Genetic Analysis of the Interface Between Cdc42p and the CRIB Domain of Ste20p in *Saccharomyces cerevisiae*

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ABSTRACT

Mutagenesis was used to probe the interface between the small GTPase Cdc42p and the CRIB domain motif of Ste20p. Members of a cluster of hydrophobic residues of Cdc42p were changed to alanine and/or arginine. The interaction of the wild-type and mutant proteins was measured using the two-hybrid assay; many, but not all, changes reduced interaction between Cdc42p and the target CRIB domain. Mutations in conserved residues in the CRIB domain were also tested for their importance in the association with Cdc42p. Two conserved CRIB domain histidines were changed to aspartic acid. These mutants reduced mating, as well as responsiveness to pheromone-induced gene expression and cell cycle arrest, but did not reduce *in vitro* the kinase activity of Ste20p. GFP-tagged mutant proteins were unable to localize to sites of polarized growth. In addition, these point mutants were synthetically lethal with disruption of *CLA4* and blocked the Ste20p-Cdc42p two-hybrid interaction. Compensatory mutations in Cdc42p that reestablished the two-hybrid association with the mutant Ste20p CRIB domain baits were identified. These mutations improved the pheromone responsiveness of cells containing the CRIB mutations, but did not rescue the lethality associated with the CRIB mutant *CLA4* deletion interaction. These results suggest that the Ste20p-Cdc42p interaction plays a direct role in Ste20p kinase function and that this interaction is required for efficient activity of the pheromone response pathway.

A member of the small GTPase superfamily, Cdc42p (Bourne *et al.* 1990), has been implicated in control of polarized growth in a variety of cell types (Chant 1996; Bishop and Hall 2000). Cdc42p interacts with a variety of downstream effector molecules, such as PAR6 (Lin *et al.* 2000), WASP (Symons *et al.* 1996), and Bni1p (Evangelista *et al.* 1997). Many of these effectors interact with Cdc42p through a conserved motif, known as the CRIB or GBD domain (Burbelo *et al.* 1995). Among the Cdc42p targets are members of the STE20/PAK kinase family, a class of kinases identified in mammalian cells because of their ability to bind GTPases of the Cdc42p and Rac families (Manser *et al.* 1994). In mammalian cells the binding of the CRIB domain region of the PAK kinases activates kinase activity (Manser *et al.* 1994). The crystal structure of PAK suggests that this activation requires a structural rearrangement of the kinase molecule (Lei *et al.* 2000).

Cdc42p function has been extensively studied in the yeast *Saccharomyces cerevisiae*, where there are three members of the STE20/PAK kinase family: Ste20p, the founding member (Leberer *et al.* 1992); Cla4p (Cvrckova *et al.* 1995); and Skm1p (Martin *et al.* 1997). Ste20p functions in a variety of signaling pathways in the yeast cell. These pathways include the pheromone response pathway (Leberer *et al.* 1992), the pseudohyphal growth pathway (Leberer *et al.* 1997), and the osmotic response or HOG pathway (Raitt *et al.* 2000). In addition, Ste20p and Cla4p have been shown to phosphorylate and activate members of the myosin I subfamily of motor proteins *in vitro* (Wu *et al.* 1996). Ste20p and Cla4p together provide an essential function, as double mutants are not viable (Cvrckova and Nasmyth 1993). Cells that are defective in both functions show loss of polarized growth and disruptions in cortical actin localization (Eby *et al.* 1998; Holly and Blumer 1999). Although loss of myosin I function in yeast can be lethal, myosin I activation is not the only essential function of Ste20p and Cla4p, as replacement of the myosin I phosphorylation site with an aspartate residue does not rescue the lethal phenotype of the Cla4 Ste20 double mutant (Wu *et al.* 1997).

The role of Cdc42p in activation of the pheromone response pathway has been controversial. Initial evidence suggested a direct role because Cdc42p was required for pheromone induction of gene expression (Simon *et al.* 1995). However, deletion of the CRIB domain of Ste20p did not affect mating (Peter *et al.* 1996; Leberer *et al.* 1997), and the apparent requirement for Cdc42p function in pheromone-induced gene expression could

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be accounted for by the fact that the cells were trapped at G_1 (Oehlen and Cross 1998). These results for the CRIB deletion showed that localization of Ste20p to the bud sites was dependent on Cdc42p binding, but that mating-induced localization was Cdc42p independent (Peter *et al.* 1996; Leberer *et al.* 1997). However, more recent evidence suggests that Cdc42p does play a role in the pheromone response pathway (Moskow *et al.* 2000). In the current study we have analyzed specific point mutants that influence the interaction between Cdc42p and the CRIB domain of Ste20p. The analysis of these mutants shows that the association of Cdc42p with Ste20p plays a role in the mating process. It appears that cellular localization of Ste20p controlled by Cdc42p is required for efficient mating. This role of the Ste20p CRIB domain residues in the mating process has been independently shown in a recent publication (Lamson *et al.* 2002).

MATERIALS AND METHODS

DNA purification: Total yeast DNA was isolated as described (Philippsen *et al.* 1991). Plasmid DNA was isolated from *Escherichia coli* using a Biorobot 9600 (QIAGEN, Valencia, CA) following the manufacturer's instructions.

DNA sequencing: The concentration of the plasmid DNA to be sequenced was determined by fluorimetry using the DyNA Quant 220 (Hoefer, San Francisco) following the manufacturer's protocol. Sequencing was done with the dRhodamine kit (Perkin-Elmer, Norwalk, CT) and was analyzed on a 377 XL DNA sequencer (Applied Biosystems, Foster City, CA).

PCR: PCR was done using the standard protocol from the Expand High Fidelity PCR kit (Roche, Indianapolis).

Oligonucleotides: The following oligonucleotides were used:

KOLI22: CCACTACAATGGATGATG
ORL19: TTTGTATACACTTATTTTTTTTATAAC
ORL72: CTACAAAATaGtACtTTTTTTACTTTTCTTG, *Sca*I
ORL71: CAAGAAAAGTAAAAAaGTaCtATTTTGTAG, *Sca*I
ORL59: CAATGCCAAGgATATCCACCATG, *Eco*RV
ORL60: CATGGTGGATATcCTTGGCATTG, *Eco*RV
ORL61: GCATATCCACgAcGTcGGCGTGGACTCC, *Aat*II
ORL62: GGAGTCCACGCCgACgTcGTGGATATGC, *Aat*II
KOLI42: GATCCTCGACTAAAGATCTTAATGAGCAATGATCC, *Bgl*II
OAF3: CTTCCAATGCATTGGCTGCAGCTAAGAATTCATGAC GGGTGATTT, *Pst*I
OJA33: CCATATGCAAACGCgtAAGTGTGTTGTTGTCG, *Mlu*I
OJA34: CGACAACAACACACTTacGCGTTTGCATATGG, *Mlu*I
OJA37: CTATGCGGTGACgcgtATGATTGGTGATGAACC, *Mlu*I
OJA38: GGTTCATCACCAATCATAcgcGTCACCGCATAG, *Mlu*I
OJA41: GGTGATGAACCAcgTACGTTAGGTTTG, *Bsi*WI
OJA42: CAAACCTAACGTAcgTGGTTCATCACC, *Bsi*WI
OJA43: GGTGATGAGgCCggcTACGTTAGGTTTG, *Nae*I
OJA44: CAAACCTAACGTAgccGGcTCATCACC, *Nae*I
OJA45: CGATGAAGCTcgaGTGGCCGCCTTG, *Xho*I
OJA46: CAAGGCGGCCACtcgAGCTTCATCG, *Xho*I
OJA47: CGATGAAGCTgcaGTGGCCGCCTTG, *Pst*I
OJA48: CAAGGCGGCCACtgcAGCTTCATCG, *Pst*I.

