Cdc42 Regulation of Kinase Activity and Signaling by the Yeast p21-Activated Kinase Ste20

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The Saccharomyces cerevisiae kinase Ste20 is a member of the p21-activated kinase (PAK) family of protein kinases. It was originally identified for its signaling role in the yeast mating pathway (2, 26, 49), though subsequently it has been found to function in other signaling pathways that regulate filamentous growth and osmotic stress response (31, 43, 48, 51), as well as in the control of actin organization and polarized growth (13, 16, 22, 55, 63). In the mating pathway, Ste20 mediates activation of a mitogen-activated protein (MAP) kinase cascade in response to extracellular mating pheromones (reviewed in references 15, 17, and 20). These pheromones bind to G protein-coupled receptors and trigger release of Gβγ dimers, which activate the MAP kinase cascade in a manner involving recruitment of the kinase cascade scaffold protein Ste5 to the plasma membrane (18, 36, 47, 62). This is thought to bring the Ste5-associated kinase Ste11 (a MAP kinase kinase) into close proximity with Ste20, which phosphorylates and activates Ste11 (60, 65). Ste20 is enriched at the cell periphery in both growing and mating cells via interaction with the membrane-bound GTPase Cdc42 (27, 39, 45, 47, 64). In addition, Ste20 binds the pheromone-activated Gβγ complex (29), potentially endowing Ste20 with increased kinase activity, increased access to Ste11, or both.

PAKs are commonly activated by small GTPases of the Cdc42/Rac family (4, 14). While Ste20 binds the GTPase Cdc42, there have been conflicting reports regarding the role of this interaction in mating pathway signaling. Early studies suggested that Cdc42 and its guanine nucleotide exchange factor Cdc24 were required for pheromone response (58, 67) and that GTP-bound Cdc42 could stimulate Ste20 kinase activity in vitro (58). Later studies suggested that the apparent requirement for Cdc24 and Cdc42 in pheromone response was an artifact of using conditional mutants that arrest at a nonresponsive position in the cell cycle (42). Furthermore, other studies concluded that while Cdc42 binding was required for proper localization of Ste20 and for its role in the filamentation and osmotic response pathways (27, 45, 48), it was not required for Ste20 kinase activity or for its role in pheromone response (27, 45). In these latter studies, the role of the Cdc42-Ste20 interaction was tested by removal of the entire Cdc42/Rac interaction binding (CRIB) domain from Ste20.

Recent studies of other PAK family members have suggested that these kinases are frequently regulated by an autoinhibitory mechanism that involves the CRIB domain. For some members of this family, including human PAK1/α-PAK and Schizosaccharomyces pombe Pak1/Shk1, it has been observed that the CRIB domain can bind to and inhibit the kinase domain (21), which has been confirmed recently for human PAK1 by crystallography (30). Furthermore, mutations in either domain that disrupt this intramolecular binding can lead to kinase activation (9, 59, 66, 68). In accord with this regulatory mechanism, recent mutational studies of yeast Cdc42 have reasserted its involvement in activating Ste20 for pheromone signaling, because mutant forms of Cdc42 that are impaired for binding Ste20 show defects in pheromone response, and these defects can be rescued by deletion of the Ste20 CRIB domain (39).

In this study, we have reinvestigated the role of the Ste20...
CRIB domain in mediating Cdc42 binding and pheromone-responsive signaling. Our results indicate that Ste20 kinase activity and signaling are limited by inhibitory binding between the CRIB and kinase domains. They further suggest that binding of Cdc42 to the Ste20 CRIB domain activates the kinase activity and signaling ability, indicating that Ste20 adheres to the generic model for PAK family kinases in which they are activated by small GTPases through relief of autoinhibition.

Table 1. Yeast strains used in this study

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<th>Strain background</th>
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<tr>
<td>W303&lt;sup&gt;a&lt;/sup&gt;</td>
<td>KBY211</td>
<td>MATa ste20::ADE2 clp4::LEU2 + YCP TRPI-1::75&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Other</td>
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</tr>
</tbody>
</table>

<sup>a</sup> W303 is ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1.

Yeast strains. Yeast strains are listed in Table 1. All two-hybrid assays were performed using strain PYP760 (11), a derivative of strain L40 (45). Filamentous assays used strains in the Σ1278b background, generated by crosses involving strains L5585 (51), L6149 (53), and meiotic segregants of strain L5978 (35). KBY211 (22) was described previously, as were PPY496, PPY640, PPY866, and PT2α (47). PPY913 was derived from YEL206 (27) by integration of a LEU2::FUS1-lacZ::ade2::hisG trp1::hisG ura3-52::TRP1 cassette from pSL2 (54) into the HI–HI fragment of pPP1219.

The materials and methods

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β-Galactosidase assays. For liquid assays, cells (usually 1 mL of culture) were harvested by centrifugation and then resuspended in 0.5 mL of Z-buffer (5) and processed as described before (46).

Pheromone response assays. Halo assays of growth arrest were performed by spreading 3 × 10^5 cells/plate on −Ura plates, then applying sterile filter disks containing 20 μl of 1 mM α-factor, and incubating for 3 days at 30°C.

