Role for the Rho-family GTPase Cdc42 in yeast mating-pheromone signal pathway

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In the budding yeast *Saccharomyces cerevisiae*, the process of conjugation of haploid cells of genotype *MATa* and *MATα* to form *MATa/α* diploids is triggered by pheromones produced by each mating type. These pheromones stimulate a cellular response by interaction with receptors linked to a heterotrimeric G protein. Although genetic analysis indicates that the pheromone signal is transmitted through the Gβγ dimer, the initial target(s) of G protein activation remain to be determined. Temperature-sensitive cells with mutations of the *CDC24* and *CDC42* genes, which are incapable of budding and of generating cell polarity at the restrictive temperature[1–3], are also unable to mate[4]. Cdc24 acts as a guanylyl-nucleotide-exchange factor for the Rho-type GTPase Cdc42[5], which has been shown to be a fundamental component of the molecular machinery controlling morphogenesis in eukaryotic cells[6–10]. Therefore, the inability of *cdc24* and *cdc42* mutants to mate has been presumed to be due to a requirement for generation of cell polarity and related morphogenetic events during conjugation. But here we show that Cdc42 has a direct signalling role in the mating-pheromone response between the G protein and the downstream protein kinase cascade.

In order to determine whether the role of Cdc24/Cdc42 in mating is limited to morphogenetic functions, we determined the integrity of other aspects of the mating-pheromone response in *cdc24* and *cdc42* temperature-sensitive (*ts*) mutants. Induction of pheromone-responsive genes such as *FUS1* constitutes one of the immediate responses to the pheromone signal and can occur at all stages of the cell cycle except late G1[11]. Figure 1*a* shows that *cdc24* and *cdc42* mutants shifted to their restrictive temperature were unable to induce a *FUS1 LacZ* fusion gene after addition of α-factor. Thus, inactivation of Cdc24 or Cdc42, in addition to interfering with morphogenetic events required for mating, prevents gene induction by pheromone, suggesting a role for these proteins in the signal transduction pathway itself. If Cdc24 and Cdc42 are required for mating-pheromone signalling, then inactivation of the proteins encoded by *ts* alleles should also release pheromone-arrested cells from their G1 block. To test this, *cdc24*, *cdc42* and wild-type cells were arrested in G1 at permissive temperature by treatment with α-factor, after which they were shifted to restrictive temperature while continuing incubation with the mating pheromone. Flow cytometric analysis showed that wild-type cells remained arrested in G1, as expected, but that *cdc24* and *cdc42* cells underwent DNA replication, indicating release from the G1 block (Fig. 1*b*). This result indicates that continued activity of Cdc24 and Cdc42 is required to maintain the pheromone signal after it has been established.

If activation of Cdc42 by guanylyl nucleotide exchange is required for pheromone signalling, mutational activation of Cdc42 might be expected to enhance the pheromone response. Expression of the activated $CDC42^{Val12}$ allele before addition of α-factor led to hyperinduction of a *FUS1–lacZ* reporter, reflecting a potentiation of the pheromone response pathway compared to the parental strain (Fig. 2*a*). However, expression of the activated Cdc42 was not sufficient to activate the pathway before the addition of α-factor (Fig. 2*a*, time 0). A possible explanation for this failure is that Cdc42 function requires mobilization of upstream elements for purposes other than guanylyl nucleotide exchange, for example, for colocalization of the active GTPase with its target. To test this idea, we coexpressed a dominant negative allele of *STE4* (encoding the β-subunit of the heterotrimeric G protein (Gβ)) simultaneously with $CDC42^{Val12}$, reasoning that this signalling-defective Gβ might be capable of supplying other essential upstream functions. Coexpression of *STE4dn-1* and $CDC42^{Val12}$ triggered a mating-pheromone response detectable using the *FUS1–lacZ* reporter construct in the absence of mating pheromone (Fig. 2*b*). Thus, activated Cdc42 can initiate a mating-pheromone response *de novo* if other upstream requirements are met.

