Role of Cdc42p in Pheromone-Stimulated Signal Transduction in Saccharomyces cerevisiae

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CDC42 encodes a highly conserved GTPase of the Rho family that is best known for its role in regulating cell polarity and actin organization. In addition, various studies of both yeast and mammalian cells have suggested that Cdc42p, through its interaction with p21-activated kinases (PAKs), plays a role in signaling pathways that regulate target gene transcription. However, recent studies of the yeast pheromone response pathway suggested that prior results with temperature-sensitive cdc42 mutants were misleading and that Cdc42p and the Cdc42p-PAK interaction are not involved in signaling. To clarify this issue, we have identified and characterized novel viable pheromone-resistant cdc42 alleles that retain the ability to perform polarity-related functions. Mutation of the Cdc42p residue Val36 or Tyr40 caused defects in pheromone signaling and in the localization of the Ste20p PAK in vivo and affected binding to the Ste20p Cdc42p-Rac interactive binding (CRIB) domain in vitro. Epistasis analysis suggested that they affect the signaling step at which Ste20p acts, and overproduction of Ste20p rescued the defect. These results suggest that Cdc42p is in fact required for pheromone response and that interaction with the PAK Ste20p is critical for that role. Furthermore, the ste20ΔCRIB allele, previously used to disrupt the Cdc42p-Ste20p interaction, behaved as an activated allele, largely bypassing the signaling defect of the cdc42 mutants. Additional observations lead us to suggest that Cdc42p collaborates with the SH3-domain protein Bem1p to facilitate signal transduction, possibly by providing a cell surface scaffold that aids in the local concentration of signaling kinases, thus promoting activation of a mitogen-activated protein kinase cascade by Ste20p.

In the budding yeast Saccharomyces cerevisiae, cells of the a and α mating types secrete pheromones that bind to G-protein-coupled receptors on the surfaces of cells of the opposite mating type, initiating a signaling cascade in which the G subunits of the G protein promote the activation of a mitogen-activated protein kinase (MAPK) cascade (4). This process appears to involve the binding of at least two proteins, the upstream kinase Ste20p and the scaffold protein Ste5p, to the liberated Gβγ subunits (11, 25, 40, 51). MAPK activation then promotes cell cycle arrest in G1 and stimulates the expression of several genes, including FUS1, leading ultimately to fusion of the mating partners (22).

The small GTPase Cdc42p and its guanine nucleotide exchange factor Cdc24p are essential for polarity establishment and subsequent bud formation (1, 38). In addition to their roles in cell polarity, these proteins have been proposed to play roles in signal transduction in response to mating pheromones (46, 47, 53). Temperature-sensitive cdc42 and cdc24 mutants have defects in α-factor-stimulated transcription of FUS1 and in maintaining G1 arrest at the restrictive temperature (46, 53). In addition, an interaction was detected by two-hybrid analysis between Gβγ and Cdc24p (53), and Cdc42p-GTP was shown to bind to and activate Ste20p (46). These findings led to the hypothesis that Gβγ activated Cdc24p, causing GTP loading of Cdc42p and consequent activation of Ste20p, as an important part of the pheromone signaling pathway.

More recent experiments have cast doubt upon the existence of a Gβγ-Cdc24p-Cdc42p-Ste20p pathway. Mutations in CDC24 that abolished detectable interaction with Gβγ did not cause any defects in α-factor-stimulated FUS1 transcription or G1 arrest but rather were specifically defective in orientation of the mating projection towards the mating partner (33). Furthermore, mutations in STE20 that abolished detectable interaction with Cdc24p were also reported to be wild type with regard to α-factor-stimulated FUS1 transcription, G1 arrest, and mating (23, 37). Together, these studies indicated that neither the Gβγ-Cdc24p interaction nor the Cdc42p-Ste20p interaction was important for α-factor signaling. Furthermore, the polarity defect exhibited by temperature-sensitive cdc24 and cdc42 mutants triggers the morphogenesis checkpoint to delay the cells in G2 with abundant G1 cyclins (26), a state known to render cells unresponsive to α-factor (34). This raised the possibility that the signaling defect of these mutants might be an indirect consequence of their accumulation at a nonrestrictive stage of the cell cycle. Indeed, the transcriptional induction of FUS1 was found to be quite normal in cdc24 and cdc24 mutants that were first arrested in G1 by deprivation of G1 cyclins (35), raising the question of whether Cdc24p and Cdc42p play any role at all in α-factor signaling.

As illustrated by these studies, the interpretation of cdc42 and cdc24 phenotypes is complicated by the possibility of indirect effects stemming from the well-characterized polarity defects caused by these mutants at the restrictive temperature. To circumvent these problems, we performed a screen to identify α-factor-resistant alleles of CDC42 that could still perform polarization functions. In this paper we report the isolation and characterization of such mutants, supporting the notion that Cdc42p has some direct role in pheromone signaling. Our results further suggest that this signaling function operates through Cdc42p-dependent activation and localization of Ste20p.
vector sequences with the corresponding fragment from pRS305 [45]). Ura
CDC42Sc [55] generated by replacing the
ing an additional copy of
this case, integrated at
LEU2 open reading frame). The PCR fragment was transformed into DLY1, replacing
DJL42-2 it is the reverse complement of nucleotides 204 to 226 in the
G1-phase cells was performed as described previously (27).

