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Pheromone Signalling in *Saccharomyces cerevisiae* Requires the Small GTP-Binding Protein Cdc42p and Its Activator *CDC24*

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Pheromone signalling in *Saccharomyces cerevisiae* is mediated by the *STE4-STE18* G-protein βγ subunits. A possible target for the subunits is Ste20p, whose structural homolog, the serine/threonine kinase PAK, is activated by GTP-binding p21s Cdc42 and Rac1. The putative Cdc42p-binding domain of Ste20p, expressed as a fusion protein, binds human and yeast GTP-binding Cdc42p. Cdc42p is required for α-factor-induced activation of *FUS1*. *cdc24*^{ts} strains defective for Cdc42p GDP/GTP exchange show no pheromone induction at restrictive temperatures but are partially rescued by overexpression of Cdc42p, which is potentiated by Cdc42p^{12V} mutants. Epistatic analysis indicates that *CDC24* and *CDC42* lie between *STE4* and *STE20* in the pathway. The two-hybrid system revealed that Ste4p interacts with Cdc24p. We propose that Cdc42p plays a pivotal role both in polarization of the cytoskeleton and in pheromone signalling.

In eukaryotic cells, many growth and differentiation signals are channelled through protein phosphorylation cascades that involve the mitogen-activated protein (MAP) kinase family. The similarity of MAP kinase cascades in multicellular organisms to those in the yeast *Saccharomyces cerevisiae* is a subject of great interest (7, 17, 42, 50). One of the best studied of these cascades at the genetic level is the pheromone-dependent pathway in *S. cerevisiae*, which links occupation of pheromone receptors by **a**- or α-factor to a variety of events that lead to mating (for reviews, see references 3, 27, and 41). The pheromone-induced transcriptional activation of mating-specific genes requires a kinase cascade that includes the protein kinases encoded by *STE20*, *STE11*, *STE7*, *KSS1*, and *FUS3*. Activation of the pathway is initiated by the heterotrimeric G-protein βγ subunits encoded by *STE4* and *STE18* (71, 72); the *STE20* gene product is a kinase which may lie at the top of the kinase cascade and has been implicated as a target of the *STE4*-encoded β subunit (32). In mammalian cells, the activation of MAP kinase by various proto-oncogenic tyrosine kinases requires small GTP-binding proteins (p21s) of the Ras family; more recently, heterotrimeric G-protein signalling to MAP kinase has been shown to be mediated by βγ subunits via Ras (13). While the mating pathway in *Schizosaccharomyces pombe* parallels this pathway in requiring *ras1* (73), there has been no evidence for the involvement of a p21 in the *S. cerevisiae* pheromone-induced kinase cascade.

We have described a number of proteins that appear to be the target of the Rho subfamily of p21s (39, 40). One of these proteins was purified and shown to be a serine/threonine kinase that is directly activated by GTP-binding Cdc42 (GTP-Cdc42p) and GTP-Rac1. This brain-enriched kinase, designated PAK (40), is related to Ste20p both in the kinase domain and in a separate region identified as the p21-binding domain. These findings suggested that Cdc42p, a p21 first identified as a gene product required for polarized budding (29), participates in the mating response (Rac has not been found in *S. cerevisiae*). *S. cerevisiae* Cdc42p (Cdc42Sc) has been localized to both the bud site and the mating projection (77) and is closely related to its mammalian homologs (48, 61). Other yeast polarization genes such as *BEM1* have been found to be required for mating, and indeed mating-defective alleles which do not affect vegetative growth have been identified (10). Recently a gene encoding an *S. cerevisiae* RhoGAP-like protein has been shown to affect mating efficiency (47), suggesting that a specific signal from a p21 of this family (Rho1/2/3/4 or Cdc42p) is required.

If the similarity of Ste20p to PAK extends to its mode of activation, GTP-Cdc42p could be required for the signalling of MAP kinase cascade which leads to transcriptional changes and cell cycle arrest (27). This possibility, in turn, implies that the guanine nucleotide exchange/release factor for Cdc42p, namely, the gene product of *CDC24* (75), might also play a role in this pathway; temperature-sensitive *cdc24* (*cdc24*^{ts}) strains have long been known to show deficiencies in mating (56). Recently homologs of *CDC24* and *BEM1* have been identified in *S. pombe*; these genes, designated *scd1* and *scd2*, respectively, are required for both normal morphology and mating (8). This requirement is probably reflected in the interaction of *scd1* with *ras1*, which is a component of the mating MAP kinase cascade in this organism.

We have investigated the role of Cdc42p in the pheromone-induced mating response of *S. cerevisiae*, using *cdc42*^{ts} and *cdc24*^{ts} strains. The complete absence of signalling at restrictive temperatures indicated that loss of either *CDC42* or *CDC24* function prevents pheromone signalling. The nature of the defect in *FUS1* induction upon α-factor treatment in these strains has been investigated by the use of constructs which behave as dominant activated alleles of *STE4* and *STE20* in the absence of pheromone. Protein-protein interaction assays indicate that Ste4p binds to Cdc24p and that GTP-Cdc42p binds to Ste20p. These findings suggest that signalling to the MAP kinase cascade from βγ subunits may require coactivation of the Cdc42Sc p21, which could thus allow coupling of nuclear and cytoskeletal events.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1*	62
W303	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL psi*$^+$	65
DJTD2-16A (*cdc42-1*^{ts})	*MAT***a** *cdc42-1 ura3 his4 leu2 trp1 gal2*	29
Y147 (*cdc24-4*^{ts})	*MAT***a** *cdc24-4 ura3 leu2-3,112 his3 gal2*	5
cdc24-1^{ts}	*MAT***a** *cdc24-1 ura1 tyr1 arg4 thr4 ade1 ade2 gal1*	25
HF7c	*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2*::*GAL1-HIS3 URA3*::(*GAL*4 17-mers)$_3$-*CYC1-lacZ*	Clontech

MATERIALS AND METHODS

Reagents. [α-^{32}P]dCTP (~3,000 Ci/mmol) and Hybond-N hybridization membranes were from Amersham. [γ-^{32}P]GTP (~6,000 Ci/mmol) was from Dupont-NEN. D-(+)-glucose was purchased from BDH Laboratory Supplies (Poole, United Kingdom). The constituents used in the synthetic media, α-factor, D-(+)-raffinose, D-(+)-galactose, and sand (white quartz, −50+70 mesh), were purchased from Sigma Chemical Company (St. Louis, Mo.).

Preparation of recombinant p21-binding domain proteins and analysis by [γ-^{32}P]GTP Cdc42 overlays. The GST-Ste20p fusion was constructed in the pGEX-2T (Pharmacia) vector cloned into the *Bam*HI and *Eco*RI sites as follows. PCR (25 cycles) was used to amplify the Ste20p coding sequence (residues 327 to 378) with a 5′ primer containing a *Bam*HI site (underlined) (gact gga TCC TCA TCT TCT ATA ACC ACC G; uppercase letters refer to *STE20* codons) and a 3′ primer containing an *Eco*RI site (gact gaa ttc TCT TTT GGA AAT ACC AC) and using a template W303 genomic DNA. The cDNA corresponding to PAK residues 69 to 149 was amplified (25 cycles of PCR) by using a 5′ primer (gat gga tcc GAG AGG GAG CGG CAC GAG) and a vector 3′ primer, and the product was cut at the internal *Pvu*II site and cloned into pGEX-4T-1 (*Bam*HI-*Sma*I). The insert was confirmed by sequencing. Human ACK residues 499 to 570 were derived from a truncated human ACK cDNA (clone 5) cut at the 5′ end with *Bst*EII (filled in) and at the 3′ end with *Eco*RI (plasmid cloning site); the fragment was cloned into pGEX-3X (*Sma*I-*Eco*RI). Cdc42p coding sequences were cloned in pGEX-2T as described previously (38). GST fusion proteins were purified from *Escherichia coli* BL21 induced with 0.5 mM isopropylthiogalactopyranoside (IPTG) at room temperature for 4 h as described previously (40) and diluted to 1 mg/ml for storage.

After sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis on 12% gels, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (NEN). Filters were blocked for 2 h in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 5 mM dithiothreitol, 0.5 mM $MgCl_2$, and 0.1% Triton X-100 at 4°C prior to overlay analysis (39). Briefly, [γ-^{32}P]GTP was incorporated into the p21s, using 5 μg of each protein in 50 μl of exchange buffer (50 mM NaCl, 25 mM morpholineethanesulfonic acid [MES]-NaOH [pH 6.5], 2.5 mM EDTA, 0.05% Triton X-100) containing 10 μCi of [γ-^{32}P]GTP (~6,000 Ci/mmol; NEN) and incubated for 4 min at 24°C. This solution was added to 2 ml of binding buffer (50 mM NaCl, 25 mM MES-NaOH [pH 6.5], 2.5 mM dithiothreitol, 1.25 mM $MgCl_2$, 0.05% Triton X-100) containing 0.5 mM GTP. Each PVDF membrane was evenly covered with the appropriate p21 solution and then transferred to a 1% agarose plates for 10 min at 4°C. The filters were then washed three times for 1 min each in PBS containing 25 mM MES-NaOH (pH 6.5), 5 mM $MgCl_2$, and 0.05% Triton X-100.

Yeast strains, media, and genetic manipulation. The *S. cerevisiae* strains used in this study are listed in Table 1. Standard genetic protocols were used to manipulate yeast strains (21, 58). The compositions of the rich medium and the synthetic medium were as described previously (21). Yeast transformation was carried out by the alkali cation method (60). To induce genes under control of the *GAL1* promoter, cells were pregrown in SC selective medium with 2% raffinose to log phase (optical density at 600 nm [OD_{600}] = 0.8) and then in SC selective medium with 2% galactose for the indicated time.

Modification of vectors. The yeast galactose-inducible expression vectors were constructed from the yeast-*E. coli* shuttle vector YCplac111, YEplac181 (20), pRS425, or pRS426 (12). The *GAL1/10* (0.8-kb) promoter region was amplified from W303 genomic DNA by PCR with primers 5′-aattcgacaggttatcagcaacaaca cag-3′ and 5′-tatagtttttctccttgacg-3′. The PCR product and vectors were treated with T4 DNA polymerase and deoxynucleoside triphosphate and inserted into the *Hin*dIII-*Sph*I sites in YEplac181 and YCplac111 (gifts from R. D. Gietz) and the *Sac*I site in pRS425 and pRS426 (obtained from the American Type Culture Collection; catalogue no. 77106 and 77107). The sequence and orientation of the *GAL1/10* promoter in the resultant vectors were verified by double-stranded DNA sequencing using the Sequenase 2.0 system (U.S. Biochemical). The galactose-inducible vectors used in this study were pGAL45 (YCplac111-GAL1 [with *LEU2* marker and *CEN* origin of replication]), pGAL48 (YEplac181-GAL1 [with *LEU2* marker and 2μm origin of replication]), pGAL51 (pRS425-GAL1 [with *LEU2* marker and 2μm origin of replication]), and pGAL52 (pRS426-GAL1 (with *URA3* marker and 2μm origin of replication]).