FIG. 1 Cdc24 and Cdc42 are required for pheromone signalling. *a*, α-factor-induced *FUS1* expression in *cdc24-1* and *cdc42-1* temperature-sensitive mutant strains. Both *ts* strains and the parental strain, as well as two other *ts cdc* mutant strains as controls (*cdc28-13* and *cdc34-3*), were transformed with a centromeric vector containing a *FUS1–lacZ* reporter gene. Wild-type cells (open bars), *cdc24-1* cells (shaded bars, top), *cdc42-1* cells (black bars, top), *cdc28-13* cells (shaded bars, bottom), and *cdc34-3* cells (black bars, bottom) were grown to mid-log phase at 25 °C and shifted to restrictive temperature (36 °C) for 15 min before addition of 60 ng ml^{-1} α-factor. Aliquots were taken at the indicated times and β-galactosidase activity was measured after permeabilization of cells. Time 0 corresponds to the time of addition of α-factor. Values are means of duplicates for each point. The different maximal levels of *FUS1–lacZ* induction in the two sets of experiments correspond to different reporter plasmids used, which differ by about a factor of two in efficiency. The wild-type control serves as an internal standard. *b*, Release from pheromone-induced G1 arrest in *cdc24-1* and *cdc42-1* strains. Cellular DNA content was analysed by flow cytometry[25] for wild-type (column 1), *cdc24-1* (column 2) and *cdc42-1* (column 3) cell populations growing asynchronously, treated with α-factor (60 ng ml^{-1}) for 3 h at 26 °C, and shifted to 36 °C for 1 and 2 h. The first peak (at 200 arbitrary units) corresponds to cells in G1 (1*N* DNA content), whereas the second peak (at 400 arbitrary units) corresponds to cells with a 2*N* (replicated) DNA content. The 1*N* peak for the wild-type culture arrested with α-factor tended to drift to higher values with time because of increasing cell size and thus autofluorescence; some non-growing cells remained at about 200 arbitrary units forming a small extra peak. METHODS. *cdc24-1*, *cdc42-1*, *cdc28-13* and *cdc34-3* were congenic with our wild-type strain[28]. Transformations of yeast were according to ref. 29. β-Galactosidase activity was measured according to ref. 30. Miller units are defined as $(A_{420} \times 1{,}000) / (A_{600} \times V(\text{ml}) \times t(\text{min}))$.

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TABLE 1 Two-hybrid interaction between Cdc42 and Ste20