The specific restriction sites noted are underlined, and the nucleotides changed for the mutagenesis are in lowercase letters.

Two-hybrid plasmids and the construction of site-directed mutants in *CDC42*: Plasmid pVL7 consists of the entire coding sequence of *KSS1* (Courchesne *et al.* 1989) inserted into pBTM116 as an *Eco*RI to *Sal*I fragment. Plasmid pRL39 (Leberer *et al.* 1997) consists of full-length *CDC42* from *S. cerevisiae* cloned in frame in the two-hybrid activation vector pGAD424 (Chien *et al.* 1991). Cysteine 188 of pRL39 was changed to serine by PCR-mediated site-directed mutagenesis. The mutagenic strategy employed involved complementary oligonucleotides containing the desired nucleotide substitution and the use of outside oligonucleotides to direct production of the rest of the gene and flanking sequences (Higuchi *et al.* 1988). In all site-directed modifications of *CDC42* the flanking oligonucleotides were KOLI22 and ORL19, which encode sequences from the *ADH1* promoter and *ADH1* terminator regions of pGAD424, respectively. Oligonucleotides ORL72 (coupled with KOLI22 for the first cycles) and ORL71 (coupled with ORL19 for the first cycles), together with the flanking oligonucleotides, were used to generate a DNA fragment with cysteine 188 mutated to a serine. This PCR product was transformed into yeast strain L40a (Marcus *et al.* 1994) together with plasmid pGAD424 linearized with *Bam*HI and *Eco*RI, and recombinant plasmids were generated by *in vivo* recombination. Recombinant plasmids were isolated as total yeast DNA, transformed into *E. coli*, purified, tested for the introduction of the *Sca*I site engineered along with the serine substitution, and then sequenced, and a plasmid with the correct substitution was designated pRL202. Subsequent mutations of specific hydrophobic residues of $CDC42^{C188S}$ were performed similarly, except that the starting PCR template was pRL202 rather than pRL39. The oligonucleotides for generating L4R were OJA33 and OJA34; for V44R, OJA37 and OAJ38; for Y51R, OJA41 and OAJ42; for Y51A, OJA43 and OAJ44; for I173R, OJA45 and OAJ46; and for I173A, OJA47 and OAJ48.

Construction of CRIB domain mutants: Plasmid pAF3 (Leberer *et al.* 1997) contains the N terminus of *STE20* encompassing the CRIB domain cloned as an *Eco*RI to *Sal*I fragment in the LexA DNA-binding domain plasmid pBTM116 (Chien *et al.* 1991). Derivatives containing the H345D and H348D alleles were created by site-directed PCR-mediated mutagenesis. PCR products containing the mutations were created by a first-round synthesis using primers ORL60 plus KOLI42 and ORL59 plus OAF3 for H345D and, for H348D, ORL62 plus KOLI42 and ORL61 plus OAF3. This amplification was followed by second-round synthesis using the outside primers KOLI42 and OAF3 for both constructs. The template plasmid in each case was pDH37 (Leberer *et al.* 1997). These PCR products were cut with *Bgl*II and *Sal*I and cloned into *Bam*HI- and *Sal*I-cut pRL99, and candidate H345D and H348D substitutions were identified through the coordinate introduction of restriction sites (*Eco*RV for H345D and *Aat*II for H348D). Selected plasmids were sequenced; a correct plasmid with the H345D mutation was named pRL155, and a correct plasmid with the H348D substitution was named pRL156.

Construction of CRIB domain mutant-GFP fusions: Plasmid pRL116 (Leberer *et al.* 1997) contains a functional Ste20-green fluorescent protein (GFP) fusion protein that localizes to new buds and to the tips of growing buds during polarized bud growth (Leberer *et al.* 1997; Wu *et al.* 1998). The H345D and H348D substitutions within the CRIB domain of Ste20p were introduced by replacing the *Bam*HI to *Sal*I fragment containing the CRIB domain of Ste20 with the equivalent fragment from pRL155 and pRL156 to create plasmids pJA82 and pJA83, respectively.

Construction of random mutants of *CDC42*: Plasmid pRL202 was used as a template for Taq polymerase-mediated PCR using oligonucleotides KOLI22 and ORL19. The PCR products were gel purified and cloned into *Eco*RI- and *Bam*HI-linearized pGAD424 by *in vivo* recombination to generate a library of *CDC42* genes mutated at the frequency characteristic of *Taq* polymerase nucleotide misincorporation. The library

was generated in strain L40 already transformed with the pBTM116 derivative plasmid pRL155, and *LEU2*$^+$ *TRP1*$^+$ transformants were selected. These transformants were screened by replica plating to identify colonies that generated a plasmid-based growth on medium lacking histidine and containing 5 mM 3-aminotriazole (3-AT).

Separation of double mutants: Plasmid pMJ13 contained two mutations, N39S and P69T. These were separated by digesting pMJ13 with *Eag*I and *Sna*BI, which generates a 1.65-kb fragment containing the N39S mutation and a 5.6-kb fragment with the P69T mutation. An equivalent digestion of pRL202 was performed, and the fragment pairs were ligated together to generate plasmids with the separated mutations. Plasmids containing the independent mutations were sequenced, and a plasmid with N39S was designated pJA49, and P69T was designated pJA50.

Transfer of N39S and P69T mutations: The N39S and P69T mutations were transferred to the wild-type *CDC42* gene. In plasmid pRS315 *CDC42* (Adams *et al.* 1990), a kind gift of D. Johnson (University of Vermont), the *Not*I (*Eag*I) site of the multiple cloning site was destroyed. The resulting plasmid was then digested with *Eag*I to cleave the *CDC42* gene between amino acids 39 and 69 (at position 58). Protruding extremities were digested with mung bean nuclease and this linear plasmid was cotransformed with a PCR fragment of the mutant (N39S, P69T) *CDC42* gene. Following *in vivo* recombination, plasmids were extracted and sequenced. A double mutant (N39S, P69T) was designated pJA55, while the single amino acid changes were called pJA53 (N39S) and pJA54 (P69T).

Strain constructions: Strains are listed in Table 1. Strains JAY25 and JAY26 were derived from strain W303-1A by replacing, respectively, the histidines 345 and 348 of *STE20* with aspartic acid. *Sal*I to *Bam*HI fragments of 1.1 kb containing the mutated region of *STE20* from pRL155 and pRL156 were subcloned into pRS306 cut with *Sal*I and *Bam*HI to create plasmids pJA45 and pJA46. Plasmids pJA45 (H345D) and pJA46 (H348D) were targeted to the *STE20* locus by cleavage with *Cla*I, leading to duplication of the *STE20* locus flanking the vector and *URA3* marker. Loop-outs of the *URA3* marker were identified by growth on medium with 5-fluoroorotic acid (5-FOA), and strains with the histidines replaced with asparagines were detected as showing reduced mating in 4-hr mating tests. These replacement alleles were confirmed by digesting PCR products generated from strains JAY25 and JAY26 using oligonucleotides ORL1 and ODH77 with the diagnostic restriction enzymes *Aat*II for JAY25 and *Eco*RV for JAY26. Subsequently, strains JAY25 and JAY26, together with W303-1A, were transformed to *sst1::URA3* using plasmid pJGsst1 (Reneke *et al.* 1988) cut with *Sal*I and *Eco*RI. These strains were then grown on 5-FOA medium to select loss of the *URA3* marker and then transformed to *fus1::LacZ* using plasmid pDH17 cut with *Hin*dIII. The W303-1A derivative containing the *sst1* disruption and the *fus1::lacZ* reporter was named JAY54, while the JAY25 and JAY26 derivatives were JAY39 and JAY40, respectively.

