A Novel Cdc42-interacting Domain of the Yeast Polarity Establishment Protein Bem1

IMPLICATIONS FOR MODULATION OF MATING PHEROMONE SIGNALING*

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In Saccharomyces cerevisiae, the Rho-type small GTPase Cdc42 is activated by its guanine-nucleotide exchange factor Cdc24 to polarize the cell for budding and mating. A multidomain protein Bem1 interacts not only with Cdc42 but also with Cdc24 and the effectors of Cdc42, including the p21-activated kinase Ste20, to function as a scaffold for cell polarity establishment. Although Bem1 interacts with Cdc24 and Ste20 via its PB1 and the second SH3 domains (SH3b), respectively, it is unclear how Bem1 binds Cdc42. Here we show that a region comprising the SH3b and its C-terminal flanking segment termed CI (SH3b-CI) directly interacts with Cdc42. A dual-bait reverse two-hybrid approach revealed that the CI is critical to the interaction: N253D substitution in the CI abolishes the binding of the SH3b-CI to Cdc42 but not to the proline-rich region of Ste20, whereas W192K substitution in the SH3b has the opposite effect. Nevertheless, the SH3b-CI interacts with Ste20 proline-rich region and Cdc42 in a mutually exclusive manner. The N253D substitution renders cellular growth temperature-sensitive and suppresses mating. The W192K-induced mating defect is exacerbated by the N253D substitution and suppressed by increasing the dosage of Ste20 provided that the CI is intact. Intriguingly, Cdc42 can mediate an indirect interaction of the SH3b-CI to the CRIB domain of Ste20. These results suggest that the SH3b and the CI collaborate in tethering of Ste20 to Bem1 to ensure efficient mating pheromone signaling.

It becomes increasingly evident that signaling proteins assemble into a multiprotein complex to function in various aspects of cellular regulation (1). Accordingly, a novel class of proteins composed of multiple protein-binding domains plays integral roles in signaling by serving as scaffolds for the assembly (2). For instance, the budding yeast Ste5 tethers the members of the MAPkinase cascade, namely the MAP kinase kinase Ste11, the MAP kinase kinase Ste7, and the MAP kinases Fus1 and Kss1, as well as the βγ dimer (Ste18–Ste4) of the trimeric G-protein, thereby ensuring the efficiency and specificity of mating pheromone signal transduction (3). Similarly, several potential scaffold proteins are reported for mammalian MAP kinase cascades (4).

A scaffold is also used in the signaling for cell polarity establishment (5, 6). The yeast cells polarize at budding in vegetative growth and at shmoo formation in mating. These processes are controlled by the Rho-type small GTPase Cdc42. The Cdc42 is activated by its guanine nucleotide exchange factor Cdc24. The activated, GTP-bound form of Cdc42 interacts with its various effectors, including the p21-activated kinases Ste20 and Cla4, to transduce signals for cytoskeletal reorganization.

Bem1 is a multidomain protein that lacks any catalytic domain but binds both the activator Cdc24 and the effectors Ste20 and Cla4 (7–11), likely functioning as a scaffold for Cdc42 signaling in cell polarity establishment. Bem1 is also assumed to constitute a part of positive feedback loop to stabilize polarized localization of activated Cdc42 (12–14). We previously identified a novel modular domain termed PB1 (Phox and Bem 1) at the C terminus of Bem1 and demonstrated that it mediates a homotypic interaction with another PB1 domain occurring at the C terminus of Cdc24 (9). Functional importance of this PB1-PB1 interaction was evidenced by the bem1 and cdc24 mutants defective in the interaction: they displayed temperature-sensitive growth and a mating defect (9). It was also shown that Bem1 with a defective PB1 domain fails to maintain Cdc24 at the site of polarized growth (15). In addition to PB1-mediated association with Cdc24, we and others showed that Bem1 uses its second SH3 domain to bind the p21-activated kinases Cla4 and Ste20 (7, 9, 10, 15). The Cla4 recruited to the bud tip phosphorylates Cdc42 to induce its dissociation from Bem1, thereby serving as a negative feedback loop (8). The importance of Ste20-binding in mating was demonstrated using mutants defective in the interaction (16).

Besides the activator and effectors of Cdc42, the GTP-bound form of Cdc42 per se was repeatedly reported to bind Bem1. Many authors observed a two-hybrid interaction between Cdc42 and Bem1 (10, 12, 15, 17). An in vitro interaction was also shown between purified Cdc42 and Bem1 (7, 18). Although maltose-binding protein; GTPγS, guanosine 5′-3′-(thio)triphosphate; PRR, proline-rich region; CI, Cdc 42-interacting; NLS, nuclear localization signal; Ste20-N, N-terminal portion of Ste20.
Bem1-Cdc42 Interaction

Ste20 and Cla4 as well as other Cdc42 effectors such as Gic1/2 share the CRIB (Cdc42/Rac interactive binding) domain for binding to the GTP-bound form of Cdc42, Bem1 has no CRIB domain and its Cdc42-binding domain has remained to be identified. Similarly, the biological role of the interaction is totally elusive.

