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Functional analysis of the interaction between the small GTP binding protein Cdc42 and the Ste20 protein kinase in yeast

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***STE20* encodes a protein kinase related to mammalian p65[Pak] which functions in several signal transduction pathways in yeast, including those involved in pseudohyphal and invasive growth, as well as mating. In addition, *Ste*20 plays an essential role in cells lacking *Cla*4, a kinase with significant homology to Ste20. It is not clear how the activity of Ste20 is regulated in response to these different signals *in vivo*, but it has been demonstrated recently that binding of the small GTP binding protein Cdc42 is able to activate Ste20 *in vitro*. Here we show that Ste20 functionally interacts with Cdc42 in a GTP-dependent manner *in vivo*: Ste20 mutants that can no longer bind Cdc42 were unable to restore growth of *ste20 cla4* mutant cells. They were also defective for pseudohyphal growth and agar invasion, and displayed reduced mating efficiency when mated with themselves. Surprisingly, however, the kinase activity of such Ste20 mutants was normal when assayed *in vitro*. Furthermore, these alleles were able to fully activate the MAP kinase pathway triggered by mating pheromones *in vivo*, suggesting that binding of Cdc42 and Ste20 was not required to activate Ste20. Wild-type Ste20 protein was visualized as a crescent at emerging buds during vegetative growth and at shmoo tips in cells arrested with α-factor. In contrast, a Ste20 mutant protein unable to bind Cdc42 was found diffusely throughout the cytoplasm, suggesting that Cdc42 is required to localize Ste20 properly *in vivo*.**

Keywords: actin/Cdc42/MAP kinase pathway/Ste20/ yeast mating

Introduction

Mitogen-activated protein kinase (MAPK or ERK) signal transduction pathways are critical for many developmental events in eukaryotes, and are mediated by the sequential activation and phosphorylation of a series of protein kinases. Activation of MAPK requires phosphorylation by MAPKK/ERK kinase (MEK or MAPKK), which in turn is regulated by another kinase—Raf, Mos or a group of structurally related kinases termed MEKK or MAPKKK (for review, see Blumer and Johnson, 1994; Herskowitz, 1995). In mammalian cells, MAPKs are important for a wide array of cytokine and growth factor responses (Marshall, 1994). For example, the protein kinase Raf is regulated downstream of receptor tyrosine kinases in a process involving the small GTPase Ras and its regulator Sos (Blenis, 1993).

In yeast *Saccharomyces cerevisiae*, several independent MAP kinase pathways have been characterized (Ammerer, 1994; Levin and Errede, 1995). One pathway mediates the response to hyperosmotic conditions through the MAP kinase pathway homologs Pbs2 and Hog1, which stimulate glycerol accumulation (Brewster *et al.*, 1993). A second pathway controls cell wall biosynthesis through Pkc1 and, moreover, a MAP kinase pathway is required during sporulation (Krisak *et al.*, 1994). The best studied MAP kinase pathway controls mating of haploid cells; induction of the kinase cascade by pheromones leads to transcriptional activation of mating-specific genes, cell cycle arrest in G_1 and changes in cell morphology as a prelude to conjugation. Binding of pheromone to a seven transmembrane receptor results in GDP–GTP exchange on a heterotrimeric G-protein, which then activates the MAP kinase cascade composed of Ste11 (MEKK), Ste7 (MEK) and two partially redundant MAPKs, Fus3 and Kss1 (Herskowitz, 1995). Either of these two MAPKs is able to regulate the transcription factor Ste12, which, together with Mcm1, induces the expression of several mating-specific genes (Dolan *et al.*, 1989; Errede and Ammerer, 1989; Oehlen *et al.*, 1996). In addition, Fus3 also phosphorylates the inhibitor Far1, required for cell cycle arrest (Peter *et al.*, 1993; Tyers and Futcher, 1993).

It is unclear how the heterotrimeric G-protein is coupled to the MAP kinase module. Ste5 recently has been shown to interact directly with the G-protein, raising the possibility that Ste5 might function upstream of the kinase cascade (Whiteway *et al.*, 1995). Ste5 also interacts with several proteins of the MAP kinase cascade and is thus thought to provide a structural scaffold during signal transduction (Choi *et al.*, 1994; Marcus *et al.*, 1994; Printen and Sprague, 1994). The protein kinase Ste20 has been implicated in the activation of Ste11; cells lacking *STE20* exhibit a severe mating defect and fail to activate the mating pathway in response to α-factor (Leberer *et al.*, 1992; Ramer and Davis, 1993). Epistasis analysis shows that Ste20 functions between the heterotrimeric G-proteins and the MAP kinase module (Leberer *et al.*, 1992; Akada *et al.*, 1996). Furthermore, Ste20 is able to phosphorylate Ste11 directly *in vitro* (Wu *et al.*, 1995).

Components of the mating signal transduction pathway are also required for the formation of pseudohyphae and invasive growth behavior (Liu *et al.*, 1993; Roberts and Fink, 1994). When starved for nitrogen, diploid cells undergo a developmental transition from a yeast form to

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a filamentous pseudohyphal form. These filaments are composed of chains of elongated cells that radiate away from the colony and penetrate the agar substrate on which they are grown (Gimeno *et al.*, 1992). Mutations in *STE20*, *STE11*, *STE7* or *STE12* block pseudohyphal growth, whereas mutations in the pheromone receptors and the G-protein components do not affect pseudohyphal development, suggesting that other molecules generate and transduce the signal that triggers filamentous growth (Liu *et al.*, 1993). Under certain conditions, haploid cells are also capable of invasive growth which shows several similarities to diploid pseudohyphal development. Haploids switch their growth pattern and consequently form pseudofilaments, which penetrate the agar substrate (Roberts and Fink, 1994).

Kinases with significant homology to Ste20 have been identified in yeast and are thought to function in signaling upstream of MAP kinase modules or in cell morphology. *CLA4* was found in a genetic screen for mutations lethal in a G1-cyclin-deficient background (Cvrckova *et al.*, 1995). Deletion of *CLA4* in a wild-type background does not affect viability but produces morphological abnormalities (Cvrckova *et al.*, 1995). However, such *CLA4* deletion is lethal in a *ste20Δ* background, implying that in addition to its function in pheromone signaling Ste20 can play a role in vegetative growth that is redundant with that of Cla4. A third member of the Ste20 family in yeast has been identified recently by the genome sequencing project. Its structure is more similar to Cla4 then to Ste20, but its function remains to be determined (Pringle *et al.*, 1995). Finally, Spk1, a kinase with significant homology to Ste20, is thought to be required to activate a MAP kinase cascade during sporulation (Friesen *et al.*, 1994; Krisak *et al.*, 1994).

Ste20-like kinases are not unique to yeast: homologs have been discovered in a wide variety of mammalian systems. For example, human *PAK1* is able to functionally complement *STE20* in yeast (Brown *et al.*, 1996). Interestingly, human $p65^{Pak}$ was first identified by virtue of its specific interaction with the small GTP binding protein Cdc42, which stimulated the kinase activity of $p65^{Pak}$ *in vitro* (Manser *et al.*, 1994; Martin *et al.*, 1995). These results suggest that Cdc42 might play a role in signal transduction *in vivo* by directly activating $p65^{Pak}$. Consistent with this hypothesis, expression of mammalian Cdc42 was found to regulate the activity of the Jun N-terminal kinase (JNK) MAP kinase pathway (Coso *et al.*, 1995; Hill *et al.*, 1995; Minden *et al.*, 1995); this pathway is also termed the stress-activated protein kinase (SAPK) pathway. Mammalian Cdc42 has also been implicated in polarization of T cells (Stowers *et al.*, 1995) and in the formation of filopodia in fibroblasts (Kozma *et al.*, 1995; Nobes and Hall, 1995). In addition to small GTP binding proteins, $p65^{Pak}$ isoforms can also be activated by heterotrimeric G-proteins (Knaus *et al.*, 1995; Teo *et al.*, 1995).

In yeast, Cdc42 is involved in the control of several morphogenetic events during the cell cycle and mating, in particular in the establishment of cell polarity. Cdc42 is a member of the Rho family of low molecular weight GTP binding proteins, and is localized at the bud site and at shmoo tips (Johnson and Pringle, 1990; Ziman *et al.*, 1993). Bud emergence or mating site selection results from several distinct events (for review, see Chant, 1994; Chenevert, 1994; Pringle *et al.*, 1995). First, the cell must choose a site on its surface and direct growth towards this site. A group of non-essential genes (*BUD1–BUD5*) localizes the bud site during vegetative growth, whereas during mating the polarization site is determined by the mating partner by a mechanism which involves the *FAR1* gene (Dorer *et al.*, 1995; Valtz *et al.*, 1995). The first necessary step for budding and shmoo formation during mating—polarization site establishment—requires the products of multiple genes including *BEM1*, *CDC24* and *CDC42*. Cdc24 encodes a GDP–GTP exchange factor for Cdc42 (Sloat and Pringle, 1978; Zheng *et al.*, 1994). Bem1 contains two SH3 domains and directly interacts with Cdc24 (Peterson *et al.*, 1994; Park *et al.*, in preparation) and Ste20 (Leeuw *et al.*, 1995). Cells lacking either Cdc24 or Cdc42 function arrest their cell cycle as large unbudded cells (Johnson and Pringle, 1990), whereas cells deleted for *BEM1* are viable but exhibit severe morphological abnormalities (Chenevert *et al.*, 1992).

In yeast, as in mammalian cells, Cdc42 has been shown to interact directly with both Ste20 and Cla4, and cells lacking Cdc42 function display defects in signal transduction during mating (Simon *et al.*, 1995; Zhao *et al.*, 1995). Moreover, *CLA4* and *CDC42* are synthetically lethal, suggesting that they functionally interact *in vivo* (Cvrckova *et al.*, 1995). To investigate the significance of Cdc42 for the activation and function of Ste20 *in vivo*, we analyzed alleles of *STE20* that could no longer bind Cdc42. We found that such *STE20* alleles were unable to rescue the viability of cells defective in both *STE20* and *CLA4* and were deficient in supporting agar invasion and pseudohyphal growth. Surprisingly, however, interaction between Ste20 and Cdc42 was dispensable to trigger the MAP kinase pathway activated by mating pheromones. Our results demonstrate that Cdc42 is required to localize Ste20 *in vivo*.

