the wild-type and point mutants of GST-Scd2(1–266) (1 μg). B, His₆-Scd2(1–266) was tested in co-precipitation experiments with the wild-type and P364R mutant of GST-Scd2(257–536). The co-precipitating His₆-tagged proteins were detected by Western blotting with ECL development.

(data not shown). This agrees with the result that the deletion of the amino-terminal 234 amino acid residues of Bem1p impairs the binding to Cdc42p (33). However, our results are inconsistent with the report that the deletion of the carboxyl-terminal 35 amino acid residues of Bem1p impairs Cdc42p binding (33). Considering that Scd2(1–450), lacking the PB1 domain, was insoluble, a possible explanation for this observation could be that the PB1 domain might be important for the conformational stability of the full-length Scd2.

Binding Activity of the Scd2(1–87)/W62R Mutant Fragment to Cdc42—As described above, the GTP₆S-bound Hisᵦ-Cdc42 co-precipitated with either GST-Scd2(1–109) or GST-Scd2(1–87). However, GST-Scd2(25–87), which lacks the amino-terminal 24 amino acid residues, did not bind to Hisᵦ-Cdc42. To determine whether Scd2(1–24) is sufficient for the binding to Hisᵦ-Cdc42, we tried to express GST-Scd2(1–24). However, GST-Scd2(1–24) was insoluble (data not shown). Thus, Scd2(1–87) was the shortest soluble Cdc42-binding fragment tested.

The three-dimensional structures of many SH3 domains have been determined using NMR and x-ray crystallography, revealing that the well conserved tryptophan residues in the hydrophobic ligand-binding sites of the SH3 domains directly contact the ligands (35–38). Therefore, we replaced the conserved Trp₁₆₀ of the SH3(C) domain with an arginine residue (Fig. 1A). As a result, the GTP₆S-bound Hisᵦ-Cdc42 did not co-precipitate with GST-Scd2(1–87)/W62R (Fig. 3A). This indicates that Scd2(1–87), including the amino-terminal 24 amino acid residues and the SH3(N) domain, composes a functional region that binds to the GTP-bound form of Cdc42. Thus, we designated this region, Scd2(1–87), as CB1 (Fig. 1A).

Binding Activities of the Scd2(110–536/W160R) and Scd2(110–536/P364R) Mutant Fragments to Cdc42—As described above, GST-Scd2(110–536) did not bind to the GTP₆S-bound Hisᵦ-Cdc42, whereas GST-Scd2(186–536) was able to bind to the GDP- and GTP₆S-bound Hisᵦ-Cdc42. Therefore, Scd2 might have a second Cdc42-binding site. To determine whether the region of Scd2 containing amino acid residues 186–266 binds to Cdc42, we used the Scd2(1–266/W62R) mutant. We found that the W62R mutation decreased the amount of Hisᵦ-Cdc42 that co-precipitated with GST-Scd2(1–266) but that the GTP₆S-dependent binding was still detectable (Fig. 3B). In contrast, the same mutation, W62R, completely impaired the Cdc42 binding of GST-Scd2(1–87) depending only on CB1 (Fig. 3A). Consequently, Scd2(1–266/W62R) retains the ability to bind to the GTP₆S-bound Cdc42, probably within amino acid residues 88–266.

In this context, it was reported that a subunit of the human NADPH oxidase, the p47(phox) protein, exhibits an intramolecular binding between the PX and SH3 domains (23). The p47(phox) protein is an adaptor protein containing one PX domain and two SH3 domains. The PX domain of the p47(phox) protein intramolecularly binds to the second SH3 domain. We investigated whether the intramolecular binding between the SH3(C) and PX domains of Scd2 inhibits the second Cdc42-binding site. Hisᵦ-tagged forms of Scd2(1–266) and Scd2(267–536) were prepared (Fig. 1C) and were tested for their bindings to the GST fusions of Scd2(267–536) and Scd2(1–266), respectively. We found that Scd2(1–266) and Scd2(267–536) bind to each other (Fig. 4, A, lane 1, and B, lane 1) and, furthermore, that the substitution of an Arg residue for either the conserved Trp₁₆₀ residue of the SH3(C) domain or the conserved Pro₃₆⁴ residue of the polyproline motif in the PX domain impairs this binding (Fig. 4, A, lanes 2 and 3, and B, lane 2). These results clearly demonstrate that Scd2 has the intramolecular association between the ligand-binding site of the SH3(C) domain and the polyproline motif of the PX domain.