To answer these questions, we mapped a Cdc42-binding domain of Bem1 to reveal that a region comprising the second SH3 domain (SH3b) and its C-terminal flanking region, the latter of which was termed CI (Cdc42-interacting) region, is responsible for the interaction. We isolated a separation-of-function mutant of the SH3b-CI that is specifically defective in Cdc42 binding. The mutant not only highlighted the importance of CI in Cdc42 binding but also allowed us to examine a biological role of the Bem1-Cdc42 interaction. The results indicate that the CI likely cooperates with the SH3b to recruit Ste20 to Bem1, thereby ensuring efficient mating pheromone signaling.

EXPERIMENTAL PROCEDURES

Yeast Strains and General Yeast Methods—The yeast strains used in this study were summarized in Table 1. We used standard media and methods for the growth and manipulation of yeast cells (19). For transformation, the protocol of Gietz and Schiestl (20) was adopted except for the addition of 10% Me2SO prior to the heat shock step. For gene disruption, the manufacturer’s instructions. The affinity-purified Cdc42 was loaded with GTPγS prior to the binding assays. For an in vitro pull-down assay, GST fusion proteins were incubated with other proteins in Tris-buffered saline containing 5 mM MgCl2, 0.5 mg/ml bovine serum albumin, and 0.1% Triton X-100. Following the incubation at 4 °C for 30 min, the proteins were precipitated with glutathione-Sepharose 4B beads. The beads were washed and subjected to SDS-PAGE. The precipitated proteins were detected by an immunoblotting procedure using an anti-GST, T7, or MBP monoclonal antibody and the ECL Western blotting detection system (Amersham Biosciences) in LAS-3000 image analyzer (Fuji Film). A synthetic 24-mer peptide corresponding to Ste20-(463–486) was purchased from Sigma.

β-Galactosidase Assay—The yeast cells harvested from 1.0 ml of culture by centrifugation were permeabilized using Y-PEP (Pierce) and resuspended in 700 μl of Z-Buffer (19). The reaction was started by adding 160 μl of o-nitrophenyl-β-d-galactoside (4 mg/ml) to the suspension and was stopped by adding 400 μl of 1 m Na2CO3. Following the centrifugal removal of cell debris, the A420 of the supernatant was measured.

Mating and Pheromone Response Assay—Quantitative mating and pheromone response assays were performed as previously described (24). In brief, the same numbers of a- and α-type cells were combined and collected onto a nitrocellulose filter by filtration. The filter was placed on wet YPAD (19) plate at 30 °C for overnight to allow mating. The cells were washed off from the filter and plated to an appropriate medium to select diploid cells. Mating efficiency between the wild-type cells was taken as 100%. For the FUS1-lacZ reporter assay, YYF003 cells (bem1Δ bar1Δ) having an episomal copy of BEM1 or a certain bem1 allele were grown to mid-log phase in an appropriate selective medium and stimulated with 25 nm α-factor.

Yeast Three-hybrid Assay—The yeast three-hybrid assay was performed using P69-2A cells and a pBridge vector (BD Biosciences) according to the instructions of the manufacturer.

RESULTS

Mapping a Cdc42-binding Domain of Bem1—Although Bem1 was reported to bind the GTP-bound form of Cdc42, the region responsible for the interaction has remained to be identified. To address this issue, we examined various portions of Bem1 for the interaction with Cdc42-(G12V/C188S), an activated form of Cdc42, using a yeast two-hybrid system (Fig. 1A). The C-terminal half of Bem1 spanning amino acid residues 283–551 or Bem1-(283–551), which contains both the PX/PB2 region responsible for the interaction has remained to be identified. The N-terminal half or Bem1-(1–256), which contains two SH3 domains, showed a two-hybrid interaction with Cdc24 (9) but failed to interact with Cdc42. In contrast, the N-terminal half of Bem1-(1–140) and Bem1-(1–256), which contain the first and second SH3 domains, respectively. Although the former failed to interact with Cdc42, the latter displayed a two-hybrid interaction with Cdc42 comparable to the one shown by Bem1-(1–256).

The second SH3 domain (SH3b) of Bem1 was previously shown to bind the PRRs of Ste20, Cla4, and Boi1/2 (9, 10, 16, 25, 26). Because all of these proteins were reported to bind Cdc42,
Dual-bait Reverse Two-hybrid Screen for Separation-of-function bem1 Alleles—To pinpoint the region responsible for the Cdc42 binding, we tried to subdivide Bem1-(140–256) into smaller segments. However, all of our trials have so far failed (data not shown). Thus, we decided to isolate separation-of-function mutants that can interact with Ste20 but not with Cdc42. For this purpose, we conducted a dual-bait reverse two-hybrid screening (Fig. 2A).