β-Galactosidase assays: For the assay with strains JAY39, JAY40, and JAY54, induction with 0.4 μg/ml of α-mating factor was done for 2 hr prior to the β-gal assays. Standard conditions were used for β-gal assays (Jarvis *et al.* 1988); the values shown are Miller units based on the following formula: units = $1000 \times OD_{420}/T \times V \times OD_{600}$, where T is the reaction time (in minutes) and V is the volume of culture used for the test (in milliliters).

Quantitative mating: Quantitative matings were done on filter discs as described (Leberer *et al.* 1993).

Kinase assays: *In vitro* kinase assays were performed essentially as described (Wu *et al.* 1995), except that the washed immune complexes were incubated at 30° for 15 min with 30 μl kinase buffer containing 100 μM ATP and then washed three times with kinase buffer, 1 ml each, without ATP. The kinase reactions with labeled ATP were started by addition of 30 μl of kinase buffer containing 10 μM ATP and 1 μl of [γ-^{32}P]ATP (10 μCi/μl at 6000 Ci/mmol) and 2 μg of myelin basic protein (MBP), and the reactions were incubated at 30° for 15 min. One set of mock reactions processed without addition of [γ-^{32}P]ATP was used for Western blot analysis to reveal the amount of Ste20p in the immune complexes.

Preparation of total yeast cell extracts: Overnight cultures were diluted 1:20 and grown in selective media to an A_{600} of 1.0. A total of 1 ml of these cultures was harvested by centrifugation at 3000 × *g* and resuspended in 100 μl of loading buffer. Cells were then disrupted by boiling for 5 min. The extracts were clarified by centrifugation in a microcentrifuge at top speed for 10 min at room temperature. Twenty microliters of the supernatants was loaded on 12% SDS-PAGE gels.

Western blot analysis: Protein samples were resolved on 12% SDS-PAGE and transferred on Immobilon-P membrane (Millipore, Bedford, MA). The membranes were probed first with affinity-purified anti-CDC42 antiserum, incubated with anti-rabbit HRP (Santa-Cruz), and developed using Lumilight Plus chemiluminescent substrate (Roche). The anti-Cdc42p antibody was raised in rabbits against purified bacterially expressed glutathione S-transferase (GST)-Cdc42p. The antibody was affinity purified against GST-Cdc42p immobilized on nitrocellulose membranes essentially as described (Talian *et al.* 1983).

Fluorescence microscopy: Cells of strain YCW563 containing the plasmids pRL116C.4, pJA82, pJA83, pRS315:CDC42, and pJA55 were grown overnight in selective medium. GFP was visualized with a Leica DMIRE2 inverted microscope (Leica Microsystemes, Montreal) equipped with a Hamamatsu cooled CCD camera and a Ludl motorized stage at ×630 magnification. Openlab software (Improvision, Lexington, MA) and Adobe Photoshop were used for image acquisition and manipulation. Pheromone treated cells were incubated in the presence of 74 μg/ml α-factor for 3 hr to trigger shmoo formation.

RESULTS

Recent structural studies (Guo *et al.* 1998; Stevens *et al.* 1999; Lei *et al.* 2000; Morreale *et al.* 2000) have provided a framework for the interface between members of the Cdc42p class of proteins and the CRIB domain of members of the Ste20/Pak kinase family. These studies have implicated specific residues as likely playing important roles in directing the association of these proteins. We have used classical yeast genetics coupled with the powerful two-hybrid protein association assay to investigate aspects of the interaction between Cdc42p and Ste20p.

Hydrophobic residues of Cdc42p are involved in association with Ste20p: NMR analyses have identified residues of Cdc42p whose resonances are modified through an interaction with CRIB domain peptides (Guo *et al.* 1998; Stevens *et al.* 1999; Morreale *et al.* 2000). These residues are likely involved, either directly or indirectly, with the association of the GTPase and its CRIB domain containing target protein. Several of these residues are nonpolar, and a set of these residues forms a hydrophobic cluster within the Cdc42 protein. We used the two-hybrid protein association assay to test whether the residues of Cdc42p forming this hydrophobic cluster are involved in the interaction with the CRIB domain region of Ste20p. Mutations L4R, V44R, Y51A, Y51R, I173A,

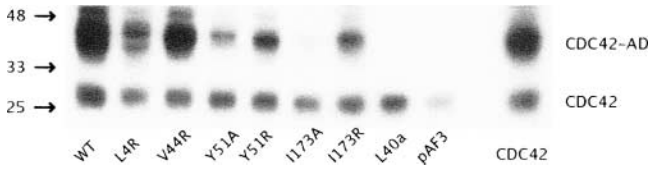

FIGURE 1.—Western blot of total protein extract detected with Cdc42p antibodies. The lower band represents the endogenous Cdc42p and the higher band is Cdc42p fused to the Gal4 activation domain. The endogenous protein serves as an internal control for sample loading, which shows that the amounts of the fusion proteins containing the amino acid substitutions are quite variable. TheV44R substitution protein is as abundant as the wild-type protein, while the I173A fusion protein is almost undetectable. The lane labeled L40a is the two-hybrid analysis strain with no plasmids, while the lane labeled pAF3 represents L40 transformed with the STE20 CRIB domain plasmid fused to the LexA DNA-binding domain. The lane labeled CDC42 contains endogenous Cdc42p as well as Cdc42p fused to the Gal4 activation domain in plasmid pGAD424.

and I173R were created by PCR-mediated site-directed mutagenesis as described in MATERIALS AND METHODS. These mutant constructs were introduced into plasmid pRL202, which contains a C188S mutant version of Cdc42p fused in frame to the LexA DNA-binding domain of plasmid pBTM116. As shown in Figure 1, the protein levels generated from the various Cdc42p two-hybrid plasmids varied considerably. In particular, the I173A protein was expressed dramatically less than the wild-type protein or the other mutants. The V44R substitution protein was expressed as well as wild type, while the L4R, Y51A, Y51R, and I173R proteins were reduced relative to wild type. These mutant constructs were compared with the starting fusion protein in pRL202 for the level of activation of the reporter constructs *HIS3* and *LacZ*. Although the V44R protein was well expressed, the substitution dropped the two-hybrid association measurement to close to background levels, suggesting that V44 plays an important role in Cdc42p association with the CRIB domain. In addition, the L4R and Y51R substitution proteins were expressed as well as the I173R mutant protein, but had reduced two-hybrid interactions (Figure 2). The very low expression of the I173A protein precluded interpretation of the reduced two-hybrid interaction; however, the lack of an influence of the I173R modification may suggest that the I173 residue is not critical for the two-hybrid association.

Conserved residues in the Ste20p CRIB domain are necessary for protein function: An alignment of CRIB domains from several proteins has defined a core consensus region of 12 amino acids (BURBELO *et al.* 1995). Within this conserved region are several invariant amino acids whose conservation in Ste20/Pak kinases from yeast to humans suggests that they play important functional roles. We used site-directed mutagenesis to test the importance of a pair of closely located invariant histidine residues found near the center of the CRIB domain. These histidine residues were modified to aspartates by oligonucleotide-directed PCR mutagenesis as described in MATERIALS AND METHODS. The two mutant alleles, H348D and H345D, were both introduced into the genome of strain W303-1A to replace the wild-type *STE20* gene to create strains JAY25 and JAY26 (Table 1).