Results

Ste20 binds Cdc42 in vitro in a GTP-dependent manner through a conserved binding motif

STE20 encodes a protein kinase composed of a carboxy-terminal catalytic domain and a large, non-catalytic sequence at its amino-terminus (Figure 1A). A segment within the amino-termini of several recently identified Ste20-like kinases is highly conserved (Figure 1B); this segment in $p65^{Pak}$ has been shown to interact with human Cdc42 (Manser *et al.*, 1994; Burbelo *et al.*, 1995). To test directly whether yeast Cdc42 is able to bind to Ste20, we used an antibody affinity column to purify epitope-tagged wild-type and mutant forms of Ste20 and tested their ability to bind *in vitro* to Cdc42 purified from *Escherichia coli*. Figure 1C shows that Cdc42 in its active, GTP-bound state readily bound to Ste20 (lane 2) but was unable to bind to Ste20 when Cdc42 is bound to GDP (lane 1). Binding of Cdc42 was abolished if a segment within the putative Cdc42 binding site of Ste20 was deleted (Ste20-Δ335–370, lane 3). Cdc42 could also interact in a GTP-dependent manner with a kinase-inactive mutant of Ste20 (Ste20-K649R; lane 4). No interaction was detected when the Cdc42 binding site of this mutant was deleted (data not shown). Taken together, these results demonstrate that

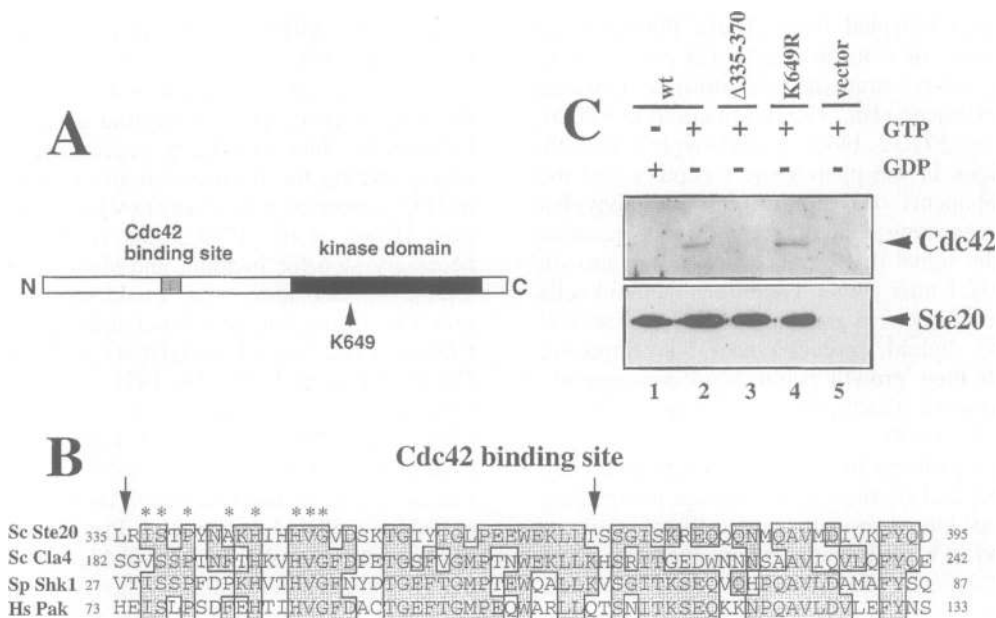

Fig. 1. Ste20 specifically binds to GTP-Cdc42 *in vitro* through an amino-terminal motif. (**A**) Schematic representation of Ste20. Black bar: kinase domain; gray bar: conserved Cdc42 binding site as shown in (B); K649 indicates the position of the Lys residue in the ATP binding site. (**B**) Alignment of the putative Cdc42 binding sites of *S.cerevisiae* Ste20 (Leberer *et al.*, 1992; Ramer and Davis, 1993), *S.cerevisiae* Cla4 (Cvrckova *et al.* 1995), *Schizosaccharomyces pombe* Shk1 (Marcus *et al.*, 1995) and human Pak (Manser *et al.*, 1994). The two arrows mark the segment deleted in the Ste20-Δ335–370 mutant; asterisks show conserved residues implicated in Cdc42 binding (Burbelo *et al.*, 1995). (**C**) Equal amounts of purified wild-type and mutant forms of Ste20 were incubated with Cdc42 expressed in *E.coli* bound either to GDP or GTP-γS. After extensive washing of the immobilized Ste20, bound Cdc42 (upper panel) was detected by immunoblotting using an affinity-purified peptide antibody specific for Cdc42 (Park *et al.*, in preparation). The same blot subsequently was reprobed with an affinity-purified polyclonal antibody against Ste20 (Ste20-XI; Kinetek Biotechnology Corp., Vancouver) to ensure equal amounts of Ste20 proteins in each reaction (lower panel). Arrowheads point to the position of Cdc42 (upper panel) and Ste20 (lower panel). The following Ste20 proteins were analyzed: lanes 1 and 2: wild-type; lane 3: Ste20-Δ335–370; lane 4: Ste20-K649R; lane 5: vector.

Cdc42 is able to bind to Ste20 *in vitro* in a GTP-dependent manner, and suggest that the conserved amino-terminal segment of Ste20 is necessary for this interaction.

Cdc42 functionally interacts with Ste20 in vivo

A genetic suppression assay provided evidence that Ste20 functionally interacts with GTP-bound Cdc42 *in vivo* (Figure 2). Cells expressing Ste20-K649R from the inducible *GAL* promoter were unable to grow under inducing conditions, whereas cells expressing wild-type Ste20 grew normally (Figure 2A). The cells arrested with a phenotype reminiscent of cells lacking *STE20* and *CLA4* (data not shown; Cvrckova *et al.*, 1995), suggesting that Ste20-K649R might interfere with the overlapping essential function of these kinases. Deletion of 483 amino acids of the amino-terminal, non-catalytic domain of Ste20-K649R restored the ability to grow, indicating that an essential factor is titrated by binding to this domain (Figure 2A). Interestingly, overexpression of Cdc42 suppressed this growth defect (Figure 2B), suggesting that Cdc42 might interact with Ste20 *in vivo*. In contrast, growth was not restored by overproduction of Rho1, another small GTP binding protein of the Cdc42 subfamily (Madaule *et al.*, 1987), or by overexpression of the two polarity establishment proteins, Bem1 and Cdc24 (Chenevert, 1994). Overexpression of a mutant form of Cdc42 which lacks the carboxy-terminal CaaX motif (C188S) was unable to restore growth (Figure 2C), indicating that the ability of Cdc42 to interact with membranes is necessary for suppression. Membrane association may be important for activation of Cdc42, since the exchange factor Cdc24 is localized at the plasma membrane (Pringle *et al.*, 1995). To corroborate this hypothesis, we combined the C188S mutation with either of two mutant forms of Cdc42: one is Cdc42-D118A, which cannot be converted to its active GTP-bound state and the other, Cdc42-Q61L, is locked into a GTP-bound state and is thought to be constitutively active (Ziman *et al.*, 1991). Interestingly, the ability of the Cdc42-C188S mutant to allow growth of cells overexpressing Ste20-K649R could be restored if the C188S mutation was combined with the activating Q61L mutation but not if combined with D118A (Figure 2C). These results support the view that conversion of Cdc42 to its active, GTP-bound form occurs at the plasma membrane. Importantly, these observations suggest that Ste20 interacts specifically with the GTP-bound form of Cdc42 *in vivo*.

Binding of Ste20 and Cdc42 requires an intact effector domain

Alignments of small GTP binding proteins identified a highly conserved region which is known to mediate interactions with several targets (called the effector domain, Bourne *et al.*, 1991; Figure 3A). To determine whether the interaction of yeast Cdc42 and Ste20 requires an intact effector domain of Cdc42, we mutated the conserved Thr35 of Cdc42 to an alanine residue (Cdc42-T35A). The Cdc42-T35A protein was then expressed as a 6His fusion in *E.coli* and tested for its ability to bind to Ste20 purified from yeast as described above. Interestingly, Cdc42-T35A mutant protein failed to bind Ste20 (Figure 3B, lane 1), whereas wild-type Cdc42 protein bound

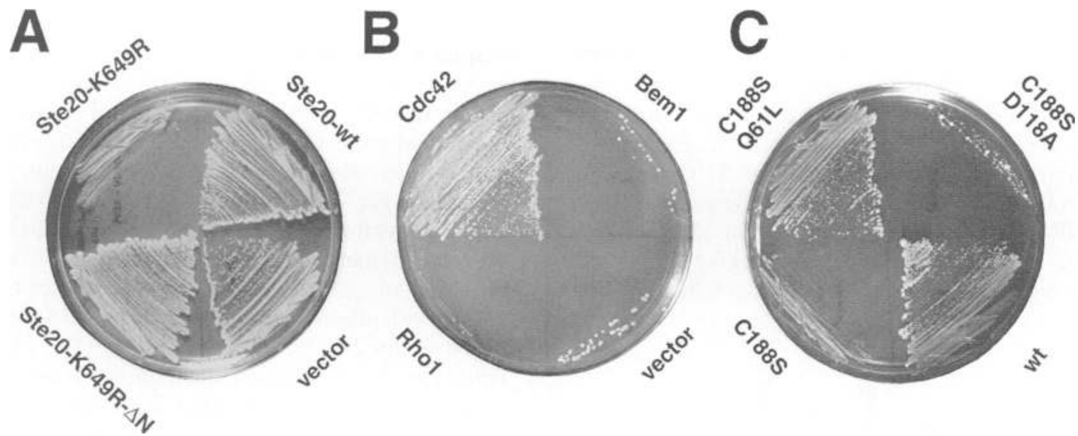

Fig. 2. Ste20 specifically interacts with GTP-Cdc42 *in vivo*. (**A**) Cells expressing Ste20-K649R from the inducible *GAL* promoter were unable to grow on plates containing galactose, whereas cells expressing wild-type or Ste20-K649R lacking 483 amino acids of the amino-terminal, non-catalytic domain (Ste20-K649R/ΔN) grew normally. (**B**) The growth defect of cells expressing Ste20-K649R was suppressed by a high copy plasmid carrying *CDC42* but not by high copy plasmids carrying either *RHO1* or *BEM1*. (**C**) Suppression of the growth defect of cells expressing Ste20-K649R requires that Cdc42 can be converted into its GTP-bound form. Plasmids carrying *CDC42-C188S* which is no longer able to localize to the plasma membrane were only able to restore growth when combined with an activating Q61L mutation but not when combined with the inactive D118A mutation.

normally (lane 2). Similar amounts of Cdc42 proteins were added to the reactions as confirmed by immunoblotting (Figure 3B, lower panel). The mutant protein was also unable to suppress the lethality caused by the dominant-negative Ste20-K649R mutant (Figure 3C, upper panel), indicating that Cdc42-T35A was no longer able to bind to Ste20 *in vivo*. Overexpression of the Cdc42-T35A mutant protein in wild-type cells did not interfere with growth (Figure 3C, lower panel). In addition, the Cdc42-T35A mutant protein was unable to complement a temperature-sensitive *cdc42* strain at the restrictive temperature (data not shown). Taken together, these results demonstrate that the interaction of Cdc42 and Ste20 requires an intact effector domain.