The yeast two-hybrid vectors pGBT9M (GAL4 DNA-binding domain) and pGAD424M (GAL4 activation domain) are derivatives of pGBT9 and pGAD424 (purchased from Clontech Laboratories, Inc.) by replacing the polylinker (*Eco*RI-*Pst*I) with the complementary oligonucleotides 5′-aattgggatccccgaattcccg ggtcgactcgagcggccgcctgca-3′ and 5′-ggcggccgctcgagtcgacccgggaattcgggatccc-3′, which contain restriction sites in the following order: *Bam*HI, *Eco*RI, *Xma*I, *Sma*I, *Sal*I, *Xho*I, *Not*I, and *Pst*I. The modified polylinkers of both pGAD424M and pGBT9M contain a *Bam*HI site whose reading frame is GGA TCC. The expression of fusion proteins in these vectors is directed by a crippled *ADH* promoter (22).

Gene cloning and Cdc42p mutagenesis. The plasmid constructs used in this study are listed in Table 2. The coding regions of *CDC24*, *STE4*, *STE20*, and *CDC42Sc* were amplified by using *Taq* DNA polymerase (Cetus) and W303 genomic DNA through 25 cycles of PCR. The ULTma DNA polymerase with proofreading activity (Cetus) was used to generate derivatives of the parent sequence by 15 cycles of PCR. The pGAL::STE4 clone was tested by lethality and *FUS1* induction, the pGBT::Cdc24 clone was tested by its ability to complement *cdc24-4*^{ts} at 37°C, and the pGAL::STE20 full-length clone was tested by complementation of the Δ*ste20* mutant strain. The wild-type and mutant constructs of human Cdc42 (Cdc42Hs) and Cdc42Sc were completely sequenced.

A two-round PCR-based mutagenesis method was used to introduce mutations into CDC42Hs. In the first round, the N-terminus region of *CDC42Hs* was amplified by PCR using plasmid (39) pGEX-CDC42Hs(*Bam*HI-*Eco*RI) as the template and vector forward primer and phosphorylated oligonucleotide 5′-tttaccaacagcaccatc-3′. The adjacent 3′ *CDC42Hs* sequence was amplified by another PCR using vector reverse primer and phosphorylated mutagenic oligonucleotide 5′-aattgtctcgtgatatcctac-3′, which contains a threonine-to-asparagine mutation at amino acid residue 17 position (the nucleotide changes in the oligonucleotide are underlined). The purified PCR products were ligated by using T4 DNA ligase (Bethesda Research Laboratories) at 14°C overnight and used as the template for the second-round PCR using vector forward and reverse primers. The second-round PCR product was purified, digested with *Bam*HI-*Eco*RI, and subcloned into pGAL48(*Bam*HI-*Eco*RI). The Cdc42Hs^{17N} mutant was verified by double-strand sequencing.

Northern (RNA) analysis. Yeast total RNA was isolated by a single-step phenol-chloroform-isoamyl alcohol method (15). For α-factor induction, 20 ml of cells cultured to log phase (OD_{600} = 0.8, with pH adjusted to 4.0 with HCl) was treated with α-factor at a final concentration of 5 μM for 15 min at 37°C, 25 min at 30°C, or 30 min at 24°C. These cells or untreated cells were harvested by pouring the culture into a 50-ml Falcon tube containing crushed ice and centrifuging it at 4,000 rpm for 2 min. The cells were transferred into a 2-ml Eppendorf tube with a safe lock cap in 1 ml of ice-cold PBS for RNA isolation. Total RNA (50 μg) from each sample was denatured and separated on 1% agarose formaldehyde gel as described previously (59). The RNA was transferred onto Hybond-N (Amersham) and UV cross-linked onto the membrane, and the membrane was baked for 2 h at 80°C. The filter was hybridized with [α-^{32}P]dCTP-labelled *FUS1* probe in buffer containing 7% SDS, 0.23 M Na_2HPO_4-0.27 M NaH_2PO_4 (pH 6.8), and 10% formamide at 60°C for 18 h, washed twice with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 25°C for 15 min, and washed once with 0.2× SSC–0.1% SDS at 60°C for 10 min.

The *FUS1* DNA fragment (50 to 571 bp of coding region) was amplified by PCR from W303 genomic DNA with oligonucleotides 5′-cgcaatgtctactacc-3′ and 5′-ccagaccaagcatatg-3′, and the 0.5-kb PCR product was subcloned into cloning vector PCRII (Invitrogen) and sequenced. The *Bam*HI-*Xba*I 0.5-kb *FUS1* DNA fragment from plasmid PCRII-FUS1 was labelled by random priming with [α-^{32}P]dCTP (Amersham). Comparative RNA loading was determined either by methylene blue staining of rRNA (59) before hybridization or by probing filters with an *ACT1* fragment (derived by PCR with oligonucleotides 5′-cgggatctggcat catacttc-3′ and 5′-acaaagcttctggggctcgga-3′) or a *URA1* fragment (derived by PCR using oligonucleotide 5′-gtccatatggtatcttcccaccctaaa-3′ and 5′-attgccggcat taaatgctgttcaact-3′) under conditions as described for *FUS1*.

Morphological analysis. Yeast cells were fixed with 150 mM NaCl–3.7% formaldehyde for 10 min at the relevant culture temperature and sonicated for 10 s before examination under a microscope. The photomicrographs of yeast cells were taken with a phase II contrast lens (×20) on a Nikon Microphot FXA microscope.

Halo and complementation assays. *cdc24-4*^{ts} cells containing plasmids pGAL::Cdc42Hs, pGAL::Cdc42Sc, and pGAL::Cdc42^{12V} were streaked on glucose selective plates and grown at 24°C for 24 h. These plates were replica plated to a selective plate containing 2% galactose and 1 M sorbitol (48) and incubated at 36°C for 2 days. A second replica on the selective Gal plate showed growth rescue clearly. The cells from the rescue plate were resuspended and diluted in

TABLE 2. Plasmid constructs used in this study

Plasmid	Vector	Insert	Comment Reference or derivation
pGAL::Cdc42Hs	pGAL48	*CDC42Hs* (*Bam*HI-*Eco*RI)	36a
pGAL::Cdc42Hs^{17N}	pGAL45	*CDC42Hs^{17N}*	This report
pGAL::Cdc42Hs^{12V}	pGAL48	*CDC42Hs^{12V}* (*Bam*HI-*Eco*RI)	36a
pGAL::Cdc42Hs^{5R12V}	pGAL48	*CDC42Hs*^{5R12V} (*Bam*HI-*Eco*RI)	36a
pGAL::Cdc42Sc	pGAL48	*CDC42Sc* (*Bam*HI-*Eco*RI)	PCR using oligonucleotides 5′-gatggatccaaaatgcaaacgctaaagtg-3′ and 5′-gtgaattctacaaaattgtacatttttac-3′
pGAL::Cdc42Sc^{12V}	pGAL48	*CDC42Sc^{12V}* (*Bam*HI-*Eco*RI)	PCR using oligonucleotides 5′-cgggatccaaaatgcaaacgctaaagtgtg ttgtcggtgatgttgc-3′ and 5′-gtgaattctacaaaattgtacatttttac-3′
pGAL::Rac1^{12V}	pGAL48	*RAC1^{12V}* (*Bam*HI-*Eco*RI)	2
pGAL::STE4	pGAL48	*STE4* (*Bam*HI-*Eco*RI)	PCR using oligonucleotides 5′-acggatccatggcagcacatcagatg-3′ and 5′-aggaattccaattcgaagctattg-3′
pGAL::ΔN^{494}Ste20	pGAL52	Δ*NSTE20*(495–937) (*Bam*HI-*Xho*I)	PCR using oligonucleotides 5′-gcatggatccgcttcagctccaat-3′ and 5′-ccgctcgagtttacttttgtttatcatc-3′
pGAL::STE20	pGAL51	*STE20* (*Bgl*II-*Xho*I) (full length)	PCR using oligonucleotides 5′-gatcctcgactaaagatcttaatgagcaatg atcc-3′ and 5′-ccgctcgagtttacttttgtttatcatc-3′
pGBT::Cdc24	pGBT9M	*CDC24* (*Bam*HI-*Pst*I) (full length)	PCR using oligonucleotides 5′-cgggatccatggcgatccaaacccgttttg-3′ and 5′-ttctgcagtcaatacagacgaatgttcaag-3′
pGAD::Cdc24	pGAD424M	*CDC24* (*Bam*HI-*Pst*I)	The *Bam*HI-*Pst*I fragment from pGBT::Cdc24 was cloned into *Bam*HI-*Pst*I-linearized pGAD424M
pGBT::Cdc24(1-289)	pGBT9M	*CDC24*(1–289) (*Bam*HI-*Pst*I)	PCR using oligonucleotides 5′-cgggatccatggcgatccaaacccgttttg-3′ and 5′-ttctgcagcgttgcaacgaattcctt-3′
pGBT::Cdc24(1-487)	pGBT9M	*CDC24*(1–487) (*Bam*HI-*Pst*I)	PCR using oligonucleotides 5′-cgggatccatggcgatccaaacccgttttg-3′ and 5′-ttctgcagatataataactcaccgaac-3′
pGBT::Cdc24(1-779)	pGBT9M	*CDC24*(1–779) (*Bam*HI-*Bgl*II)	The larger fragment from pGBT::Cdc24 cut with *Bgl*II and *Pst*I was recircularized by blunt-end ligation
pGBT::Cdc24(280-487)	pGBT9M	*CDC24*(280–487) (*Bam*HI-*Pst*I)	PCR using oligonucleotides 5′-cgggatccgttaaaattatcaaggaatt-3′ and 5′-ttctgcagatataataactcaccgaac-3′
pGBT::Cdc24(280-854)	pGBT9M	*CDC24*(280–854) (*Bam*HI-*Pst*I)	PCR using oligonucleotides 5′-cgggatccgttaaaattatcaaggaatt-3′ and 5′-ttctgcagtcaatacagacgaatgttcaag-3′
pGBT::Cdc24(473-854)	pGBT9M	*CDC24*(473–854) (*Bam*HI-*Pst*I)	PCR using oligonucleotides 5′-gatggatccaactggaagggttatagaatt-3′ and 5′-ttctgcagtcaatacagacgaatgttcaag-3′
pGBT::Cdc24(473-701)	pGBT9M	*CDC24*(473–701) (*Bam*HI-*Xho*I)	PCR using oligonucleotides 5′-gatggatccaactggaagggttatagaatt-3′ and 5′-gatctcgagttgtggtgggtgacatca-3′
pGBT::Cdc24(766-854)	pGBT9	*CDC24*(766–854) (*Eco*RV-*Pst*I)	The 280-bp *Eco*RV-*Pst*I fragment from pGBT::Cdc24 was cloned into *Sma*I-*Pst*I-linearized pGBT9
pGBT::ΔN^{494}Ste20	pGBT9	Δ*NSTE20*(495–939) (*Bam*HI-*Xho*I)	The *Bam*HI-*Xho*I (blunt-ended) fragment from pGAL::ΔNSte20 was cloned into *Bam*HI-*Eco*RI (blunt-ended)-linearized pGBT9

YEP-Gal medium prewarmed to 36°C, and 10^6 cells in 400 μl were spread evenly on a 36°C YEP-Gal plate, which was then air dried. Eight micrograms (1 μg/μl) of α-factor (Sigma) dissolved in 90% methanol was dotted on Whatman 3MM filter discs (diameter = 6 mm) on the cell spread plate. The plate was incubated at 36°C for 2 days before photography.