Previous work had shown that the CRIB domain of the Ste20p kinase was essential for the Ste20p kinase to support cellular growth in the absence of Cla4p (PETER *et al.* 1996; LEBERER *et al.* 1997). An identical result was obtained when either the H345D or the H348D allele was crossed to a *CLA4* deletion strain. Strains JAY26 and JAY25 were crossed to W303-1B *cla4::TRP1* strain EL252-1B to generate diploids DM98001 and DM98014 (Table 1). These diploids were sporulated and dissected and the pattern of viable spores was observed. In 18 tetrads from DM98001 and 9 tetrads from DM98014 one-half of the expected *cla4::TRP1* spores were missing. This pattern of inviability was that expected for the independent segregation of a mutation that would render the *cla4::TRP1* cells inviable. Evidence that this mutation was either *ste20*^{H345D} or *ste20*^{H348D} was obtained by introducing plasmid pVT-*STE20* into the strains DM98001 and DM98014 prior to dissection. Several Trp$^+$ spores containing the *URA3* plasmid with *STE20* were unable to grow on 5-FOA-containing medium, as expected for strains in which the *STE20* plasmid covered a lethal mutation. This establishes that a single point mutation in the CRIB domain of *STE20* can render the kinase incapable of complementing the loss of *CLA4* function.

Strains containing the H345D and H348D alleles were assayed for the effects of the mutations on Ste20p function in the pheromone response pathway. In contrast to the complete deletion of the CRIB domain, which had essentially no effect on the response of cells to mating (PETER *et al.* 1996; LEBERER *et al.* 1997), the H345D and H348D alleles caused detectable defects in mating. Strains JAY39, JAY40, and JAY54 (Table 1) were tested for pheromone-induced *fus1::LacZ* induction and for mating. As shown in Figure 3 (rows 1, 6, and 11), the H345D and H348D alleles reduced mating to approximately one-tenth the level of the wild-type strain in the absence of any modification to Cdc42p. *Fus1::LacZ* induction was similarly compromised by the H345D and H348D mutations. After a 2-hr induction with α-factor, strains JAY39 and JAY40 generated 13 and 11 Miller units of activity, respectively, while the wild-type control strain JAY54 generated 120 Miller units under the same conditions. Thus the CRIB domain mutations also reduced pheromone-responsive gene induction ~10-fold (Figure 4, rows 1, 6, and 11).

We determined the catalytic activity of the Ste20p kinase containing the modified histidines. The Ste20p kinase was immunoprecipitated from strains W303-1A, JAY25, and JAY26 as described (WU *et al.* 1995). The immunoprecipitated protein was assayed for autophosphorylation and for activity on MBP. As shown in Figure 5, the Ste20p immune complexes had a strong *in vitro*

pBTM116 (BD)	pGAD424 (AD)	β-gal
STE20 aa 1-436	*CDC42:C188S*	66 ± 2
	CDC42:C188S:L4R	9 ± 3
	CDC42:C188S:V44R	0 ± 0
	CDC42:C188S:Y51A	35 ± 6
	CDC42:C188S:Y51R	19 ± 1
	CDC42:C188S:I173A	2 ± 1
	CDC42:C188S:I173R	48 ± 9

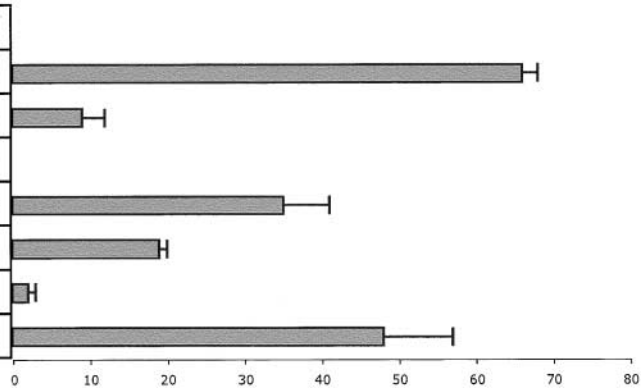

FIGURE 2.—Two-hybrid interactions between Ste20p and Cdc42p. Interactions were determined between the STE20 gene (binding domain, BD) and the CDC42 gene (activation domain, AD) as described in MATERIALS AND METHODS. The β-galactosidase determinations are the average of three independent samples. (Right) β-Galactosidase readings with standard deviation error bars.

kinase activity on MBP, whereas the immune complexes containing the catalytically inactive Ste20pK649R did not phosphorylate MBP. The Ste20p mutants from JAY25 and JAY26 had intracellular concentrations similar to the wild-type Ste20p as judged by the Western blot (Figure 5A) and also showed similar kinase activity on the phosphorylation of MBP as judged by autoradiography (Figure 5B, bottom). The residual amount of ^{32}P labeling of Ste20p by autophosphorylation after the immune complexes were incubated with high concentration of nonlabeled ATP was also very similar among the mutants and the wild-type Ste20p. These results indicated that the Ste20p mutants and the wild-type Ste20p immune complexes prepared from total cell extracts had very similar *in vitro* kinase activities.

The loss of biological function in the absence of modification to the kinase activity suggested that some other aspect of Cdc42p function was compromised. We constructed GFP fusions to the CRIB domain point mutants of Ste20p to analyze cellular localization. Previous work had established that wild-type Ste20p localized to sites of polarized growth and was concentrated in small buds and at the tips of larger buds (PETER *et al.* 1996; LEBERER *et al.* 1997; WU *et al.* 1998). The single point mutants of the CRIB domain prevented this localization, and the GFP signal was observed throughout the cells with no evidence for localization to the buds or bud tips (Figure 6). This confirms the result of the previous localization studies undertaken with CRIB deletion mutants (PETER *et al.* 1996; LEBERER *et al.* 1997). We also examined the localization of the Ste20p point mutants during the mating process. In contrast to the deletion mutants of the Ste20p CRIB domain (PETER *et al.* 1996; LEBERER *et al.* 1997), the Ste20-GFP fusion protein con-

TABLE 1

Yeast strains

Strain	Genotype	Source
W303-1A	*ade2 ura3 his3 leu2 trp1 can1*	R. Rothstein
JAY25	W303-1A with *ste20*^{H348D}	This study
JAY26	W303-1A with *ste20*^{H345D}	This study
JAY39	JAY25 with *sst1::ura3 fus1::LacZ::URA3*	This study
JAY40	JAY26 with *sst1::ura3 fus1::LacZ::URA3*	This study
JAY54	W303-1A with *sst1::ura3 fus1::LacZ::URA3*	This study
DM98001	W303a/α but *cla4::TRP1*/+ and *ste20*^{H345D}/+	This study
DM98014	W303a/α but *cla4::TRP1*/+ and *ste20*^{H348D}/+	This study
EL254-1B	W303-1B with *cla4::TRP1*	This study
M98001-2A	W303-1A *cla4::TRP1ste20*^{H345D}(p*STE20*::URA3)	This study
M98014-1A	W303-1A *cla4::TRP1ste20*^{H348D}(p*STE20*::URA3)	This study
YCW563	W303-1A *sst1::hisG*	This study
	With the following plasmids:	
	pRL116C.4 (*STE20*:GFP)	
	pJA82 (*ste20*^{H345D}:GFP)	
	pJA83 (*ste20*^{H348D}:GFP)	
	pRS315:*CDC42*	
	pJA55 (*CDC42*$^{N39S:P69T}$)	