A Ste20 mutant unable to bind Cdc42 displays full kinase activity when purified from yeast

To examine whether the ability of Ste20 to bind to Cdc42 is required for Ste20 kinase activity, wild-type and mutant forms of Ste20 were purified from yeast and assayed for their ability to phosphorylate myelin basic protein (MBP) as an exogenous substrate (Figure 4, upper panel) or to autophosphorylate (Figure 4, middle panel). Interestingly, both wild-type and Ste20-Δ335–370 displayed similar kinase activity against MBP and were able to autophosphorylate (compare lanes 2 and 3). In addition, both kinases phosphorylated a potential *in vivo* substrate, Ste11, with equal efficiency (data not shown). No kinase activity could be detected for Ste20 proteins carrying the K649R mutation (lane 5) or if expression of Ste20 was not induced (lane 1, –Gal). These results suggest that binding of GTP-Cdc42 to Ste20 is not required for kinase activity of Ste20 *in vitro*. Consistent with this finding, a truncated form of Ste20 lacking the entire amino-terminal regulatory domain was not only fully active as a protein kinase *in vitro* (data not shown) but is also weakly constitutive *in vivo* (Ramer and Davis, 1993; E.Leberer, personal communication).

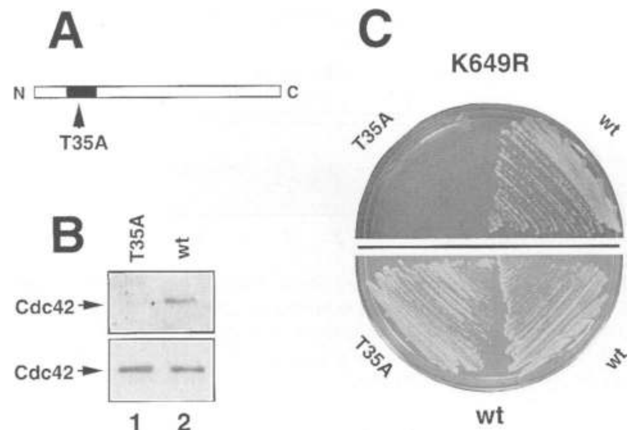

Fig. 3. Interaction of Ste20 and Cdc42 requires an intact effector domain of Cdc42. (**A**) Schematic representation of Cdc42. Small GTP binding proteins share a conserved effector domain, which is thought to mediate interactions with targets (Bourne *et al.*, 1991). Black bar: effector domain; T35A indicates the position of the conserved Thr residue which was mutated to an Ala residue. (**B**) Equal amounts of GTP-γS-bound wild-type and T35A mutant form of Cdc42 expressed in *E.coli* were incubated with purified Ste20 as described in Figure 1C. Bound Cdc42 (upper panel) and total Cdc42 (lower panel) were detected by immunoblotting using an affinity-purified peptide antibody specific for Cdc42. Arrowheads point to the position of Cdc42. The following Cdc42 proteins were analyzed: lane 1, T35A; lane 2, wild-type. (**C**) The growth defect of cells expressing Ste20-K649R was suppressed by a high copy plasmid carrying wild-type *CDC42* but not by a high copy plasmid carrying *CDC42-T35A* (upper panel). Overexpression of Cdc42 and Cdc42-T35A did not interfere with growth of wild-type cells (lower panel).

Interaction between Ste20 and Cdc42 is essential in vivo for the viability of ste20 cla4 mutant cells and for invasive growth behavior

We next examined the physiological consequences of a Ste20 mutant unable to interact with Cdc42 *in vivo*. Cells lacking *STE20* are viable, but *STE20* becomes essential in cells deleted for *CLA4*, encoding a protein with homology to Ste20 (Cvrckova *et al.*, 1995). Plasmids encoding

wild-type and mutant forms of *STE20* were transformed into a strain deleted for both *STE20* and *CLA4* which carries a temperature-sensitive allele of *CLA4* (K4580, Cvrckova *et al.*, 1995). These cells are viable when grown at 25°C but inviable when incubated at 37°C. Expressing wild-type Ste20 fully restored growth at 37°C, whereas vector controls (data not shown) and catalytically inactive Ste20-K649R were unable to complement (Figure 5A). Interestingly, Ste20-Δ335–370 protein lacking the Cdc42 binding site was unable to function *in vivo* when assayed for its ability to restore growth at 37°C (Figure 5A), despite normal kinase activity *in vitro* (see above). These results indicate that binding of Ste20 and Cdc42 is physiologically relevant *in vivo* for cell growth.

Haploid yeast cells change their growth properties and morphology when starved, and, as a result, are able to penetrate agar. The ability of cells to exhibit agar invasion requires functional Ste20 protein (Roberts and Fink, 1994). To test whether binding of Cdc42 to Ste20 is necessary for invasive growth, we transformed a strain deleted for *STE20* with plasmids expressing either wild-type or mutant forms of Ste20. As shown in Figure 5B, expression of wild-type Ste20 restored the ability to invade agar; in contrast expression of neither Ste20-K649R nor Ste20-Δ335–370 was able to restore invasion. Similarly, interaction of Cdc42 and Ste20 was also necessary for the formation of pseudohyphae (data not shown, E.Leberer, personal communication). Thus, interaction between Cdc42 and Ste20 appears to be required for both invasive growth and the formation of pseudohyphae.

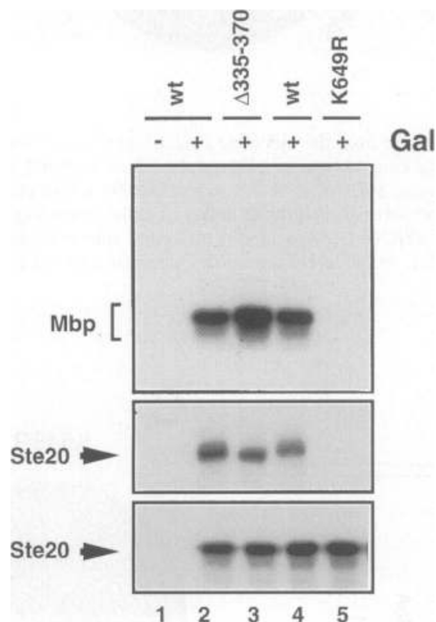

Fig. 4. Kinase activity of purified Ste20 proteins. Wild-type or mutant forms of Ste20 were expressed from the inducible *GAL* promoter, purified as described and subsequently assayed for their ability to either phosphorylate myelin basic protein (Mbp, upper panel) or to autophosphorylate (middle panel). Similar amounts of Ste20 proteins were added to each reaction as shown by immunoblotting (lower panel). Plus sign '+' indicates addition of galactose (Gal); minus sign '–' indicates galactose not added (*GAL* promoter off). Arrowheads point to the position of Ste20; the bracket marks the position of Mbp. The following Ste20 proteins were analyzed: lanes 1, 2 and 4, Ste20 wild-type; lane 3, Ste20-Δ335–370; lane 5, Ste20-K649R.

Interaction between Ste20 and Cdc42 is not required in vivo for signal transduction in response to mating pheromones

Ste20 functions in a signal transduction pathway triggered by yeast mating pheromones leading to the formation of a diploid cell. Ste20 is thought to function between Gβγ and the MEKK, Ste11 (Leberer *et al.*, 1992). To test whether binding of Cdc42 to Ste20 is necessary for mating, we transformed a strain deleted for *STE20* with plasmids expressing either wild-type or mutant forms of Ste20. Cells lacking *STE20* are not completely sterile, possibly because another Ste20-like kinase, Cla4, is able to function weakly in the mating pathway. Consistent with this notion, overexpression of Cla4 suppresses the mating defect of a strain lacking *STE20* (data not shown). As expected, expression of wild-type Ste20 fully restored mating to either a wild-type or weakened mating testers, whereas expression of the catalytically inactive Ste20-K649R protein was unable to do so (Figure 5C). Surprisingly, however, expression of Ste20-Δ335–370 was able to almost fully restore mating to wild-type cells (Figure 5C,

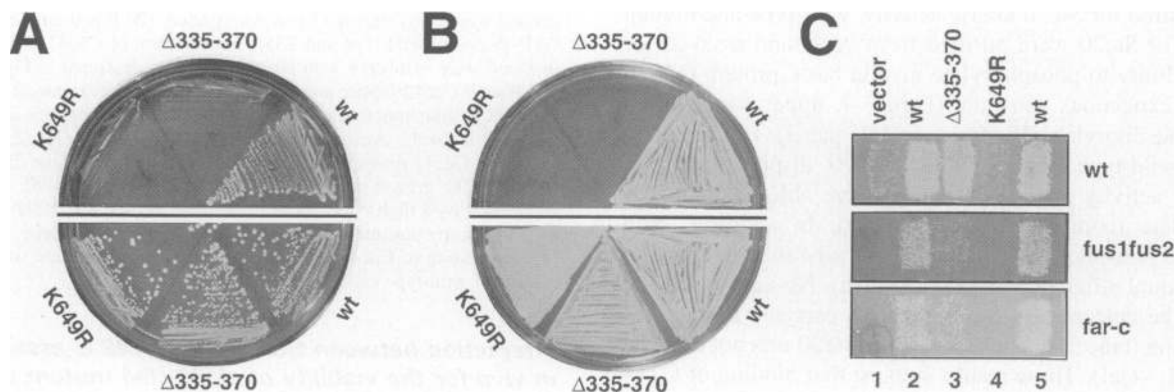

Fig. 5. Interaction of Cdc42 and Ste20 is relevant *in vivo*. (**A**) A *cla4-75 ste20* temperature-sensitive mutant strain (K4580) expressing either wild-type or mutant forms of Ste20 was grown at 37°C (restrictive temperature, upper panel) or at 25°C (permissive temperature, lower panel). The following Ste20 mutants were analyzed: wt: Ste20 wild-type; Δ335–370: Ste20-Δ335–370; K649R: Ste20-K649R. (**B**) A strain deleted for *STE20* (L5585) expressing either wild-type or mutant forms of Ste20 was analyzed for the ability to invade agar. Upper panel, after washing plates; lower panel, before washing plates. (**C**) Wild-type or mutant forms of Ste20 were expressed in a strain lacking *STE20* (IH2735) and assayed *in vivo* for their ability to mate to either wild-type (IH1793, upper panel), *fus1fus2* (IH2351, middle panel) or *far1-c* (IH2625, lower panel) mating testers.