Two-hybrid protein interaction assay. HF7c competent cells were cotransformed with 8 μg of each GAL4 DNA-binding domain fusion plasmid DNA and GAL4 activation domain fusion plasmid DNA; equal amounts of the cell mix were plated on an SC agar plate for selection of uracil, leucine, tryptophan, and histidine and on a plate for selection of uracil, leucine, and tryptophan. The colony numbers on both plates were scored after 3 days of incubation at 30°C.

RESULTS

Mammalian and yeast GTP-Cdc42s bind to recombinant Ste20p. The significant homology between a region in Ste20p and the PAK binding/activation domain, both N terminal to their highly conserved kinase domains (40), led us to test whether the Ste20p region binds [γ-^{32}P]GTP-Cdc42. The sequence in Ste20p of 52 amino acids (bracketed in Fig. 1A) corresponding to the minimal p21-binding region of the tyrosine kinase ACK (39) was expressed as a glutathione *S*-transferase (GST) fusion protein (Fig. 1B) and analyzed for binding to human and yeast Cdc42p. The p21s, labelled with [γ-^{32}P]GTP under identical conditions, were overlaid onto PVDF filters containing the immobilized GST fusion proteins. The putative p21-binding domain of Ste20p indeed bound to [γ-^{32}P]GTP-Cdc42Hs (Fig. 1B, middle) with similar affinity as the proteins containing the p21-binding domains of the two mammalian kinases. The small proteolytic breakdown products in the Ste20p lane do not bind p21. Interestingly, labelled Cdc42Sc interacted more strongly with the Ste20p fusion protein than the mammalian sequences. This domain of PAK appears to regulate autophosphorylation and activation of the kinase domain upon binding of the activated Cdc42. None of the fusion proteins bound to the related mammalian p21 RhoA or RhoG (not shown).

The GTPase activities of recombinant Cdc42Hs and the two putative dominant mutants Cdc42Hs^{12V} and Cdc42Hs^{5R,12V} used in this study are shown in Fig. 1C. The slower rate of GTP hydrolysis of the mutant proteins relative to wild-type Cdc42 (with a half-life of 5 min at 30°C) parallels the G-12→V mutation in the related p21 Rac1 (57), which gives a dominant-activated phenotype in vivo because the mutant p21 tends to remain in its GTP-bound active conformation.

***FUS1* transcriptional activation in response to α-factor is defective in *cdc42^{ts}* and *cdc24^{ts}* strains.** Pheromone activation leads to *STE12*-mediated transcriptional activation of a number of yeast mating-specific genes (16), including *FUS1*, whose product is required for cell fusion (14). To assay activation of the kinase cascade, we directly monitored *FUS1* mRNA levels following a short α-factor treatment (15 to 30 min, depending on the temperature), since this is a highly induced and widely used reporter gene for the function of the pathway. Where

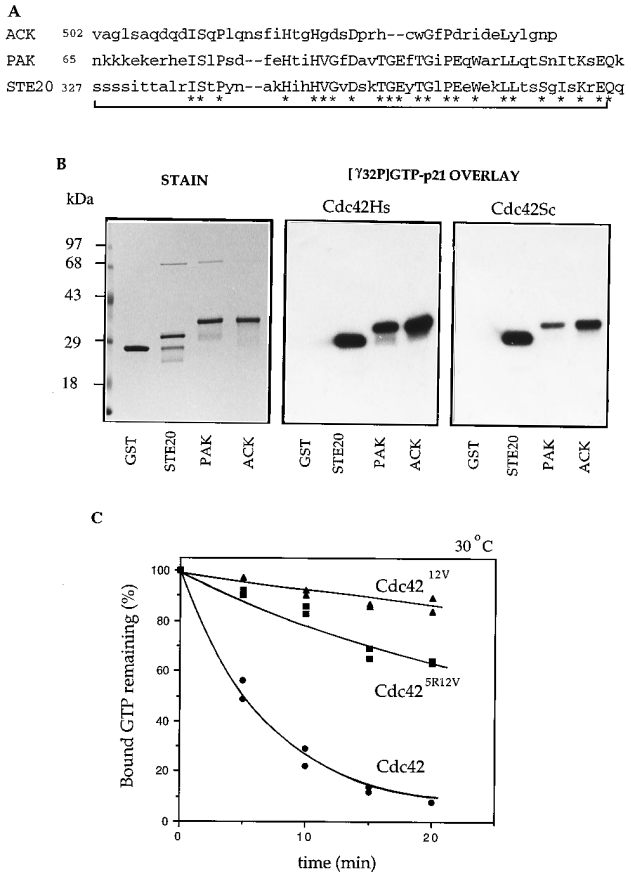

FIG. 1. Ste20p contains a GTP-Cdc42p binding domain. (A) Alignment of the p21-binding domains of PAK and ACK with the homologous region of Ste20p. Residues conserved between PAK and Ste20p are in uppercase and marked (*). The Ste20p residues expressed in the GST fusion construct are bracketed. (B) Purified recombinant GST fusion proteins (adjusted to ~1 μg per band) were separated on a 12% polyacrylamide gel and Coomassie blue stained (left); the proteins corresponded to GST-Ste20p(327–379), GST-PAK(69–150), and GST-ACK(499–570). For the p21 overlay assay, duplicate sets were transferred to PVDF membranes (NEN). Recombinant p21s were each labelled with 10 μCi of [γ-^{32}P]GTP and overlaid at a concentration of 2 μg/ml onto the corresponding filters. Binding on 1% agarose was allowed for 10 min. Filters were washed and exposed to hyperfilm (Amersham) without an intensifying screen for 2 h at −70°C. (C) Hydrolysis of bound GTP by recombinant human Cdc42- and GTPase-impaired mutants. [γ-^{32}P]GTP was exchanged into the p21 in the presence of 2.5 mM EDTA, then transferred to buffer containing 1.25 mM $MgCl_2$, and incubated at 30°C. At each time point, 20-μl aliquots (in duplicate) were removed, absorbed onto nitrocellulose, washed, and assayed for bound [γ-^{32}P]GTP. The average counts were expressed as a percentage of counts at $t = 0$. Data shown are from two independent experiments, and curves were drawn by hand.

possible, the temperature-sensitive strains were maintained for as short a time as necessary at the lethal restrictive temperature to avoid pleiotropic effects of the gene deficiency. The level of *FUS1* mRNA induction (following 15 min of exposure to α-factor) declined with time after transfer of *cdc42-1^{ts}* cells (1) to restrictive temperatures (Fig. 2A). To confirm this involvement of Cdc42 in the mating response, we expressed the 17N putative dominant negative Cdc42Hs mutant in wild-type cells. Induction of this protein under a *GAL* promoter led to the expected phenotype of large unbudded cells (at 30°C; Fig. 2C) and a concomitant decrease in level of α-factor-induced *FUS1* mRNA (Fig. 2A). Thus, the pheromone signalling defect correlates with phenotypic loss of function produced by the putative dominant negative Cdc42. Similarly, the *cdc24-1^{ts}* (25) and *cdc24-4^{ts}* (63) strains, which exhibit a similar morphological defect genetically upstream of *CDC42* (5) and have a normal pheromone response without preincubation at restrictive temperatures (Fig. 2B, lanes 1 and 4), completely lose the ability to respond to pheromone after 2 h at 37°C. Wild-type cells show no change in the extent of *FUS1* induction with prolonged incubation at 37°C, while *cdc24-1^{ts}* and *cdc24-4^{ts}* strains remain unresponsive on this time scale, as expected (Fig. 2B). Because the *cdc24-4^{ts}* strain required the shorter time at restrictive temperatures for complete loss of α-factor signalling, it was used for most of the subsequent experiments.

***CDC24* is required for *STE4* signalling.** To rule out the possibility that *cdc42^{ts}* and *cdc24^{ts}* cells become nonresponsive to pheromone because of down-regulation of some component in the receptor complex or a failure in coupling of pheromone receptor to the G-protein α and βγ subunits, *STE4* (encoding the β subunit) placed under a galactose-inducible promoter was introduced into the *cdc24-4^{ts}* strain. *FUS1* mRNA levels at various times after addition of galactose to cells at 24°C were compared with those in cells preincubated at 37°C for 2 h before galactose addition (Fig. 3). The data demonstrate that the constitutive induction of *FUS1* mRNA by Ste4p overexpression in the absence of pheromone occurs in the *cdc24-4^{ts}* strain at the permissive but not restrictive temperature. This *STE4*-mediated induction of *FUS1* achieves levels ~50% of that obtained with α-factor, as previously reported (72). Wild-type W303 cells containing the pGAL::STE4 construct showed similar *FUS1* induction at 37°C (data not shown). Phenotypically irregularly shaped cells are seen at permissive temperatures in galactose, while at restrictive temperatures, *cdc24-4^{ts}* pGAL::STE4 cells remain rounded and enlarged (data not shown). Thus, *CDC24* appears to act at the same level or downstream of *STE4* in the pheromone-induced kinase cascade.

Effects of Cdc42p overexpression on *FUS1* induction in *cdc24^{ts}* cells. The growth defect in *cdc42^{ts}* strains at restrictive temperatures can be rescued by overexpression of human or worm Cdc42 (9, 61). Similarly, the *cdc24-4^{ts}* strain has been rescued by overexpression of Cdc42Sc and Cdc42Hs (5, 48) (as also shown in Fig. 5A). Figure 4A shows that galactose-induced overexpression of Cdc42Sc partially restored (~30%) α-factor-mediated *FUS1* induction in *cdc24-4^{ts}* cells at the restrictive temperature compared with the non-temperature-sensitive *cdc24-4^{ts}*::*CDC24* strain, in which the temperature-sensitive allele has been rescued by homologous recombination with wild-type *CDC24* coding sequence. Thus, *CDC42* appears to act downstream of *CDC24* in the α-factor-initiated signalling pathway. Overexpression of Cdc42Sc and Cdc42Hs allowed budding and thus viability at the restrictive temperature (Fig. 5A). The partially defective pheromone signalling in these cells cannot be ascribed to secondary effects, such as cell cycle arrest at an inappropriate stage for the mating response. Galactose-induced expression of dominant positive (GTPase-defective) Cdc42Sc^{12V} and of the Cdc42Hs^{5R,12V} mutant (originally characterized with respect to its ability to activate PAK in vivo) in *cdc24-4^{ts}* cells were equally effective in promoting induction of *FUS1* mRNA in response to α-factor at 37°C (Fig. 4B). No *FUS1* induction was observed in any case without pheromone treatment, implying that another factor additional to activated Cdc42p was required for stimulating the pathway. Expression of the human activated Cdc42Hs^{12V} and Cdc42Hs^{5R,12V} was lethal at all temperatures and gave rise to cells with an irregular multibudded phenotype (Fig. 4C) similar to that described by Ziman et al. (76) for the Cdc42Sc^{12V} mutant.