Strain	Plasmid	Mating *	Relative mating (%)
JAY54 (W303-1A, sst1::ura3 fus1::LacZ::URA3)	pRS315	1.16 ± 0.35	
JAY54 " "	pRS315:*CDC42*	1.14 ± 0.11	
JAY54 " "	pRS315:*CDC42:N39S*	1.45 ± 0.46	
JAY54 " "	pRS315:*CDC42:P69T*	0.46 ± 0.16	
JAY54 " "	pRS315:*CDC42:N39S:P69T*	1.09 ± 0.50	
JAY39 (W303-1A, ste20 H348D, sst1::ura3 fus1::LacZ::URA3)	pRS315	0.18 ± 0.15	
JAY39 " "	pRS315:*CDC42*	0.39 ± 0.03	
JAY39 " "	pRS315:*CDC42:N39S*	1.07 ± 0.21	
JAY39 " "	pRS315:*CDC42:P69T*	0.16 ± 0.03	
JAY39 " "	pRS315:*CDC42:N39S:P69T*	2.61 ± 0.91	
JAY40 (W303-1A, ste20 H345D, sst1::ura3 fus1::LacZ::URA3)	pRS315	0.15 ± 0.08	
JAY40 " "	pRS315:*CDC42*	0.38 ± 0.05	
JAY40 " "	pRS315:*CDC42:N39S*	0.65 ± 0.20	
JAY40 " "	pRS315:*CDC42:P69T*	0.14 ± 0.06	
JAY40 " "	pRS315:*CDC42:N39S:P69T*	1.12 ± 0.52	

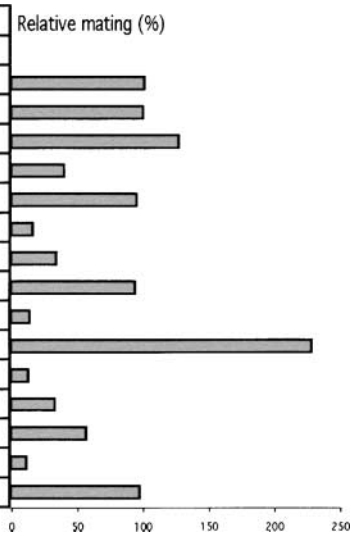

FIGURE 3.—Matings involving STE20 wild-type and mutant strains were done as described in MATERIALS AND METHODS. The strains contained pRS315-based plasmids that were either the control vector or recombinant plasmids containing a version of CDC42. The matings were the average of three independent measurements. The bars on the right are a graphical representation of the relative mating levels; the absolute levels are low due to the effects of the sst1 and fus1 mutations. (*) Values represent the number of diploids formed for each 100 in-put haploid cells.

taining the histidine mutation failed to localize to the shmoo tip (Figure 6).

Conserved residues in the Ste20p CRIB domain are necessary for association with Cdc42p: Both histidine substitution mutants dramatically affected the Ste20p-Cdc42p interaction as measured by the two-hybrid assay. Two-hybrid DNA-binding domain chimeras of the *STE20* N terminus containing the wild-type sequence (pAF3), the H345D allele (pRL155), or the H348 allele (pRL156) were tested with the Cdc42p activation domain chimera pRL202. As shown in Figure 7, both the H345D and the H348D mutations reduced the two-hybrid association

Strain	Plasmid	β-gal
JAY54 (W303-1A, sst1::ura3 fus1::LacZ::URA3)	pRS315	120 ± 7
JAY54 "	pRS315:*CDC42*	123 ± 5
JAY54 "	pRS315:*CDC42:N39S*	133 ± 5
JAY54 "	pRS315:*CDC42:P69T*	122 ± 15
JAY54 "	pRS315:*CDC42:N39S:P69T*	125 ± 18
JAY39 (W303-1A, ste20 H348D, sst1::ura3 fus1::LacZ::URA3)	pRS315	13 ± 3
JAY39 "	pRS315:*CDC42*	39 ± 10
JAY39 "	pRS315:*CDC42:N39S*	63 ± 8
JAY39 "	pRS315:*CDC42:P69T*	29 ± 6
JAY39 "	pRS315:*CDC42:N39S:P69T*	100 ± 13
JAY40 (W303-1A, ste20 H345D, sst1::ura3 fus1::LacZ::URA3)	pRS315	11 ± 4
JAY40 "	pRS315:*CDC42*	28 ± 3
JAY40 "	pRS315:*CDC42:N39S*	56 ± 6
JAY40 "	pRS315:*CDC42:P69T*	21 ± 1
JAY40 "	pRS315:*CDC42:N39S:P69T*	88 ± 12

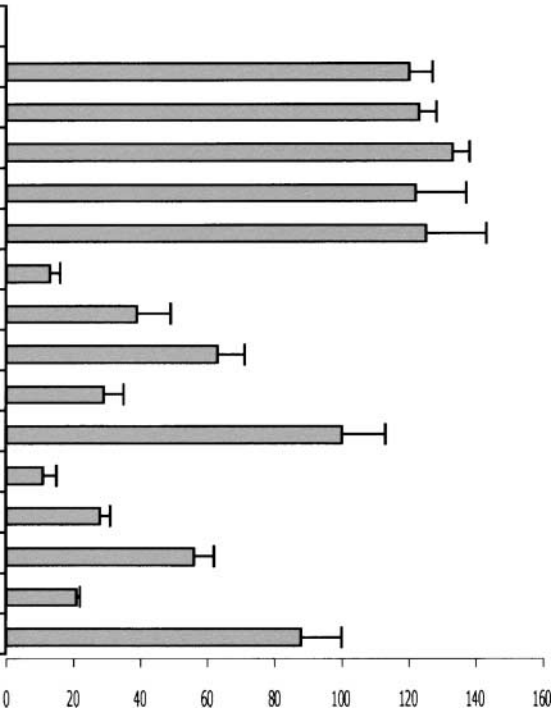

FIGURE 4.—Pheromone-induced expression of the *fus1::LacZ* constructs in STE20 wild-type and mutant strains. β-Gal expression was measured as described in MATERIALS AND METHODS. The strains contained pRS315-based plasmids: either the control vector or recombinant plasmids containing a version of CDC42. The β-gal values were the average of three independent measurements; the bars on the right represent the β-gal values graphically.

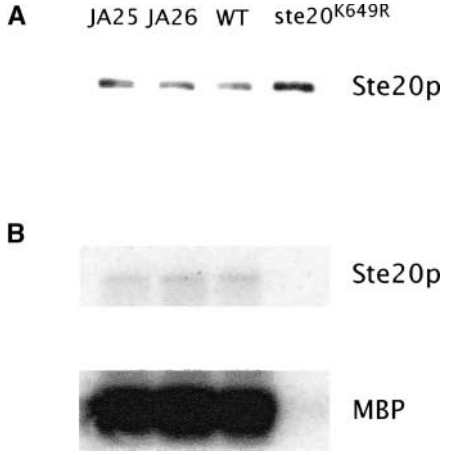

FIGURE 5.—*In vitro* kinase assay of Ste20p. Ste20p was isolated as immune complexes from yeast strains W303-1A (Ste20WT), JA25 (Ste20H345D), JA26 (Ste20H348D), and W303-1A (ste20K649R) expressing catalytically inactive Ste20p. The immune complexes were assayed for kinase activity using MBP as a substrate as described (WU *et al.* 1995). (A) The relative amount of Ste20p in each assay detected by Western blotting. (B) MBP phosphorylation (bottom) and Ste20p autophosphorylation (top).

signal to background levels, showing that the residues are involved in the interaction between the two proteins.