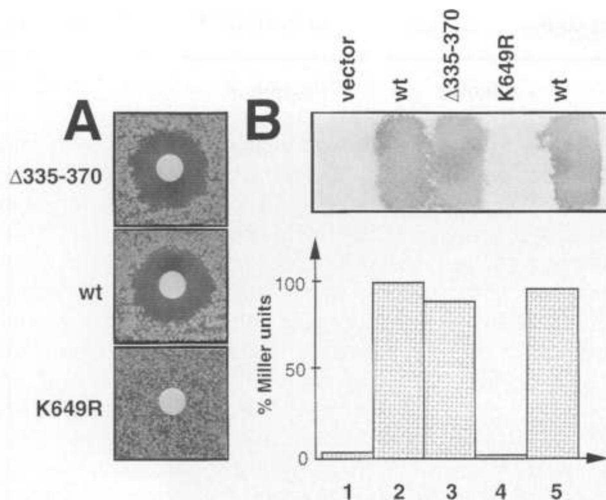

Fig. 6. Cell cycle arrest and signal transduction in response to α-factor. Strains lacking *STE20* (YMP1044) and expressing either wild-type or mutant forms of Ste20 were analyzed by halo assay for their ability to arrest the cell cycle in response to α-factor (**A**) or to induce expression of the reporter construct *FUS1–LacZ* (**B**). The activity of the mating pathway was quantified and expressed as % Miller Units relative to wild-type controls. The following Ste20 proteins were analyzed: lane 1, vector; lane 2 and 5, Ste20-wild-type; lane 3, Ste20-Δ335–370; lane 4, Ste20-K649R.

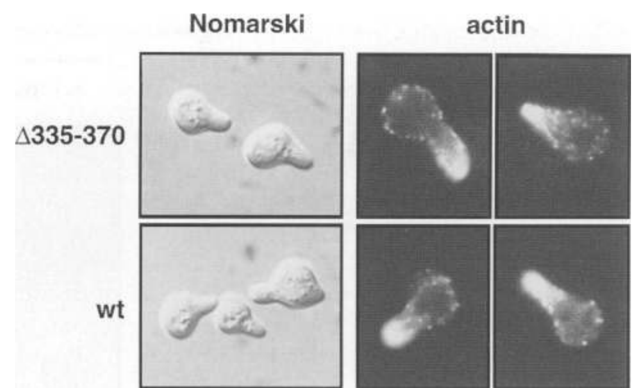

Fig. 7. Polarization of Ste20 mutant cells in the presence of α-factor. The shmoo morphology of cells deleted for *STE20* (IH2735) expressing either wild-type (lower panel) or Ste20-Δ335–370 treated with α-factor for 3 h at 30°C was indistinguishable when analyzed by Normarski microscopy (left panels). Actin distribution is directed towards the shmoo tip as visualized after staining with rhodamine–phalloidin (right panels).

upper panel). A weak mating defect only became apparent when cells expressing Ste20-Δ335–370 were mated with themselves (data not shown), or to enfeebled mating testers such as *far1-c* or *fus1fus2* (Figure 5C). Quantitative mating experiments indicated that the mating efficiency was reduced ~20-fold when mated to *far1-c* and 50-fold when mated to *fus1fus2*.

To corroborate these results further, we next tested whether Ste20-Δ335–370 was able to restore cell cycle arrest and signal transduction triggered by mating pheromones. As shown in Figure 6A, cells expressing wild-type (middle panel) or Ste20-Δ335–370 proteins (upper panel) were unable to grow around a filter disk containing α-factor, leading to the formation of a halo, thereby demonstrating that both proteins are able to signal cell cycle arrest. In contrast, no halo was observed with cells expressing a catalytically inactive Ste20 protein (Figure 6, lower panel).

We next determined whether Ste20-Δ335–370 was able to induce transcription of the *FUS1–LacZ* reporter in response to α-factor in a strain lacking *STE20* (IH2735). Expressing wild-type Ste20 fully restored induction of *FUS1–LacZ*, whereas vector controls and catalytically inactive Ste20-K649R were unable to complement, demonstrating that kinase activity of Ste20 is required for signal transduction (Figure 6B). Importantly, cells expressing Ste20-Δ335–370 protein unable to bind Cdc42 displayed only a minor defect in signal transduction in response to mating pheromones, and induced the *FUS1–LacZ* reporter to ~80% of wild-type levels (Figure 6B). *FUS1–LacZ* was not induced in the absence of α-factor (data not shown), indicating that the Ste20-Δ335–370 protein was still properly regulated *in vivo* in an α-factor-dependent manner. In addition, expression of a GTP-locked, active form of Cdc42 could only weakly activate *FUS1–LacZ in vivo* (data not shown; Simon *et al.*, 1995; Akada *et al.*, 1996). Taken together, these results indicate that formation of GTP-Cdc42 is neither necessary nor sufficient to fully activate Ste20 in response to α-factor *in vivo*.

Finally, we tested whether cells expressing Ste20-Δ335–370 exhibit a morphological defect in response to mating pheromones. Cells exposed to α-factor polarize and grow towards the source of pheromone, resulting in an elongated cell shape called shmoo (Chenevert, 1994). As shown in Figure 7, cells expressing Ste20-Δ335–370 were able to form projections which were morphologically indistinguishable from wild-type shmoos (Figure 7, left panels). Furthermore, the actin cytoskeleton was polarized properly, as visualized by phalloidin staining (Figure 7, right panels). Thus, binding of Ste20 and Cdc42 was not necessary for proper shmoo morphology and actin reorganization.

The interaction between Ste20 and Cdc42 is required for localization of Ste20

The observation that binding of Ste20 and Cdc42 was not required for Ste20 kinase activity *per se*, but nevertheless played an important role in Ste20 function *in vivo*, raised the possibility that Cdc42 might be involved in localizing Ste20. To test this possibility, we fused green fluorescent protein (GFP) to both wild-type Ste20 and the Ste20-Δ335–370 mutant which is unable to interact with Cdc42. Both GFP fusion proteins were expressed in yeast from the *GAL* promoter and were present in similar amounts (Figure 8A). The GFP–Ste20 fusions were fully functional *in vivo* and were able to complement the mating defect of cells deleted for *STE20* (data not shown). The localization of GFP–Ste20 was first analyzed in living cells which were not exposed to α-factor. Interestingly, GFP–Ste20 was concentrated at the emerging bud in a crescent (Figure 8C). This crescent-shaped staining remained clearly visible in small budded cells, but staining gradually disappeared when the bud grew larger (Figure 8C). In large budded cells, GFP–Ste20 was dispersed throughout the cytoplasm. No staining was detected with control constructs lacking GFP (Figure 8B) and no bud tip staining could be detected with GFP alone (data not shown). In cells exposed to α-factor, GFP–Ste20 was localized in a crescent at the shmoo tip (Figure 8C). In contrast, GFP–Ste20-Δ335–370, which is unable to interact with Cdc42,

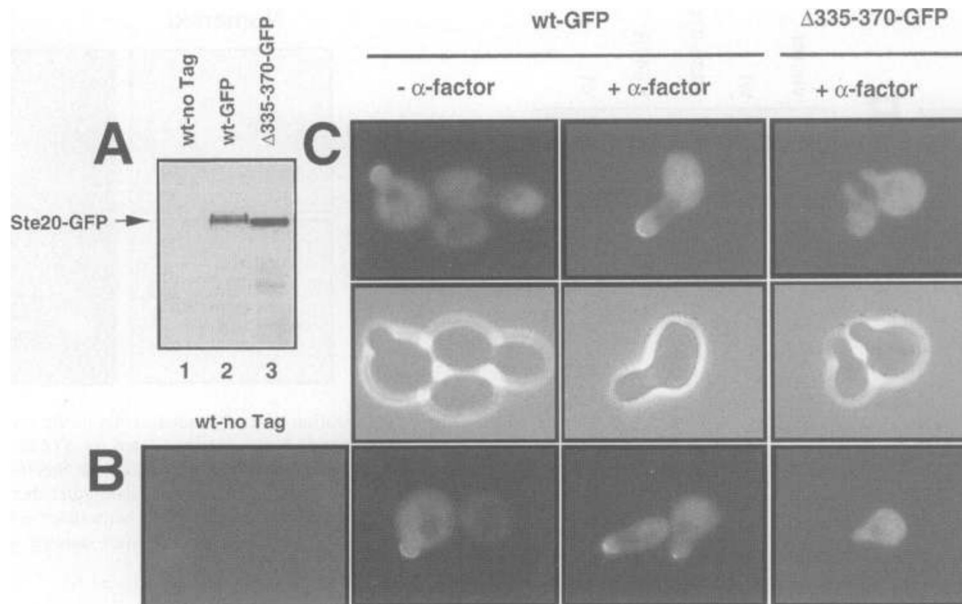

Fig. 8. Subcellular localization of GFP–Ste20. Wild-type or mutant Ste20 were fused to GFP and expressed in wild-type cells from the inducible *GAL* promoter. Proteins were monitored either by immunoblotting using antibodies specific for GFP (**A**) or by microscopy (**B** and **C**). (B) Cells expressing Ste20 without the GFP tag. Where indicated, cells were treated with α-factor for 3 h. The arrow marks the position of GFP–Ste20. Note that wild-type GFP–Ste20 but not GFP–Ste20-Δ335–370 is localized in a crescent at the tip of small budded cells and at the shmoo tip in mating projections.

was no longer localized in small buds and in shmoo tips, but instead was found dispersed throughout the cytoplasm (Figure 8C). This result suggests that binding of Ste20 and Cdc42 is necessary to localize Ste20 properly at bud emergence during the cell cycle and at the shmoo tip during mating. Consistent with these results, Cdc42 is localized in a similar fashion and is found at the emerging bud and at shmoo tips (Ziman *et al.*, 1993). Finally, we found that inappropriate targeting of wild-type Ste20, by fusing the myristoylation sequence of Gpa1 to the amino-terminus of Ste20, was lethal for cells (data not shown). This result supports the notion that proper localization of Ste20 is functionally important *in vivo*. Inappropriate localization of Ste20 was not sufficient, however, to cause activation of the mating pathway, because cells expressing myristoylated Ste20 did not induce expression of the *FUS1–LacZ* reporter in the absence of mating pheromones (data not shown).