A pheromone halo assay on galactose plates (Fig. 5B) showed that the *cdc24-4^{ts}* cells overexpressing either yeast or

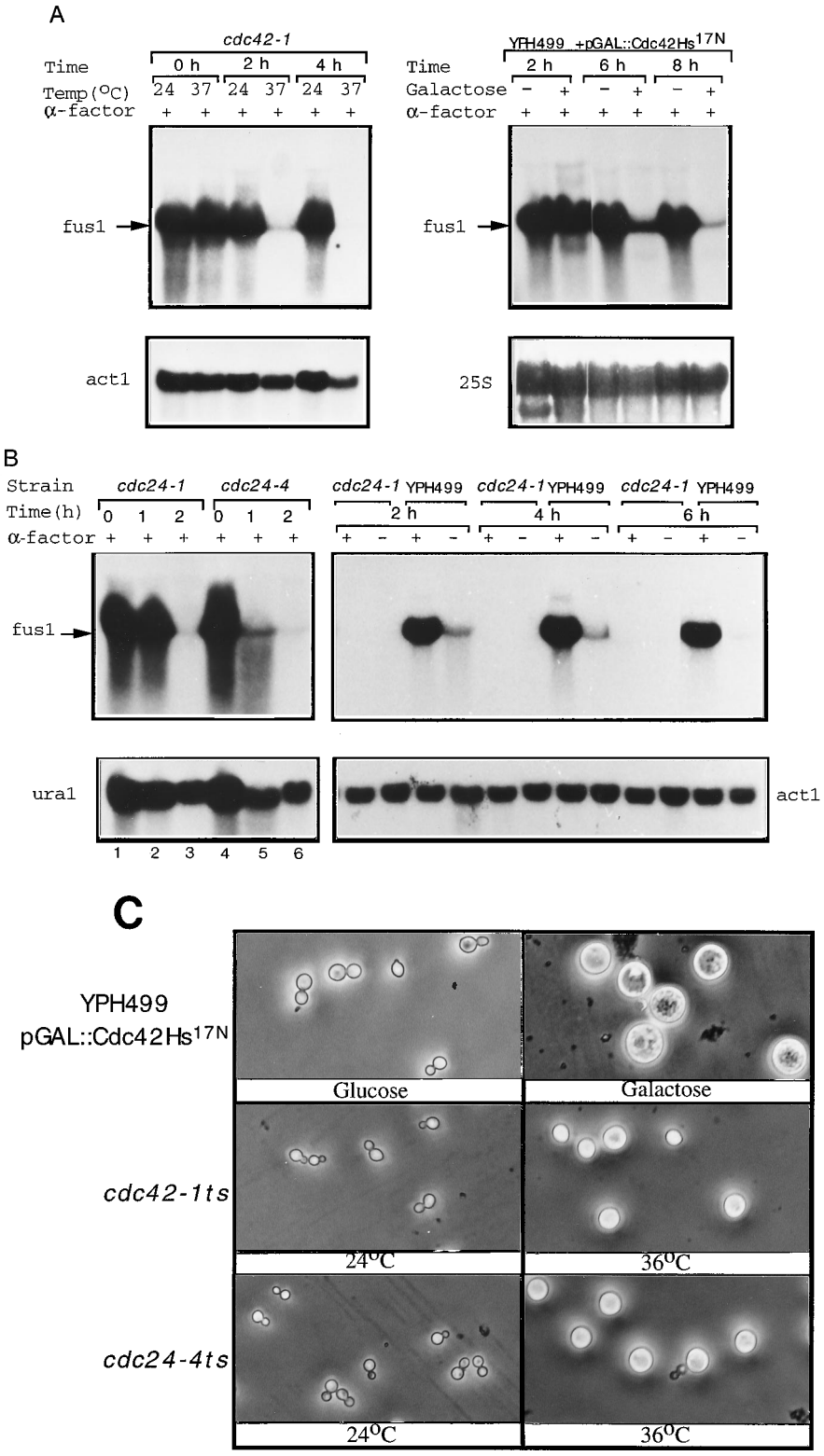

FIG. 2. The *cdc42*^{ts} and *cdc24*^{ts} strains are defective in α-factor induction of *FUS1* at the restrictive temperature. (A) The *cdc42-1*^{ts} strain was grown at 24°C in YEPD medium; cells at an OD of 0.7 were transferred to 37°C at the start of the experiment. Total RNA was isolated from cells (10^7/ml) treated with α-factor (5 μM) for 15 min and subjected to Northern analysis using a *FUS1* probe. Strain YPH499 was transformed with the putative dominant negative construct pGAL::Cdc42Hs^{17N}. Cells were grown in raffinose to an OD of 0.8 and then transferred to medium containing either galactose or glucose at 30°C. At the time shown, 10^7 cells per ml were treated with 5 μM α-factor for 20 min. (B) The *cdc24-1*^{ts} and *cdc24-4*^{ts} strains were grown at 24°C and then transferred to 37°C. At the appropriate time, α-factor was added as described above. (C) Comparison of the phenotype of YPH499 cells overexpressing Cdc42Hs^{17N} from a galactose-inducible plasmid at 30°C with those of *cdc42-1*^{ts} and *cdc24-4*^{ts} cells grown at 37°C. Cells were incubated for 8 h as indicated, fixed, sonicated, and observed under phase-contrast microscopy. All fields were photographed at the same magnification.

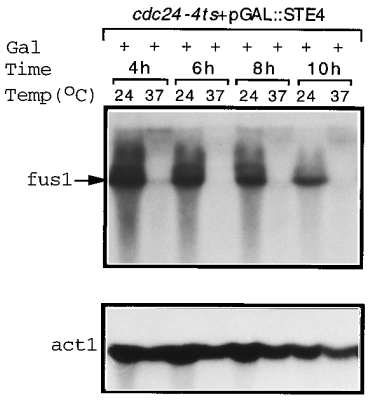

FIG. 3. Constitutive induction of *FUS1* by *STE4* overexpression is defective in the *cdc24-4*^{ts} strain at the restrictive temperature. The *cdc24-4*^{ts} strain harboring plasmid pGAL::STE4 was grown to an OD_{600} of 0.8 in raffinose at 24°C. At the start of the experiment, either galactose was immediately added to the cells at 24°C or the cells were transferred to 37°C for 2 h prior to galactose addition. At the times shown after the start of the experiment, cells were harvested on ice and analyzed for *FUS1* mRNA levels.

human Cdc42 displayed defective pheromone signalling at the restrictive temperature. Halos from *cdc24-4*^{ts} cells rescued by constitutive expression for pGAD::Cdc24 were like those of the wild-type strain W303. The smaller halos from *cdc24-4*^{ts} cells overexpressing Cdc42Sc indicate an attenuated pheromone-induced G_1 arrest which appears to reflect the lower level of *FUS1* induction (Fig. 4A and B). However, we cannot discount induction of protease activity for this effect; the use of *bar*$^-$ strains would resolve this issue.

Signalling from truncated Ste20p is independent of *CDC24*. The expression of Ste20p as an N-terminally truncated form (containing residues 495 to 939) has been shown to activate constitutively the mating pathway in the absence of pheromone, as assayed with a Fus1p::LacZ reporter construct (54). We placed this truncated *STE20* sequence under a *GAL* pro-