In this screening, we expressed Ste20-PRR and Cdc42 as fusions with LexA and Gal4 DNA-binding domain (Gal4-DBD), respectively, in Mav\(\chi\) cells (Table 1). LexA-Ste20-PRR binds the lexA operator sequences embedded upstream of HIS3 reporter gene, whereas Gal4-DBD-Cdc42 binds the Gal4-binding site located upstream of URA3 reporter gene. On the other hand, we introduced random mutations into the DNA fragment encoding Bem1-(140–256) by means of error-prone PCR and cloned the products into a prey vector that expresses its insert as a fusion with the Gal4 activation domain (Gal4-AD), thereby constructing a library of mutated Bem1 fused with Gal4-AD in PJ69-4A\(\Delta\) cells (Table 1). The Mav\(\chi\) cells (MATa) and PJ69-4A\(\Delta\) cells (MATa) carrying the bait and library plasmids, respectively, were mated to form diploid cells (MATa/\(\alpha\)), which were selected on a medium containing both 3-aminotriazole and 5-fluoroorotic acid. Because the expression of HIS3 and URA3 reporter genes confers 3-aminotriazole-resistant and 5-fluoroorotic acid-sensitive phenotypes, respectively, the diploid cell would not survive the selection unless it bears a mutated Bem1 that is capable of binding to Ste20 but not to Cdc42 (Fig. 2A).

Using this screening strategy, we successfully isolated three single-point mutants encoding Bem1 that can interact with Ste20 but not with Cdc42 (Fig. 2B). Intriguingly, all of these mutations occurred in the C-terminal flanking region of the SH3b but not in the SH3b per se. These results are consistent with the result of the W192K substitution described above (Fig. 1B), underscoring a critical role of the non-SH3 region in Cdc42 recognition. Thus, we designated the C-terminal flanking segment of the SH3b as the CI region.

Among the identified substitutions, we selected N253D for further analysis because of its prominent effect on the interaction: it abolished the binding of Bem1-(140–256) to Cdc42-(G12V) but not to Ste20-PRR (Fig. 2C). The effect of N253D substitution was specific to the activated form of Cdc42, because Bem1-(140–256) failed to interact with Cdc42-(D57Y), an inactivated form of Cdc42, even in the presence of this substitution (Fig. 2C). To confirm the importance of this residue in the context of the full-length protein, we examined Bem1-(1–551) with and without the N253D substitution (Table 1). These results are consistent with the result of the W192K substitution described above (Fig. 1B), underscoring a critical role of the non-SH3 region in Cdc42 recognition. Thus, we designated the C-terminal flanking segment of the SH3b as the CI region.

Direct Interaction between Bem1 and Cdc42—To examine whether the interaction described above is direct or not, we performed an in vitro binding assay using recombinant proteins expressed in and purified from E. coli cells (Fig. 3A). We expressed Bem1-SH3b-CI and Cdc42-(G12V) as a GST fusion and a His\(_6\)/T7-tagged protein, respectively. To improve the solubility, the SH3b-CI used in this assay was slightly extended toward its C terminus (amino acids 140–271) compared with the original one (amino acids 140–256) used in the two-hybrid
Bem1-Cdc42 Interaction

A

Error-prone PCR & gap repair

bem1

LEU2

Gal4-AD-fusion

MATα cell

MATα cell

<->
mating

Bem1

3-AT

5-FOA

Gal4-DBD-fusion

Ste20

Cdc42

LexA-fusion

STE20

ZEO

CDC42

TRP1

B

Bem1

ScBem1

SpScd2

YiBem1

CaBem1

AgAELE241Wp

CgBem1

ScBem1

SpScd2

YiBem1

CaBem1

AgAELE241Wp

CgBem1

SH3-2

140. GSNSRRKNDLNGSGLYIAVLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG
108. NSSTRTRGISEHLSQGFPLIGPGDFAFAPDPBIHACKAEAIITARSNHMLVAKPT
162. PSMAAGSRSQPGSPFPLGMLYDFPAERPQDPAQLSAQKEMIEIQAQSHNHMFVAKPVG
117. NSSSQPQQGSRNSQGLYAVLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG
130. SLTSPQQQVMPVFGSGLYAVLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG
113. ALGRSVSTSSRNGEKMCTLYVGLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG

RLOGGPLVPVFGSGLYAVLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG
RLOGGPLVPVFGSGLYAVLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG
RLOGGPLVPVFGSGLYAVLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG
RLOGGPLVPVFGSGLYAVLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG
RLOGGPLVPVFGSGLYAVLYDFKAEKADLTYVGHNLPCACACNYCBWPIAKPIG

GSUWSEQQQQGQITPKQ.271
GFSDFEHTQTMPSLQ.239
GNDPEPCQSHQQH.292
GZBIWQAPPVSSQTO.251
GSUWSEQQQQGQITPKQ.260
GAVYVPFNEGPTTRQ.261

C

Bem1

Bem1

N253D

Ste20

Ste20

Ste20

SC-Trp-Leu

SC-Trp-Leu-His-Ade

D

β-galactosidase (U)

Ste20

100

500

100

100

Ste20

Cdc42

Cdc42

G12V

G12V

G12V

PB1

PB1

PB1

Bem1

Bem1-N253D

Mock
assay. We confirmed that both forms show comparable binding to Cdc42 in the two-hybrid system (data not shown).