For FUS1-lacZ transcriptional induction assays, cells were grown overnight at 30°C to mid-exponential phase (optical density at 660 nm [OD_{660}] = 0.3 to 0.6) in selective (plasmidless) or complete (integrated alleles) glucose medium and then incubated with and without 5 μM α-factor for 2 h before harvesting 1 mL for the β-galactosidase assay. For FUS1-lacZ activated by galactose-inducible constructs, cells were grown overnight in selective raffinose medium to the mid-exponential phase and then induced with 2% galactose for 3 h before harvest.

For quantitative mating assays, cells were grown overnight to the mid-exponential phase in selective or complete glucose medium, as appropriate, and then 5 × 10^4 a cells were mixed with 10^7 PT2a partner cells and collected onto filters, and the filters were placed onto complete glucose plates. After 4 h of mating at 30°C, cells on the filters were suspended in liquid, and serial dilutions were plated on medium selective for diploids. Mating efficiency was calculated as the percentage of input a cells that formed diploids; the number of input cells was determined by plating a dilution of the cell cultures on selective (plasmid) or complete (integrated) plates at the start of the mating assay.

Patch mating assays were performed by patching a cell transformants directly onto a lawn of PT2a partner cells, incubating overnight at 30°C, replicating to minimal medium to select for diploids, and then incubating for 2 days at 30°C.

Yeast cell lyases and kinase assays. Transformants were grown in selective medium overnight at 30°C to an OD_{660} of 0.5 to 1.0. For kinase assays involving Cdc42^{G12V}, transformants were first grown in −His/raffinose medium and then induced with galactose (2% final) for 90 min. Equivalent numbers of cells (usually 1 × 10^6 to 4 × 10^6) were harvested by centrifugation at 4°C, suspended in residual supernatant, transferred to an ice-cold microcentrifuge tube, and recentrifuged for 30 s in the cold; the supernatant was aspirated, and the pellets were stored at −80°C. Pellets were thawed by addition of 1 ml of ice-cold buffer A (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM dithiothreitol [DTT], 2 mM EDTA, 10% glycerol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF]), washed once with 1 ml of buffer B (buffer A containing 0.1% Triton X-100 and 1 mM NaVO_3, plus protease inhibitors [PIs; 20 μg of 4-(2-aminoethyl)-benzenesulfonyl fluoride, 2 μg of leupeptin, 1 μg of pepstatin, and 1 μg of aprotinin per ml]) kept cold throughout. Approximately 0.4 ml of glass beads was added, followed by three 1-min pulses at full speed in a multitube vortexer (VWR Scientific Products Corp.), with 30 s on ice between pulses. Additional Triton X-100 was added (40 μl of a 10% solution), and the lysate was mixed on a nutator for 10 min.

Following 2 min of centrifugation, the supernatant was transferred to a fresh tube and centrifuged for 4 min, and the new supernatant containing the clarified lysate was transferred to a fresh tube. For kinase assays involving Cdc42^{G12V/C188S}, transformants were first grown in −His/raffinose medium and then induced with galactose (2% final) for 90 min. Equivalent numbers of cells (usually 1 × 10^6 to 4 × 10^6) were harvested by centrifugation at 4°C, suspended in residual supernatant, transferred to a fresh tube, and centrifuged for 90 min. Equivalent numbers of cells (usually 1 × 10^6 to 4 × 10^6) were harvested by centrifugation at 4°C, suspended in residual supernatant, transferred to a fresh tube.
containing extra adenine (0.008%) to inhibit accumulation of flade2 camera (DAGE-MTI, Inc.).

and images were captured using a cooled black and white charge-coupled device function. A polyadenylation sequence was predicted to lie 149 bp downstream of the open reading frame (26).

c for at least 3 h before harvest.

° fl/H11003 epi trients to maintain plasmid selection) to an OD660 of 0.2 to 0.3 and incubating at diluting overnight cultures into fresh glucose medium (lacking appropriate nu-
reporter expression was measured in exponentially growing cultures, prepared by and after being rinsed under a gentle stream of deionized water. Filamentation C and then for 2 days at room temperature. Plates were photographed before

2942 LAMSON ET AL. MOL. CELL. BIOL.

2344 LAMSON ET AL. MOL. CELL. BIOL.

C for at least 3 h before harvest.

° fl/H11022

Microscopy. Transformsants were grown at 30°C in -Ura/glucose medium containing extra adenine (0.008%) to inhibit accumulation of fluorescent pig-
ment in ade2 cells. Cells were examined without fixation under a Nikon E600 epifluorescence microscope equipped with a 50× Plan oil-immersion objective, and images were captured using a cooled black and white charge-coupled device camera (DAGE-MTI, Inc.).

RESULTS

Pheromone-responsive signaling requires Cdc42-Ste20 interaction. Previous studies tested the role of the Cdc42-Ste20 interaction in pheromone response by deleting the entire Ste20 CRIB domain (27, 45). To reexamine this issue, we made point mutations at two highly conserved residues in the Ste20 CRIB domain (Fig. 1A and B), changing Ser338 to Ala (S338A) or His345 to Gly (H345G). Alteration of the homologous residues in human and S. pombe PAKs disrupts Cdc42 binding (59, 68). The Ste20 mutations were also combined to make an S338A/H345G double mutant.