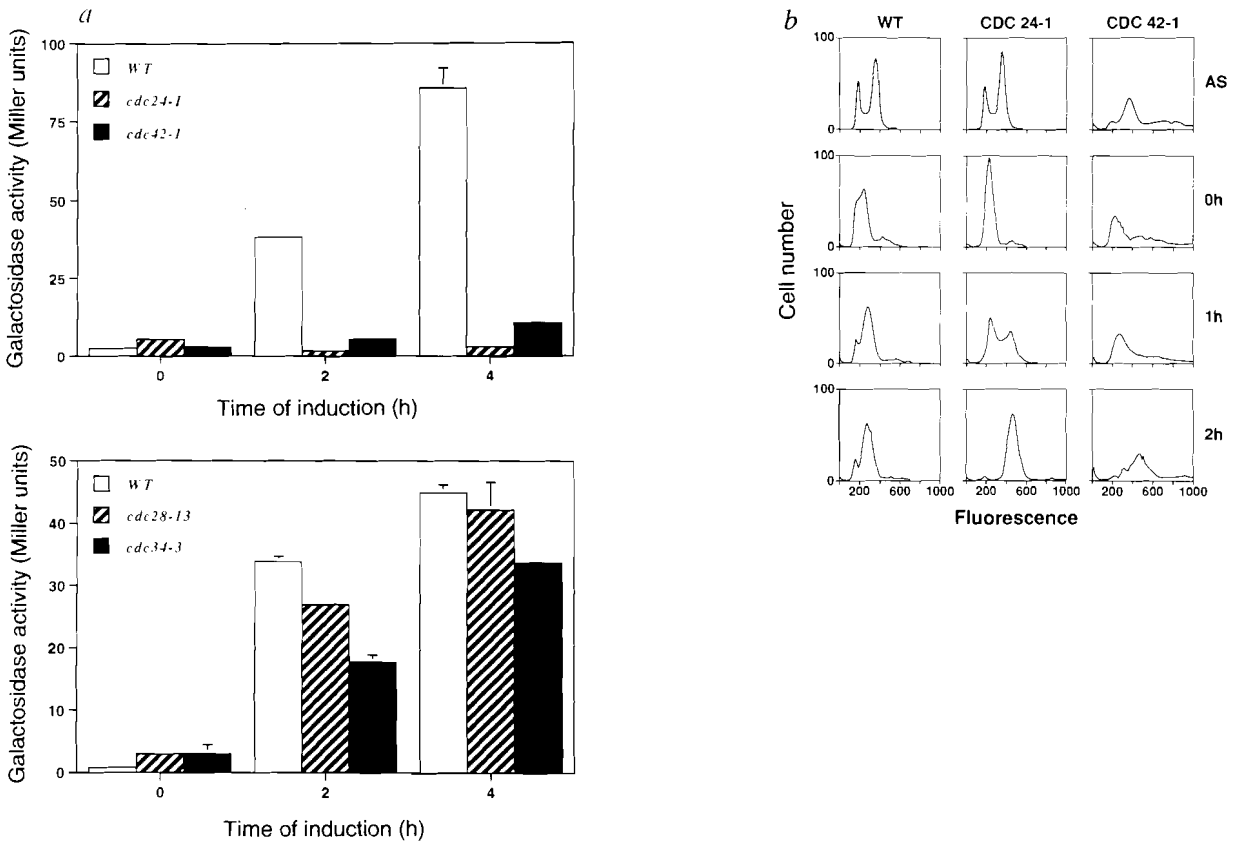
(*a*) Cdc42 interacts specifically with an amino-terminal domain of Ste20

	pJG4-5-*STE20*-1	pJG4-5-*STE20*-2	pJG4-5-*STE20*-3	pJG4-5-*MSB2*	pJG4-5
pEG202-*CDC42*	105	88	232	4	2
pEG202-*CDC42*^{Ser188}	2,111	2,741	3,506	41	40
pEG202-*RHO1*	10	9	16	7	7
pEG202-*RHO1*^{Ser206}			22	33	30
pEG202-*RHO2*$^{Ser188\ 189}$			28	23	26
pEG202-*RHO3*^{Ser228}			4	14	10
pEG202-*RHO4*^{Ser288}			43	97	69
pEG202-*RSR1*	21	17	12	10	5
pEG202-*RAS2*	9	10	7	6	4

(*b*) Interaction between Ste20 and activated Cdc42

	pJG4-5-*CDC42*	pJG4-5-*CDC42*^{Ser188}	pJG4-5-*CDC42*$^{Val12\ Ser188}$	pJG4-5-*CDC42*$^{Leu61\ Ser188}$	pJG4-5-*MSB2*	pJG4-5
pEG202-*STE20-4*	781 ± 278	2490 ± 500	4895 ± 537	5196 ± 622	20 ± 10	38 ± 3
pEG202-*MSB2*	2	7	3	3	2	2
pEG202	20	18	26	18	17	17