Together with the
TRP1 was excised from pMOSB36 and transformed into MOSY0090 to-

The
S. cerevisiae

The

To create pDLB644, the recipient plasmid for expressing the mutant alleles of
CDC42, CDC42 promoter sequences were fused (via a unique BglII site) to the
transcription terminator sequences from TDH3 and cloned into the vector
RNAi3 (45). The oligonucleotides DLJL2-3 (see above) and DLJL2-5 (5
AGAATTCTGAGATCATTTCGAAGATGTTCTGAATTCATATTCGATGC
AGG-3') were first
used to amplify a DNA fragment containing the LEU2 gene, the
GAL1 promoter, and flanking CDC42 sequences using PCR with the plasmid
pGAL-CDC42Sc (55) as a template (the underlined sequence in DJL42-1 is the
LEU2 promoter and coding sequences were fused to the transcription terminator se-

To generate the
cdc42::URA3 null allele, the oligonucleotides CDC42-5' (5'-
AGGGTC-3') and CDC42-3' (5'GAGGCTCTAAGGGCTAATCAGGAT
TCACCAGATGATTTCATCGAATACCTTTCCTGATGCGGTATTTTC-3') were used
as an additional copy of
the G1 phase of cell cycle. The

To create pDLB643 was also cloned into the corresponding sites of pRS315 (45) to
make pMOSB55.

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FIG. 1. Isolation of α-factor-resistant cdc42 alleles. The results of growth arrest assays of strain DLY302 (Galp-cdc42) containing plasmid pMOSB55 (CDC42), pMOSB36 (cdc42-md1), pMOSB37 (cdc42-md2), or pMOSB38 (cdc42-md3) are shown. The photographs show growth after 3 days at 30°C with (+) or without (−) α-factor.

To express the Ste20p Cdc42p-Rac interactive binding (CRIB) domain as a glutathione S-transferase (GST) fusion protein, the oligonucleotides ste20-1 (5′-ATGTCACTTCTTAAACCAATGCGG-3′) and ste20-2 (5′-TGTGCGCGCCGTTTTGGA-3′) were used to amplify a DNA fragment encoding ste20-1 (5′-ATGTCATCTTCTATAACCACCGC-3′) and ste20-2 (5′-AACCGTGCAACCGTGAAG-3′) to determine whether the excision event left STE20 (558-bp PCR product) or ste20-CRIB (446-bp PCR product) as the sole remaining allele.

To generate the α-factor-resistant cdc42 mutant strain pMOSB36 from a cross between MOSY0095 and BOY774 (cdc42-TRP1) or BOY489 (ste20-TRP1), respectively. BOY489 and BOY774 were obtained from F. Cross (6). The bem1-URA3 allele was generated by one-step gene replacement using an EcoRI-BamHI fragment from the plasmid pkO1 (9).

The results of growth arrest assays of strain DLY302 with Spht1-digested pFl-HIS2. pFl-HIS2 contains a 2-kb HIS2 HindIII fragment in place of the HindIII/Hinfl TRP1 fragment in pFl-TRP, which itself contains a 0.9-kb TRP1 EcoRI/StuI fragment (along with pBluescript polynucleotides from EcoRI-HinflII) of the HindIII/Ura3 URA3 fragment of pS826 (39).

Screen to identify α-factor-resistant cdc24 mutants. The oligonucleotides CDC42-4 and CDC42-6 (see above) were used to amplify CDC42 (and TDH3 terminator sequences) by mutagenic PCR using pDLB644 as a template. Mutagenic PCRs were similar to standard PCR except for the addition of 0.2 mM MnCl2 and 10 pmol of an unbalanced deoxynucleoside triphosphate triphosphate (Mitogen, 0.5 mM dTTP, 0.1 mM dGTP, and 0.1 mM dCTP [final concentrations]). The PCR products were transformed into DLY3067 together with pBlp1-digested (‘gapped’ plasmid) pDLB644, and Ura+ transforms were selected on dextrose-containing plates (to repress transcription of the genomic GAL1p-CDC42 allele) coated with 10 μg of α-factor (to select for α-factor-resistant cdc42 mutants).

This amount of α-factor (custom synthesized by Research Genetics, Huntsville, Ala.) was more than 10 times the amount needed to completely inhibit growth of the Ura1 parent strain. α-Factor-resistant colonies could in theory arise due to genomic mutations in other genes. To distinguish cdc42 mutants, colonies were tested for α-factor-resistant growth on galactose-containing plates, where the genomic (wild-type) CDC42 is expressed. Plasmids were isolated from α-factor-resistant colonies that were α-factor resistant when grown in dextrose-containing medium but not when grown in galactose-containing medium and were then retransformed into fresh DLY3067 to confirm that the plasmids were responsible for the α-factor-resistant growth phenotype. To generate the TRP1-containing plasmids pMOSB42 to -45, CDC42 alleles were transferred from URA3 plasmids (pMOSB36 to -38 and pMOSB55) as 2.1-kb BamHI-Stu1 fragments to the corresponding sites of pRS314 (45).

Spot assays for growth arrest. Cells harboring plasmid-borne CDC42 alleles were grown in synthetic medium lacking the corresponding plasmid (to repress growth on the genomic CDC42 locus that was introduced by transformation of the BY4741 strain) or with 10 μg/mL α-factor. Plates were scored for growth arrest.

After spotting the control plasmid, the strain was grown on a filter containing 2% raffinose and 0.1% 2-deoxytrehalose to an optical density of 0.600 at 660 nm of 0.3 to 0.6, preincubated for 2 h at 38.5°C, and then induced for 4 h with 2% galactose with or without 0.01 μM α-factor. Assays in CDC42 strains were performed at 30°C. The presence of recombining α-factor-resistant cdc42 alleles were described previously (10, 41, 42). The FUS1 probe was made from a 880-bp internal EcoR1/HindIII fragment from the FUS1 gene, and the ACTI probe was made from the entire ACTI open reading frame, using [α-32P]dCTP (ICN Pharmaceuticals, Costa Mesa, Calif.) and the Prime-it II kit (Stratagene) accorded to the manufacturer’s recommendations.