To test for effects on binding, the mutations were introduced into a two-hybrid fusion construct containing Ste20 residues 1 to 499. To assay function, they were introduced into low-copy-number vectors encoding either native Ste20 or a GFP-Ste20 fusion protein, each expressed from the STE20 promoter. We found it useful to study the mutations in both expression contexts, as they appeared to confer different levels of function—e.g., the native constructs gave lower signaling and thus revealed the subtle defects of mild mutants, whereas the GFP constructs gave higher signaling and thus revealed the residual function of severe mutations (see Table 3, Materials and Methods, and also Fig. 2 below). For comparison to the point mu-
mutations, a CRIB domain deletion (Δ334–369) studied previ-
ously (27, 45) was introduced into all of the same vectors.

Cdc42 binding, measured by a two-hybrid assay (Fig. 1C), was disrupted by the point mutations to various degrees: S338A showed the mildest defect, H345G showed a stronger defect, and the S338A/H345G double mutant showed the strongest defect, which approximated that observed with the complete CRIB domain deletion (Δ334–369). These effects were specific to Cdc42, as the mutations did not interfere with binding to Bem1 in a similar assay (Fig. 1C). The subcellular localization of GFP-Ste20 harboring these mutations was consistent with loss of Cdc42 binding (27, 39, 45), as all mutants showed loss of the sharp peripheral fluorescence at bud tips (Fig. 1D), including that with the weakest Cdc42-binding de-
fect, S338A.

Importantly, we found that the point mutants showed defects in mating pathway function that paralleled their Cdc42-binding defects (Fig. 2A), with the S338A mutant showing mild defects, H345G showing stronger defects, and the S338A/ H345G mutant showing very strong defects that approached the null phenotype. This pattern was observed in all assays of mating pathway function, including growth arrest, transcriptional induction (FUS1-lacZ), and mating ability. In stark con-
trast to these point mutants, the mutant lacking the entire CRIB domain (Δ334–369) was largely signaling competent (e.g., roughly 50-fold-higher FUS1-lacZ induction and 1,000-fold-higher mating efficiency than the S338A/H345G mutant) despite negligible Cdc42 binding. These results suggest that interaction with Cdc42 is normally critical for Ste20 to function in the mating pathway, whereas deletion of the CRIB domain bypasses this requirement.

In order to rule out the possibility that these pheromone response defects result from disruption of binding between Ste20 and Gβγ (29), we assayed signaling that was initiated by a membrane-targeted derivative (Ste5-CTM) of the kinase cascade scaffold protein Ste5, which bypasses the requirement for Gβγ but still depends on Ste20 (47). In this setting (Fig. 2B), the CRIB domain mutants showed a spectrum of defects similar to those found when signaling was initiated by pheromone; in the most extreme case, FUS1-lacZ induction in the S338A/ H345G double mutant was reduced to roughly 1% of the wild-type level. These data are consistent with the pheromone response assays and show that the signaling defects are not caused by defective Ste20-Gβγ interaction. In addition, be-
cause of the absence of Ste4 (Gβ) and pheromone in these experiments, they demonstrate that the signaling role of the Cdc42-Ste20 interaction operates independently of receptor activation and does not require regulatory input from pheromone or Gβγ.

We also integrated into the genomic STE20 locus the two

<table>
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<tr>
<th>Strain</th>
<th>Plasmid*</th>
<th>Native STE20 sequence (bp)</th>
<th>STE20 allele</th>
<th>Mean FUS1-lacZ expressiona (U) ± SD</th>
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<td>2</td>
<td>Vector</td>
<td>0.05 ± 0.04</td>
</tr>
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</table>

a In some plasmids (*), STE20 was inserted into the pRS316 vector in an opposite orientation from the others. Conceivably, transcriptional termination is governed by vector sequences in all plasmids except pST2E0-5, and thus different downstream vector sequences in the two orientations may contribute to the different levels of function. A polyadenylation sequence was predicted to lie 149 bp downstream of the open reading frame (26).

b Mean β-galactosidase units ± SD (n = 12), measured after 2 h of exposure to 5 μM α-factor.

The STE20 open reading frame in pBTL56 is extended by two codons at the C terminus.
alleles with the strongest Cdc42-binding defects, S338A/H345G and Δ334–369. In both mating and transcriptional induction assays, these alleles showed opposing phenotypes: the S338A/H345G mutant was nearly null, while the Δ334–369 mutant was nearly wild type (Fig. 2C). These results largely confirm the results with plasmid-borne alleles, though one difference was notable: the ability of the Δ334–369 allele to approach wild-type levels of function was greatest when integrated (e.g., 76% of wild-type FUS1-lacZ), and when it was plasmid-borne, function was greater for the GFP fusion (40% of wild-type level) than for the native, non-GFP derivative (14% of wild-type level). Thus, whether the CRIB deletion form behaves as largely competent or as significantly defective can depend on the expression context (see also Table 3). This pattern is important when comparing the effects of CRIB domain deletion on different pathways (such as mating versus filamentation pathways; see below). For example, the conclusion that Ste20Δ334–369 is defective at diploid pseudohyphal growth (27) derived from a comparison of plasmids (pSTE20–5 versus pRS316-STE20A334–369) in which wild-type and mu-