Discussion

The protein kinase Ste20 functions in several signal transduction pathways in yeast; it is required for pseudohyphal and invasive growth, as well as during mating (Herskowitz, 1995). In addition, Ste20 plays an essential role in cells lacking *CLA4*, encoding a kinase with significant homology to Ste20 (Cvrckova *et al.*, 1995). Recent evidence suggests that the activity of Ste20 in response to these different signals is regulated by direct binding to the small GTP binding protein Cdc42 (Simon *et al.*, 1995; Zhao *et al.*, 1995). To test this hypothesis directly, we have analyzed Ste20 mutants which can no longer bind Cdc42. We found that such alleles were defective in triggering a subset of the *in vivo* functions of Ste20. Surprisingly, the kinase activity of such Ste20 mutants was normal when purified from yeast, and these alleles were able to activate the MAP kinase pathway triggered by mating pheromones *in vivo*. Our results suggest that the interaction between Cdc42 and Ste20 is important for the localization of Ste20 *in vivo*.

Direct interaction between Ste20 and Cdc42

Several lines of evidence demonstrate that Cdc42 physically interacts with Ste20. First, binding of GTP-bound, active Cdc42 and Ste20 can be detected *in vitro* and also in a two-hybrid assay *in vivo* (Simon *et al.*, 1995). Second, Cdc42 was able to restore growth of cells arrested by overexpression of a kinase-inactive form of Ste20. This suppression was specific for Cdc42 because other small GTP binding proteins of the Rho family were unable to restore growth. A mutant in the CaaX box of Cdc42 (C188S), which is required for its membrane localization, was unable to suppress, but growth was restored when the CaaX box mutation was combined with a mutation that mimicks the GTP-bound form of Cdc42. Taken together, these data suggest that only GTP-bound Cdc42 is able to restore growth and, furthermore, that Cdc42 can only be converted into its GTP-bound, active form when it is properly targeted to the plasma membrane. Consistent with this observation, the Cdc42 exchange factor, Cdc24, has been shown recently to be localized at the plasma membrane (Pringle *et al.*, 1995). Alignment of several Ste20-like kinases identified a consensus binding site for Cdc42 in the amino-terminal domain of Ste20 (Figure 1B, Burbelo *et al.*, 1995). As shown here, deletion of this putative Cdc42 binding site abolished the binding of Cdc42 and Ste20 both *in vitro* and *in vivo*. We cannot rigorously exclude the possibility that the Ste20-Δ335–370 mutant protein is still able to interact weakly with Cdc42, possibly through a binding site other than the amino-terminal domain. However, both direct *in vitro* binding assays (Figure 1C) and two-hybrid assays

(E.Leberer *et al.*, personal communication) were unable to detect any remaining association between Cdc42 and Ste20-Δ335–370. In addition, the Ste20-Δ335–370 mutant protein was unable to function *in vivo* to trigger invasive (Figure 5B) and pseudohyphal growth (E.Leberer *et al.*, personal communication), as well as cell cycle progression of *cla4 ste20* double mutant cells (Figure 5A).

Interestingly, the interaction of Ste20 and Cdc42 requires an intact effector domain of Cdc42. A point mutation which changes a conserved threonine residue in this effector domain of Cdc42 no longer binds to Ste20 *in vitro*, and concomitantly was no longer able to restore growth of cells expressing the Ste20-K649R mutant *in vivo*.

Binding of Ste20 and Cdc42 is required for proper localization of Ste20

We found that wild-type Ste20 is localized in a crescent at the emerging bud and at the shmoo tip of cells arrested with α-factor. This localization is strikingly similar to Cdc42 which is found at the presumptive bud site early in the cell cycle, and at the tips and sides of enlarging buds. In addition, Cdc42 is localized at shmoo tips in cells arrested with α-factor (Ziman *et al.*, 1993). Importantly, mutant Ste20 unable to bind to Cdc42 remained diffuse throughout the cytoplasm both in small budded cells and mating projections. Thus, these data suggest that binding of Cdc42 and Ste20 appears to be necessary to target Ste20 to the site of growth *in vivo*. Moreover, our results demonstrate that proper localization of Ste20 by Cdc42 is important only for a subset of the functions of Ste20 *in vivo*.

Small GTP binding proteins function as targeting molecules in many biological responses. For example, Rab proteins deliver specific vesicles to their target membranes within the cell (Zerial and Stenmark, 1993). Likewise, mammalian Ras binds to the protein kinase Raf and thereby recruits Raf to the plasma membrane (Hall, 1994). Analogously to Ste20 and Cdc42, Ras does not appear to be required for the activation of Raf itself, because expression of Raf fused to a CaaX box is sufficient to transform 3T3 cells in a Ras-independent manner (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Several other kinases have also been shown to interact directly with small GTP binding proteins. For example, the yeast small GTP binding protein Rho1 binds to protein kinase C (Pkc1) and this interaction does not appear to activate the catalytic activity of Pkc1 (Nonaka *et al.*, 1995). It is tempting, therefore, to speculate that small GTP binding proteins might generally play important roles in the localization of protein kinases (Mochly-Rosen, 1995).

Functional relevance of the interaction of Ste20 and Cdc42

The interaction between Ste20 and Cdc42 appears to be important for several of its functions *in vivo*. Ste20 plays an essential role in cells lacking *CLA4*, encoding a protein kinase with significant homology to Ste20 (Cvrckova *et al.*, 1995). Cells lacking *STE20* and *CLA4* display severe defects in early bud morphogenesis and fail to undergo cytokinesis, suggesting that these kinases are necessary for proper localization of cell growth (Cvrckova *et al.*, 1995). Normal bud neck development and cytokinesis require the establishment of a ring composed of septin-containing microfilaments in late G_1 (Sanders and Field, 1994). Assembly of the septin ring occurs normally in *cla4 ste20* mutant cells, but the ring appears unstable and the septin-containing structure fails to stay at the bud neck (Cvrckova *et al.*, 1995). Interestingly, as shown here, binding of Cdc42 and Ste20 was required for Ste20 to support growth of a *ste20 cla4* double mutant strain, suggesting that correct localization of Ste20 by Cdc42 is necessary to stabilize the septin ring at the bud neck. The substrate(s) of Ste20 required for stabilizing the septin ring is not known, but could include the septins themselves or associated proteins.

Yeast cells are able to change their growth properties when starved for nitrogen: diploid cells adopt a filamentous pseudohyphal growth pattern, whereas haploid cells switch their budding pattern and form filaments capable of invading agar plates (Roberts and Fink, 1994). The protein kinase Ste20 is essential to trigger both of these developmental transitions, suggesting that the function of Ste20 in these pathways is not redundant with Cla4 (Roberts and Fink, 1994). Interestingly, cells expressing a Ste20 protein which can no longer bind Cdc42 were unable to invade agar and also failed to form pseudohyphae (data not shown; E.Leberer *et al.*, personal communication), suggesting that proper localization of Ste20 by Cdc42 is important for the formation of these filaments. In addition to Ste20, pseudohyphal and invasive growth require many of the same signaling components as the pheromone response pathway, including Ste11 and Ste7, and it is thought, therefore, that Ste20 triggers a MAPK signaling pathway. Despite this overlap, neither pseudohyphal nor invasive growth conditions induce expression of the mating-specific reporter gene *FUS1–LacZ* (Roberts and Fink, 1994). Recently, a target gene that is specifically activated by these signaling pathways has been identified (Mösch *et al.*, 1996).

Activation of Ste20 in response to mating pheromones

We found that mutants in Ste20 that no longer bind to Cdc42 are still regulated normally in an α-factor-dependent manner *in vivo*. This result was surprising because previous work suggested that Cdc42 is able to activate purified Ste20 *in vitro* (Simon *et al.*, 1995). Based on these findings, a linear model was proposed: in response to mating pheromones, Cdc42 is converted to the GTP-bound form, possibly by the guanine nucleotide exchange factor Cdc24 (Figure 9, Zheng *et al.*, 1994). In turn, GTP-Cdc42 binds to Ste20, rendering it able to activate the MAP kinase cascade. At least two lines of evidence strongly argue against such a linear pathway: first, we demonstrate here that binding of Cdc42 and Ste20 is not required to activate Ste20 in response to α-factor *in vivo*. Likewise, overexpression of an amino-terminal truncation of Ste20 lacking the Cdc42 binding site is able to constitutively activate the mating pathway even in the absence of α-factor (Ramer *et al.*, 1992). Second, both overexpression of an activated, GTP-locked mutant form of Cdc42 or deletion of a protein that is thought to function as a GTPase-activating protein (GAP) for Cdc42 only weakly activate the mating signaling pathway *in vivo* (data not shown, Simon *et al.*, 1995; Stevenson *et al.*, 1995; Akada *et al.*, 1996). Cells lacking Cdc42 function exhibit a