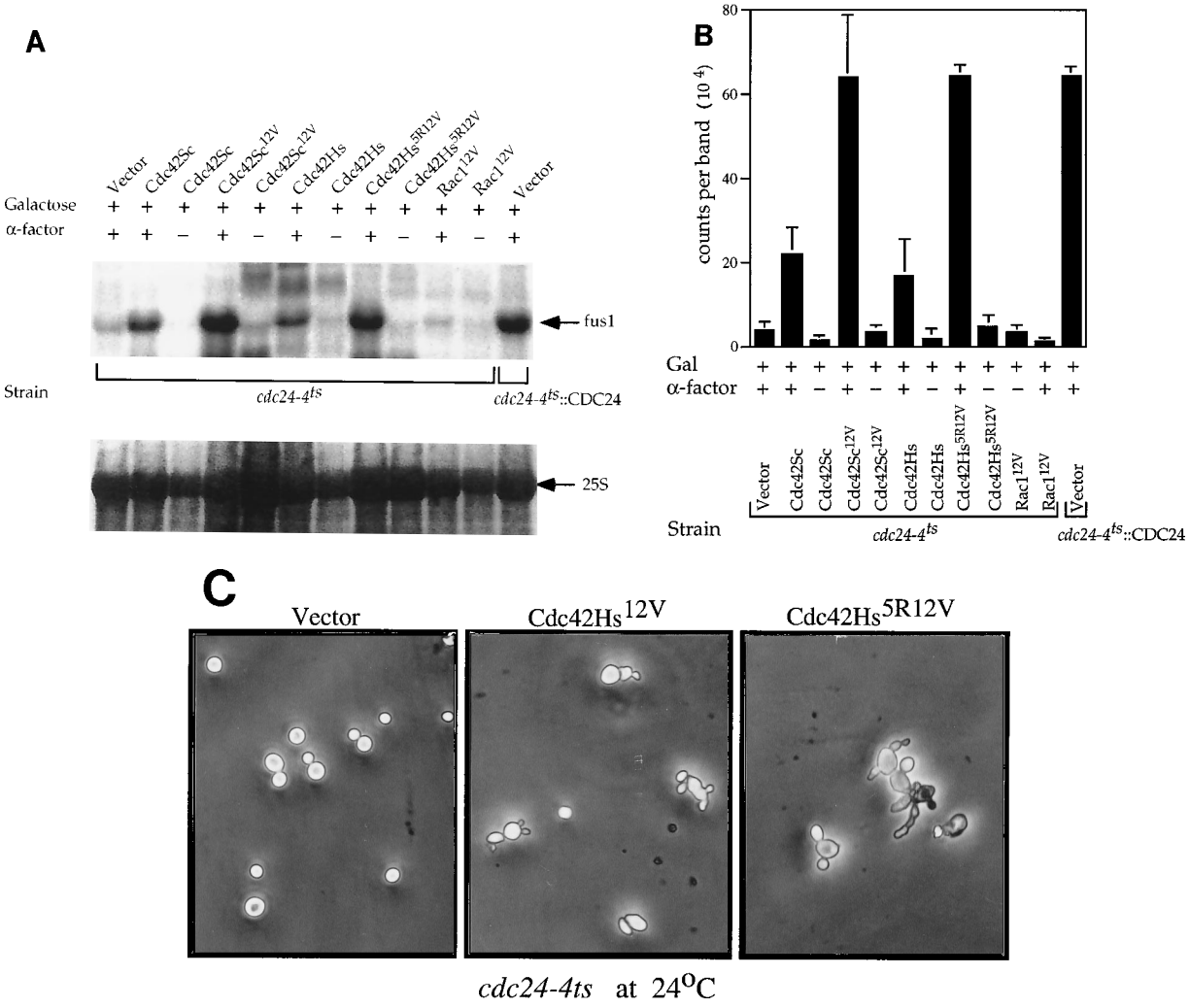

FIG. 4. The pheromone response in the *cdc24-4*^{ts} strain is potentiated by overexpression of Cdc42p. (A) Galactose-inducible yeast and human Cdc42p constructs in the *cdc24-4*^{ts} strain were induced for 10 h at 24°C. Following heat shock at 37°C for 2 h, cells were treated with 5 μM α-factor under standard conditions. The *FUS1* mRNA levels in α-factor-treated and untreated cells were examined by Northern analysis. The last lane corresponds to RNA isolated from the *cdc24-4*^{ts}*::CDC24* (non-temperature-sensitive) strain transformed with an empty vector plasmid. (B) The *FUS1* mRNA levels were determined quantitatively, using a PhosphorImager (Molecular Dynamics). The values shown represent the averages from two independent experiments; error bars indicate the higher value of the two. (C) The phenotype of the *cdc24-4*^{ts} strain at 24°C overexpressing the dominant active mutants $Cdc42Hs^{12V}$ and $Cdc42Hs^{5R,12V}$.

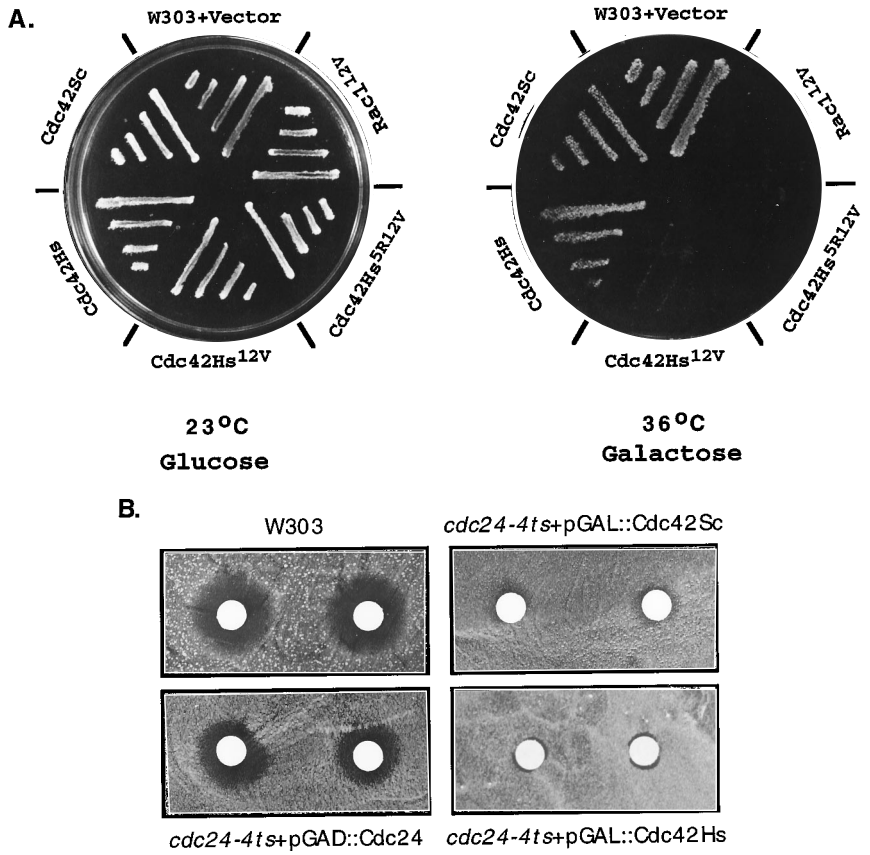

FIG. 5. Overexpression of wild-type yeast and human Cdc42p does not rescue the transmission of the growth arrest signal in the *cdc24-4*^{ts} strain at 36°C. (A) Growth rescue in the *cdc24-4*^{ts} strain by overexpression of human or yeast Cdc42p from a galactose-inducible vector. Cells were grown on glucose for 2 days at room temperature, then replica plated twice onto a galactose plates, and grown for 2 days at 36°C on each occasion. (B) The *cdc24-4*^{ts} strain transformed with a Cdc42Sc or Cdc42Hs plasmid, under the GAL promoter, or the constitutive pGAD::Cdc24 vector were spread on galactose plates. Each circle of filter paper placed on the lawn contained 8 μg of α-factor; the photograph shows plates incubated for 2 days at 36°C.

moter and tested the ability of such as plasmid to induce *FUS1* mRNA. Because the constitutive induction was ~2-fold lower in W303 than in *cdc24-4*^{ts} strains at restrictive temperatures (not shown), a variation attributable to a strain differences, we compared the effects of Δ*N*^{494}*STE20* in *cdc24-4*^{ts} and *cdc24-4*^{ts} pGBT::Cdc24 cells (in which viability and pheromone responsiveness are normal at 37°C). The levels of *FUS1* mRNA produced in response to galactose-induced expression of ΔSte20p at 37°C were essentially identical in cells with and without *CDC24* (Fig. 6, lanes 1 and 2). Maximal levels of *FUS1* mRNA were achieved only after relatively long periods of galactose induction, corresponding to ~5% of that resulting from α-factor treatment at the same temperature (by quantification of *FUS1* band). This constitutive induction was weaker at 37°C than at 30°C (data not shown) perhaps because of instability or down-regulation of ΔN^{494}Ste20p at higher temperatures. Interestingly, cells expressing ΔN^{494}Ste20p were found to respond to α-factor treatment at restrictive temperatures, achieving levels of *FUS1* mRNA (Fig. 6, lanes 3 and 4) like those in wild-type cells. Again, the absence or presence of *CDC24* did not affect the level of *FUS1* induction in this case. However, overexpression of full-length Ste20p did not lead to *FUS1* induction either in the presence or in the absence of pheromone (Fig. 6, lanes 7 and 8) at restrictive temperatures. We have found that W303 *ste20*$^{-}$ cells containing pGAL::ΔN^{494}Ste20 can also respond to pheromone (not shown), indicating that in *cdc24-4*^{ts} cells expressing ΔN^{494}Ste20p, the

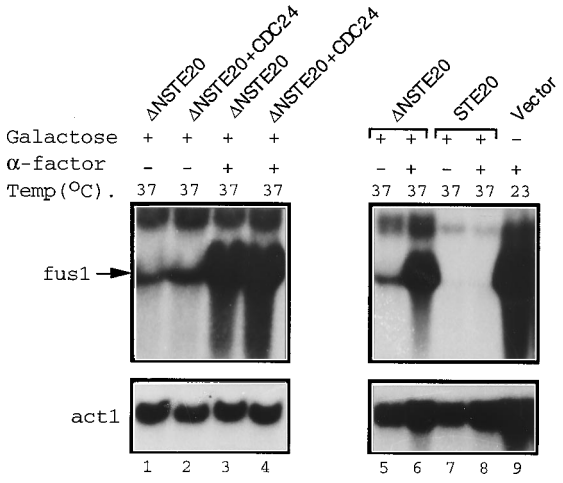

FIG. 6. Constitutive and α-factor-dependent *FUS1* induction mediated by the truncated Ste20p kinase is not dependent on *CDC24* function. Overexpression of a truncated kinase from plasmid pGAL:ΔN^{494}Ste20 (as indicated) leads to weak constitutive induction of *FUS1* mRNA as determined by Northern analysis. Cells were moved to 37°C in raffinose for 2 h before addition of galactose for 8 h. The first two lanes show this induction to be equivalent in the *cdc24-4*^{ts} strain with or without functional Cdc24p expressed from plasmid pGBT::Cdc24. In both cases, α-factor (added under standard conditions) led to further *FUS1* induction. At the restrictive temperature, the truncated but not full-length Ste20p allowed the *cdc24-4*^{ts} strain to respond to pheromone.

pheromone response is not indirectly mediated by wild-type Ste20p. Although the level of *FUS1* induction is relatively low, our results show that the truncated kinase ΔSte20p bypasses the normal requirement for active Cdc42p for pheromone signalling, consistent with deletion of the Cdc42p-binding domain (Fig. 1A). Since *FUS1* mRNA is not detectable when full-length Ste20p is overexpressed (Fig. 6, lane 8), the sequence containing residues 1 to 494 of Ste20p clearly suppresses the activity of the kinase domain in the absence of pheromone signalling. The properties of the N-terminally deleted *STE20* construct assayed in the *cdc24*^{ts} strain therefore indicate the existence of another pheromone-responsive element on the kinase in addition to the p21-binding domain, which in the full protein responds to GTP-Cdc42p. The mammalian PAK when expressed in the *ste20*$^{-}$ strain is unable to complement the mating defect (Fig. 7), suggesting that the mammalian kinase is unable to phosphorylate appropriate downstream targets or that it is insufficiently activated by Cdc42Sc (Fig. 1B).

Ste4p may interact with the N-terminus of Cdc42p. Recent reports of an interaction between the β-ARK pleckstrin homology domain and mammalian βγ subunits (67) suggested the possibility that the STE4-encoded β subunit interacts with Cdc42p, which, in common with all mammalian Dbl-like guanine nucleotide exchange factors, also contains a flanking pleckstrin homology domain (49). We detected an interaction between these two yeast proteins by the two-hybrid system (18), using hybrid constructs pGAD::STE4 and pGBT::Cdc24 driven by a weak truncated *ADH* constitutive promoter in strain HF7c (Fig. 8A). Viability of the cotransformants in the absence of histidine indicates a positive interaction, although β-galactoside activity is much lower than with other promoters (36); under these conditions, no false-positive colonies were observed under His^{-} selection. Plasmid pGBT::Cdc24, which contained the complete coding sequence of *CDC24* (*CLS4* [45]), using the corrected initiation site (46), rescued both growth and pheromone response defects in the *cdc24-4*^{ts} strain, indicating the fusion protein to be functional (Fig. 5). Unlike the inducible expression from pGAL::STE4, the pGAD::STE4 vector was not deleterious to growth, perhaps because of the lower expression level and nuclear targeting signal of the product (this was also true for the pGAD::ΔN^{494}Ste20 product). The specificity of the in vivo Cdc24p-Ste4p interaction assay was tested by using a variety of *CDC24* deletion constructs (Fig. 8B). To normalize results, the interaction was assayed by cotransformation of the plasmids and comparative analysis of viability on His-containing and His-deficient plates (Fig. 8C). The data suggest that an N-terminal domain upstream of the guanine nucleotide exchange domain of Cdc24p was sufficient for the observed interaction with the β subunit. Whether the interaction requires the presence of endogenous γ subunits or other proteins remains to be tested. The absence of colonies by cotransformation of plasmids pGAD::STE4 and pGBT::ΔN^{494}Ste20 indicated lack of a direct interaction of ΔN^{494}Ste20p with Ste4p, or that such an interaction was weaker than can be detected by this method. Similarly, we were unable to detect interaction between Cdc42p and Ste4p or between Cdc24p and Ste20p (data not shown).