FIG. 1. Mutations to test whether the Ste20 CRIB domain behaves as a Cdc42-regulated autoinhibitory domain. (A) Model for activation of Ste20, supported by this study, involving transition between low-activity (closed) and high-activity (open) conformations. The CRIB domain (grey) is indicated as having regions that bind Cdc42 (notch) or the kinase domain (bump). Asterisks denote residues mutated in this study. The Gβγ-binding domain was defined previously (29). (B) Alignment of CRIB domains in PAKs from S. cerevisiae (S.c.), S. pombe (S.p.) and Homo sapiens (H.s.). Residues identical in three or more proteins are boxed in black, and mutated residues are indicated (+). Also indicated are human PAK1 residues (30, 37, 66, 68) involved in binding Cdc42 (caret) or the PAK1 kinase domain (+). A consensus CRIB motif is shown at the bottom; sequences C-terminal to this motif are well conserved among PAKs (68) but not between PAKs and other Cdc42 targets, such as mammalian WASP and ACK or yeast Gic1 and Gic2 (8, 37). (C) Effects of CRIB domain mutations on binding to Cdc42 and Bem1, measured by two-hybrid assay. Wild-type (pB20N2) and mutant (pPP1059, pPP1060, pPP1061, and pPP1062) derivatives of a DNA-binding-domain fusion to Ste201–499 were coexpressed in PPY760 with activation domain fusions to Bem1157–551 (pRL51.1) or Cdc42G12V/C188S (pPP1027), or with vector (pGAD424). β-Galactosidase measurements (mean ± standard deviation [SD]; n = 4) were normalized to the wild-type (WT) Ste20 allele (= 100%) for each binding partner; values in parentheses are the mean units for the wild-type interactions. The negative control (control) gives the basal signal of pB20N2 with pGAD424 (mean units = 0.04); the mutant derivatives of pB20N2 gave similar basal signals (not shown). (D) Effects on subcellular localization. Representative fluorescence images of cells (PPY913) expressing the indicated GFP-Ste20 mutants or carrying only a vector (pRS316).
tant alleles were expressed in different contexts, and which we found also differed by roughly 15-fold for FUS1-lacZ induction (see Table 3). Overall, our observations indicate that deletion of the CRIB domain creates a hypomorphic form of Ste20 that is mildly compromised for mating pathway signaling, to a degree that may be enhanced in contexts of reduced expression.

Cdc42 binding is required to antagonize autoinhibitory interaction between Ste20 CRIB and kinase domains. To address the possibility that something special about the extensive size of the Δ334–369 deletion allows it to bypass the requirement for Cdc42 binding or that something detrimental about the point mutations makes them defective for reasons unrelated to Cdc42 binding, we tested whether the S338A/H345G double mutant could be rescued by a third point mutation at Ste20 residue Leu369 (L369G), at the downstream end of the CRIB domain (see Fig. 1A and B). In other PAKs, mutation at the analogous residue interferes with intramolecular interaction between the CRIB and kinase domains (9, 59, 66). Thus, we predicted that if Ste20 can adopt an autoinhibited conformation, the L369G mutation might disrupt this conformation and render Ste20 Cdc42 independent.

Indeed, the L369G mutation on its own had little effect on either Cdc42 binding or signaling by Ste20, but it showed a dramatic ability to suppress the signaling defects of the S338A/H345G mutation, and did so without restoring Cdc42 binding (Fig. 3A). This indicates that the Cdc42-Ste20 interaction is primarily required to relieve the negative influence of Leu369 and that loss of Leu369 is sufficient to explain the Cdc42-independent behavior of the larger Δ334–369 deletion.

Consistent with these observations, we were able to detect a specific interaction between the Ste20 C-terminal kinase domain and an N-terminal fragment containing the CRIB domain in a two-hybrid assay (Fig. 3B and C). Importantly, this interaction was eliminated by each of the CRIB domain mutations that conferred Cdc42-independent signaling—i.e., the large deletion (Δ334–369) and the L369G point mutation. In contrast, the interaction was not disrupted by the double mutation (S338A/H345G), which produced the most severe pheromone response defect (and which actually increased the CRIB-kinase interaction signal, perhaps by reduced interference from endogenous Cdc42). Therefore, the Ste20 CRIB domain interacts both with Cdc42 (via S338 and H345) and

FIG. 2. Interaction between the Ste20 CRIB domain and Cdc42 is required for mating pathway function, in a manner that is bypassed by CRIB domain deletion. (A) CRIB domain point mutations reduce pheromone response in proportion to their Cdc42-binding defect. Strain PPY913 harbored a vector (pRS316) or the indicated STE20 plasmids in either native (pPP1001-based) or GFP fusion (pRL116-based) contexts. Tests of pheromone-induced growth arrest (left) and FUS1-lacZ expression (middle) and of mating efficiency (right) were done as described in Materials and Methods. (B) Signaling role of the Cdc42-Ste20 interaction is independent of pheromone and Gβγ. FUS1-lacZ induction was stimulated without addition of pheromone and in the absence of Gβγ (Ste4) by galactose-induced synthesis of Ste5-CTM (pH-G55-CTM) in an ste4Δ ste5Δ ste20Δ strain (PPY866). The strain also harbored a vector (pRS316) or the indicated GFP-STE20 mutant plasmid. (C) Mating and FUS1-lacZ results using alleles integrated at the genomic STE20 locus. Strains were PPY496, PPY640, PPY1205, and PPY1203. αF, α-factor. In A to C, bars indicate mean ± SD for three or more repeats.
with the kinase domain (via L369), and the role of the former (Cdc42-CRIB) interaction is to antagonize the negative effect of the latter (CRIB-kinase) interaction.