The GST-Bem1-SH3b-Cl immobilized to glutathione-Sepharose 4B beads was incubated with His6/T7-Cdc42-(G12V). The proteins bound to the beads were eluted and detected by an immunoblotting procedure using anti-GST and anti-T7 antibodies. Effects of the W192K substitution in the SH3b and the N253D substitution in the CI on Cdc42 binding were also examined.

Bem1-Cdc42 Interaction

A Biological Role of the Bem1-Cdc42 Interaction——To reveal a biological role of the Bem1-Cdc24 interaction mediated by the SH3b-Cl, we compared the phenotypes of two bem1Δ strains, one bearing an epistatic copy of BEM1 and the other bearing bem1-N253D, in an FY1679 background. A temperature-sensitive growth phenotype of bem1Δ cells was comparably suppressed by the BEM1 and bem1-N253D, both of which were expressed from a centromeric vector using their own BEM1 promoter (data not shown). We also failed to find any difference in growth between the cells bearing bem1-W192K and bem1-W192K/N253D alleles (data not shown).

Since the deletion of BUD1 was reported to render BEM1 essential for growth (13), we reasoned that bud1Δ background may sensitize the assay. Although the synthetic lethality was not observed in the FY1679 background, cells deleted for both BEM1 and BUD1 displayed a severe temperature-sensitive growth (Fig. 4A). As expected, bem1-W192K suppressed the phenotype less effectively than BEM1. Notably, the defective suppression by bem1-W192K was exacerbated by the N253D substitution (Fig. 4A). Further examination of other genetic backgrounds led us to find that bem1-N253D is defective in suppression of temperature-sensitive growth of W303 cells deleted for BEM1 (Fig. 4B).

Many bem1 mutants are known to cause a bilateral mating defect. We thus examined mating efficiency of the bem1Δ cells bearing an epistatic copy of various bem1 alleles (Fig. 4C). In this assay, we mated a- and α-type cells sharing the same bem1 allele. We prepared bem1-W192K, -N253D, and -I489A alleles, which bear a loss-of-function mutation in the SH3b, CI, and PB1, respectively. We also generated three additional bem1 alleles by combining two of these mutations.

The importance of the SH3b and PB1 domains in mating was demonstrated previously (9, 13). In accordance with these findings, both the W192K and I489A substitutions substantially inhibited the mating (Fig. 4C). We had previously used K482A substitution for the PB1, which almost completely eliminates the binding of Bem1 to Cdc24 and hence confers a severe defect in mating (9). However, in this study, we employed the I489A substitution that causes ~50% inhibition of the interaction (29), because the effect of additional mutations would be masked when combined with such a strong mutation as the K482A (see below).

In contrast with the substitutions in the SH3b and PB1, the effect of the N253D substitution in the CI was rather modest,
although it reproducibly suppressed mating (Fig. 4C). We thus examined the synthetic effect between the N253D and W192K or 1489A substitutions. Although the N253D substitution affected the I489A-induced mating defect only modestly, it considerably exacerbated the defect caused by the W192K substitution (Fig. 4C). Thus, the CI may affect the function mediated by the SH3b but not that by the PB1.

The results described above demonstrate the significance of the Bem1-Cdc42 interaction in both budding and mating. Because the effect of defective Bem1-Cdc42 interaction on budding heavily depends on the genetic background, we pursued in this study the biological significance of the interaction in the mating process.

Cooperation between the SH3b and CI in Ste20 Recruitment for Efficient Mating—The interaction between Bem1-SH3b and Ste20-PRR was shown to be crucial for efficient mating (16). We thus hypothesized that the defect induced by the W192K substitution would be suppressed by increasing the dosage of Ste20. To test this hypothesis, we overexpressed STE20 using the inducible GAL1 promoter in the cells that carry various bem1 alleles and examined these cells for α-factor-induced expression of FUS1-lacZ, a reporter of mating pheromone signaling. In the presence of overexpressed Ste20, the BEM1 and bem1-W192K cells displayed comparable FUS1-lacZ induction (Fig. 5). By contrast, the cells bearing bem1-W192K/N253D allele failed to induce the reporter gene so efficiently as the BEM1 and bem1-W192K cells did (Fig. 5). These results indicate that the intact CI or the Bem1-Cdc42 interaction is required for the Ste20-mediated suppression of signaling defects induced by the W192K substitution.