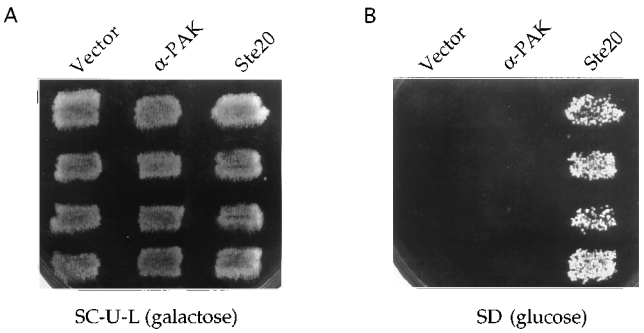

FIG. 7. Overexpression of mammalian PAK is insufficient to complement the mating defect in an *ste20*$^{-}$ strain. (A) The *ste20*$^{-}$ strain (74) was transformed with pGAL::STE20, pGAL::PAK (40), and vector (with *LEU2* marker), and four individual transformants of each were patched onto an SC − Ura − Leu [SC-U-L (galactose)] plate and grown at 30°C for 2 days. (B) The plate shown in panel A was replicated on a YEP-Gal plate containing a lawn of STX318-1A mating tester cells (Yeast Genetic Stock Center). Following incubation at 30°C for 10 h, the plate was replicated on an SD plate without amino acids to select for the diploid cells (growth at 30°C for 20 h is shown).

DISCUSSION

In this report, we have examined the significance of the homology between the Cdc42/Rac-binding domain of the mammalian p21-regulated kinase PAK (40) and predicted sequence of a similar region in the *S. cerevisiae* mating response kinase Ste20p. Binding of GTP-p21 to p65-PAK leads to activation of the kinase by an autophosphorylation mechanism, while GDP-p21 is ineffective. The kinase domains of Ste20p and PAK share 70% amino acid identity, suggesting that they may be regulated similarly. As predicted from the amino acid sequence homology, the recombinant Cdc42-binding domain of Ste20p binds [γ-^{32}P]GTP-Cdc42 as effectively as the related region of α-PAK (Fig. 1).

The substrates of Ste20p are as yet unresolved. *STE5* was isolated as a multicopy suppressor in an *ste20-1* strain (33), and its ability to rescue mating appeared to be related to the absence of any disruption in the Ste20p kinase domain. *STE5* acts closely downstream of the G-protein β and γ subunits (26). While Ste5p forms multicomponent complexes with Ste11p, Ste7p, and Fus3p, it is apparently unable to bind Ste20p (11). Ste7p has been shown to be directly activated by Ste11p in vitro (51). Within the limits of our current knowledge of the pheromone pathway, the simplest model would place the Ste11p kinase as directly regulated by Ste20p, with Ste5p providing a scaffolding for the kinase cascade (11).

The experiments described here provide evidence that *CDC42* is required for transducing the pheromone signal to the nucleus. *CDC24* clearly acts upstream of *CDC42*, and it is likely that *STE4* acts upstream of these genes although the alternative scenario that they can modulate *STE4* signalling is not ruled out. The ability of the partially active ΔN^{494}Ste20 construct to induce *FUS1* mRNA to the same extent in *cdc24-4*^{ts} with or without wild-type Cdc24p (at restrictive temperatures) shows the truncated kinase to be independent of GTP-Cdc42p, placing *CDC42* upstream of *STE20* in the pheromone response. This placement is consistent with the normal *STE20* gene product binding to, but acting downstream of, *CDC42*. One interpretation of these interactions is illustrated in the model shown in Fig. 9. The *STE4*-encoded β subunit may activate Cdc24p, which would explain the biochemical observation that Cdc42p is translocated to the plasma membrane fraction upon pheromone treatment (77). Activated Cdc42p is probably required for formation of the polarized structures called shmoos; however, expression from pGAL::STE4 in *ste20*$^{-}$ cells is not lethal and does not lead to abnormally shaped cells (not shown), suggesting the morphological changes caused by Ste4p overexpression have requirements at or below the level of *STE20*. These results together with those of studies of PAK indicate that one function of GTP-Cdc42p, via an interaction with the p21-binding domain of *STE20*, is

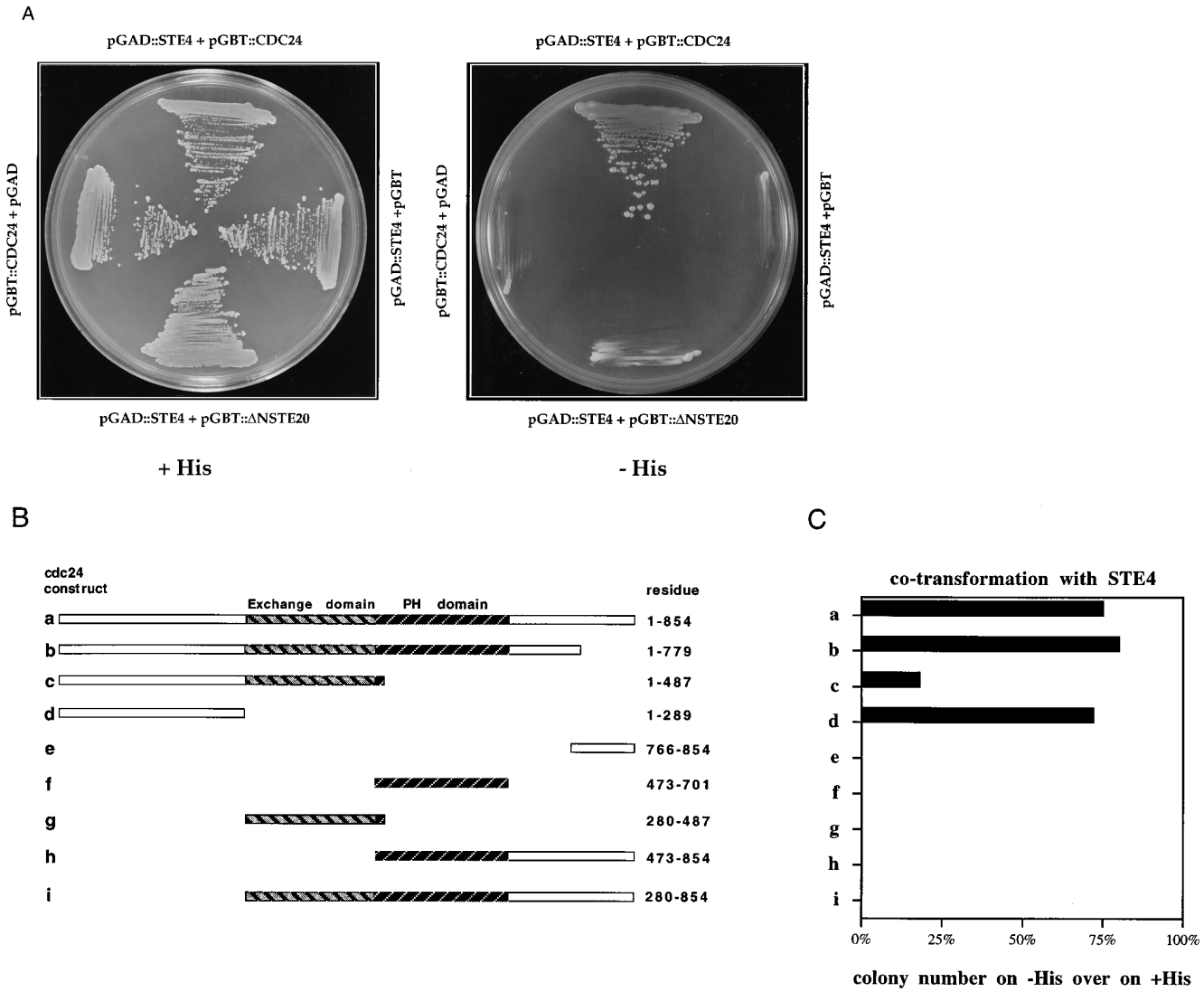

FIG. 8. The G-protein β subunit interacts with an N-terminal region of Cdc24p. (A) Reporter strain HF7c containing the indicated plasmids was selected for cotransformants on His-containing medium; from these cells, a single colony was streaked onto a His-deficient plate shown (in every case, several colonies were tested with the same result). Only pGAD::STE4 and pGBT::Cdc24 allowed cell growth. (B) Schematic diagram of the regions of Cdc24p tested for interaction with Ste4p in the two-hybrid system. *CDC24* DNA corresponding to the amino acid residues shown was cloned in frame to the pGBT vector for cotransformation with pGAD::STE4. (C) For each analysis, 8 μg of each plasmid was transformed with 200 μl of cells; 40-μl equivalents of cells were spread onto His-containing or His-deficient agar plates. Colonies were counted after 3 days at 30°C. In every case, more than 100 colonies were scored on the His-containing plate. This analysis indicated that the interaction occurs within the first 289 residues of Cdc24p.

likely to involve activation of the Ste20p kinase by stimulating autophosphorylation. In the absence of *CDC24*, it is clear that the activated yeast and human mutant Cdc42ps are more effective in signalling to Ste20p than wild-type proteins. This finding provides evidence that the behavior of mutant Cdc42p in the pheromone signalling pathway is similar to its effects in the budding pathway, i.e., $Cdc42^{12V}$ is hyperactive.