**Cdc42 binding stimulates Ste20 kinase activity by relief of autoinhibition.** We tested the kinase activity of all of the Ste20 mutants upon immunoprecipitating the GFP fusion derivatives from cell lysates using anti-GFP antibodies (Fig. 4A). We found that kinase activity was elevated three- to fourfold for the three mutants whose signaling properties suggested they are Cdc42 independent, namely, Ste20<sub>H334–369</sub>, Ste20<sub>H338A/H345G/L369G</sub>, and Ste20<sub>L369G</sub>. This hyperactivity supports the contention that these mutations disrupt an autoinhibited conformation of Ste20, although it was not observed in previous studies of Ste20 deleted for the CRIB domain (see Discussion).

Surprisingly, we did not observe reduced kinase activity with the signaling-deficient mutants (Fig. 4A), even for the mutant showing the strongest signaling defect in vivo, S338A/H345G. We considered the possibility that only a small fraction of wild-type molecules are fully active at any one time in the cell, and thus any reduction in this fraction may be masked by an excess of low-activity molecules or perhaps by molecules that become activated by unfolding during cell lysis. Therefore, we tested whether the level of active Ste20 in vivo could be boosted by increasing the amount of GTP-bound Cdc42, using the GTPase-deficient mutant Cdc42<sub>Q61L</sub> (Fig. 4B). Indeed, galactose-induced expression of Cdc42<sub>Q61L</sub> stimulated the kinase activity of wild-type Ste20 (Ste20<sub>WT</sub>). In contrast, Ste20<sub>S338A/H345G</sub> could not be stimulated, whereas Ste20<sub>H334–369</sub> activity was constitutively elevated and was not further increased by Cdc42<sub>Q61L</sub>. These results show clearly that the kinase activity of Ste20 can be stimulated by Cdc42 in vivo. Furthermore, they suggest that the signaling defects of Ste20<sub>S338A/H345G</sub> result from an inability to become stimulated by Cdc42, whereas the Cdc42-independent kinase activity of Ste20<sub>H334–369</sub>(or Ste20<sub>H338A/H345G/L369G</sub>) allows signaling without binding Cdc42.

We also took advantage of our ability to detect Cdc42-mediated stimulation of Ste20 to revisit the issue of whether pheromone stimulates Ste20 kinase activity, but we found no effect (Fig. 4B, far right lane). This is consistent with a previous report (65), and hence there remains no evidence that pheromone can stimulate Ste20 kinase activity. Moreover, because it did not mimic expression of Cdc42<sub>Q61L</sub>, pheromone treatment also does not produce an obvious increase in the levels of GTP-bound Cdc42. While it remains possible that such effects do occur (see Discussion), the alternative scenario raised by these observations is that Cdc42 and Ste20 activities per se may be uninfluenced by pheromone.

**L369G highlights separable roles for Cdc42 in Ste20 kinase activity and function.** It has been observed previously that overexpression of a fragment encoding only the kinase domain of Ste20 (Ste20ΔN) is toxic due to disruption of actin organization (27, 49). Consistent with their hyperactive ki-

![FIG. 3](http://mcb.asm.org/10.1128/MCB.22.10.2940-2953.2002)
nase activity, we found that Ste20\textsuperscript{S334–369}, Ste20\textsuperscript{L369G}, and Ste20\textsuperscript{S338A/H345G/L369G} were also toxic when overexpressed from the \textit{GAL1} promoter, whereas wild-type Ste20 and the signaling-deficient mutant Ste20\textsuperscript{S338A/H345G} were not (Fig. 5A).

Despite their similar kinase activities and toxicities, however, the L369G mutant differs from the other two hyperactive mutants in that it retains the ability to bind Cdc42. This prompted us to ask whether Cdc42 binding might still contribute to Ste20 function—e.g., by localization—even when kinase activity is deregulated. Two phenotypes indicate that it does.

**FIG. 4. Ste20 kinase activity and stimulation by Cdc42-GTP.** (A) Hyperactive kinase activity displayed by Cdc42-independent Ste20 mutants. Strain PPY913 was transformed with the indicated \textit{GFP}-\textit{STE20} plasmids or pRS316 (vector). Ste20 mutant proteins were immunoprecipitated with anti-GFP antibodies and tested for kinase activity by using myelin basic protein (MBP) as the substrate. Portions of the kinase reactions were also analyzed by anti-GFP immunoblot to determine protein levels. Bottom, quantification of two independent experiments (mean ± range), normalized in each experiment to the level of \textsuperscript{[32P]}MBP in the wild-type (WT) sample (= 1). (B) Cdc42-GTP stimulates kinase activity of wild-type but not mutant Ste20 derivatives. Ste20 kinase activity was assayed using strains PPY1234, PPY1238, and PPY1236, expressing Myc\textsubscript{12}-tagged wild-type, S338A/H345G, and \textDelta S34–369 derivatives of Ste20, respectively. The strains harbored a vector (pRS413) or a galactose-inducible \textit{CDC42Q61L} construct (pHG42-L61), as denoted by – and + \textit{GAL-CDC42Q61L}, respectively. Transformants were induced with 2% galactose for 90 min in the absence (−) or presence (+) of 10 μM α-factor as indicated. The Myc\textsubscript{12}-tagged proteins were immunoprecipitated, assayed for kinase activity, and tested for Ste20–Myc protein levels, as for panel A. Bottom, quantification of two independent experiments as in panel A.