CI-mediated Ternary Complex Formation among Bem1, Cdc42, and Ste20—To gain further insight into the CI function in terms of molecular interactions, we examined whether a ternary complex can be formed among Bem1-SH3b-CI, Cdc42, and Ste20-CRIB (Fig. 6A). We incubated GST-Bem1-SH3b-CI with His6/T7-Cdc42-(G12V) and MBP-Ste20-CRIB, and precipitated the proteins using glutathione beads. Intriguingly, Bem1-SH3b-CI precipitated not only Cdc42-(G12V) but also Ste20-CRIB (Fig. 6A). Of note, the co-precipitation was observed only in the presence of the GTP-bound form Cdc42-(G12V) but not the GDP-bound form Cdc42-(D118A). Furthermore, Bem1-SH3b-CI-(N253D) failed to precipitate Ste20-CRIB even in the presence of Cdc42-(G12V).

We also performed a reverse-tagging experiment in which GST-Ste20-CRIB was incubated with Cdc42 and MBP-Bem1-SH3b-CI prior to precipitation by glutathione beads. As shown in Fig. 6B, Ste20-CRIB precipitated Bem1-SH3b-CI, but not Bem1-SH3b-CI-(N253D), in a Cdc42-(G12V)-dependent manner. These results indicate that the GTP-bound form of Cdc42 can bind Bem1 and Ste20 simultaneously to form a ternary complex among the three proteins in vitro.

This finding was further supported by a yeast three-hybrid experiment, in which an interaction was examined between Gal4-DBD-Ste20-CRIB and Gal4-AD-Bem1-SH3b-CI in the presence or absence of Cdc42-(G12V/C188S) targeted to the nucleus by a nuclear localization signal (NLS) (NLS-Cdc42-(G12V/C188S)) (Fig. 6C). Note that, in this three-hybrid system, the expression of NLS-Cdc42 is regulated by MET25 pro-
moter that is suppressed by methionine. In the presence of methionine or in the absence of NLS-Cdc42, Ste20-CRIB failed to bind Bem1-SH3b-CI (Fig. 6C, lower right panel, sector 1). By contrast, in the absence of methionine or in the presence of NLS-Cdc42, Ste20-CRIB displayed an interaction with Bem1-SH3b-CI (Fig. 6C, upper right panel, sector 1). It should be noted that the three-hybrid interaction was dependent on both the intact CI of Bem1 and the GTP-bound form of Cdc42: neither Bem1-SH3b-CI (N253D) nor the GDP-bound form Cdc42-(D118A/C188S) supported the three-hybrid interaction (Fig. 6C, sectors 2 and 3).

Simultaneous binding of Cdc42 to Ste20-CRIB and Bem1-SH3b-CI suggests that Cdc42 uses different surfaces to interact with these two proteins. We thus examined several Cdc42 mutants for differential binding to Ste20-CRIB and Bem1-SH3b-CI using the two-hybrid system (Fig. 7A). The results of G12V and D118A mutants confirmed that both Ste20 and Bem1 recognize the GTP-bound, but not the GDP-bound form of Cdc42: neither G12V nor D118A impaired the binding of Cdc42 to Ste20-CRIB but barely impaired its binding to Bem1-SH3b-CI (Fig. 7A, sector 3). Conversely, substitutions of the C-terminal basic residues (i.e. Lys at positions 183, 184, 186, and 187) abolished the binding of Cdc42 to Ste20-CRIB but not to Bem1-SH3b-CI (Fig. 7A, sector 3). Notably, an effector loop mutation Y40C abolished the binding of Cdc42 to Ste20-CRIB and Bem1-SH3b-CI (N253D), and no insert, respectively. Bem1-SH3b-CI-(N253D) and the GDP-bound form Cdc42-(D118A/C188S) supported the three-hybrid interaction (Fig. 7A, sector 3).

Bem1-Cdc42 Interaction

FIGURE 7. Effects of Cdc42 mutations on its binding to Bem1 and Ste20. A, two-hybrid interactions between Gal4-DBD-Bem1-SH3b-CI (upper panels) or Gal4-DBD-Ste20-CRIB (lower panels) and Gal4-AD-Cdc42 bearing various mutations were examined by histidine- and adenine-independent growth. The transfectants in sectors 1–7 contained a prey plasmid to express Cdc42-(G12V/C188S), -(D118A/C188S), -(G12V/Y40C/C188S), -(G12V/K183A,K184A,K186A,K187A), -(G12V/K183Q,K184Q,K186Q,K187Q), -(G12V/185–191K-A), and no insert, respectively. B, purified GST-Ste20-CRIB was incubated with MBP-Bem1-SH3b-CI in the presence of His6/T7-Cdc42 bearing the indicated substitutions. Note that K-A, K-Q, and ΔC7 on individual lanes indicate Cdc42-(G12V/K183A,K184A,K186A,K187A), -(G12V/K183Q,K184Q,K186Q,K187Q), and -(G12V/185–191Δ), respectively. Proteins were pulled down using glutathione-Sepharose 4B beads and visualized by an immunoblotting procedure using the indicated antibodies. Right panels show 5% of the His6/T7-Cdc42 mutants and MBP-Bem1-SH3b-CI used for the assays.