Flow cytometry. Cells were processed for fluorescence-activated cell sorter (FACS) analysis as described (14), except that the DNA was stained with 1 μM Sytox (Molecular Probes, Eugene, Ore.) in 50 mM Tris-HCl (pH 8.0) (instead of propidium iodide).

DIC microscopy. Cells were viewed on an Axioskop apparatus (Zeiss, Thornwood, N.Y.) equipped with epifluorescence and differential interference contrast (DIC) optics. Images were captured by using a cooled-model charge-coupled device camera (Princeton Instruments, Princeton, N.J.).

Analysis of FUS1-lacZ expression. β-Galactosidase assays were performed as described previously (40). Transfomers in cdc42-2 strains were grown over night at 28°C in selective synthetic medium containing 2% raffinose and 0.1% dextrose to an optical density of 0.600 nm of 0.3 to 0.6, preincubated for 2 h at 38.5°C, and then induced for 4 h with 2% galactose with or without 0.01 μM α-factor. Assays in CDC42 strains were performed at 30°C.

Construction of recombinant α-factor-resistant cdc42 alleles. Wild-type and mutant蛛myc-tagged CDC42 alleles were expressed in Escherichia coli BL21(DE3) (Stratagene). Extracts were prepared in bacterial lysis buffer (750 mM sucrose, 100 mM NaCl, 100 mM Tris-HCl [pH 8.0], 5 mM EDTA) containing the protease inhibitor cocktail (Sigma), 10 μg/mL leupeptin (Sigma), 10 μg/mL aprotinin (Sigma), and 10 μg/mL pepstatin (Sigma). Protein was extracted by using glass beads (Sigma) and analyzed by SDS-PAGE (22) followed by fluorography.
of lysozyme/ml for 20 min on ice. To remove genomic DNA, MgCl₂ was added to 15 mM and DNase I was added to 50 μg/ml. The cells were lysed by 20 min of incubation at 4°C with 2 mg of deoxycholic acid/ml. Insoluble material was removed by centrifugation at 4°C for 10 min. GST-Ste20-CRIB was expressed in the protease-deficient E. coli BL21, extracts were prepared as described above, and the protein was purified using glutathione Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, N.J.) as specified by the manufacturer.

Binding assays were performed by incubating the bacterial extracts containing Cdc42p-myc with either GST or GST-Ste20-CRIB immobilized on glutathione beads in 200 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 85 mM NaCl, 6 mM MgCl₂, 10% glycerol) at 4°C for 3 h. Binding reaction mixtures were washed three times at room temperature with wash buffer (10 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 1 mM dithiothreitol, 0.1% Triton X-100). Bead-bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to Immobilon-P nylon membranes (Millipore, Bedford, Mass.), and immunoblotted with monoclonal anti-myc antibodies (9E10; Santa Cruz Biotechnology, Santa Cruz, Calif.) using standard procedures (2). To confirm equal loading, the amount of GST-Ste20-CRIB in each lane was visualized by staining the membrane with India ink (42).

Localization of GFP-Ste20p. Cells containing pRL116 or pPP828 were propagated for at least 24 h in selective liquid medium containing 2% dextrose and 0.008% adenine (to inhibit accumulation of red pigment in ade1 cells) at 30°C (GAL1p-CDC42 strains) or at 36°C (cdc42-1 strains). The cells were examined without fixation using a Nikon E600 epifluorescence microscope with 100× Plan...
Identification of recessive α-factor-resistant alleles of CDC42. A hypothetical cdc-42 mutant specifically defective in pheromone signal transduction should retain all functions necessary for cell polarity and proliferation but would fail to arrest in G₁ upon exposure to α-factor. Thus, cells expressing such an allele as the sole source of Cdc42p should proliferate and form colonies on plates containing a growth-inhibitory dose of α-factor. We employed an error-prone PCR mutagenesis strategy to generate random mutations in CDC42 and recombined the resulting alleles into a low-copy-number plasmid in cells whose
endogenous \textit{CDC42} was transcriptionally repressed (using the regulatable \textit{GALI} promoter; see Materials and Methods for details). Transformants that proliferate on \alpha-factor-containing plates were selected. Plasmids containing putative \textit{CDC42} mutants were isolated from the rare \alpha-factor-resistant colonies and retransformed into the starting strain to confirm that the \alpha-factor resistance was due to the plasmid and not to a fortuitous genomic mutation. Three independently derived mutants (\textit{cdc42-md1}, \textit{cdc42-md2}, and \textit{cdc42-md12} [md for mating pathway defective]) were selected for further characterization (Fig. 1).