Based on these results, we next investigated how the mutations that disrupt the intramolecular binding affect the inter-
molecular binding of Scd2 to Cdc42. The W160R mutation did not affect the Cdc42 binding activity of Scd2(1–266) (Fig. 3B). This means that the ligand-binding site, or at least the Trp 160 residue, in the SH3(C) domain is not directly involved in the binding to the GTP\textsuperscript{S}-bound His\textsubscript{6}-Cdc42. On the other hand, the W160R mutation enabled Scd2(110–536) to bind to the GTP\textsuperscript{S}-bound His\textsubscript{6}-Cdc42 (Fig. 3C). We also found that the P364R mutation within the PX domain has the same effect on the binding activity of Scd2 to Cdc42 (Fig. 3C). These results suggest that the intramolecular binding between the SH3(C) and PX domains prevents Scd2(110–536) from binding to Cdc42. It should be noted here that GST-Scd2(110–536/W160R) and GST-Scd2(110–536/P364R) bound to the GTP\textsuperscript{S}-bound form, but not the GDP-bound form, of His\textsubscript{6}-Cdc42 (Fig. 3C). These results indicate that amino acid residues 110–266, including the SH3(C) domain, compose a functional region that binds to the GTP-bound form of Cdc42, independently of CB1. Thus, we designated the region containing amino acid residues 110–266 as CB2 (Fig. 1A). The GTP-bound form of Cdc42 binds to regions containing CRIB motifs in many Cdc42 targets (2, 16). On the other hand, no CRIB motif exists in several Cdc42 targets, such as phosphatidylinositol 3-kinase (39, 40), phospholipase D (41), phospholipase C\textsuperscript{β2} (42), Cdc42-interacting protein 4 (43), IQGAP (3, 44), and the \( \gamma \)-subunit of the coatomer complex (45). In the present study, we identified two Cdc42-binding regions, each containing an SH3 domain but lacking a CRIB motif. Among other Cdc42 target proteins lacking the CRIB motif, however,
the p85 subunit of phosphatidylinositol 3-kinase and Cdc42-interacting protein 4 both contain one SH3 domain (39, 40, 43). In the case of the p85 subunit, the Rho-GAP homology domain has been reported to be responsible for the binding to the GTP-bound Cdc42 (39). On the other hand, it is unclear whether the Cdc42-interacting protein 4 SH3 domain binds to Cdc42-GTP.

**Scd2(110–536) Binding to Cdc42 Is Dependent on Scd1(760–872)**—It was reported that Scd2 binds to a carboxyl-terminal fragment of Scd1, Scd1(710–872) (13). However, we found that GST-Scd1(710–872) was unstable and sensitive to protease (data not shown). This instability might be caused by an inappropriate position for the deletion. In fact, the secondary structure prediction algorithm suggests that the amino-terminal region of Scd1(710–872) forms a long loop structure. On the other hand, Scd1 contains the PC motif within amino acid residues 782–809 that binds to the PB1 domain (24, 26, 32). Therefore, we tried to express a shorter fragment, Scd1(760–872). The expression level of GST-Scd1(760–872) was much more stable than GST-Scd1(710–872) (data not shown). We used this Scd1(760–872) fragment (Scd1-PC) in experiments to investigate how the PB1-PC motif association affects the Cdc42 binding activity of Scd2.

We incubated GST-Scd2(1–536) with both His$_{8}$-Cdc42 and Scd1(760–872). Scd1(760–872) was obtained by thrombin cleavage from GST-Scd1(760–872). The amount of the His$_{8}$-Cdc42-GTPyS complex that co-precipitated with GST-Scd2(1–536) increased in the presence of Scd1(760–872) (Fig. 5A). This means that Scd1(760–872) has a positive role in the Cdc42 binding activity of Scd2, which has both CB2 but not CB1. The GTPyS-bound His$_{8}$-Cdc42 co-precipitated with GST-Scd2(110–536) in the presence of Scd1(760–872), and the effect of Scd1(760–872) was much more significant for GST-Scd2(110–536) than for GST-Scd2(1–536) (Fig. 5B). This result clearly shows that, in the presence of Scd1(760–872), the PB1-PC motif association enables CB2 to bind to the GTP-bound form of Cdc42.