FIGURE 6. CI-mediated ternary complex formation among Bem1, Cdc42, and Ste20. A, purified GST-Bem1-SH3b-CI, either with or without the N253D substitution, was incubated with MBP-Ste20-CRIB in the presence of His6/T7-Cdc42-(G12V) or His6/T7-Cdc42-(D118A), Proteins were pulled down using glutathione-Sepharose 4B beads and visualized by an immunoblotting procedure using the indicated antibodies. The right three lanes contain 5% of the His6/T7-Cdc42-(D118A), His6/T7-Cdc42-(G12V), and MBP-Ste20-CRIB used for each assay. B, purified MBP-Bem1-SH3b-CI, either with or without the N253D substitution, was incubated with GST-Ste20-CRIB in the presence of His6/T7-Cdc42-(G12V) or His6/T7-Cdc42-(D118A), Proteins were pulled down using glutathione-Sepharose 4B beads and visualized by an immunoblotting procedure using the indicated antibodies. The right four lanes contain 5% of the His6/T7-Cdc42-(D118A), H3/T7-Cdc42-(G12V), MBP-Bem1-SH3b-CI, and MBP-Bem1-SH3b-CI-(N253D) used for each assay. C, a yeast three-hybrid assay was performed in PJ69-2A cells to examine an in vivo interaction between Gal4-DBD-Ste20-CRIB and Gal4-AD-Bem1-SH3b-CI in the presence or absence of NLS-Cdc42 (i.e. Cdc42 bearing a nuclear localization signal). Note that the expression of NLS-Cdc42 was induced and suppressed by the presence and absence of 1 μM metionine, respectively, Sector 1, Gal4-DBD-Ste20-CRIB, NLS-Cdc42-(G12V/C188S), and Gal4-AD-Bem1-SH3b-CI; sector 2, Gal4-DBD-Ste20-CRIB, NLS-Cdc42-(G12V/C188S), and Gal4-AD-Bem1-SH3b-CI-(N253D); and sector 3, Gal4-DBD-Ste20-CRIB, NLS-Cdc42-(D57Y/C188S), and Gal4-AD-Bem1-SH3b-CI.

that the three-hybrid interaction was dependent on both the intact CI of Bem1 and the GTP-bound form of Cdc42: neither Bem1-SH3b-CI (N253D) nor the GDP-bound form Cdc42-(D118A/C188S) supported the three-hybrid interaction (Fig. 6C, sectors 2 and 3).
We also examined the effect of these Cdc42 mutations on the in vitro ternary complex formation among Bem1-SH3b-CI, Cdc42, and Ste20-CRIB (Fig. 7B). The results of the in vitro binding assays were consistent with those obtained using the two-hybrid system, except for Cdc42-(G12V/185–191Δ). These results indicate that the mode of Cdc42 recognition is clearly different between Bem1 and Ste20.

**SH3b-mediated Ternary Complex Formation among Bem1, Cdc42, and Ste20** —The results described above demonstrated that a ternary complex can be formed among Bem1-SH3b-CI, Cdc42, and Ste20-CRIB. We wondered if another ternary complex can be formed among Bem1-SH3b-CI, Cdc42, and Ste20-PRR. Thus, we examined whether Bem1-SH3b-CI can simultaneously bind Cdc42 and Ste20-PRR. We first incubated MBP-Ste20-PRR with GST-Bem1-SH3b-CI and His6/T7-Cdc42-(G12V) to examine whether MBP-Ste20-PRR can precipitate not only GST-Bem1-SH3b-CI but also His6/T7-Cdc42-(G12V). However, we failed to obtain any evidence for the ternary complex formation (data not shown). We next performed a yeast three-hybrid assay to examine an interaction between Gal4-DBD-Ste20-PRR and Gal4-AD-Cdc42-(G12V/C188S) in the presence of NLS-Bem1-SH3b-C but again failed to detect the interaction (data not shown).

Conversely, we found that the nuclear-targeted Ste20-PRR (NLS-Ste20-PRR) rather inhibits the interaction between Bem1-SH3b-CI and Cdc42 (Fig. 8A). Notably, Ste20-PRR-(P477A,P480A), which is defective in SH3b-binding, failed to inhibit the interaction (Fig. 8A, sector 2). It should be also noted that Ste20-PRR failed to inhibit the binding of Cdc42 to Bem1-SH3b-CI-(W192K), which is defective in PRR binding (Fig. 8A, sector 6). These results indicate the inhibitory effect of Ste20-PRR depends on its ability to interact with the SH3b. Furthermore, the inhibitory effect was also demonstrated in vitro: a synthetic 24-mer peptide corresponding to Ste20-(463–486) inhibited the Cdc42 binding of Bem1-SH3b-CI but not that of Bem1-SH3b-CI-(W192K) (Fig. 8B). It is thus likely that Ste20-PRR and Cdc42, albeit recognizing different sites on Bem1-SH3b-CI (Fig. 3), bind it in a mutually exclusive manner, thereby preventing formation of the ternary complex among Ste20-PRR, Bem1-SH3b-CI, and Cdc42.