In an early study of mating in 19 *cdc* mutants (56), two temperature-sensitive alleles of *CDC24* were shown to exhibit the greatest deficiency in diploid formation at 35°C (<0.01% of that at the permissive temperature) when mixed with a wild-type α tester strain. The *CDC24* gene product has a complex role during bud formation and growth (63) and is a putative calcium-regulated protein (46). Most of the steps of the pheromone response pathway have been defined genetically by null alleles of so-called sterile (*STE*) genes that block mating. Other genes involved include two alleles of *BEM1* (10) obtained by screening for cells that were unable to form shmoos when treated with pheromone. Interestingly, *bem1* null mutants have a disordered actin cytoskeleton. *BEM1* codes for a protein containing two SH3 domains that was also isolated as a synthetically lethal gene with *MSB1*, a multicopy suppressor of the budding defect in $cdc42^{ts}$ and $cdc24^{ts}$ strains (6). It is likely that one role of *CDC24* and *CDC42* in mating is to coordinate the downstream cytoskeletal reorganization accompanying the mating response and allowing cells to override the normal program of cell polarization. Evidence for the presence of bud proteins at the site of shmoo formation comes from the observation that cells which form such a projection (but do not mate) produce a bud at the site if they resume mitotic growth (66). In *S. pombe*, the protein product of the *CDC24* homolog *scd1* complexes to the Bem1p homolog protein Scd2p (8). In

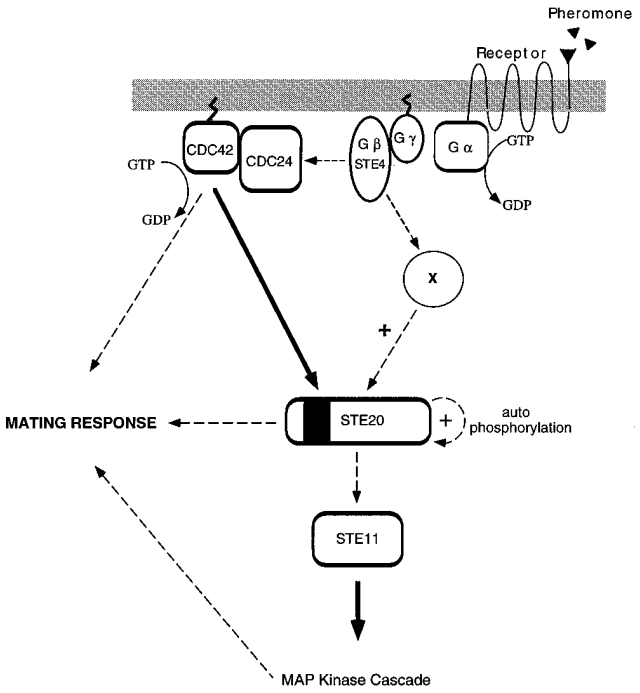

FIG. 9. Proposed role for *CDC42* and *CDC24* in the pheromone-activated signalling pathway of *S. cerevisiae*. Receptor-mediated activation of the G-protein α subunit occurs through the exchange GDP for GTP and a proposed dissociation of α and βγ subunits. Solid lines indicate the protein-protein interactions that have been identified. The broken lines indicate pathways that have yet to be established. GTP-Cdc42p interacts with N-terminal region of Ste20p (black), which appears to suppress the activity of the kinase.

contrast to the *S. pombe* homologs (8, 44) our analysis clearly placed *CDC42* downstream of *CDC24*.

The mating/starvation response pathway in *S. pombe* utilizes the *ras1* gene product for both morphological and MAP kinase responses to pheromone (for a review, see reference 52). As is the case with overexpression of $Cdc42Sc^{12V}$ in *S. cerevisiae* (Fig. 4), the activated $Ras1p^{17V}$ in *S. pombe* does not cause a mating response in the absence of pheromone (or bypass the requirement for nitrogen starvation [53]). Targets of GTP-Ras1p in *S. pombe* appear to be the *STE11* kinase homologs encoded by *byr2* (68) and *scd1* (8). Whether a Ste20p-like gene product is linked to *S. pombe* Cdc42 activation downstream of *ras1* remains to be resolved. The response to nutrient starvation in this case might parallel regulation of Ste20p in diploid *S. cerevisiae* cells leading to polarized pseudohyphal growth (37).

Our data suggest that part of the pheromone-induced pathway can operate independently of Cdc42p with N-terminally truncated Ste20p. Together with the failure to detect an interaction between Ste4p and ΔN^{494}Ste20p, this finding implies that there is a mediator (X factor) between the heterotrimeric G protein and Ste20p (Fig. 9). One such candidate might be *STE50* (55). The mediator could be a coactivator of Ste20p with GTP-Cdc42p; we have so far been unable to show activation of full-length recombinant Ste20p with GTP-Cdc42p in vitro (not shown). Since without *CDC24-CDC42* signalling no *FUS1* induction can be observed in response to α-factor or Ste4p overexpression (even with overexpressed full-length Ste20p), *CDC24* and *CDC42* appear to act as a checkpoint in the pathway. Although overexpression of full-length Ste20p was shown to rescue the effects of a dominant negative mutant of *STE4* (32, 34), it cannot relieve the block to α-factor signalling in $cdc24\text{-}4^{ts}$ cells at restrictive temperatures.

The mating deficiency of *ste20*$^-$ strains cannot be rescued by overexpression of mammalian PAK (Fig. 7). It is probable that the additional domains in Ste20p (the protein is ~40 kDa larger than PAK) are required for appropriate activation, coupling, or targeting of the kinase to other proteins involved in the mating response. Experiments with chimeric mammalian-yeast kinases may resolve this issue.

A number of kinases which interact with activated p21s are being identified. Most effort has been put into understanding the Ras-Raf interaction in mammalian cells (69, 70), although no close homolog exists in *S. cerevisiae*; in this case, it appears that the p21 is responsible for the localization but not the direct activation of Raf (35, 64), in contrast to the in vitro activation of PAK by GTP-Cdc42 and GTP-Rac. We suggest, on the basis of its similarity to PAK, that GTP-Cdc42p binding to Ste20p might fulfill both functions, although other coactivators are probably involved. Ste20p may not be the only Cdc42p-associated kinase in *S. cerevisiae*: it has recently been reported that the *CLA4* gene product is a kinase similar to Ste20p (7) and contains a region similar to the Cdc42p-binding domain of PAK (15a). The *SPS1*-encoded kinase which is required for spore formation is also related to Ste20p and PAK in the kinase domain (19). Similarly, there appear to be a number of PAK-like proteins in mammalian tissue extracts (40).

The involvement of *CDC24* and *CDC42* in nuclear signalling has implications for the signalling of Rho family p21s in mammalian cells. Oncogenes such as *dbl* (23), *ect2* (43), and *ost* (28) have been identified as containing functional *CDC24*-like guanine nucleotide exchange/release domains that act on Rho family p21s. For *dbl*, its ability to transform NIH 3T3 cells correlates with the functional activity of the nucleotide exchange domain (24). Constitutively active (GTPase-deficient) RhoA has been shown to be transforming (4) and is required for cytoplasmic division of *Xenopus* embryos (30), though its mechanism is unknown. Significantly, tyrosine phosphorylation and phosphatidylinositol 3-kinase activities are modulated by RhoA in fibroblasts (31). The direct involvement of Cdc42p in a yeast mating response pathway will prompt the search for a parallel mammalian Rho-p21-dependent MAP kinase cascade.

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REFERENCES

1. **Adams, A. E., D. I. Johnson, R. M. Longnecker, B. F. Sloat, and J. R. Pringle.** 1990. CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. J. Cell Biol. **111:**131–142.
2. **Ahmed, S., J. Lee, L.-P. Wen, Z. Zhao, J. Ho, A. Best, R. Kozma, and L. Lim.** 1994. Breakpoint cluster region gene product-related domain of n-chimaerin. J. Biol. Chem. **269:**17642–17648.
3. **Ammerer, G.** 1994. Sex, stress and integrity: the importance of MAP kinases in yeast. Curr. Biol. **4:**90–95.
4. **Avraham, H., and R. A. Weinberg.** 1989. Characterization and expression of the human rhoH12 gene product. Mol. Cell. Biol. **9:**2058–2066.
5. **Bender, A., and J. R. Pringle.** 1989. Multicopy suppression of the *cdc24* budding defect by CDC42 and three newly identified genes including the ras-related gene RSR1. Proc. Natl. Acad. Sci. USA **86:**9976–9980.

6. **Bender, A., and J. R. Pringle.** 1991. Use of a screen for synthetic lethal and multicopy suppressor mutants to identify two new genes involved in morphogenesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**1295–1305.
7. **Blumer, K. J., and G. L. Johnson.** 1994. Diversity in function and regulation of MAP kinase pathways. Trends Biochem. Sci. **19:**236–240.
8. **Chang, E. C., M. Barr, Y. Wang, V. Jung, H.-P. Xu, and M. H. Wigler.** 1994. Cooperative interaction of *S. pombe* proteins required for mating and morphogenesis. Cell **79:**131–141.
9. **Chen, W., H.-H. Lim, and L. Lim.** 1993. The CDC42 homologue from *Caenorhabditis elegans*. J. Biol. Chem. **268:**13280–13285.
10. **Chenevert, J., K. Corrado, A. Bender, J. Pringle, and I. Herskowitz.** 1992. A yeast gene (BEM1) necessary for cell polarization whose product contains two SH3 domains. Nature (London) **356:**77–79.
11. **Choi, K.-Y., B. Satterberg, D. M. Lyons, and E. A. Elion.** 1994. Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. Cell **78:**499–512.
12. **Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119–122.
13. **Crespo, P., N. Xu, W. F. Simonds, and J. S. Gutkind.** 1994. Ras-dependent activation of MAP kinase pathway mediated by G-protein βγ subunits. Nature (London) **369:**418–420.
14. **Cross, F., L. H. Hartwell, C. Jackson, and J. B. Konopka.** 1988. Conjugation in *Saccharomyces cerevisiae*. Annu. Rev. Cell Biol. **4:**429–457.
15. **Cross, F. R., and A. H. Tinkelenberg.** 1991. A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. Cell **65:**875–883.

15a. **Cvrckova, F.** Personal communication.