In addition to promoting growth on \alpha-factor-containing plates (Fig. 1), strains bearing the \textit{cdc42-md} alleles were significantly, though not completely, defective in \alpha-factor-induced \textit{FUS1} transcription (see below) and in mating to a wild-type partner (data not shown). A possible explanation for the defect in \textit{FUS1} induction observed previously in temperature-sensitive \textit{cdc42} mutants is that the mutants accumulate at a nonrespiring stage (post-\textit{G}1) of the cell cycle (35). To address whether a similar situation might apply with \textit{cdc42-md} mutants, we isolated early-\textit{G}1-phase cells from \textit{cdc42-md1} and isogenic wild-type strains using centrifugal elutriation and monitored cell cycle progression and \textit{FUS1} mRNA accumulation upon treatment with different doses of \alpha-factor. As shown in Fig. 2A, \textit{cdc42-md1} cells synchronized in early \textit{G}1 phase displayed a defect in \textit{FUS1} induction compared to similarly treated wild-type cells, suggesting that these mutants are defective in responding to \alpha-factor even when the cells are in a responsive stage of the cell cycle. Flow cytometric analysis confirmed that both mutant and wild-type cells remained in \textit{G}1 for the duration of the 30-min \alpha-factor treatment (Fig. 2B). In addition, \textit{cdc42-md1} mutants progressed into \textit{S} phase (data not shown) and formed buds (Fig. 2C) with unaltered kinetics in the continuous presence of 0.015 \muM \alpha-factor, a concentration sufficient to completely arrest wild-type cells. However, 0.06 \muM \alpha-factor did cause \textit{cdc42-md1} cells to delay briefly in \textit{G}1 (Fig. 2C), indicating that the signaling defect is not complete (consistent with the \textit{FUS1} induction data). In other experiments, we have found that \textit{cdc42-md} mutants also show a \textit{FUS1} induction defect in cells arrested in \textit{G}1 by depletion of the \textit{G}1 cyclins \textit{Cln1p-Cln3p} (data not shown). Together, these data strongly suggest that the signaling defect of \textit{cdc42-md} mutants is not due to arrest at a nonresponsive stage of the cell cycle.

In principle, the \textit{cdc42-md} mutants could be defective for a normal function of Cdc42p in \alpha-factor signaling or they could encode forms of Cdc42p that can interfere with \alpha-factor signaling even though wild-type Cdc42p might not play a role in signaling. To distinguish between these options, we performed a dominance test to determine whether cells containing both wild-type and mutant forms of \textit{CDC42} were \alpha-factor resistant. As shown in Fig. 3A, the mutants were all recessive, in that cells containing plasmids expressing both wild-type and mutant Cdc42p were \alpha-factor sensitive. Intriguingly, very tiny colonies were reproducibly observed in strains containing both \textit{cdc42-md1} and \textit{CDC42} expressing plasmids. This appears to be a dose-dependent effect such that cells with a high \textit{cdc42-md1/CDC42} plasmid ratio are chosen for on \alpha-factor plates: when this experiment was repeated in cells with an integrated copy of \textit{cdc42-md1} and a plasmid-borne copy of \textit{CDC42}, no pheromone-resistant growth was observed (data not shown). The recessive behavior of these alleles suggests that Cdc42p wild-type function is required for an efficient pheromone response.

\textbf{Intragenic complementation analysis of the \textit{cdc42-md} mutants.} We took advantage of the recessive nature of these alleles to determine whether they had defects in the same or different functions required for the \alpha-factor response. Cells containing all pairwise combinations of plasmids bearing different \textit{cdc42-md} alleles were uniformly resistant to \alpha-factor (Fig. 3B), indicating that no mutant could provide the signaling function(s) defective in another and suggesting that all three alleles were defective for the same function.

In addition to the \alpha-factor-resistant phenotype, two of the mutants (\textit{cdc42-md2} and \textit{cdc42-md12}) displayed a slow-growth phenotype (Fig. 1A and 3A) (interestingly, growth of these mutants was actually stimulated by \alpha-factor), and all three mutants displayed aberrant cell morphologies when grown in the absence of \alpha-factor (Fig. 3C) (a detailed analysis of the morphological phenotypes will be presented elsewhere). Like \alpha-factor resistance, the slow growth and morphological phenotypes were recessive (Fig. 3A and data not shown). However, cells containing two plasmids expressing \textit{cdc42-md1} and \textit{cdc42-md2} or \textit{cdc42-md1} and \textit{cdc42-md12} but not \textit{cdc42-md2} and \textit{cdc42-md12} grew at wild-type rates and did not display morphological abnormalities (Fig. 3B and C and data not shown). This suggests that \textit{cdc42-md2} and \textit{cdc42-md12} share similar defects in vegetative growth while \textit{cdc42-md1} has a distinct defect: in cells containing both \textit{cdc42-md1} and \textit{cdc42-md2}, or \textit{cdc42-md1} and \textit{cdc42-md12}, all vegetative functions are provided by one of the alleles and cells grow normally. This finding of intragenic complementation for the vegetative-growth defects is in contrast to the lack of such complementation for the pheromone signaling defect (Fig. 3B), suggesting that each \textit{cdc42-md} mutation affects the same function(s) required for the \alpha-factor response.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Defective interaction of \textit{cdc42-md} mutants with Ste20p. (A) Altered localization of Ste20p in \textit{cdc42-md} strains. (B) The V36A substitution confers \alpha-factor resistance. Growth arrest assays of strain DLY3067 (\textit{GAL1}\textit{-}\textit{CDC42}) containing plasmid pMOSB55 (\textit{CDC42}), pMOSB36 (\textit{cdc42-md1}), or pMOSB177 (\textit{cdc42-V36A}) were performed. The photographs show growth after 3 days at 30°C with (+) or without (−) \alpha-factor. (C) Binding of Cdc42p to the Ste20p CRIB domain. The binding conditions were as described in Materials and Methods. The top row shows the amount of the indicated myc-tagged Cdc42p mutant that bound to immobilized GST-CRIB, the second row shows the amount of each Cdc42p mutant that bound to immobilized GST (to control for nonspecific adsorption), and the third row shows the amount of each Cdc42p mutant added to the binding reaction. India ink staining of the blots confirmed that equal amounts of GST or GST-CRIB were present in each lane. Similar results were obtained in four independent experiments. Recombinant Cdc42p was generated from pDLB1234 (D57Y, mimicking GDP-bound Cdc42p, pDLB1235 (Q61L, mimicking GTP-bound Cdc42p), pDLB1240 (V36A and Q61L), or pDLB1242 (Y40C and Q61L).}
\end{figure}
In the "effector" domain of Cdc24p: a valine-to-alanine substitution at residue 77 (Fig. 5A). Because the Y40C substitution was the only change in cdc42-md2, we assume that the α-factor resistance of cdc42-md12 also derives from this same change. Presumably, the V77A mutation confers the somewhat-improved growth properties observed in cdc42-md12 compared to those of cdc42-md2. In a recent study, cdc42Y40C was reported to be nonviable (18). The discrepancy appears to be due to the fact that in our case the mutant is expressed from a low-copy-number (CEN ARS) plasmid while in that study it was integrated into the genome. To determine which changes were responsible for the α-factor resistance of the cdc42-md1 mutant, we constructed a mutant that contained only the V36A substitution. This mutant also conferred an α-factor-resistant phenotype (although not as strong as that conferred by cdc42-md1 [Fig. 4 and 5B]). We conclude that a mutation at either V36 (to A) or Y40 (to C) in the effector domain of Cdc42p renders the protein signaling defective.