**Cdc42 Mutants**—To examine which regions of the fission yeast Cdc42 protein bind to CB1 and CB2 of Scd2, we introduced mutations into the His$_{8}$-Cdc42 protein. Considering that the SH3(N) domain in CB1 is essential for the binding to Cdc42, we substituted Ala for the Pro residues that were supposed to be on the protein surface on the basis of the solution structures of the GTP-bound form of the human Cdc42 protein (46–48). The Pro$_{26}$ residue is in the α1 helix (Fig. 6A). The
tected and quantified with the LAS-1000plus image analyzer. The regulatory region of the Shk1 protein consists of several polyproline motifs and the CRIB motif (Fig. 6).

Among the mutated residues, only the Asp38 residue was expected to be on the interface with the CRIB motif on the basis of the complex structures of the CRIB motifs and the human Cdc42 protein (46, 47, 49, 50). In fact, only the D38A mutation impaired the binding of Cdc42 to Shk1(141–216) (Fig. 6D). GST-Shk1(141–216) bound to the other mutants as much as the wild-type His$_6$-Cdc42 (Fig. 6D), and these bindings were GTP$_S$-dependent (data not shown). Our results are consistent with previous reports that the D38A mutation drastically impairs the binding of human and budding yeast Cdc42 proteins to CRIB motifs (49, 51). Thus, the six novel Cdc42 mutants (Y32A, P34A, P99A/P106A, P123A, and P179A) retain GTP-dependent Shk1-CRIB binding activity.

CB1 and CB2 Binding Activities of the Cdc42 Mutants—To examine the CB1 binding activities of the Cdc42 mutants, we incubated GST-Scd1(760–872) (1 µg) with 10 pmol of His$_6$-Scd2(1–536), the GTP$_S$-bound His$_6$-Cdc42, His$_6$-Shk1(141–216), and His$_6$-Shk1(1–536). The co-precipitating His$_6$-tagged proteins were detected with the LAS-1000plus image analyzer. Lanes 1–4 are approximately one-fifth of the input His$_6$-tagged proteins. The arrowheads denote the locations of the His$_6$-tagged proteins.

Pro$_{34}^*$ residue is in switch I, and the Pro$_{69}^*$ and Pro$_{73}^*$ residues are in switch II. In addition to these common switch regions, the region containing the α3 helix and the adjacent β4 strand also exhibits significant changes between the solution structures of the GDP- and GTP-bound forms of the human Cdc42 protein and is therefore called “switch III” (48). Thus, the Pro$_{34}^*$ and Pro$_{106}^*$ residues in the α3 helix were mutated. The Pro$_{123}^*$ residue is in the α helix, which is the insert helix specific to the Rho family of small GTP-binding proteins (48). The Pro$_{179}^*$ residue is in the carboxyl-terminal variable loop region. In addition to these proline residues, we replaced the Tyr$_{32}^*$ and Asp$_{99}^*$ residues in switch I with Ala residues, because many effector proteins bind to the switch I regions of small GTP-binding proteins (4, 49). The P26A and P73A mutants of the His$_6$-Cdc42 proteins were insoluble (data not shown). The expression levels of the P99A and P106A mutants were relatively low (<0.1 mg from 250 ml of cell culture), whereas those of the other mutants were as high as that of the wild-type His$_6$-Cdc42 (0.4–0.5 mg from 250 ml cell culture) (data not shown). We also constructed the P99A/P106A mutant of His$_6$-Cdc42, and unexpectedly, the expression level of the P99A/P106A mutant was as high as that of the wild-type His$_6$-Cdc42 (data not shown). Therefore, we used the P99A/P106A mutant, instead of the P99A and P106A mutants. Thus, these seven mutants of the His$_6$-Cdc42 proteins were prepared for the experiments to examine the binding activities of the Cdc42 mutants to Scd2 as well as Shk1 (Fig. 6B).