The two-hybrid interaction assay was used to identify mutant versions of Cdc42p that could reestablish protein-protein interaction with a bait plasmid containing the H345D version of the Ste20p CRIB domain. Plasmid pRL202 was subjected to random PCR mutagenesis as described in MATERIALS AND METHODS. The mutagenized PCR product was cotransformed with *Bam*HI- and *Sal*I-linearized plasmid pGAD424 (CHIEN *et al.* 1991) in strain L40 containing the bait plasmid pRL155 to create a library of mutagenized Cdc42p constructs. Cells were selected on −his + 5 mM 3-AT to detect significant two-hybrid interactions between the mutant Cdc42p and the H345D version of the Ste20p CRIB domain, as this concentration of 3-AT was the minimum that was capable of eliminating all background growth of the pRL155/pRL202 combination. One mutant derivative of pRL202 was identified in ~2000 transformants screened that reproducibly provided plasmid-dependent *HIS3*$^+$ activity in strain L40 containing pRL155. Sequencing this plasmid (pMJ13) established that it contained two nucleotide substitutions (A234 to C234 and A567 to C567) that resulted in two amino acid substitutions in Cdc42p, N39S, and P69T. We tested whether both substitutions were necessary for the reestablishment of the two-hybrid interaction with pRL155 by constructing derivatives of pRL202 with the two single mutations. Neither plasmid pJA49, containing the N39S substitution, nor plasmid pJA50, containing the P69T substitution, gave a strong two-hybrid interaction with pRL155. We also tested the specificity of the double mutant. pMJ13 had a normal two-hybrid interaction with pAF3, showing that the amino acid substitutions did not block interaction between the mutant Cdc42 protein and the wild-type CRIB domain. In addition, the double mutant was capable of interacting with pRL156, which contains the H348D substitution. However, the protein was still specific for interaction with the Ste20 N terminus; the mutant Cdc42p construct did not show any interaction with pVL7, which contains the Kss1p kinase (COURCHESNE *et al.* 1989) as negative control (Figure 7).

We investigated whether other combinations of mutations could reestablish the two-hybrid interaction with pRL155 containing the H435D substitution. Plasmids pJA49 and pJA50 were used as templates for a round of PCR mutagenesis as described. Mutant plasmids that conferred growth of L40 transformed with pRL155 on plates with 5 mM 3-AT were identified and sequenced (Table 2). When pJA49 (N39S) was used as a template, we identified two plasmids in which P69 was mutated to T, regenerating the combination identified in pMJ13.

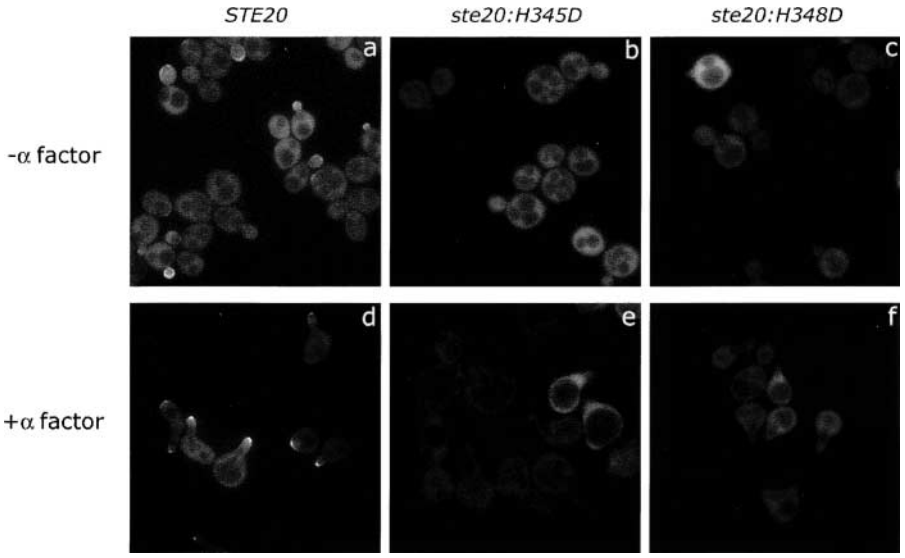

FIGURE 6.—GFP:Ste20p fusion protein localization. Yeast strains expressing the wild type (a and d), the H345D (b and e), or the H348D (c and f) CRIB domain mutant GFP:Ste20p fusion proteins were analyzed by fluorescence microscopy to determine the cellular localization of the GFP protein. The wild-type protein localizes to bud (a) or shmoo (d) tips. The CRIB domain mutant proteins fail to localize to either bud (b and c) or shmoo (e and f) tips and show instead a generalized cytoplasmic staining.

pGAD424 (AD)	pBTM116 (BD)	β-gal
pMJ13 (*CDC42:C188S:N39S:P69T*)	pAF3 (*STE20 aa 1-436*)	49.62 ± 2.54
pMJ13 "	pRL155 (*STE20:H345D*)	7.93 ± 4.14
pMJ13 "	pRL156 (*STE20:H348D*)	5.05 ± 1.43
pMJ13 "	pVL7 (*Kss1*)	0.06 ± 0.07
pJA49 (*CDC42:C188S:N39S*)	pAF3 (*STE20 aa 1-436*)	44.27 ± 8.06
pJA49 "	pRL155 (*STE20:H345D*)	0.07 ± 0.08
pJA49 "	pRL156 (*STE20:H348D*)	0.06 ± 0.07
pJA49 "	pVL7 (*Kss1*)	0.06 ± 0.06
pJA50 (*CDC42:C188S:P69T*)	pAF3 (*STE20 aa 1-436*)	117.31 ± 10.89
pJA50 "	pRL155 (*STE20:H345D*)	0.19 ± 0.22
pJA50 "	pRL156 (*STE20:H348D*)	0.16 ± 0.01
pJA50 "	pVL7 (*Kss1*)	0.05 ± 0.06
pRL202 (*CDC42:C188S*)	pAF3 (*STE20 aa 1-436*)	56.90 ± 7.90
pRL202 "	pRL155 (*STE20:H345D*)	0.05 ± 0.05
pRL202 "	pRL156 (*STE20:H348D*)	0.04 ± 0.05
pRL202 "	pVL7 (*Kss1*)	0.06 ± 0.07

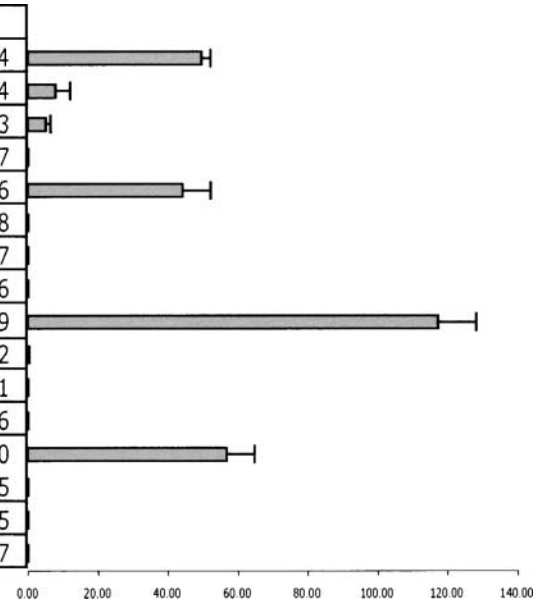

FIGURE 7.—Two-hybrid assay for the association of Cdc42 and the Ste20 CRIB domain. CAAX box mutant Cdc42p constructs fused to the Gal4 activation domain (pGAD424 derivatives) were combined with pBTM116 (DNA-binding domain) versions of either the N terminus of Ste20p or mutant derivatives or with the Kss1p kinase. Values represent the average of three to six independent assays.