Defective interaction of Ste20p with cdc42-md mutants. The Y40C mutation has been described in human CDC42 (which is 80% identical to yeast CDC42 and complements a cdc42 null mutation in yeast [44]), where it was found to render Cdc42p defective in binding to and activating p21-activated kinase (PAK), a mammalian Ste20p homologue (19). Combined with epistasis analysis (Fig. 4), this suggested that the cdc42-md mutants might be defective in binding to Ste20p. Binding of PAK family kinases to Cdc42p is mediated by the CRIB domain in these proteins (8). We produced the Ste20p CRIB domain as a GST fusion protein in E. coli and purified it using glutathione Sepharose beads. The wild type or V36A or Y40C mutants of Cdc42p were produced as myc-tagged proteins in E. coli and tested for binding to the GST-Ste20p-CRIB beads or to GST beads as a negative control. A Q61L substitution, which inhibits Cdc42p GTPase activity, was also engineered into the constructs to ensure that the proteins were GTP bound (a prerequisite for CRIB domain binding). As shown in Fig. 5C, the V36A substitution conferred a mild defect in Ste20p binding while the Y40C substitution conferred a severe defect.

Ste20p shares a redundant essential function with the related PAK-family kinase Cla4p. The Cdc42p-Ste20p interaction has been reported to be critical for this function (23, 37). We found that cells containing cdc42-md mutants as the sole source of Cdc42p were synthetically lethal with cla4Δ mutants (but not with ste20A mutants [Fig. 6]), suggesting that these mutants fail to interact with Ste20p in vivo as well as in vitro.

Defective localization of Ste20p in cdc42-md mutants. Ste20p is concentrated at the bud tips of many cells during vegetative growth, and at the shmoo tips of cells responding to a α-factor and did not make shmooes. Suppression of the cdc42-md signaling defect by overexpression of Ste20p. If the α-factor resistance phenotype of the cdc42-md mutants is due to defective interaction with Ste20p, then overexpression of Ste20p might suppress that phenotype. Indeed, overexpression of STE20 substantially suppressed the α-factor-resistant growth of cdc42-md mutants (Fig. 8). In contrast, overexpression of the related CLA4 did not suppress the cdc42-md signaling defect, although it did suppress the growth defect of cdc42-md2 mutants in the absence of α-factor (Fig.
These findings could be accommodated by at least two models: the observed suppression may be due to a bypass of the _cdc42-md_ defects by the overexpressed kinases or to a restoration of a sufficiently effective interaction between the mutant Cdc42p and the kinases. In the latter model, the CRIB-binding defect of _cdc42-md2_ causes a vegetative growth defect due to impaired interaction with Cla4p and a signaling defect due to impaired interactions with Ste20p.

Restoration of pheromone signaling in _cdc42-md_ mutants by the _ste20ΔCRIB_ allele. The findings presented above are consistent with the simple hypothesis that the Cdc42p-Ste20p interaction is a critical step in the α-factor signaling pathway: the _cdc42-md_ mutants are defective for this interaction in vitro and in vivo, epistasis analysis suggests that Cdc42p acts at the same level of the pathway as Ste20p, and overexpression of Ste20p suppresses that the _cdc42-md_ signaling defect. Nevertheless, the hypothesis that the Cdc42p-Ste20p interaction is important for signaling is contradicted by previous studies showing that a _ste20_ mutant lacking the CRIB domain (and therefore defective in Cdc42p interaction) did not affect signaling (23, 37). A possible resolution of this apparent paradox is suggested by recent evidence that the CRIB domain of PAK family kinases is part of an autoinhibitory domain, leading to a model in which Cdc42p (or Rac) proteins activate PAK family kinases by “relief of autoinhibition” (3, 49, 52, 54). For example, in the Pak1 kinase of _Schizosaccharomyces pombe_, a region overlapping the CRIB domain of Pak1 binds intramolecularly to the catalytic domain, yielding a “closed” conformation that can be “opened” either by binding to Cdc42p or by mutations in the CRIB domain (49). By analogy, the _ste20ΔCRIB_ mutant may be competent for signal transduction because it no longer requires a normally critical interaction with Cdc42p for its activation. This hypothesis predicts that the _ste20ΔCRIB_ mutant should bypass the requirement for Cdc42p in signaling, rendering _cdc42-md_ strains sensitive to pheromone. Indeed, we found that _ste20ΔCRIB_ restored significant α-factor signal-
ing to the cdc42-md mutants, as assayed by growth inhibition (Fig. 9A) or FUS1 induction (Fig. 9B). This suggests that the ste20ΔCRIB allele encodes an activated form of Ste20p and supports the hypothesis that the Cdc42p-Ste20p interaction is normally important for pheromone signaling. We noticed, however, that ste20ΔCRIB did not completely restore α-factor sensitivity to cdc42-md mutants. This suggests that cdc42-md mutants have some defect in addition to the Ste20p activation defect.