16. **Dolan, J. W., and S. Fields.** 1990. Overproduction of the yeast STE12 protein leads to constitutive transcriptional induction. Genes Dev. **4:**492–502.
17. **Errede, B., and D. E. Levin.** 1993. A conserved kinase cascade for MAP kinase activation in yeast. Curr. Opin. Cell Biol. **5:**254–260.
18. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
19. **Friesen, H., R. Lunz, S. Doyle, and J. Segall.** 1994. Mutation of the SPS1-encoded protein kinase of *Saccharomyces cerevisiae* leads to defects in transcription and morphology during spore formation. Genes Dev. **8:**2162–2175.
20. **Gietz, R. D., and A. Sugino.** 1988. New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:**527–534.
21. **Guthrie, C., and G. R. Fink (ed.).** 1991. Guide to yeast genetics and molecular biology. Methods Enzymol. **194:**3–251.
22. **Hannon, G. J., D. Demetrick, and D. Beach.** 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev. **7:**2378–2391.
23. **Hart, M. J., A. Eva, T. Evans, S. A. Aaronson, and R. A. Cerione.** 1991. Catalysis of guanine nucleotide exchange on the Cdc42Hs protein by the *dbl* oncogene product. Nature (London) **354:**311–313.
24. **Hart, M. J., A. Eva, D. Zangrill, S. A. Aaronson, T. Evans, R. A. Cerione, and Y. Zheng.** 1994. Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the *dbl* oncogene product. J. Biol. Chem. **269:**62–65.
25. **Hartwell, L. H., R. K. Mortimer, J. Culotti, and M. Culotti.** 1973. Genetic control of the cell division cycle in yeast: genetic analysis of cdc mutants. Genetics **74:**267–286.
26. **Hasson, M. S., D. Blinder, J. Thorner, and D. D. Jennness.** 1994. Mutational activation of the STE5 gene product bypasses the requirement for G protein β and γ subunits in the yeast pheromone response pathway. Mol. Cell. Biol. **14:**1054–1065.
27. **Hirsch, J. P., and F. R. Cross.** 1992. Pheromone response in yeast. Bioessays **14:**367–373.
28. **Horii, Y., J. F. Beler, K. Sakaguchi, M. Tachibana, and T. Miki.** 1994. A novel oncogene, *ost*, encodes a guanine nucleotide exchange factor that potentially links Rho and Rac signaling pathways. EMBO J. **13:**4776–4786.
29. **Johnson, D. I., and J. R. Pringle.** 1990. Molecular characterization of cdc42, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. J. Cell. Biol. **111:**143–152.
30. **Kishi, K., T. Sasaki, S. Kuroda, T. Itoh, and Y. Takai.** 1993. Regulation of cytoplasmic division of Xenopus embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). J. Cell Biol. **120:**1187–1195.
31. **Kumagai, N., N. Morii, K. Fujisawa, Y. Nemoto, and S. Narumiya.** 1993. ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured swiss 3T3 cells. J. Biol. Chem. **268:**24535–24538.
32. **Leberer, E., D. Dignard, D. Harcus, D. Y. Thomas, and M. Whiteway.** 1992. The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein βγ subunits to downstream signalling components. EMBO J. **11:**4815–4824.
33. **Leberer, E., D. Dignard, D. Harcus, L. Hougan, M. Whiteway, and D. Y. Thomas.** 1993. Cloning of *Saccharomyces cerevisiae* STE5 as a suppressor of a ste20 protein kinase mutant: structural and functional similarity of Ste5 to Far1. Mol. Gen. Genet. **241:**241–254.
34. **Leberer, E., D. Dignard, L. Hougan, D. Y. Thomas, and M. Whiteway.** 1992. Dominant-negative mutants of a yeast G-protein β subunit identify two functional regions involved in pheromone signalling. EMBO J. **11:**4805–4813.
35. **Leevers, S. J., H. F. Paterson, and C. J. Marshall.** 1994. Requirement for Ras in Raf activation is overcome by targetting Raf to the plasma membrane. Nature (London) **369:**411–414.
36. **Legrain, P., M.-C. Dokhelar, and C. Transy.** 1994. Detection of protein-protein interactions using different vectors in the two-hybrid system. Nucleic Acid Res. **22:**3241–3242.

36a. **Leung, T., et al.** Unpublished data.

37. **Liu, H., C. A. Styles, and G. R. Fink.** 1993. Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science **262:** 1741–1744.
38. **Manser, E., T. Leung, C. Monfries, M. Teo, C. Hall, and L. Lim.** 1992. Diversity and versatility of GTPase activating proteins for the p21*rho* subfamily of *ras* G proteins detected by a novel overlay assay. J. Biol. Chem. **267:**16025–16028.
39. **Manser, E., T. Leung, H. Salihuddin, L. Tan, and L. Lim.** 1993. A non-receptor tyrosine kinase that inhibits the GTPase activity of $p21^{cdc42}$. Nature (London) **363:**364–367.
40. **Manser, E., T. Leung, H. Salihuddin, Z.-S. Zhao, and L. Lim.** 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature (London) **367:**40–46.
41. **Marsh, L., A. M. Neiman, and I. Herskowitz.** 1991. Signal transduction during pheromone response in yeast. Annu. Rev. Cell Biol. **7:**699–728.
42. **Marshall, C.** 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Gen. Dev. **4:**82–89.
43. **Miki, T., C. L. Smith, J. E. Long, A. Eva, and T. P. Fleming.** 1993. Oncogene *ect2* is related to regulators of small GTP-binding proteins. Nature (London) **362:**462–465.
44. **Miller, P. J., and D. I. Johnson.** 1994. Cdc42p GTPase is involved in controlling polarized cell growth in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **14:**1075–1083.
45. **Miyamoto, S., Y. Ohya, Y. Ohsumi, and Y. Anraku.** 1987. Nucleotide sequence of the CLS4 (CDC24) gene of *Saccharomyces cerevisiae*. Gene **54:** 125–132.
46. **Miyamoto, S., Y. Ohya, Y. Sano, S. Sakaguchi, H. Iida, and Y. Anraku.** 1991. A DBL-homologous region of the yeast CLS4/CDC24 gene product is important for Ca^{2+}-modulated bud assembly. Biochem. Biophy. Res. Commun. **181:**604–610.
47. **Muller, L., G. Xu, R. Wells, C. P. Hollenberg, and W. Piepersberg.** 1994. LRG1 is expressed during sporulation in *Saccharomyces cerevisiae* and contains motifs similar to LIM and rho/racGAP domains. Nucleic Acid Res. **22:**3151–3154.
48. **Munemitsu, S., M. A. Innis, R. Clark, F. McCormick, A. Ullrich, and P. Polaskis.** 1990. Molecular cloning and expression of a G25K cDNA, the human homolog of the yeast cell cycle gene *CDC42*. Mol. Cell. Biol. **10:** 5977–5982.
49. **Musacchio, A., T. Gibson, P. Rice, J. Thompson, and M. Sarasre.** 1993. The PH domain: a common piece in the structural patchwork of signalling proteins. Trends Biochem. Sci. **18:**343–348.
50. **Neiman, A. M.** 1993. Conservation and reiteration of a kinase cascade. Trends Genet. **9:**390–394.
51. **Neiman, A. M., and I. Herskowitz.** 1994. Reconstitution of a yeast protein kinase cascade in vitro: activation of the yeast MEK homologue STE7 by STE11. Proc. Natl. Acad. Sci. USA **91:**3398–3402.
52. **Nielsen, O.** 1993. Signal transduction during mating and meiosis in *S. pombe*. Trends Cell Biol. **3:**60–65.
53. **Nielsen, O., J. Davey, and R. Egel.** 1992. The ras1 function of *Schizosaccharomyces pombe* mediates pheromone-induced transcription. EMBO J. **11:** 1391–1395.
54. **Ramer, S. W., and R. W. Davis.** 1993. A dominant truncation allele identifies a gene, STE20, that encodes a putative protein kinase necessary for mating in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **90:**452–456.
55. **Ramezani-Rad, M., G. Xu, and C. P. Hollenberg.** 1992. STE50, a novel gene required for activation of conjugation at an early step in mating in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **236:**145–154.
56. **Reid, J. B., and L. H. Hartwell.** 1977. Regulation of mating in the cell cycle of *Saccharomyces cerevisiae*. J. Cell Biol. **75:**355–365.
57. **Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall.** 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell **70:**401–410.
58. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
60. **Schiestl, R. H., and R. D. Gietz.** 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. **16:**339–346.
61. **Shinjo, K., J. G. Koland, M. J. Hart, V. Narashiman, D. I. Johnson, T.**

Evans, and R. Cerione. 1990. Molecular cloning of the gene for the human placental GTP-binding protein Gp (G25K): identification of this GTP-binding protein as the human homolog of the yeast cell-division-cycle protein CDC42. Proc. Natl. Acad. Sci. USA **87:**9853–9857.

62. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
63. **Sloat, B. F., A. Adams, and J. R. Pringle.** 1981. Roles of the CDC24 gene product in cellular morphogenesis during the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. **89:**395–405.
64. **Stokoe, D., S. G. Macdonald, K. Cadwallader, M. Symons, and J. F. Hancock.** 1994. Activation of Raf as a result of recruitment to the plasma membrane. Science **264:**1463–1467.
65. **Surana, U., H. Robotsch, C. Price, T. Schuster, I. Fitch, A. B. Futcher, and K. Nasmyth.** 1991. The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. Cell **65:**145–161.
66. **Tkacz, J. S., and V. L. MacKay.** 1979. Sexual conjugation in yeast. J. Cell Biol. **80:**326–333.
67. **Touhara, K., J. Inglese, J. A. Pitcher, G. Shaw, and R. J. Lefkowitz.** 1994. Binding of G protein βγ-subunits to pleckstrin homology domains. J. Biol. Chem. **269:**10217–10220.
68. **Van Aelst, L., M. Barr, S. Marcus, A. Polverino, and M. Wigler.** 1993. Complex formation between RAS and RAF and other protein kinases. Proc. Natl. Acad. Sci. USA **90:**6213–6217.
69. **Vojtek, A. B., S. M. Holienberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
70. **Warne, P. H., V. P. Riciana, and J. Downwood.** 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature (London) **364:**352–355.
71. **Whiteway, M., L. Hougan, D. Dignard, D. Y. Thomas, L. Bell, G. C. Saari, F. J. Grant, P. O'Hara, and V. L. MacKay.** 1989. The STE4 and STE18 genes of yeast encode potential β and γ subunits of the mating factor receptor-coupled G protein. Cell **56:**467–477.
72. **Whiteway, M., L. Hougan, and D. Y. Thomas.** 1990. Overexpression of the STE4 gene leads to mating response in haploid *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**217–222.
73. **Xu, H.-P., M. White, S. Marcus, and M. Wigler.** 1994. Concerted action of RAS and G proteins in the sexual response pathways of *Schizosaccharomyces pombe*. Mol. Cell. Biol. **14:**50–58.
74. **Zhao, Z., E. Manser, T. Leung, and L. Lim.** 1995. Unpublished work.
75. **Zheng, Y., R. Cerione, and A. Bender.** 1994. Control of the yeast bud-site assembly GTPase Cdc42: catalysis of guanine nucleotide exchange by Cdc24 and stimulation of GTPase activity by BEM3. J. Biol. Chem. **269:**2369–2372.
76. **Ziman, M., J. M. O'Brien, L. A. Ouellette, W. R. Church, and D. I. Johnson.** 1991. Mutational analysis of CDC42Sc, a *Saccharomyces cerevisiae* gene that encodes a putative GTP-binding protein involved in the control of cell polarity. Mol. Cell. Biol. **11:**357–354.
77. **Ziman, M., D. Preuss, J. Mulholland, J. M. O'Brien, D. Botstein, and D. I. Johnson.** 1994. Subcellular localization of Cdc42p, *Saccharomyces cerevisiae* GTP-binding protein involved in the control of cell polarity. Mol. Cell. Biol. **4:**1307–1316.