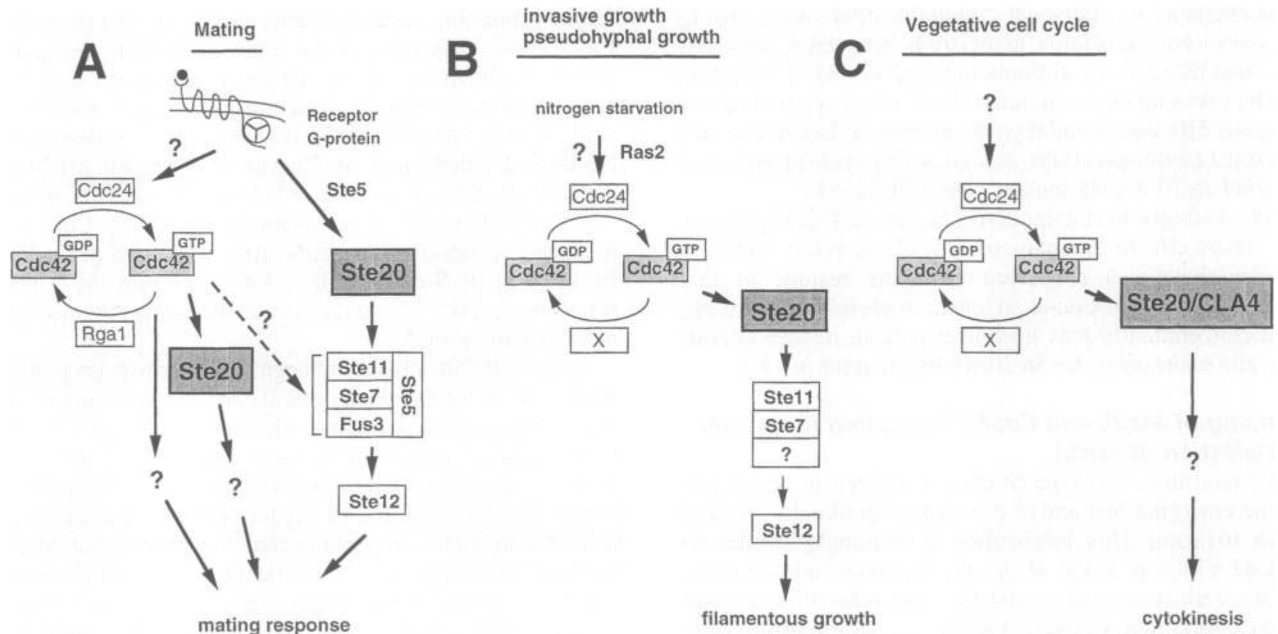

Fig. 9. Proposed roles for Ste20 and Cdc42 during mating, invasive growth and cell cycle progression. Several extracellular and intracellular signals lead to the conversion of inactive GDP-bound Cdc42 to the active GTP-bound form, possibly by activation of the exchange factor Cdc24. GTP-Cdc42 is able to bind and thereby localize Ste20. Binding of Cdc42 and Ste20 is required for invasive and pseudohyphal growth (**B**) and for the control of cytokinesis (**C**). In contrast, binding of Cdc42 and Ste20 is not required to activate the MAP kinase signaling pathway triggered by pheromones (**A**). Cdc42 might function independently of Ste20 in signal transduction in response to α-factor. We propose that Ste20 is regulated by a process involving Ste5, which interacts directly with the βγ subunits of the G-proteins (Whiteway *et al.*, 1995). Ste20 plays an additional role during mating, which is dependent on its interaction with Cdc42. Cdc42 also functions independently of Ste20 in organizing the actin cytoskeleton and in morphogenesis.

reduced ability to induce signal transduction in response to α-factor (Simon *et al.*, 1995; Zhao *et al.*, 1995). Cdc42 might, therefore, play a role in signal transduction independent of Ste20 (Figure 9A). Alternatively, these signaling defects might result, in part, from perturbation of cell polarity, or from the particular cell cycle arrest point of *cdc42* mutants.

Taken together, our results suggest that the activation of Ste20 in response to pheromones does not require Cdc42. Instead, Ste20 might be activated directly by the βγ-subunits of the heterotrimeric G-protein (Figure 9A). This view is supported by the observed genetic interaction between *STE20* and *STE4* (Leberer *et al.*, 1992). Activation of Ste20 might also involve Ste5 (Figure 9A), which has been shown to co-immunoprecipitate with Ste20 (Leeuw *et al.*, 1995) and directly associates with the β-subunit of the heterotrimeric G-protein (Whiteway *et al.*, 1995). It is interesting to note that the human Ste20 homolog Pak1 is able to complement the mating defect of a strain lacking *STE20*, indicating that the sequences required for the signaling function of Ste20 remained functionally conserved (Brown *et al.*, 1996).

Whereas binding of Ste20 and Cdc42 is dispensable for signal transduction, Ste20 plays a second role during mating, which is dependent on its interaction with Cdc42 (Figure 9A). This result is consistent with genetic evidence which supports a branched pathway (Akada *et al.*, 1996). The nature of this mating defect remains to be determined. It appears unlikely that the defect occurs at steps after cell–cell contact (such as cell fusion) because cells expressing Ste20-Δ335–370 accumulate predominantly as shmoos in a mating reaction (data not shown, E.Leberer *et al.*, personal communication). An attractive possibility is that these cells fail to orient and repolarize their mating machinery towards the mating partner (Segall, 1993; Dorer *et al.*, 1995; Valtz *et al.*, 1995). However, both pheromone confusion assays (Dorer *et al.*, 1995; Valtz *et al.*, 1995) and direct orientation assays (Segall, 1993) indicate that cells expressing Ste20-Δ335–370 were able to orient normally in a gradient of α-factor (M.Peter, unpublished results; E.Leberer *et al.*, personal communication). It is possible that Ste20-Δ335–370 fails to activate or localize properly the cell wall components or agglutinins. Interestingly, recent evidence suggests that Ste20 not only triggers the mating signaling pathway, but is also required to activate the Slt2(Mpk1) pathway in response to α-factor (Zarzov *et al.*, 1996). This signaling pathway is necessary for polarized cell growth during both mating and cell cycle progression, and cells lacking *SLT2(MPK1)* fail to stabilize their cell wall properly (Levin and Errede, 1995). Similarly to cells expressing Ste20-Δ335–370, cells deleted for *SLT2(MPK1)* form morphologically normal shmoos when exposed to α-factor but display a slight mating defect (Errede *et al.*, 1995; J.Gray, personal communication). Furthermore, like Ste20, Slt2(Mpk1) is present at the shmoo tip in α-factor-arrested cells (S.Gutierres, personal communication).

Ste20 is regulated by multiple mechanisms

Our results suggest that Ste20 is regulated by distinct mechanisms; during the cell cycle and filamentous growth the function of Ste20 requires binding to Cdc42, whereas the interaction of Cdc42 and Ste20 is dispensable for pheromone signaling mediated by heterotrimeric G-pro-

teins (Figure 9). Consistent with these results, recent experiments demonstrate that Cdc42 and Ras2, but not the heterotrimeric G-proteins, are necessary to activate the MAP kinase pathway triggered by pseudohyphal growth conditions (Mösch *et al.*, 1996). These observations are striking because both signaling pathways share the downstream components Ste11 and Ste7 (Herskowitz, 1995). Mammalian Cdc42 is proposed to link extracellular growth factors to the assembly of actin stress fibers and focal adhesion complexes by directly activating kinases of the Ste20/Pak family (Kyriakis and Avruch, 1996; Nagata and Hall, 1996). Cdc42 also plays a role in Ras-mediated malignant transformation (Symons, 1995). In addition, mammalian Ste20/Pak isoforms are regulated downstream of G-protein-coupled receptors (Knaus *et al.*, 1995; Teo *et al.*, 1995). In light of the results reported here, it will be interesting to determine whether these functions of Pak require Cdc42 as an effector.

Finally, several members of the Ste20/Pak family lack an obvious binding site for Cdc42 (Kyriakis and Avruch, 1996). Examples include Sps1, which is required for transcription and morphology during spore formation in yeast (Friesen *et al.*, 1994), and mammalian germinal center kinase (GCK) implicated in B-cell differentiation (Katz *et al.*, 1994; Pombo *et al.*, 1995). Nevertheless, both Pak1 and GCK are able to trigger the JNK/SAPK signal transduction pathway when expressed in mammalian cells (Pombo *et al.*, 1995; Brown *et al.*, 1996), suggesting that, as in yeast, Cdc42-dependent and independent mechanisms may exist to regulate the same MAP kinase pathway.

Regulation of the guanine nucleotide exchange factor Cdc24

Available results indicate that in yeast the formation of active, GTP-bound Cdc42 is essential not only to establish cell polarity during vegetative growth but also for pseudohyphal and invasive growth triggered by nitrogen starvation (Mösch *et al.*, 1996). Furthermore, GTP-Cdc42 plays an important role during mating (Johnson and Pringle, 1990; Simon *et al.*, 1995). Thus, a variety of different extracellular and intracellular signals are able to regulate Cdc42. Activation of Cdc42 is mediated by the guanine nucleotide exchange factor Cdc24 (Figure 9). Conversely, inactivation of GTP-bound Cdc42 is catalyzed by a GAP. Recent evidence suggests that Rga1 functions as a GAP for Cdc42 in the mating pathway (Stevenson *et al.*, 1995). It is not clear whether the activity of Cdc24, the GAP protein or both is regulated during the cell cycle and in response to these different signals. Interestingly, an interaction between Cdc24 and the heterotrimeric G-protein has been reported recently, suggesting that Cdc24 might be regulated by the G-proteins in response to mating pheromones (Zhao *et al.*, 1995). Consistent with this view, the Ras exchange factor p140 is activated directly by a G-protein-coupled mechanism in mammalian cells (Mattingly and Macara, 1996). Yeast cells lacking the pheromone receptor or the heterotrimeric G-proteins are still able to grow normally and are capable of forming pseudohyphae and invading agar (Roberts and Fink, 1994), indicating that under these circumstances activation of Cdc24 does not require the function of the G-proteins. Instead, activation of the pseudohyphal pathway requires Ras2 (Mösch *et al.*, 1996). It appears, therefore, that Cdc24 might be regulated by different signals, and it will be interesting to isolate alleles of Cdc24 that are specifically defective for mating or pseudohyphal response.