In addition to P69T, we found that G60S, G12S, G12H, D65G, and Y64H mutations in combination with N39S could confer growth in the presence of 5 mM 3-AT. When pJA50 (P69T) was used as the template, we identified three plasmids with the N39S mutation. In addition, we identified the Y64H change that also was found together with the N39S substitution. Finally, we identified one triple-mutant combination, which had both the N39S and the G12S substitutions, together with the template P69T change.

We determined whether the N39S P69T allele of Cdc42p was able to improve the function of Ste20p containing the H345D and H348D substitutions. Strains JAY54, JAY39, and JAY40 were transformed with pRS315 or derivatives of pRS315 plasmids containing wild-type Cdc42p or mutant versions of Cdc42p with the N39S, the P69T, or the double N39S/P69T substitutions. These transformants were tested to determine the consequence of the presence of the mutant *CDC42* constructs. As previously noted in Figure 3, in cells with the vector plasmid pRS315, the H345D and H348D substitutions reduced mating ~10-fold relative to that of the wild-type strain. These reductions were sensitive to the level of Cdc42p; introduction of an extra copy of wild-type *CDC42* in strains JAY39 and JAY40 improved mating ~2-fold, although introduction of an extra copy of the $CDC42^{P69T}$ allele generated no improvement in mating in these strains. Intriguingly, the presence of the $CDC42^{P69T}$ allele had a detrimental effect on mating in an otherwise wild-type strain, while the other plasmids had essentially no effect on mating levels in JAY54. In both strains JAY39 and JAY40, the presence of the N39S/P69T double mutant raised the mating level to essentially that of the wild-type strain with the double-mutant plasmid.

Similar results were obtained for pheromone-induced expression of the *fus1::LacZ*. The presence of the various *CDC42* alleles had little effect on *fus1::LacZ* expression in strain JAY54, but the N39S/P69T allele improved induction in both JAY39 and JAY40 (Figure 7). Finally, we tested whether the N39S/P69T allele could rescue the inviability of the *cla4::TRP1* allele coupled with either the ste20H345D or the ste20H348D allele. Strains M98001-2A (pVT STE20) and M98014-1A (pVT STE20; Table 1) were transformed with plasmid pSTE20,

TABLE 2

CDC42 interaction-enhancing mutations

Template mutation	Additional mutations
N39S	P69T (2)
N39S	G60S (2)
N39S	G12S
N39S	G12H
N39S	Y64H
N39S	D65G
P69T	N39S (3)
P69T	N39S, G12S
P69T	Y64H

Mutations that enhanced the two-hybrid interaction between Cdc42p and the Ste20p CRIB domain containing the H345D substitution were identified after mutagenesis of templates containing either the N39S or the P69T mutations. The most frequent event reconstructed the N39S P69T double mutation, but other changes were also identified.

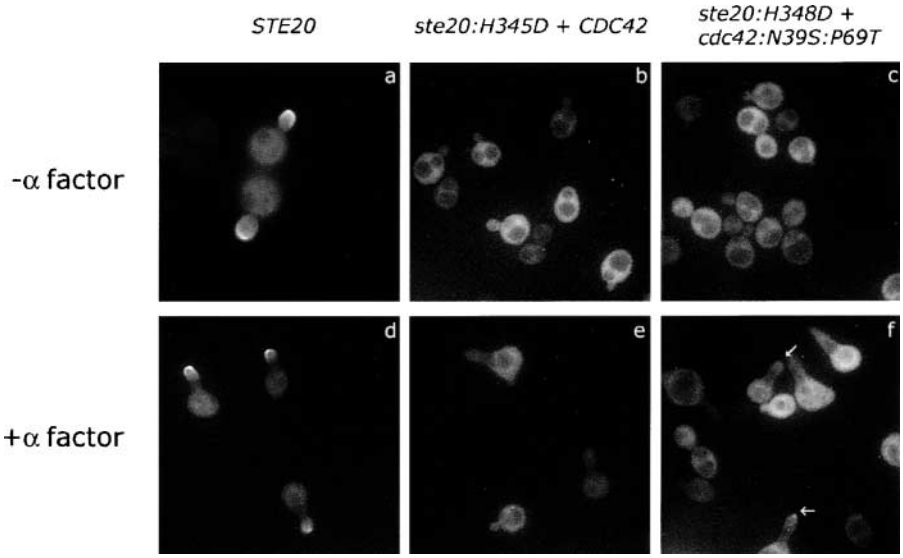

FIGURE 8.—GFP:Ste20p fusion protein localization controlled by Cdc42p. Yeast strains expressing either the wild type or the H345D CRIB domain mutant GFP:Ste20p fusion proteins were analyzed by fluorescence microscopy to determine the cellular localization of the GFP protein. The wild-type protein localizes to bud (a) or shmoo (d) tips (see also Figure 3). The CRIB domain mutant protein is not localized to sites of polarized growth, and this mislocalization is not corrected by increasing the amount of Cdc42p for either buds (b) or mating projections (e). The N39S, P69T allele of Cdc42p did not dramatically improve the localization to sites of polarized growth in budding cells (c), although some mating-factor-treated cells exhibited weak localization to the shmoo tip (arrows in f).

pRS315CDC42, or pRS315cdc42$^{N39S/P69T}$, and the resulting transformants were challenged on 5-FOA medium to determine whether the wild-type *STE20* allele was still necessary to support viability. The introduction of the *STE20* gene on the *HIS3* plasmid allowed the growth of the transformants on 5-FOA medium, whereas the introduction of either the wild-type or the N39S/P69T versions of Cdc42p did not permit growth on 5-FOA plates. This establishes that although the N39S/P69T allele is able to suppress the mating and gene expression defects of the Ste20^{H3345D} and Ste20^{H348D} alleles, the modified Cdc42 protein was not capable of rescuing the need for Ste20p CRIB function in the absence of Cla4p (data not shown).

We tested whether the double-mutant version of *CDC42* was able to improve the localization of the H345D and H348D versions of Ste20p. The wild-type and double-mutant versions of *CDC42* were introduced into strains containing the mutant versions of Ste20GFP, and cellular localization was analyzed. As shown in Figure 8, the presence of the mutant Cdc42p did not dramatically change the cellular mislocalization of Ste20GFP during either normal vegetative growth or shmoo formation. In some cells there is evidence of improved localization of the Ste20GFP signal to sites of polarized growth, but overall the H345D- and H348D-substituted Ste20GFP fusions are poorly localized even when coexpressed with the modified Cdc42p.

Localization of the mutant residues on structures (Abdul-Manan *et al.* 1999; Mott *et al.* 1999; Gizachew *et al.* 2000; Morreale *et al.* 2000) of the Cdc42p-CRIB domain (and related GTPase-CRIB interactions) show that the N39S substitution is close to the conserved histidine residues of the CRIB domain (Figure 9). The P69T substitution is not closely associated with the CRIB domain in most of the determined structures, but direct assessment of the actual *S. cerevisiae* Cdc42p-Ste20p structure will be necessary to confirm this interpretation.

DISCUSSION

Ste20p is the founding member of the PAK family of protein kinases (Leberer *et al.* 1992). PAK kinases are defined by the ability to associate with small GTPases of the Rac and Cdc42 families through a binding region termed a CRIB domain. Mammalian PAK kinases require this binding for their activity, and recent studies have defined the structure of the binding interaction and have suggested a mechanism for the role of this association in kinase activation (Lei *et al.* 2000). In this study we have identified point mutants in both the Cdc42p GTPase and the CRIB domain of the Ste20p kinase that influence the association of these proteins and modify their function.