a, Interaction between Cdc42 and fragments of Ste20 was detected using the two-hybrid system[18]. Numbers correspond to average β-galactosidase activities from 3 to 6 different isolates. *CDC42*^{Ser188}, *RHO1*^{Ser206}, *RHO2*$^{Ser188\ 189}$, *RHO3*^{Ser228} and *RHO4*^{Ser288} encode mutant proteins that cannot be prenylated at their C termini and hence are not membrane-associated. METHODS. Interaction between Cdc42 and Ste20 was detected using the two-hybrid system[18] in the configuration described in ref. 19. For construction of the fusions of various GTPase proteins to the LexA DNA-binding domain (DBD) in pEG202, *S. cerevisiae CDC42*[20], *CDC42*^{Ser188} (ref. 21), *RHO1* and *RHO2* (ref. 22), *RHO3* and *RHO4* (ref. 23), *RSR1* (ref. 5), and *RAS2* (ref. 24) full-length coding sequences were amplified by polymerase chain reaction using Vent DNA polymerase (New England Biolabs). The resulting fragments were subsequently cloned either at the *Eco*RI–*Xho*I site or at the *Bam*HI–*Xho*I site of pEG202. The three plasmids pJG4-5-*STE20*-1, pJG4-5-*STE20*-2 and pJG4-5-*STE20*-3, which contain different fragments of *STE20* (ref. 12) fused to the activation domain (AD) moiety of pJG4-5, were isolated in a two-hybrid screen for proteins that interact with Cdc42. As a negative control, *MSB2* (ref. 27) was amplified by polymerase chain reaction and cloned into pJG4-5. β-Galactosidase activities were measured in three to six different isolates of each strain after growth for 16 h at 30 C in minimal medium containing 2% galactose and 1% raffinose. The average β-galactosidase activities (in Miller units) are given. *b*, The two-hybrid system was used as in *a* except that the protein domains tested for interaction were switched with respect to fusion with the DNA-binding domain and the activation domain. The segment fused to the DNA-binding domain in pEG202-*STE20-4* corresponds to amino acids 319–496 of *STE20*. For construction of the fusions of the wild-type and mutant alleles of Cdc42 to the AD in pJG4-5, *S. cerevisiae CDC42*, *CDC42*^{Ser188}, *CDC42*$^{Val12\ Ser188}$ and *CDC42*$^{Leu61\ Ser188}$ (refs 20, 21) full-length coding sequences were amplified by polymerase chain reaction as described in *a*. The double mutant genes *CDC42*$^{Val12\ Ser188}$ and *CDC42*$^{Leu61\ Ser188}$ were amplified by using plasmids containing either *CDC42*^{Val12} or *CDC42*^{Leu61} as templates and a reverse primer that specifically introduces the C to S Ser 188 mutation. All numbers are averages of two independent biological experiments performed in triplicate. Standard error is given for the average values of interaction with *STE20-4*.



Epistasis experiments have suggested that Ste20 functions at the top of a protein kinase cascade that culminates in the MAP kinase homologues Fus3 and Kss1[12,13]. It is possible, therefore, that Cdc24 and Cdc42 provide a link between the receptor-coupled G protein and Ste20. Two approaches were used to investigate a possible relationship between Cdc42 and Ste20. First, results obtained using the yeast two-hybrid system indicate that Cdc42 and Ste20 could interact directly *in vivo* (Table 1). The interaction observed was highly specific in that the same Ste20 fusions did not interact with the other small GTPase proteins tested, including four other *S. cerevisiae* Rho-family G proteins, all of which are highly homologous to Cdc42 (Table 1*a*). In addition, stronger interactions were detected when two different activated alleles of *CDC42*, *CDC42*^{Val12} and *CDC42*^{Leu61}, were