Additional targets of Cdc42

Cdc42 is known to play an essential role in establishing cell polarity during vegetative growth, possibly by promoting localized organization of the actin cytoskeleton (Johnson and Pringle, 1990; Chang *et al.*, 1994; Li *et al.*, 1995). Similarly, members of the Rho family of GTP binding proteins are implicated in formation of focal adhesions and stress fibers in mammalian cells (Ridley, 1995). However, Ste20 or Cla4 are not the effectors of Cdc42 required for nucleation and assembly of the actin cytoskeleton during vegetative growth, because *ste20 cla4* mutant cells still nucleate and polarize actin (Cvrckova *et al.*, 1995). Likewise, shmoo morphogenesis and actin repolarization in response to α-factor occur normally in cells expressing a Ste20 mutant unable to bind Cdc42. Therefore, Cdc42 must have targets other then Ste20 and Cla4 which are involved in actin assembly during mating and vegetative growth. A third yeast member of the Cla4/Ste20 family has been identified by the genome sequencing project and appears to bind Cdc42 by two-hybrid assays (Pringle *et al.*, 1995). It will thus be interesting to examine whether cells deleted for this gene in combination with *CLA4* and *STE20* are still able to nucleate actin normally. Recently, the Wiskott–Aldrich syndrome protein (WASP) has been isolated as a novel effector of Cdc42 in mammalian cells, and this protein is implicated directly in actin polymerization (Symons *et al.*, 1996). A protein with significant homology to WASP, but lacking the putative Cdc42 binding site, is also present in yeast (encoded by *YSCLAS17*). It remains to be determined whether Las17 is a *bona fide* effector of Cdc42 in yeast and whether polymerization of actin is affected in cells lacking *LAS17*. Finally, two novel candidate effectors of Cdc42 in yeast have been identified in a database search with the Cdc42 binding motif (Burbelo *et al.*, 1995). Interestingly, these two proteins share significant sequence homology with each other and are able to bind Cdc42 in a GTP-dependent manner when assayed in the two-hybrid system (M.Jaquenoud and M.Peter, unpublished results).

Materials and methods

Yeast strains

Yeast strains are described in Table I. Standard yeast growth conditions and genetic manipulations were used as described (Rose and Fink, 1990). Yeast transformations were performed by the lithium acetate procedure (Ito *et al.*, 1983). Strains IH2735 and IH2736 were constructed by disruption of *STE20* using the plasmids pEL46.2 and pEL45, respectively as described (Leberer *et al.*, 1992).

DNA manipulations

Standard procedures were used for recombinant DNA manipulations (Sambrook *et al.*, 1989; Ausubel *et al.*, 1991). DNA fragments were eluted from agarose gels using Quiaex as recommended by the manufacturer (Quiagen Inc.). PCR primers were synthesized using a Millipore oligonucleotide synthesizer. PCRs were performed with Vent polymerase (New England Biolabs) (25 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C). PCR products were purified using the Wizard PCR purification kit according to the instructions of the manufacturer (Promega).

Table I. Yeast strains

Strain	Genotype	Source
IH1783	*MATa, ura3, leu2, his4, trp1, can1*	this study
IH2735	*MATa, ura3, leu2, his4, trp1, can1, ste20::TRP1*	this study
IH2736	*MATa, ura3, leu2, his4, trp1, can1, ste20::URA3*	this study
YMP1044	*MATa, ura3, leu2, trp1, his4, can1, ste20::TRP1, FUS1–LacZ::LEU2*	this study
IH2432	*MATα, ura3, leu2, his4, trp1, can1, cdc42-1*	this study
IH2742	*MATa, ura3, leu2, trp1, ade2, met1, can1 HMLa, HMRa, bar1-1*	collection
IH2745	*MATa, ura3, leu2, trp1, ade2, met1, can1, HMLa, HMRa, bar1-1, ste20::TRP1*	this study
IH2746	*MATa, ura3, leu2, trp1, ade2, met1, can1, HMLa, HMRa, bar1-1, ste20::URA3*	this study
L5487	*MATa, ura3-52, leu2::hisG*	R.Roberts
L5585	*MATa, ura3-52, trp1::hisG, ste20::TRP1*	R.Roberts
L5565	*MATa, ura3-52, trp1::hisG, ste11::URA3::TRP1*	R.Roberts
IH1792	*MATa, cry1, lys1*	collection
IH1793	*MATα, lys1*	collection
IH2626	*MATa, cry1, lys1, far1-c*	collection
IH2625	*MATα, lys1, far1-c*	collection
IH2351	*MATα, ura3–52, trp1Δ1, fus1Δ1, fus2Δ3*	collection
K699	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112 his3-11,15, ura3, GAL⁺, psi⁺, ssd1-d2*	K.Nasmyth
K3591	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112 his3-11,15, ura3, GAL⁺, psi⁺, ssd1-d2 cla4::LEU2*	F.Cvrckova
K4580	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112 his3-11,15, ura3, GAL⁺, psi⁺, ssd1-d2 cla4::LEU2, ste20::URA3, YCp TRP1 cla4-75*	F.Cvrckova
YMP129	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112 his3-11,15, ura3, GAL⁺, psi⁺, ssd1-d2 trp1::GAL-STE20-K649R-TRP1*	this study
YML242	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112 his3-11,15, ura3, GAL⁺, psi⁺, bar1-1 his3::GAL-PT₂-STE20-K649R-HIS3*	this study
YML243	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112 his3-11,15, ura3, GAL⁺, psi⁺, bar1-1 his3::GAL-PT₂-STE20-Δ335–370-HIS3*	this study
YML244	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112 his3-11,15, ura3, GAL⁺, psi⁺, bar1-1 his3::GAL-PT₂-STE20-HIS3*	this study

Construction of GAL–STE20 plasmids

A *Nde*I site was introduced at the start codon of *STE20* by PCR using the primers oTP302 (5'-CCTCGACTAATACAACATATGAGCAATG-ATCCA-3') and oTP131 (5'-CCCGTCATGAATTCTGCCGCCAATG-TTTCG-3'), and the plasmid pELSTE20-5 (gift from E.Leberer, Montreal), which carries a genomic fragment encoding *STE20* cloned in pRS316 (Sikorski and Hieter, 1989). The PCR product was isolated, digested with *Nde*I and *Eco*RI and ligated into pEBO112 (gift of E.O'Shea). The resulting plasmid was digested with *Nde*I, the ends filled in with T4 DNA polymerase, further digested with *Eco*RI and ligated together with the *Eco*RI–*Kpn*I fragment obtained from pELSTE20-5 into the vector pRD53 (gift of R.Deshaies) which was digested with *Bam*HI, filled in with T4 DNA polymerase and further digested with *Kpn*I. The resulting plasmid (pTP474) allows expression of Ste20 from the *GAL* promoter in yeast. To integrate *GAL–STE20* at the *TRP1* or *HIS3* locus, the plasmid pTP474 was digested with *Not*I and *Kpn*I and the fragment was ligated into either pRS304 or pRS305 respectively (Sikorski and Hieter, 1989). Plasmids were linearized with *Bsu*36I or *Pst*I respectively for integration.

Construction of Ste20 mutants

Ste20 with a deletion of the Cdc42 binding site (Ste20-Δ335–370) was constructed by PCR using pELSTE20-5 as template (Leberer *et al.*, 1992). The following primers were used: oTP103 (5'-CTCGAATAAGA-GCTCCACTGATATACG-3'), oTP134 (5'-TTGTTGAGATCTAGTG-GTATTTCC-3'), oTP135 (5'-CAAAGATCTGGTTATAGAAGATG-AGG-3') and oTP131 (5'-CCCGTCATGAATTCTGCCGCCAATGT-TTCG-3'). The primers oTP134 and oTP135 introduce a *Bgl*II site that, after ligation, restores the reading frame of *STE20*. The PCR products were gel purified, digested with *Sac*I and *Bgl*II and *Eco*RI and *Bgl*II respectively. The two PCR fragments were then ligated into CY409 digested with *Eco*RI and *Sac*I. To obtain pTP475, the plasmid was digested further with *Sac*I and ligated with the *Sac*I–*Sac*I fragment isolated from pTP474; the correct orientation of the fragment was determined after digestion with *Eco*RI and *Not*I. The correct deletion was confirmed by sequencing using the Sequenase kit (United States Biochemical Corp.) according to the manufacturer's instructions.

The K649R mutant of Ste20 was a kind gift of S.Marcus (Polverino *et al.*, 1995). The fragment was cloned as an *Eco*RI–*Kpn*I fragment either into pTP474 to yield plasmid pTP476 or into pTP475 to generate pTP477.

Construction of Ste20 plasmids expressing Ste20 mutants from their own promoter

The plasmid pELSTE20-5 was digested with *Sac*I, the *Sac*I–*Sac*I fragment was then isolated and ligated into the vectors pTP475 and pTP474, digested with *Sac*I and treated with calf intestine phosphatase, to yield pTP478 (Ste20-Δ335–370) and pTP479 (Ste20-K649R). To construct *HIS3*-marked versions of these plasmids, the *Not*I–*Kpn*I fragment was cloned into pRS313 (Sikorski and Hieter, 1989) digested with *Not*I and *Kpn*I.

Construction of epitope-tagged versions of Ste20

For construction of a GST fusion of Ste20, the plasmid pELSTE20-5 was digested with *Sna*BI and *Kpn*I and the fragment was ligated into the yeast GST expression vector pRD56 (gift of R.Deshaies) digested with *Sma*I and *Kpn*I to yield the plasmid CY409 (Polverino *et al.*, 1995). This plasmid fully complements the mating defect of IH2735. To construct a catalytically inactive GST–Ste20, the *Eco*RI–*Kpn*I fragment of CY409 was replaced by the *Eco*RI–*Kpn*I fragment of *STE20* harboring the K649R mutation to yield CY410. Ligation of the *Eco*RI–*Kpn*I fragment isolated from CY410 into the pRD56 expression vector digested with *Eco*RI and *Kpn*I yielded CY412, which allows expression of a catalytically inactive amino-terminal deletion of Ste20 as a GST fusion protein in yeast (deletes the first 483 amino acids of Ste20).