Shk1 CRIB Binding Activities of the Cdc42 Mutants—The regulatory region of the Shk1 protein consists of several polyproline motifs and the CRIB motif (Fig. 6C). We first examined the activities of the Cdc42 mutants to bind to the CRIB motif-containing fragment of Shk1, Shk1(141–216) or Shk1-CRIB. Among the mutated residues, only the Asp$_{99}^*$ residue was expected to be on the interface with the CRIB motif on the basis of the complex structures of the CRIB motifs and the human Cdc42 protein (46, 47, 49, 50). In fact, only the D38A mutation impaired the binding of Cdc42 to Shk1(141–216) (Fig. 6D). GST-Shk1(141–216) bound to the other mutants as much as the wild-type His$_6$-Cdc42 (Fig. 6D), and these bindings were GTP$_S$-dependent (data not shown). Our results are consistent with previous reports that the D38A mutation drastically impairs the binding of human and budding yeast Cdc42 proteins to CRIB motifs (49, 51). Thus, the six novel Cdc42 mutants (Y32A, P34A, P99A/P106A, P123A, and P179A) retain GTP-dependent Shk1-CRIB binding activity.

CB1 and CB2 Binding Activities of the Cdc42 Mutants—To examine the CB1 binding activities of the Cdc42 mutants, we incubated GST-Scd1(760–872) (1 µg) with 10 pmol of His$_6$-Scd2(1–536), the GTP$_S$-bound His$_6$-Cdc42, His$_6$-Shk1(141–216), and His$_6$-Shk1(1–536). The co-precipitating His$_6$-tagged proteins were detected with the LAS-1000plus image analyzer. Lanes 1–4 are approximately one-fifth of the input His$_6$-tagged proteins. The arrowheads denote the locations of the His$_6$-tagged proteins.
The Cdc42 binding competition between the quaternary complex and Shk1-CRIB. A, we incubated GST-Scd1(760–872) (1 μg) with 20 pmol of His6-Scd2(1–536), the GTPγS-bound His6-Cdc42, and His6-Shk1(1–658). After the incubation, the resin was washed once with PBS containing 5 mM MgCl2 to remove the His6-tagged proteins that did not bind to GST-Scd1(760–872), and then His6-Shk1(141–216) (20 pmol) was added. The resin was washed twice with PBS containing 5 mM MgCl2. The co-precipitating His6-tagged proteins were detected (20 pmol) was added. The resin was washed twice with PBS containing 5 mM MgCl2. The co-precipitating His6-tagged proteins were detected and quantified with the LAS-1000plus image analyzer. The arrowheads denote the locations of the His6-tagged proteins. Graphical representations of the relative intensities of the co-precipitating His6-Cdc42 are shown in B. Intensities relative to that obtained after the incubation of His6-Cdc42 with GST-Scd1(760–872) and His6-Shk1(1–536) are shown. The data represent the averages of at least three independent experiments.

These results show that switch I of the GTP-bound form of Cdc42 is involved in the CB2 binding as well as in the Shk1-CRIB binding and are consistent with the report that mutations in switch I impair the binding of the budding yeast Cdc42 protein to Bem1p (51). On the other hand, our results indicate that switch III, containing the Pro99 and Pro106 residues, is also involved in the GTP-dependent binding of Cdc42 to CB2. Therefore, the binding mechanism of Cdc42 to CB2 differs from that to the CRIB motif of Shk1. Furthermore, the two CB2-binding regions, CB1 and CB2, of Scd2 recognize different switch regions of Cdc42.

Cdc42 Binding Competition between Scd2 and Shk1-CRIB—In a yeast two-hybrid system, the co-expression of Scd2 increases the Cdc42 binding of Shk1 (12). On the other hand, our experiments using the Cdc42 mutants indicate that the Scd2- and Shk1-CRIB-binding regions of Cdc42 overlap. Therefore, we performed a Cdc42-binding competition between Scd2 and Shk1-CRIB. We incubated GST-Scd2(1–536) with the GTPγS-bound His6-Cdc42 (CB1 binding), followed by the addition of various amounts of His6-Scd2(141–216). As the His6-Scd2(141–216) concentration increased, the amount of His6-Cdc42 that co-precipitated with GST-Scd2(1–536) decreased (Fig. 7A). Similar results were obtained in the presence of Scd1(760–872) (CB1 and CB2 binding). To examine the Cdc42 binding competition of the respective Cdc42-binding regions of Scd2 with Shk1-CRIB, we incubated GST-Scd2(1–87) (CB1) or the GST-Scd2(110–536)Scd1(760–872) complex (including CB2) with the GTPγS-bound His6-Cdc42, followed by the addition of His6-Shk1(141–216). The amount of His6-Cdc42 that co-precipitated with each of the Scd2 fragments decreased with the addition of His6-Shk1(141–216), as in the case of the full-length Scd2 (Fig. 7B and C). Especially, the decrease of the Cdc42 binding of CB2 was significant.