**Contribution of Bem1p to Cdc42p- and Ste20-dependent signaling**. The BEM1 gene was discovered through its key role in polarity establishment (5, 9, 38). Subsequent studies showed that Bem1p can also modulate the pheromone response pathway, as Bem1p overexpression increases signaling while Bem1p removal decreases signaling (17, 29). Because the role of Bem1p in polarity establishment involves Cdc42p and Cdc24p (5, 38), we speculated that its role in pheromone-responsive signaling may also involve Cdc42p and therefore that the residual signaling defect observed in cdc42-md ste20ΔCRIB mutants might reflect reduced Bem1p function resulting from the cdc42-md mutations. Consistent with this hypothesis, we found that like cdc42-md ste20ΔCRIB mutants, bem1Δ ste20ΔCRIB mutants also displayed partial α-factor resistance (Fig. 10A) and reduced FUS1 induction (Fig. 10B) compared to ste20ΔCRIB single mutants. The signaling defect in bem1Δ ste20ΔCRIB mutants was also evident when the pathway was activated by membrane-targeted Ste5p but not when it was activated by the activated Ste11p derivative Ste11ΔN (Fig. 10C). This indicates that like Cdc42p, Bem1p acts downstream of Ste5p membrane recruitment to promote activation of Ste11p by Ste20p.

We also compared the effects of bem1Δ and ste20ΔCRIB mutations on signaling by Ste5p that was targeted to different subcellular locations (Fig. 10D). Remarkably, while Ste20pΔCRIB was mildly defective at activating Ste5p that was targeted to the plasma membrane (Ste5ΔN-CTM), it showed increased ability to activate Ste5p that was targeted primarily to internal membranes (Ste5ΔN-Sec22 [40]). Consequently, in ste20ΔCRIB cells, the four- to fivefold signaling advantage of plasma membrane-targeted Ste5p over internal-membrane-targeted Ste5p was eliminated. These results support the notion that Ste20pΔCRIB is an active but delocalized kinase. Loss of Bem1p did not mimic the effect of ste20ΔCRIB: instead, bem1Δ reduced the ability of Ste20pΔCRIB to support signaling by Ste5ΔN-Sec22 and Ste5ΔN-CTM to similar extents, indicating that Bem1p can affect Ste20p- and Ste5p-dependent signaling even when both components are mislocalized.

If a part of the signaling defect of cdc42-md mutants is due to defective Bem1p function, as argued above, then Bem1p overexpression might be expected to improve signaling in cdc42-md cells. Indeed, we found that a high-copy-number BEM1 plasmid substantially improved signaling in response to membrane-targeted Ste5p in cdc42-md1 and cdc42-md2 mutant strains (Fig. 11). Bem1p overexpression also suppressed the growth and morphology defects of cdc42-md mutants (data not shown). However, this cannot account for the improved signaling because a high-copy-number CLA4 plasmid (which also suppressed the growth and morphology defects) or introduction of both cdc42-md1 and cdc42-md2 (leading to complementation of the growth and morphology defects) failed to suppress the signaling defect (Fig. 11). Strikingly, Bem1p overexpression (but not CLA4p overexpression or cdc42-md combinations) also suppressed the Ste20p localization defect in cdc42-md mutants (Fig. 11). This result is consistent with previous studies indicating that Bem1p interacts with Ste20p (24) and strongly suggests that Bem1p is acting to promote signaling at the level of Ste20p, consistent with our epistasis results above. When combined, the introduction of Ste20pΔCRIB (to bypass the Ste20p activation defect) and excess Bem1p (to bypass a possible Bem1p defect) into the cdc42-md1 mutant completely suppressed its α-factor resistance phenotype (Fig. 12). Together, these data suggest that the signaling defect of cdc42-md mutants can be accounted for by their simultaneous failures to activate Ste20p and to promote Bem1p function.

**DISCUSSION**

A role for Cdc42p in signal transduction in response to α-factor. We have isolated cdc42 mutants that display recessive defects in α-factor signaling while retaining the ability to proliferate. All of these cdc42-md mutants had morphogenesis
Mants.