To fuse two copies of the polyoma epitope to the amino-terminus of Ste20, an *Nde*I site was introduced at the ATG start codon of the coding sequence of *STE20* by PCR using pELSTE20-5 (Leberer *et al.*, 1992) as template and primers oTP131 and oTP302 (see above). The PCR product was purified, digested with *Nde*I and *Eco*RI, and ligated into vector pEBO112 which encodes two copies of the polyoma tag. Tagged *STE20* was then excised by digesting with *Bam*HI and the fragment ligated into pTP474 digested with *Bam*HI and *Not*I together with the *Bam*HI–*Not*I fragment isolated from pRD53. To integrate *GAL*–PT$_2$–*STE20* at the *HIS3* locus, the plasmids encoding wild-type or mutant versions of *STE20* were digested with *Not*I and *Asp*718 and the fragment was ligated into pRS305 digested with *Not*I and partially with *Asp*718 (Sikorski and Hieter, 1989). Plasmids were linearized with *Pst*I for integration.

The myristoylation sequence of *GPA1* (Dietzel and Kurjan, 1987; Nakafuku *et al.*, 1987) was amplified by PCR using CY433 (gift from K.Matsumoto) as a template and the primers oTP313 (5'-GAAGG-ATCCATAATGGGGTGTACAGTGAGTACG-3') and oTP314 (5'-TTTGTTCTGTAGCATATGATCACTTTCG-3'). For the G2A mutant myristoylation signal, the primer oTP313 was replaced by the primer oTP315 (5'-GAAGGATCCATAATGGCGTGTACAGTGAGTACG-3'). PCR products were digested with *Bam*HI and *Nde*I and ligated together with the *Not*I–*Bam*HI fragment isolated from pRD53 into pTP474, digested with *Not*I and *Nde*I, to yield pTP72 (encoding Ste20-myr-wt) and pTP73 (encoding Ste20-myr-G2A), respectively.

Construction of GFP–Ste20 and GFP–Ste20-Δ335–370

An *Xho*I site was introduced at the ATG start codon of *STE20* by PCR using the plasmids pELSTE20-5 and pTP478 as templates and the primers oTP352 (5'-AGACTCGAGATGAGCAATGATCCATCTGCTG-3') and oTP353 (5'-CGAAACATTGGCGGCAGAATTCATGACGGG-3'), which covers the *Eco*RI site within the *STE20* coding sequence (Leberer *et al.*, 1992; Ramer and Davis, 1993). The coding sequence of GFP harboring the S35T mutation was amplified by PCR using the plasmid CMP43 (Heim *et al.*, 1995; kind gift of S.O'Rourke) as template and the primers oTP351 (5'-GAAGGATCCAAGATGAGTAAAGGA-3') and oTP350 (5'-TCCCTCGAGTTTGTATAGTTCATCCATGCC-3'). The PCR products were digested with *Xho*I and *Eco*RI or with *Xho*I and *Bam*HI and ligated with the vector pRD53 digested with *Bam*HI and *Eco*RI. The resulting plasmids were then digested with *Eco*RI and *Kpn*I and ligated with the *Eco*RI–*Kpn*I fragment isolated from pEL-STE20-5 to yield the plasmids pTP480 (GFP–Ste20) and pTP481 (GFP–Ste20-Δ335–370). The plasmid pTP480 is able to complement the mating defect of cells deleted for *STE20* (IH2735).

Construction of the Cdc42 effector domain mutant

The mutation in the effector domain of Cdc42 was introduced by PCR using the plasmid CY335 carrying a genomic fragment of CDC42 as a template and the following primers: oTP317 (5'-ATCGAACACTGC-TGGAACATAGTCGGCTGGAAATTG-3'), oTP318 (5'-TATGTTCCA-GCAGTGTTCGATAACTATGCGGTG-3'), oTP319 (5'-TCAGAATT-CCATATGCATACCGTGTCTATGCAACG-3'), oTP320 (5'-AATCCC-CACCTCGAGCACTTTCTC-3') and oTP321 (5'-AAACAAGGATCC-AACGTATTAGGTCTTCC-3'). oTP320 introduces an *Xho*I and *Nde*I site upstream of the ATG start codon of CDC42, whereas oTP321 introduces a *Bam*HI site. The PCR products were gel purified, mixed and used as a template for a second PCR with the primers oTP319 and oTP320, and oTP319 and oTP321 respectively. The PCR product was gel purified, digested with *Bam*HI and *Eco*RI and *Xho*I and *Eco*RI respectively. The fragments were then ligated either into the vector pGAL-CDC42 (gift from D.Kellogg) or HP633A (Park *et al.*, in preparation), to generate pTP214 (allowing expression of the Cdc42-T35A mutant protein from the *GAL* promoter in yeast) or pTP58 (allowing expression of 6-His tagged Cdc42-T35A protein in *E.coli*). The correct sequence of the Cdc42-T35A mutant was confirmed by sequencing.

Antibodies and Western blots

Cell extracts were prepared as described previously (Peter *et al.*, 1993). Proteins were separated by SDS–PAGE and electroblotted to nitrocellulose (Schleicher and Schuell) using the Minigel system (Bio-Rad Labs., Hercules, CA). Blots were probed as indicated with monoclonal antibodies against GFP (kindly provided by Ilan Davis), or affinity-purified polyclonal antibodies against Ste20 (Kinotek, Inc., Vancouver) or Cdc42 (Park *et al.*, in preparation) and developed using epichemiluminescence (Amersham Corp., Arlington Heights, IL). Antibodies against the polyoma tag were harvested from supernatants of the hybridoma cell line AK1310 (kindly provided by E.O'Shea).

Purification of Ste20 proteins from yeast and Cdc42 binding assays

Wild-type or mutant forms of Ste20 tagged at their amino-terminus with two copies of a polyoma virus medium T antigen epitope tag (Py) sequence (Schneider *et al.*, 1994) were expressed in yeast from the inducible *GAL* promoter. The tagged wild-type Ste20 fully complements the mating defect of a strain deleted for *STE20* (AN1021). Cell extracts were prepared in buffer TNE450 (450 mM NaCl, 10 mM EDTA, 50 mM Tris–HCl pH 7.5, 0.1% NP-40) by bead-beating essentially as described previously (Peter *et al.*, 1993). Py-tagged proteins were purified using α-Py antibodies covalently coupled to protein G–Sepharose (Pharmacia) as described (Harlow and Lane, 1988; Schneider *et al.*, 1994). Cdc42 was expressed in *E.coli* NB42 as a 6His fusion protein using the vector pTrcHis (Invitrogen) and purified on a column containing iminodiacetic acid immobilized on Sepharose-6B (Sigma) coupled with Co^{2+}. Purified Cdc42 was loaded with GDP or GTP-γS, as described previously (Park *et al.*, 1993) and incubated for 1 h at 4°C with Ste20 proteins immobilized on the Py matrix in 200 μl of buffer B (10 mM Tris pH 7.5, 85 mM NaCl, 6 mM $MgCl_2$, 10% glycerol and 0.6 mM GTP-γS or 0.6 mM GDP). After washing four times with buffer TMT (10 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100) Cdc42 bound to Ste20 was eluted twice with 20 μl of phosphate-buffered saline (PBS) containing 0.1% *N*-octylglucoside (Fluka) and 100 μg/ml Py peptide (EYMPME, kind gift of E.O'Shea).

Ste20 kinase assays

Py–Ste20 proteins for kinase reactions were purified on an α-Py antibody column as described above and eluted using Py peptide. Five μl of eluted kinase was assayed at room temperature by addition of 5 μCi of [γ-^{32}P]ATP in 50 μl of kinase buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 μM ATP) containing 250 mg/ml MBP (Sigma). Reactions were stopped after 10 min by addition of 50 μl of sample buffer and boiling. The phosphorylated proteins were analyzed by SDS–PAGE followed by autoradiography.

Pheromone response and mating assays

Constructs were transformed into IH2735 and its isogenic wild-type strain (IH1783) carrying an integrated *FUS1–LacZ* reporter. For patch mating, patches were grown on SD-URA plates and allowed to mate for 1 h at 30°C with mating testers IH1793 (MATα, lys1), IH2625 (*MATα, lys1, far1-c*) or IH2351 (*MATα, fus1fus2, lys1*); then plates were replica plated on restrictive medium, on which only diploid cells can grow, and incubated for 2 days at 30°C (Valtz *et al.*, 1995).

Quantitative matings and pheromone confusion assays were performd as described previously (Valtz *et al.*, 1995). To measure induction of the *FUS1–LacZ* reporter construct, cells were grown in SD-URA to mid log-phase and exposed to α-factor (1 μg/ml) for 1 h. β-Galactose levels were quantified as described (Stern *et al.*, 1984). Data are expressed as a percentage of Miller Units deduced from three independent experiments; the activity of cells expressing wild-type Ste20 was set to 100%.

For cell cycle arrest (halo) assays, 10^3–10^4 IH2735 cells carrying the *STE20* constructs as indicated were plated on SD-URA plates. Then 8 μg of α-factor (Sigma Chemical Co., St Louis) in 16 μl of 0.01 M HCl was spotted on a sterile filter disk (Schleicher and Schuell, Keene, NH) and placed on plates, which were then incubated for 3 days at 30°C.

Invasive growth assays

Invasive growth assays were performed as described in Roberts and Fink (1994). Briefly, strains were transformed with the indicated *STE20* constructs, patched on YEPD plates and allowed to grow for 3 days at 30°C followed by an additional 2 days at room temperature. Plates were photographed before rinsing them with a gentle stream of deionized water. Plates were allowed to dry briefly and were then photographed again.

Microscopy

Shmoo morphology was determined by the addition of 10^{-6} M α-factor to 3 ml of log phase cultures for 3 h at 30°C. Cells were sonicated, fixed with formaldehyde to a final concentration of 3.7%, and viewed by differential interference contrast microscopy. Yeast actin was visualized with rhodamine–phalloidin (Molecular probes, Inc., OR). Briefly, fixed cells were stained for 20 min on ice with rhodamine–phalloidin (diluted 1:5 in PBS), washed three times with PBS and viewed on an Olympus fluorescenz microscope.

Cells harboring plasmids encoding GFP–Ste20 were grown in Raff-URA medium (2% raffinose, 0.8% sucrose) at 30°C and expression of GFP–Ste20 was induced for 3 h by addition of galactose to 2% final concentration. Where indicated, α-factor was added (10^{-6} M final) for 3 h at 30°C. Cells were fixed with formaldehyde to a final concentration of 3.7% and GFP fluorescence was visualized using an Olympus fluorescence microscope.

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