We next incubated GST-Scd1(141–216) with the GTPγS-bound His6-Cdc42, followed by the addition of His6-Scd2(1–536). Unlike the case of the Scd2 fragments, the amount of His6-Cdc42 that co-precipitated with GST-Scd1(141–216) did not decrease with the addition of His6-Scd2(1–536) (Fig. 7D). Similar results were also obtained in the presence of Scd1(760–872). These results suggest that Scd2 and Shk1-CRIB are not able to bind simultaneously to Cdc42 but rather that the GTP-bound form of Cdc42 binds to Shk1-CRIB more strongly than to Scd2.

Enhancement of the Scd2/Shk1 Association by Scd1(760–872) and Cdc42—The SH3(C) domain of Scd2 binds to the amino-terminal polyproline-containing region of Shk1 (12). Our results indicate that the SH3(C) domain also intramolecularly binds to the PX domain. We next examined the binding of Scd2 to Shk1 in more detail. We incubated GST-Scd2(1–536) with the His6-Scd2(1–536) (Fig. 7A) or the full-length Scd2 (His6-Scd2(1–658)). His6-Scd2(1–658) co-precipitated with GST-Scd2(1–536) (Fig. 8, lane 1). Scd1(760–872) only slightly increased the binding of Shk1(1–658) to Scd2(1–536), whereas the GTPγS-bound His6-Cdc42 caused a much more significant increase (lanes 2 and 3). The amount of His6-Shk1(1–658) that co-precipitated with GST-Scd2(1–536) was much larger in the presence of both Scd1(760–872) and the GTPγS-bound His6-Cdc42 (lane 4). On the other hand, His6-Shk1(1–658) did not co-precipitate with GST-Scd2(1–536/W160R), which has a mutation in the polyproline-binding site of the SH3(C) domain, in the absence and presence of Scd1(760–872) and/or the GTPγS-bound His6-Cdc42 (lanes 5–8). These results indicate that Scd1-PC and the GTP-bound form of Cdc42 enhance the Scd2/Shk1 association and that the polyproline-binding site of the SH3(C) domain is essential for it. Considering that the SH3(C) domain is also involved in the intramolecular binding, the C2B-Cdc42 association might stabilize the open configuration of Scd2, which is required for the binding of the SH3(C) domain to the polyproline motif of Shk1.

Quaternary Complex Formation by Scd1, Scd2, Cdc42, and Shk1—As shown by the experiments described above, the Shk1-CRIB fragment decreased the Cdc42 binding of Scd2, whereas Scd1-PC and Cdc42 increased the binding of Scd2 to the SH3(C) domain to the polyproline motif of Shk1.

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
the full-length Shk1. Therefore, we examined the complex formation of Scd1, Scd2, Cdc42, and Shk1. First of all, we incubated GST-Scd1(760–872) separately with each of His6-Scd2(1–536), the GTPγS-bound His6-Cdc42, His6-Shk1(141–216) (Shk1-CRIB), and His6-Shk1(1–658) (the full-length Shk1). As a result, only His6-Scd2(1–536) co-precipitated with GST-Scd1(760–872) (Fig. 9, lanes 5–8). His6-Cdc42 co-precipitated with GST-Scd1(760–872) in the presence of His6-Shk1(1–536) (lane 9). This suggests that Scd2 links Scd1-PC and the GTP-bound form of Cdc42 and that they form a ternary complex. We next incubated His6-Scd1(141–216), containing only the CRIB motif, with this ternary complex. His6-Scd1(141–216) co-precipitated with GST-Scd1(760–872) (Fig. 9, lanes 5–8). His6-Cdc42 co-precipitated with GST-Scd1(760–872) in the presence of His6-Shk1(1–536) (lane 9). This suggests that the full-length Shk1 with the scaffolding protein Scd2 promotes the dissociation of the CRIB motif from the kinase domain, possibly by keeping Cdc42-GTP in the proximity of the CRIB motif and/or changing the conformation of Shk1.