**FIG. 5. Additional contribution of Cdc42 to Ste20 function, separable from kinase activity.** (A) Hyperactive kinase mutants are toxic when overexpressed. Strain PPY398 was transformed with pPP1219, pH166, pPP1248, pPP1272, pPP1274, or pPP1273, and then fivefold serial dilutions were spotted onto −His/glucose or −His/raffinose/galactose plates, as indicated, and incubated for 3 days at 30°C. (B) Loss of Cdc42-binding, not deregulated Ste20 activity, disrupts the Clα4-redundant essential function of Ste20. Strain KBY211 (ste20\textsuperscript{Δ} clα4\textsuperscript{Δ} YCp-TRP1-clα4–75°) was transformed with the indicated \textit{GFP}-\textit{STE20} plasmids, and then fivefold serial dilutions were spotted onto −Ura plates and incubated for 3 days at 25 or 37°C, as indicated. (C) Loss of Cdc42 binding decreases Ste20 signaling activity even when kinase activity is already made Cdc42 independent by the L369G mutation. Strain PPY866 harbored the indicated \textit{GFP}-\textit{STE20} plasmids plus either pH-GS5-CTM (Ste5-CTM) or pH-SL2 (Ste5\textsuperscript{P44L}-GST). Bars show \textit{FUS1-lacZ} induction (mean ± SD, n = 3) after galactose-induced synthesis of the indicated Ste5 derivative.
First, we compared the CRIB domain mutants for their ability to perform an essential cell polarization function (13, 22) that is redundant with a related kinase, Cla4 (Fig. 5B). Previous work showed that this function was impaired by removal of the Ste20 CRIB domain (27, 45), which, in light of our results, could indicate that cells lacking Cla4 are sensitized to either delocalization or hyperactivity of Ste20. We found that the Ste20L369G mutant was distinguishable from the other two hyperactive mutants in that it remained capable of supporting growth when Cla4 was inactivated. This suggests that it is delocalization, not deregulated kinase activity alone, that causes loss of the essential (Cla4-redundant) function in the Ste20L369G mutant. Both mutant kinases were defective in other reporter assays (e.g., (53)); it is included here to illustrate the range of transcriptional effects caused by removal of the Ste20 CRIB domain, from no effect (TyFRE and FLO11) to moderate effect (flo11[10/9]), to mild effect (flo11[10/9]), to moderate effect (YL042C and KSS1).

Our results with the CRIB deletion mutants were surprising in light of prior reports that concluded it was defective for filamentation pathway function (27, 45). Previous tests used plasmid-borne STE20 alleles, which may enhance mild signaling defects (see above). We were unable to observe robust agar invasion using plasmid-borne alleles (data not shown), but we did compare them for transcriptional reporter expression (Fig. 6C). These tests yielded a pattern similar to that found for the integrated alleles, with the S338A/H345G double mutant approximating the null phenotype and the Δ334–369 mutant showing an intermediate phenotype in which the severity was dependent on the reporter—i.e., either no defect (TyFRE), a mild defect (flo11[10/9]), or a stronger defect (YL042C).

As seen with mating pathway signaling, defects in expression of filamentation reporters (e.g., YLR042C) were more pronounced for the Δ334–369 mutant when plasmid borne (39% of wild-type level) than when integrated (72% of wild-type level). More importantly, the strong defects in filamentation pathway signaling observed for the S338A/H345G double mu-
tant were suppressed by the L369G mutation, with the resulting triple mutant matching the Δ334–369 derivative for all reporters (Fig. 6C). This shows that for filamentation, as for mating, Cdc42-Ste20 binding is primarily required to antagonize inhibitory CRIB sequences. In total, our results indicate that rather than being qualitatively distinct (27, 45), the role of the Cdc42-Ste20 interaction is highly similar between the mating and filamentation pathways.

**DISCUSSION**

In this study we have readdressed the signal transduction role of the Cdc42-binding (CRIB) domain in Ste20, a yeast PAK family kinase. We provide genetic and biochemical evidence that Cdc42-Ste20 binding regulates Ste20 kinase activity and signaling competence. Point mutations in the CRIB domain decrease pheromone response to a degree proportional to their Cdc42-binding defect (Fig. 2), suggesting that the Cdc42-Ste20 interaction is normally critical for pheromone signaling. This conclusion contrasts with that made previously from studies in which larger deletions were used to remove the Cdc42-binding site (27, 45). In retrospect, those studies appear to have bypassed the Cdc42 requirement (39) rather than show it is unnecessary, because the deletions also removed inhibitory residues immediately downstream of the CRIB consensus motif.