Because the N-terminal portion of Ste20 (Ste20-N) was shown to bind Bem1-SH3b and Cdc42 via PRR and CRIB, respectively (9, 10, 16), a ternary complex is assumed to be formed among Bem1-SH3b and Cdc42, we used the three-hybrid system to examine an interaction between Gal4-DBD-Bem1-SH3b-CI and Gal4-AD-Cdc42-(G12V/K183A,K184A,K186A,K187A) in the presence or absence of NLS-Ste20-N containing both PRR and CRIB (Fig. 8C). Note that this Cdc42 mutant is defective in the CI-mediated Bem1 binding but is fully capable of binding to Ste20-CRIB (Fig. 7A, sector 4; Fig. 8C, sector 4). The interaction between the SH3b-CI and the Cdc42 mutant

Cdc42-(G12V/K183A,K184A,K186A,K187A) (sectors 4–6) in the presence or absence of NLS-Ste20-N containing both PRR and CRIB (sectors 1 and 6), NLS-Ste20-N-(H345G) (sectors 2 and 5), or mock NLS (sectors 3 and 4). Note that Ste20-PRR-(H345G) with a defective CRIB cannot bind Cdc42.
was observed only in the presence of NLS-Ste20-N (Fig. 8C, *sector 6*). Notably, Ste20-N-(H345G), which carries a defective CRIB motif incapable of binding Cdc42 (24), failed to support the interaction (Fig. 8C, *sector 5*). As expected from the results shown in Fig. 8A, both NLS-Ste20-N and NLS-Ste20-N-(H345G) were inhibitory to the CI-mediated interaction between Bem1-SH3b-CI and Cdc42-(G12V/C188S) (Fig. 8C, *sectors 1–3*). These results indicate that Ste20-N mediates an indirect interaction between Bem1-SH3b and Cdc42.

Taken together, Bem1 can use the CI to indirectly interact with Ste20-CRIB via Cdc42. Similarly, it can use the SH3b to indirectly interact with Cdc42 via Ste20-N. However, Bem1 cannot simultaneously bind Cdc42 and Ste20-N via the CI and SH3b, respectively.

**DISCUSSION**

We revealed that a region comprising the second SH3 domain (SH3b) and its C-terminal flanking segment CI serves as a Cdc42-binding site of Bem1 (Fig. 1). The SH3b-CI region directly binds the GTP-bound, but not the GDP-bound form of Cdc42 (Figs. 2 and 3). We used a unique dual-bait reverse two-hybrid system to isolate separation-of-function alleles and demonstrated that the CI plays a critical role in the recognition of Cdc42 (Fig. 2). This approach would be useful in dissecting interactions of a protein with many binding partners. It allows us to gain initial insight into the structural basis for each interaction and to make mutants defective in the interaction to reveal its biological role as demonstrated in this study.

We assume that the SH3b and CI are linked not only functionally (see below) but also structurally, because the removal of the CI makes the SH3b insoluble and because the isolated CI fails to bind Cdc42 (data not shown). Intriguingly, it was also impossible for others to express the second SH3 domain of Scd2, the fission yeast homolog of Bem1 (27, 28), in a soluble form. The SH3b and CI likely depend on each other for their integrity to constitute a single structural unit that presumably uses different sites for binding to Ste20-PRR and Cdc42 (Figs. 2 and 3). However, Ste20-PRR and Cdc42 bind the SH3b-CI in a mutually exclusive manner (Fig. 8). Because a short synthetic Ste20-PRR peptide can inhibit the binding of Bem1-SH3b-CI to Cdc42, the inhibition may be mediated either by an allosteric effect of PRR binding on the Cdc42-binding site or by a juxtaposition of the two binding sites. The structure of SH3b-CI complexed with Ste20-PRR or Cdc42 would be of particular interest.

The fission yeast homologue of Bem1 is Scd2, which was extensively analyzed for its binding to Cdc42 using *in vitro* binding assays (27, 28). These studies identified two Cdc42-binding regions, namely CB1 and CB2, containing the first and second SH3 domains, respectively. Although we and others (7) have thus far failed to directly demonstrate Cdc42 binding at the region containing the first SH3 domain (SH3a) of Bem1, which likely corresponds to the CB1 of Scd2, the removal of the SH3a substantially compromises the binding of Bem1 to Cdc42 (7), suggesting that the N-terminal region of Bem1 is involved in mediating another stronger interaction with Cdc42. In this context, it is intriguing to note that recent reports implicate a role for the N-terminal portion of Bem1 in vacuole homeostasis (30, 31) and Sec15 binding (32).