Acknowledgments—We are grateful to M. Yamamoto for providing the scd1(A61) and scd2(A63) genes and to the members of our laboratory for comments and helpful discussions on this work.

REFERENCES

1. Kaibuchi, K., Kuroda, S., and Amano, M. (1999) Annu. Rev. Biochem. 68, 459–486
2. Bishop, A. L., and Hall, A. (2000) Biochem. J. 348, 241–253
3. Forsburg, S. L., and Chernoff, J. (2001)Curr. Opin. Cell Biol. 13, 153–157
4. Takai, Y., Sasaki, T., and Matozaki, T. (2001) Physiol. Rev. 81, 153–208
5. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) Nature 348, 125–132
6. Boguski, M. S., and McCormick, P. (1993) Nature 366, 643–654
7. Polakis, P., and McCormick, F. (1993) J. Biol. Chem. 268, 9157–9160
8. Sprang, S. R. (1997) Annu. Rev. Biochem. 66, 639–678
9. Miller, F. J., and Johnson, D. J. (1994) Mol. Cell. Biol. 14, 1075–1083
10. Ishikawa, T., Tanaka, K., and Yamada, M. (1994) Tanpakushitsu Koho 39, 429–438
11. Hughes, D. A. (1995) Semin. Cell Biol. 6, 89–94
12. Chang, E. B., Bartholomew, G., Pimental, R., Chen, J., Lai, H., Wang, L., Yang, P., and Marcus, S. (1999) Mol. Cell. Biol. 19, 8066–8074
13. Chang, E. B., Barr, M., Wang, Y., Jung, V., Xu, H. P., and Wigler, M. H. (1994) Mol Cell Biol. 14, 131–141
14. Marcus, S., Polverino, A., Chang, E., Robbins, D., Cobb, M. H., and Wigler, M. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6180–6184
15. Ottolino, S., Miller, P. J., Johnson, D. I., Creasy, C. L., Sells, M. A., Bagrodia, S., Forsburg, S. L., and Chernoff, J. (1995) EMBO J. 14, 5908–5919
16. Burrello, P. D., Drechsel, D., and Hall, A. (1995) J. Biol. Chem. 270, 29071–29074
17. Pirone, D. M., Carter, D. E., and Burrello, P. D. (2001) Trends Genet 17, 370–373
18. Zenev, F. T., King, C. C., Bohl, B. P., and Bokoch, G. M. (1999) J. Biol. Chem. 274, 32565–32573
19. Tu, H., and Wigler, M. (1999) Mol. Cell. Biol. 19, 6011–6015
20. Leb, M., Wu, X., Meng, W., Parrini, M. C., Eck, M. J., Mayer, B. J., and Harrison, S. C. (2000) Curr. Op. Cell Biol. 12, 387–397
21. Chong, C. T., Lim, L. M., and Manser, E. (2001) J. Biol. Chem. 276, 17347–17353
22. Ronald, G., Hostinova, E., Rudolph, M. G., Kraemer, A., Sickness, M. E., Hefferek, K., and Wittinghofer, A. (2001) Mol Cell Biol. 21, 5179–5188
23. Hiroaki, H., Ato, I., Tito, S., Sumimoto, H., and Kohda, D. (2001) Nat. Struct. Biol. 8, 526–530
24. Ito, T., Matsui, Y., Ato, T., Oka, K., and Sumimoto, H. (2001) EMBO J. 20, 3938–3946
25. Ponting, C. P. (1996) Protein Sci. 5, 2353–2357
26. Terasawa, N., Noda, Y., Ito, T., Hatanaka, H., Ichikawa, S., Ogura, K., Sumimoto, H., and Inagaki, F. (2001) EMBO J. 20, 3947–3956
27. Ponting, C. P., Ito, T., Masuda, J., Diaz-Meza, M. T., Inagaki, F., and Sumimoto, H. (2002) Trends Biochem Sci. 27, 10
28. Bravo, J., Karathanassis, D., Paolozzi, C. M., Paolozzi, M. E., Ellison, C. D., Anderson, K. E., Butler, R., Lavenir, J., Perus, O., Hawkins, P. T., Stephens, L., and Williams, R. L. (2001) Mol. Cell 8, 829–839
29. Song, X., Xu, W., Zhang, A., Huang, G., Liang, X., Verbass, Y. J., Czech, M. P., and Zhou, G. W. (2001) Biochemistry 40, 8014–8024