Indeed, we found here that the Ste20 CRIB domain interacts with the kinase domain and disruption of this interaction by mutation relieves the requirement for Cdc42 binding (Fig. 3). Our results overwhelmingly support a model in which Cdc42 activates Ste20 by antagonizing the negative influence of sequences within the CRIB domain (see Fig. 1A and 7). They also complement the recent data for Cdc42 mutants that show defects in both Ste20 binding and pheromone response (39) and are consistent with other recent studies on PAKs from other organisms (9, 30, 59, 66). We suggest that the basic mechanism proposed for GTPase-mediated regulation of PAKs (reviewed in reference 21) is shared by Ste20, indicating that Ste20 continues to serve as a model for PAK function. Our results with Ste20 are equally compatible with models in which autoinhibition occurs intramolecularly (in cis) or intermolecularly between members of a homodimer (in trans), as shown recently for mammalian PAK1 (44).

Interestingly, PAKs are not alone among targets of Rho family GTPases in being regulated by conversion between autoinhibited and uninhibited forms, generically termed intrasteric regulation (25). Instead, this mechanism appears to be common among many kinase and nonkinase targets of Rho, Rac, and Cdc42 (7, 21, 24, 30). It is also notable that the yeast pheromone response pathway (for a review, see reference 15) repeatedly uses a strategy in which positive activation is accomplished by antagonism of a negative regulator: (i) ligand-bound receptor activates Gβγ by inhibiting the negative effect of the Go subunit; (ii) Cdc42 activates Ste20 by inhibiting the negative effect of the Ste20 CRIB domain; (iii) Ste20 activates Ste11 by inhibiting the negative effect of the Ste11 N terminus; (iv) Fus3 and Kss1 activate Ste12-mediated transcription by inhibiting repressors of Ste12, Dig1/Rst1, and Dig2/Rst2; and (v) release of an interaction between the N and C termini of Ste5 may also contribute to activation of the MAP kinase cascade (54).

We observed hyperactive kinase activity in all Ste20 mutants with Cdc42-independent signaling ability (Fig. 4). The level of hyperactivity (three- to eightfold) is similar to that observed with another yeast PAK, Cla4 (6), whereas each of these is relatively modest compared to mammalian PAKs bearing analogous mutations (9, 66). While our results are consistent with those for other PAKs, they were not observed in previous studies of Ste20 mutants lacking the CRIB domain (27, 45). It is not clear why our results were different, though they were consistent regardless of whether an N-terminal GFP tag or a C-terminal Myc tag was used to purify Ste20. It is possible that in prior studies, the wild-type protein was artificially activated by unfolding to the “open” conformation, either during preparation of cell lysates or by binding of polyclonal anti-Ste20 antibodies (27); alternatively, overexpression may have made Ste20 resistant to negative regulation (45). It is also conceivable that in our experiments the wild-type protein became inactivated during lysate preparation by a mechanism to which the Δ334–369 mutant is insensitive (e.g., refolding); if so, this may have simultaneously obscured differences between the wild type and the signaling-deficient mutants (e.g., S338A/H345G).

Regardless of the explanation, our results indicate that there
are measurable differences in kinase properties between wild-type Ste20, signaling-deficient mutants, and Cdc42-independent mutants which are likely to be of fundamental importance to signaling. The toxicity resulting from overexpression of the hyperactive mutants (Fig. 5A) independently suggests that the kinase hyperactivity observed in vitro reflects real changes in kinase properties in vivo.

We also found that Ste20 kinase activity is stimulated by expression of GTP-bound Cdc42 in vivo. This has not been reported previously for S. cerevisiae; stimulation in vitro was observed using baculovirus-produced proteins (58), but this was not reproduced using Ste20 purified from yeast extracts (27). Most importantly, we found that the signaling defect of the S338A/H345G mutant correlated with an inability to respond to Cdc42 stimulation, while the ability of L369G or Δ334–369 mutations to confer Cdc42-independent signaling correlated with deregulated, Cdc42-independent kinase activity. Thus, our observations link the Cdc42 dependence of Ste20 kinase activity to the in vivo signaling behavior and argue that signaling by wild-type Ste20 requires that its kinase activity be stimulated by Cdc42.

How is Ste20 kinase activity harnessed by the mating pathway? It is informative that, despite their hyperactive kinase activity, the Cdc42-independent alleles Δ334–369, L369G, and S338A/H345G/L369G do not show constitutive signaling—i.e., there is no increase in either the basal or induced levels of FUS1-lacZ (Fig. 2C and data not shown; see also reference 27). While this may appear counterintuitive, it is in fact consistent with our previous arguments (39, 47) that the rate-limiting step in pheomon signaling is unlikely to be the production of active Ste20, but rather the access of Ste20 to its substrate—namely, the MAP kinase kinase kinase Ste11 associated with the scaffold protein Ste5 (18, 47)—perhaps along with other events, such as conformational changes in Ste5 (54). Consistent with this view, there is no indication that pheromone can stimulate Ste20 kinase activity, as noted previously (65) and confirmed here in parallel with experiments in which Ste20 kinase activity clearly could be stimulated by another method, expression of Cdc42Q61L (Fig. 4B). The fact that pheromone did not mimic Cdc42Q61L expression may also indicate that the levels of GTP-bound Cdc42 are not increased by pheromone, despite the known ability of Gβγ to assemble with the Cdc42 exchange factor, Cdc24. While association of Gβγ with Cdc24 helps guide cell polarization along pheomon gradients (11, 40, 41), it is unresolved whether Gβγ alters only the localization or also the activity levels of Cdc24 and Cdc42, but currently there is no evidence for the latter.