The CB2 of Scd2 likely corresponds to the SH3b-CI of Bem1. We demonstrated that the SH3b-CI directly interacts with Cdc42 and that the CI plays a critical role in the interaction. Although the importance of the region around the SH3b was suggested by the results of the previous *in vitro* binding study on Bem1 (7) and the studies on Scd2 (27, 28), this study has, for the first time, succeeded in pinpointing CI as the critical region.

Furthermore, in contrast with previous studies, this study demonstrated an *in vivo* role of the interaction using a separation-of-function allele *bem1*-N253D that encodes Bem1 capable of binding to Ste20 at the SH3b but not to Cdc42 at the CI. Phenotypic analyses of the *bem1*-N253D cells indicate that the Bem1-Cdc42 interaction is not essential but rather modulatory: *bem1*-N253D confers a modest defect in both budding and mating (Fig. 4). To our interest, the N253D substitution exacerbates the mating defect induced by the W192K substitution in the SH3b, which abolished its binding to Ste20-PRR (Fig. 4). We also demonstrated that the CI-mediated binding of Bem1 to Cdc42 is required for Ste20 to multicyclic suppress the pheromone-signaling defect induced by the W192K substitution (Fig. 5).

These observations prompted us to propose a model that postulates cooperation between the SH3b and CI in the recruitment of Ste20 to Bem1 (Fig. 9). The scaffold protein Bem1 uses its C-terminal PB1 domain to tether Cdc24 for the activation of Cdc42 (Fig. 9, *interaction 1*). The activated Cdc42 is subsequently transferred to Ste20 tethered at the SH3b for mating pheromone signaling (Fig. 9, *interaction 4*). The CI facilitates the transfer of activated Cdc42 from the activator Cdc24 to an effector Ste20 (Fig. 9, *interactions 2 and 3*).

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3 Y. Yamaguchi, K. Ota, and T. Ito, unpublished observation.
Bem1-Cdc42 Interaction

On the mechanistic aspect, two scenarios would be plausible. Cdc42 may sequentially bind Bem1-CI and Ste20-CRIB. Alternatively, Cdc42 may simultaneously interact with Bem1-CI and Ste20-CRIB to form a ternary complex. The results of in vitro binding and three-hybrid assays support the latter scenario; Cdc42 can mediate an indirect binding of Bem1-SH3b-CI to Ste20-CRIB both in vitro and in vivo (Fig. 6).

Simultaneous binding of Cdc42 to Bem1-SH3b-CI and Ste20-CRIB suggests a possibility that Cdc42 uses different surfaces to bind the two proteins. Indeed, mutational analysis revealed that the binding of Cdc42 to Bem1-SH3b-CI and Ste20-CRIB is sensitive to an effector loop mutation Y40C and mutations at the C-terminal basic residues, respectively (Fig. 7).

Because the latter mutations were reported to inhibit the homo-dimerization of Cdc42 observed in vitro (33), Bem1-CI might recognize only the dimerized form of Cdc42. In any case, the CI represents a novel mode of Cdc42 recognition distinct from that of CRIB, and its structural basis is of particular interest. It also remains to be seen whether sequences homologous to the CI are used for Cdc42 binding in proteins other than fungal Bem1 orthologues, among which the CI is well conserved (Fig. 2).

The results of this study indicate that Bem1 tethers Cdc42-Ste20 complex in two different ways: CI-mediated tethering via Cdc42 (Fig. 6) and SH3b-mediated tethering via Ste20-PRR (Fig. 8). The former seems weaker than the latter. We thus assume that the CI-mediated weak tethering of Cdc42-Ste20 complex is a transient interaction to be eventually switched to the SH3b-mediated stable interaction (Fig. 9, interactions 2–4). In accordance with this model, the effect of defective CI-mediated tethering is rather modest (Fig. 4) but becomes prominent when the direct tethering is compromised (Fig. 5). Here it should be noted that Cdc42 and Ste20-PRR likely bind different sites of Bem1-SH3b-CI but do so in a mutually exclusive manner (Figs. 3 and 8). This mutually exclusive interaction would provide the molecular basis for the switching. The two different modes of Ste20 tethering may also enable Bem1 to conduct a fine modulation of Cdc42 signaling. Bem1 may not only function as a mere scaffold but actively participate in the regulation of Cdc42 signaling.

Although Ste20 plays a key role in mating, another p21-activated kinase Cla4 has been shown to be critical for vegetative growth (7, 8, 15). The results in the budΔ and W303 cells suggest the importance of the Bem1-Cdc42 interaction in budding (Fig. 4). Similar to Ste20, Cla4 bears a PRR and a CRIB to bind the SH3b and Cdc42, respectively. It is thus intriguing to examine whether a similar mechanism is involved in the Bem1-Cla4 interaction to modulate budding of the yeast.

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