30. Wishart, M. J., Taylor, G. S., and Dixon, J. R. (2001) Cell 105, 817–820
31. Yu, J. W., and Lemenon, M. A. (2001) J. Biol. Chem. 276, 41479–41484
32. Nakamura, R., Sumimoto, H., Mizuki, K., Hata, K., Ato, T., Kitajima, S., Takekawa, K., Sakaki, Y., and Ito, T. (1999) Eur. J. Biochem. 251, 583–599
33. Bose, I., Irazoqui, J. E., Moskow, J. J., Bardes, E. S., Zyla, T. R., and Lew, D. J. (2001) J. Biol. Chem. 276, 7176–7186
34. Shirouzu, M., Motonaka, K., Koyama, H., C. D., Hirota-Tamura, N., Okada, T., Kiyori, K., Kataoka, T., Kikuchi, A., and Yskoyama, S. (1998) J. Biol. Chem. 273, 7737–7742
35. Fend, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994) Science 266, 1241–1247
36. Yu, H., Rosen, M. K., Shin, T. B., Seidel-Dugan, C., Brugge, J. S., and Schreiber, S. L. (1992) Science 258, 1665–1668
37. Ben, R., Mayer, B. J., Cipetic, P., and Baltimore, D. (1993) Science 259, 1157–1161
38. Germa, S., and Pavletich, N. P. (1996) Science 274, 1001–1005
39. Zheng, Y., Bagrodia, S., and Cerione, R. A. (1994) J. Biol. Chem. 269, 18727–18730
40. Tobias, R. F., Cantley, L. C., and Carpenter, C. L. (1995) J. Biol. Chem. 270, 17656–17659
41. Han, S. J., Kim, H. C., Chung, J. K., Kang, H. S., Donaldson, J., and Koh, J. K. (1998) Biochem. Mol. Biol Int. 45, 1089–1103
42. Danenberg, D., Schwab, F., Pines, J., Kirschen, D., Rinder, W. Maier, G., Dietrich, A., and Gierschik, P. (1998) EMBO J. 17, 6241–6249
43. Asprenstrom, P. (1997) Carf. Biol. 7, 479–487
44. Kuroda, S., Fukata, M., Nakagawa, M., and Kayakami, K. (1999) Biochem. Biophys. Res. Commun. 265, 1–6
45. Wu, W. J., Erickson, J. W., Lin, R., and Cerione, R. A. (2000) Nature 405, 800–804
46. Abdul-Manan, N., Aghazadeh, B., Liu, G. A., Majumdar, A., Ouerfelli, O., Siminovitch, K. A., and Rosen, M. K. (1999) *Nature* **399**, 379–383
47. Mott, H. R., Owen, D., Nietlispach, D., Lowe, P. N., Manser, E., Lim, L., and Laue, E. D. (1999) *Nature* **399**, 384–388
48. Falham, J. L., Dotsch, V., Raza, S., Manor, D., Cerione, R. A., Sutcliffe, M. J., Wagner, G., and Oswald, R. E. (1997) *Biochemistry* **36**, 8755–8766
49. Owen, D., Mott, H. R., Laue, E. D., and Lowe, P. N. (2000) *Biochemistry* **39**, 1243–1250
50. Morreale, A., Venkatesan, M., Mott, H. R., Owen, D., Nietlispach, D., Lowe, P. N., and Laue, E. D. (2000) *Nat. Struct. Biol.* **7**, 384–388
51. Gladfelter, A. S., Moskow, J. J., Zyla, T. R., and Lew, D. J. (2001) *Mol. Biol. Cell* **12**, 1239–1255
52. Wang, B., Zou, J. X., Ek-Rylander, B., and Russlahti, E. (2000) *J. Biol. Chem.* **275**, 5222–5227
The Cdc42 Binding and Scaffolding Activities of the Fission Yeast Adaptor Protein Scd2
Makoto Endo, Mikako Shirouzu and Shigeyuki Yokoyama

J. Biol. Chem. 2003, 278:843-852.
doi: 10.1074/jbc.M209714200 originally published online October 29, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209714200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 24 of which can be accessed free at
http://www.jbc.org/content/278/2/843.full.html#ref-list-1