It remains conceivable that pheromone effects on Cdc24, Cdc42, and Ste20 activities do occur but are difficult to detect because they either are extremely labile (e.g., GTP hydrolysis) or involve only a small fraction of molecules. Nevertheless, it seems clear that assembly of complexes involving Gβγ and Cdc24 is not necessary for pheromone to induce Ste20-dependent signaling, as mutants in which these complexes are disrupted are fully competent at pheromone response (41). Furthermore, we show here (Fig. 2B and 5C) that the effects of disrupting the Cdc42-Ste20 interaction are apparent without pheromone stimulation and in the absence of Gb (Ste4), which, along with other observations (39, 47), suggests that the Cdc42-dependent step normally precedes pathway activation rather than requiring regulation by pheromone or Gβγ. Therefore, in total these findings indicate that although both the Cdc42-Ste20 interaction and Ste20 kinase activity are required for pheromone-dependent signaling, pheromone stimulation of these states is neither required nor evident.

The simplest model would seem to be that pheromone regulates the ability of an existing pool of active Ste20 to activate the downstream MAP kinase cascade (Fig. 7). It is noteworthy in this regard that overexpression of Ste20ΔN, an “activated” form of Ste20 lacking its N-terminal 495 residues, induces FUS1-lacZ to relatively miniscule levels compared to the level when pheromone is added (27); this suggests that pheromone still triggers a critical rate-limiting event, such as phosphorylation of Ste11 by Ste20 (60, 65) or expedited signal transmission from activated Ste11 (18).

Related to the issue of how pathway stimuli harness Ste20 activity, our observations suggest that the role of the Cdc42-Ste20 interaction is not qualitatively distinct between the mating and filamentation pathways, in contrast to previous conclusions (27, 45). Instead, we found that the two pathways are impacted similarly by CRIB domain mutations: (i) precise disruption of the Cdc42-Ste20 interaction by point mutation causes a severe reduction in function, and (ii) these defects are suppressed by either the L369G point mutation or the complete CRIB domain deletion, though not to wild-type efficacy for either pathway. These observations indicate that both pathways require Cdc42 to bind Ste20 and that in each pathway a primary role for this binding is to antagonize the negative effect of the Ste20 CRIB domain (Fig. 7). In addition, when the CRIB domain is completely removed from Ste20, producing a hyperactive kinase, signaling in both pathways is hyperactive but instead is demonstrably less efficient (to a degree that is exaggerated by reduced expression). It seems likely that this handicap is due to delocalization from the plasma membrane, where activation of the mating pathway MAP kinase cascade is thought to be initiated (36, 47, 61). Whether activation of the filamentation pathway MAP kinase cascade is also initiated at the membrane is less clear, though interactions of Ste11 and Ste7 kinases with the polarity proteins Spa2 and Sph1 (52, 56) could potentially play a role in restricting filamentation pathway signaling to the cell periphery. Proper localization is also critical to the essential function shared by Ste7 and Cla4, as removal of the Cdc42-binding site disrupts this function in either kinase (6, 27, 45) (Fig. 5B). Relatedly, excess levels of hyperactive Ste20 are lethal (27, 49) (Fig. 5A) and produce a depolarized actin phenotype (27), similar to when Ste20 and Cla4 are absent (22) or when Cdc42 is inactivated (1). This requirement for not only PAK activity per se but also its proper spatial regulation is consistent with the fundamental asymmetry of cytoskeletal reorganization events regulated by Cdc42 during cell growth and division (23).

Although Ste20Δ334–369 and Ste20S338A/H345G/L369G are detectably impaired for signaling (e.g., in comparison to the Ste20Q61L/CDC24 form, which retains Cdc42-binding; cf. Fig. 2A versus 3A and see Fig. 5), it is somewhat surprising how well they do signal, given their delocalization. It is conceivable that the kinase hyperactivity of these forms compensates for their delocalization to an extent that is purely coincidental. It is also possible that these forms are still restricted to signaling in specialized subcellular locales, in a manner assisted by inter-
actions with other proteins. Indeed, the signaling efficiency of Ste20 \( ^{334} \)–\( ^{369} \) appears to depend on interaction with the SH3 domain protein Bem1, as it is reduced by deletion of BEM1 (39) and by mutation of a Bem1-binding domain within Ste20 (M. J. Winters and P. M. Pryciak, unpublished data). This may indicate that while Ste20 \( ^{334} \)–\( ^{369} \) is delocalized, it might still signal predominantly at the cell periphery, where Bem1 is abundant (3). Ste20 also binds the phenome-activated Gβγ complex (29), potentially allowing recruitment of Ste20 to its mating pathway substrates in a manner that compensates to some degree for defects in localization via Cdc42.

Further work will be required to determine the full spectrum of mechanisms involved in colocalizing activated Ste20 with its substrates and to describe the complete set of events triggered by a specific pathway stimulus that are rate limiting for Ste20-dependent signal transduction.

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