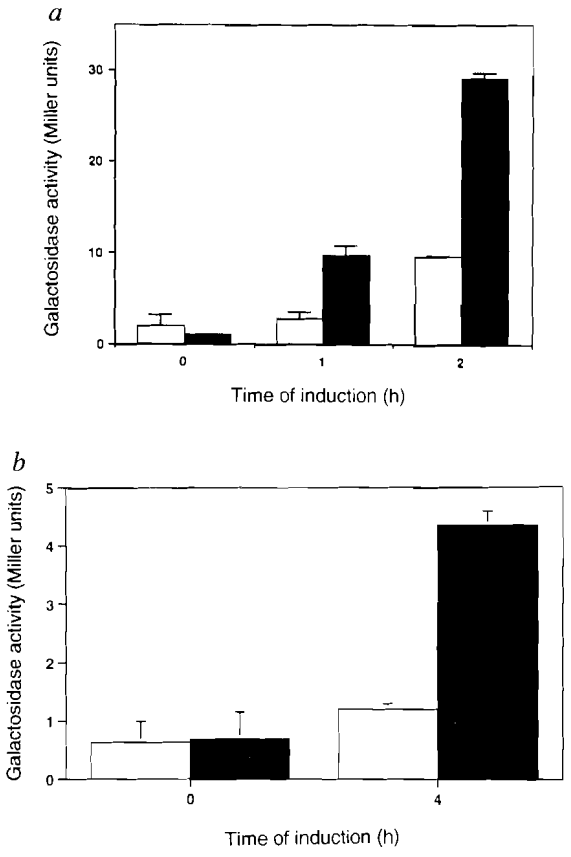


FIG. 2 Effect of *CDC42*^{Val12} expression on the pheromone response. *a*, *CDC42*^{Val12} potentiates the α-factor response. β-Galactosidase activity expressed from a *FUS1–lacZ* reporter gene was measured in the parental strain (open bars) and in the same strain containing a centromeric plasmid with the dominant activated *CDC42*^{Val12} allele under control of the *GAL1* promoter (black bars). Cells were grown in non-inducing sucrose medium to mid-log phase and then transferred to inducing medium containing 2% galactose for 1 h. α-Factor (60 ng ml^{-1}) was added to the culture at time 0 and aliquots were taken at the indicated times for measurement of β-galactosidase activity (see Fig. 1). *b*, *CDC42*^{Val12} can activate the pheromone response *de novo* in the presence of a dominant-negative allele of *STE4*. β-Galactosidase activity from a *FUS1–lacZ* reporter gene was measured in a strain containing a dominant-negative allele of *STE4* (encoding the β-subunit of the receptor-coupled G protein) and *CDC42*^{Val12} both under control of the GAL1 promoter (black bars). A control strain contained only the dominant-negative *STE4* allele (open bars). Cells were grown in non-inducing sucrose medium to mid-log phase and then transferred to inducing medium containing 2% galactose. β-Galactosidase activity was measured at the time of transfer and after 4 h of induction. Values given represent the averages from two independent experiments; error bars give the variation from the mean. The response, although not equivalent to a maximal pheromone response, indicates complementation of the signalling defect of the mutant Ste4 by activated Cdc42. The dominant negative *STE4* allele (*STE4dn-1*) was isolated from a library of plasmid-borne *STE4* alleles, randomly mutated by PCR, based on its ability to confer dominant α-factor resistence when expressed from the *GAL1* promoter (M.-N.S. and S.I.R., manuscript in preparation). It is presumed that the phenotype conferred by this allele results from an ability to interact with downstream elements in a non-productive fashion.