6-factor. The bars indicate the means were assayed in glucose medium after 90 min with (1) or without (m) FUS1-lacZ plasmid pSB231 plus either pMOSB45 (WT) or pMOSB42 (1-factor). (B) FUS1-lacZ induction by 1-factor. Strains harboring a FUS1-lacZ reporter plasmid (p3058) were treated with and without 0.1 μM 1-factor in duplicate for 1 and 2 h with similar results; the data were expressed as percent mean induced β-galactosidase activity in the wild-type (WT) strain (427 U at 1 h; 835 U at 2 h) and combined, with each bar representing the mean ± standard deviation (SD) of four measurements. (C) FUS1-lacZ induction by Ste5AN-CTM or Ste11AN was assayed in strains harboring p3058 and either pGFP-GS5-AN-CTM or pGS11AN-T after 4 h of induction with galactose; the bars indicate the means ± SD of four to six transformants, expressed as percent mean induced β-galactosidase activity in the WT strain (1,024 U for Ste5AN-CTM; 644 U for Ste11AN). (D) FUS1-lacZ induction in strains harboring p3058 and either pGFP-GS5-AN-CTM (Ste5AN-CTM) or pGFP-GS5-AN-Sec22 (Ste5AN-Sec22) was assayed after 4 h of induction with galactose; the bars indicate the means ± SD of eight transformants. The strains in all panels were DLY1 (WT), MOSY0252 (ste20ΔCRIB), JMY1128 (bem1Δ), and MOSY0270 (bem1Δ ste20ΔCRIB).

FIG. 10. BEM1 is important for signaling in ste20ΔCRIB mutant strains. (A) Growth arrest. The photographs show growth after 2 days at 30°C with (+) and without (−) 1-factor. (B) FUS1p-lacZ induction by 1-factor. Strains harboring a FUS1p-lacZ reporter plasmid (p3058) were treated with and without 0.1 μM 1-factor in duplicate for 1 and 2 h with similar results; the data were expressed as percent mean induced β-galactosidase activity in the wild-type (WT) strain (427 U at 1 h; 835 U at 2 h) and combined, with each bar representing the mean ± standard deviation (SD) of four measurements. (C) FUS1p-lacZ induction by Ste5AN-CTM or Ste11AN was assayed in strains harboring p3058 and either pGFP-GS5-AN-CTM or pGS11AN-T after 4 h of induction with galactose; the bars indicate the means ± SD of four to six transformants, expressed as percent mean induced β-galactosidase activity in the WT strain (1,024 U for Ste5AN-CTM; 644 U for Ste11AN). (D) FUS1p-lacZ induction in strains harboring p3058 and either pGFP-GS5-AN-CTM (Ste5AN-CTM) or pGFP-GS5-AN-Sec22 (Ste5AN-Sec22) was assayed after 4 h of induction with galactose; the bars indicate the means ± SD of eight transformants. The strains in all panels were DLY1 (WT), MOSY0252 (ste20ΔCRIB), JMY1128 (bem1Δ), and MOSY0270 (bem1Δ ste20ΔCRIB).

FIG. 9. ste20ΔCRIB largely restores 1-factor sensitivity to cdc42-md strains. (A) Growth arrest. Strains DLY3067 (GAL1p-CDC42 STE20) and MOSY0268 (GAL1p-CDC42 ste20ΔCRIB) were transformed with pMOB58 (wild type [WT]), pMOB36 (md1), pMOB37 (md2), or pMOB38 (md12), as indicated. The photographs show growth after 3 days at 23°C with (+) or without (−) 1-factor. (B) FUS1-lacZ induction. The same strains as in panel A harbored the FUS1-lacZ plasmid pSB231 plus either pMOSB45 (WT) or pMOSB42 (md1) and were assayed in glucose medium after 90 min with (+) or without (−) 0.01 μM 1-factor. The bars indicate the means ± standard deviations of four transformants.

defects in addition to their signaling defect. However, the signaling defect is unlikely to be an indirect result of the morphogenesis defects for the following reasons. First, overexpression of CLA4 suppressed the morphogenesis defects but not for the signaling defect. Second, tions from earlier studies with temperature-sensitive cdc42 mutants (46, 47, 53).

The conclusion that Cdc42p is important for 1-factor signaling is at odds with a study that found no signaling defect in G1-1-factor-induced signaling that is separate from its roles in morphogenesis, confirming the suggestions that Cdc42p is not required for 1-factor signaling and affects signaling indirectly as a result of cell cycle perturbation (35). However, an earlier study found Cdc42p and Cdc42p to be required for the maintenance of G1 arrest in cells that had been prearrested by α-factor (46), which is not explainable by a model in which the signaling defects of cdc42 and cdc42 mutants are due simply to accumulation of cells at a nonresponsible stage of the cell cycle.

While our observations support a role for Cdc42p in pheromone-responsive signal transduction, they do not suggest that Cdc42p is a pathway intermediate that gets activated in response to pheromone. Instead, our work is consistent with the view (40) that Cdc42p plays a permissive role in maintaining cellular competence for signaling. For instance, Cdc42p may be constitutively required for the establishment of relatively long-lived pre-signaling complexes containing active Ste20p
Transmission of the pheromone signal could then utilize this preactivated pool of Ste20p without any further immediate involvement of Cdc42p. In this hypothesis, the cdc42-md mutants have a signaling defect because they fail to establish this competent pool of Ste20p, while the temperature-sensitive cdc42-1 inactivation regimen used by Oehlen and Cross (35) did not result in a signaling defect because sufficient Ste20p activation was established prior to the temperature shift to allow subsequent signaling. Notable in this regard is evidence from human PAK family members that GTPase-dependent establishment of the open conformation allows for PAK autophosphorylation, which subsequently interferes with regeneration of the closed conformation and thus makes the kinase temporarily GTPase independent (30, 52).

Ste20p is a key Cdc42p target involved in pheromone signaling. By several criteria, the cdc42-md mutants are defective in interacting with Ste20p: they are defective (to varying extents) in binding to the Ste20p CRIB domain in vitro, they fail to localize Ste20p to bud tips in vivo, and they display synthetic lethality with cla4 mutants. Furthermore, epistasis analysis suggests that cdc42-md mutants are defective in signal transduction at the same step in the pathway as ste20 mutants (i.e., the activation of Ste11p following membrane targeting of Ste5p). Finally, the signaling defect of cdc42-md mutants is largely suppressed by overproduction of Ste20p. These data strongly support the hypothesis that the Cdc42p-Ste20p interaction is important for efficient signaling in the pheromone response pathway, as originally proposed (46, 53). However, more recent studies have argued that the Cdc42p-Ste20p interaction is dispensable for pheromone response, based on the signaling competence of a version of Ste20p (Ste20p_DCRIB) that cannot bind to Cdc42p. We found that Ste20p_DCRIB largely restored signaling competence to strains containing the cdc42-md mutants. This observation suggests that Ste20p_DCRIB is an activated version of Ste20p that no longer requires interaction with Cdc42p for its activation, consistent with current models for
PAK activation which involve a relief of autoinhibition mechanism (3, 49, 54). We therefore conclude that interaction of Cdc42p with Ste20p is normally required for Ste20p to participate in the pheromone response pathway. Importantly, however, we do not find it necessary to propose that pheromone regulates either GTP loading of Cdc42p, the Cdc42p-Ste20p interaction, or Ste20p kinase activity. Instead, the simplest model consistent with available data is that access of Ste20p to its substrates is the pheromone-regulated step, with the effect of Cdc42p on Ste20p kinase activity being a preexisting condition that is independent of pheromone exposure (see reference 40 for further discussion).

Cdc42p may play a second role together with Bem1p in pheromone signaling. Although Ste20p appears to be the major Cdc42p target for signal transduction, our results suggest the existence of a second role for Cdc42p in this process, because cdc42-md mutants displayed a residual signaling defect even in the presence of the activated ste20ΔCRIB allele. Given the strong links between Cdc42p and the SH3-domain-containing scaffold protein Bem1p that have been established through studies of cell polarity in yeast (5, 9, 38), we suspected that this second role might involve Bem1p (which has also been shown to modulate the strength of α-factor signaling [17, 29]). Consistent with this hypothesis, Bem1p overexpression partially suppressed the signaling defect of cdc42-md mutants and Bem1p overexpression together with ste20ΔCRIB could fully suppress the α-factor-resistant growth of cdc42-md mutants.

Our observations suggest that the cdc42-md mutants are defective in activation of the Ste11p-Ste7p-Fus3p MAPK cascade. This step involves the interaction of Gβγ with the Ste5p scaffold protein (associated with Ste11p, Ste7p, and Fus3p), resulting in recruitment of Ste5p to the plasma membrane (40) and also the interaction of Gβγ with Ste20p (25). These interactions likely serve to raise the local concentrations of Ste20p and its substrate Ste11p (bound to Ste5p), thereby initiating MAPK cascade activity. The previously described effects of Bem1p on pheromone signaling (20, 29) are compatible with its acting anywhere in the pathway from receptor to the MAPK Fus3p. Our experiments indicate that Bem1p affects steps following Ste5p membrane recruitment, since both loss and overproduction of Bem1p affects signaling by membrane-targeted Ste5p, even in cells with the activated ste20ΔCRIB allele. In contrast, signaling by the activated Ste11p derivative Ste11N was comparatively insensitive to the loss of Bem1p. We also observed that Bem1p can affect the localization of Ste20p, and previous studies showed that Bem1p could form complexes with both Ste20p and Ste5p (24, 29). Together, these results suggest that Bem1p influences activation of the Ste5p-associated kinase cascade by Ste20p, perhaps by helping to bring these proteins together. It should be noted that earlier work observed effects of Bem1p overproduction on the residual pheromone response of ste20Δ cells (20, 29); the mechanism for this residual response has not been determined, but it may rely on inefficient substitution for Ste20p by another PAK family kinase (e.g., Cla4p), which would be compatible with our suggestion that Bem1p affects the step in which Ste11p is activated by the Ste20p (or substitute) kinase.

The dramatic effect of Bem1p overexpression on Ste20p localization in cdc42-md mutants provides a suggestion as to how Bem1p may function. During vegetative growth, recruitment of Ste20p to the bud tip by Cdc42p may be assisted by Bem1p-mediated local retention of Ste20p. Similarly, during pheromone response, recruitment of Ste5p and Ste20p to a region of the plasma membrane by Gβγ may be assisted by local retention of these proteins promoted by Bem1p. Our results suggest some collaboration between Cdc42p and Bem1p in pheromone response. Thus, Cdc42p and Bem1p may each help to provide a cell surface scaffold that facilitates the association and/or local retention of Ste5p (with its associated kinases) and Ste20p, allowing the local concentrations of these signaling components to be raised above the threshold required for efficient signal transduction.

**Conclusions.** In aggregate, the analysis of the pheromone-resistant cdc42 alleles presented here combined with previous studies suggest a novel paradigm for the “signaling” role of Cdc42p that is quite distinct from the paradigm established for the ras GTPase. Cdc42p is required for Ste20p activation, but there is little evidence to suggest that pheromone signals stimulate this activation; instead, pheromone signaling may make use of a preexisting constitutive Ste20p activity. In addition, Cdc42p (acting with Bem1p) helps increase the local concentration of Ste20p together with other signaling components, promoting signal transduction by a proximity effect (36). Providing a surface conducive to the local concentration of signaling reactants could be a common contributor to eukaryotic signaling pathways, especially those that rely on regulatable protein-protein interactions rather than diffusible second messengers (16). It remains to be seen to what extent these types of signaling roles will be generally applicable for other Rho family GTPases and other cells.

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