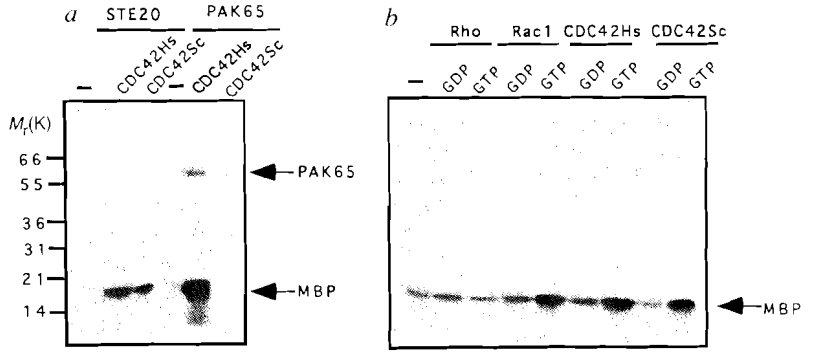


FIG. 3 GTP-bound Cdc42 activates the Ste20 kinase *in vitro*. *a*, Comparison with PAK65. Purified recombinant yeast Ste20 was incubated either in buffer alone or with purified recombinant yeast or human Cdc42 loaded with GTP-γS and analysed for myelin basic protein (MBP) kinase activity. In a parallel experiment, human PAK65 (hPAK65), a structural homologue of Ste20, was assayed in the same manner. Although human CDC42 (CDC42Hs) could activate yeast Ste20, yeast Cdc42 (CDC42Sc) could not activate hPAK65. An additional difference between activation of yeast Ste20 and hPAK65 was lack of autophosphorylation observed for Ste20. Because autophosphorylation of hPAK65 (indicated on autoradiograph) renders its activity GTPase independent[26], these data suggest that Ste20 may require continued interaction with a GTPase to maintain activity. *b*, Specificity of Ste20 activation. Ste20 was incubated with buffer alone, human RHO1, human RAC1, human CDC42, or yeast Cdc42 either loaded with GTP-γS or GDP-βS and then tested for the ability to phosphorylate myelin basic protein (MBP; indicated on the autoradiograph). Although a basal level of kinase activity is detected in the absence of added Cdc42, there is a significant GTP-dependent stimulation of this activity by human and yeast Cdc42 and human RAC1. Human RHO1 conferred no stimulation. The GTPase-independent background observed for Ste20 appears to be related to significant contamination with proteolytic fragments containing the catalytic domain but missing the complete amino-terminal regulatory domain and therefore having constitutive activity (data not shown).

METHODS. Recombinant hPAK65 or Ste20 (1–2 μg bound to protein G–Sepharose conjugated with either myc or glu–glu monoclonal antibodies) was washed with 1 ml and incubated with 40 μl of kinase buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$) with 1–2 μg of the indicated GTPase preloaded with either 100 μM GTP-γS or GDP-βS. All recombinant proteins used were generated in Sf9 cells as previously described[26]. The reaction was initiated by adding 10 μl kinase buffer containing 50 μM ATP and 5 μCi [γ^{32}P]ATP and incubated for 20 min at 30 °C. Myelin basic protein (4 μg) was included in each assay. The reaction was stopped by adding SDS–PAGE sample buffer and boiling for 5 min. Samples were applied to a 14% SDS–PAGE gel which was stained with Coomassie blue, destained, dried and exposed to film.

assayed (Table 1*b*). The results of the two-hybrid studies are consistent with the demonstration that the highly conserved human homologue of Cdc42 (Cdc42Hs), when in the GTP-bound form, can bind and activate *in vitro* a protein kinase (PAK65) with sequence similarity to Ste20[14,26]. Second, to characterize further the nature of the interaction between Cdc42 and Ste20, the respective recombinant proteins were expressed and purified. GTP-bound (but not GDP-bound) Cdc42 was found to be capable of stimulating the protein kinase activity of Ste20 *in vitro*, consistent with a direct signalling relationship (Fig. 3).

The genetic and physiological properties of Cdc24 and Cdc42 demonstrated here suggest that these proteins are components of the primary mating-pheromone signal-transduction pathway. These results potentially fill a gap in this signalling pathway, between the heterotrimeric G protein and the downstream MAP kinase cascade, that has remained refractory to elucidation by genetic analysis. The apparent dual role of Cdc42, a Rho-family GTPase, in generation of cell polarity during vegetative growth and in pheromone signal transduction raises the possibility of coregulation of the two processes. We suggest an evolutionarily conserved regulatory motif whereby Rho-family GTPases link cell differentiation to morphogenetic processes. In the yeast mating-pheromone response, polarized growth toward the source of pheromone[15] is correlated with clustering of both pheromone receptors and Cdc42 at the tips of growth projections[16,17], presumably oriented by exposure to the pheromone gradient. Thus, Cdc42 would be poised to transduce signals involved in both polarized growth and gene expression. □

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