Previous structural studies had implicated a number of residues of the human Cdc42 protein in interactions with peptides synthesized to correspond to the CRIB domain region of PAK (Guo *et al.* 1998; Stevens *et al.* 1999; Lei *et al.* 2000; Morreale *et al.* 2000). These results suggest that the binding surface of the GTPase with the CRIB domain involved a more extended surface than that observed for the Ras/Raf association (Emerson *et al.* 1995) and, in particular, implicated a block of physically adjacent residues of Cdc42p in the interaction (Stevens *et al.* 1999). We have used the two-hybrid assay to test the role of the residues (Ile4, Val44, Tyr51, and Ile173) forming this hydrophobic block in the association of the yeast Cdc42p and the CRIB domain of Ste20p. Most of the substitutions reduced protein stability as measured by the relative steady-state protein amounts determined by Western blotting, suggesting that these hydrophobic residues have a role in overall

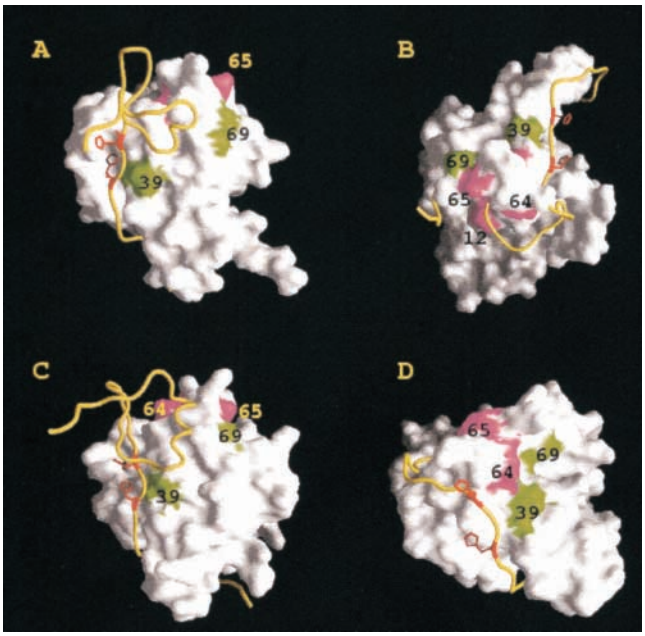

FIGURE 9.—Models of CRIB domain interactions. Mutations that enhanced the two-hybrid interactions between Cdc42p and the H245D/H348D Ste20p CRIB domain are mapped onto the representative structures of human Cdc42-CRIB complexes. Cdc42 is represented as a molecular surface and the CRIB fragment as a yellow worm. The location of the Ste20p CRIB mutants H345D/H348D are shown in red. The template mutations of Cdc42p, N39S, P69T that most frequently showed binding are colored green; other residues leading to changes are colored magenta. All four structures show the close proximity of N39 in Cdc42 to the mutated His residues in Ste20p. However, P69 in Cdc42 was consistently distant from the His mutations in Ste20p. Some mutated residues are not visible on all the structures. (A) Cdc42-PAK (MORREALE *et al.* 2000). (B) Cdc42-PAK (GIZACHEW *et al.* 2000). (C) Cdc42-WASP (ABDUL-MANAN *et al.* 1999). (D) Cdc42-ACK (MOTT *et al.* 1999). The figures were produced using GRASP (NICHOLLS *et al.* 1991).

protein structure. The most significant destabilization of the Cdc42 fusion protein was caused by the I173A substitution, while the I173R protein showed moderate stability. The I173R protein also showed an essentially wild-type two-hybrid interaction with the Ste20p CRIB domain. This suggests that the position 173 residue is important in overall protein folding, but that the I173A mutation does not have a specific role in the interaction with the PAK family kinase CRIB domain.

However, some of the hydrophobic residues appear directly implicated in the association with the CRIB domain. The V44R substitution did not influence protein stability, but eliminated the two-hybrid interaction with the Ste20p CRIB domain. Previous analysis of a V44A substitution (RICHMAN *et al.* 1999) suggested that this mutation blocked the association of Cdc42p with effectors such as Cla4p, Gic1p, and Gic2p, but did not influence the association with Ste20p. The dramatic consequence of the V44R change in the Ste20p association confirms that position 44 plays an important role in the selectivity of Cdc42p for CRIB motifs. In a manner similar to that of position V44, the L4 position appeared to modulate the CRIB-binding efficacy of Cdc42p. The protein stability was somewhat compromised by this substitution, but the fusion protein generated a weaker two-hybrid signal than that of other mutants of equivalent stability. Thus within the hydrophobic block are residues whose major contribution appears to be to protein stability, while other residues are implicated primarily in the protein association function.

We also examined the importance of residues within the Ste20p CRIB domain for binding to the Cdc42 protein. Two conserved histidine residues at positions 345 and 348 were mutated to aspartic acids. Both mutations blocked the association of the kinase and the GTPase as measured by the two-hybrid assay. This loss of association was correlated with a loss of biological function; when the H345D and H348D substitutions were introduced into the endogenous *STE20* gene, neither the *ste20*^{H345D} nor the *ste20*^{H348D} alleles were able to rescue the deletion of *CLA4*. This confirms previous results obtained for CRIB domain deletions (PETER *et al.* 1996; LEBERER *et al.* 1997). The defect in Ste20p function caused by mutation of either CRIB domain histidine does not appear to be the result of reduced kinase activity. *In vitro* assays show that the kinase activity toward myelin basic protein of Ste20p immunoprecipitated from the mutant cells was identical to that of Ste20p immunoprecipitated from wild-type cells. This is perhaps surprising as the structure of the mammalian PAK suggests that conformational changes associated with Cdc42p binding are required for kinase catalytic activity (LEI *et al.* 2000). Either the yeast protein has a less critical requirement for Cdc42p-induced conformational change or the protein isolation conditions are sufficient to activate catalytic activity in the absence of Cdc42p. Recent data (LAMSON *et al.* 2002) suggest that even an "activated" Ste20p allele fails to complement the *CLA4* deletion when it lacks Cdc42p-binding capacity.

Even though the kinase activity was normal, the strains containing the *ste20*^{H345D} and the *ste20*^{H348D} alleles were compromised in *STE20* function in the pheromone response pathway. Strains containing the alleles showed reduced mating and reduced ability to induce the *fus1::lacZ* reporter construct when treated with mating pheromone. These phenotypes appeared to result from the defect in the association of Cdc42p with the Ste20p kinase. Mutations that reestablished the two-hybrid association with the H345D allele of the *STE20* CRIB domain with Cdc42p were selected in Cdc42p. This strategy identified a double-mutant *CDC42*^{N39S,P69T} that was capable of a more effective association with both the H345D and the H348D CRIB mutations. The double mutant and the two single mutations were transferred to a nonfusion version of Cdc42p to test the consequences of the changes on the function of Cdc42p. The double mutant provided a significant improvement in the mat-

ing response of cells containing either the H345D or the H348D mutation. Similarly, the double mutant was able to restore essentially normal pheromone responsiveness to the H345D and H348D mutant strains. Analysis of the single mutants showed that neither was able to reestablish the two-hybrid association on its own. In the biological assays the P69T mutation actually reduced mating and pheromone response relative to the wild-type *CDC42*, while the N39S substitution caused a moderate improvement.

Although the double mutant was necessary for the enhanced association with the mutant CRIB domain, other double-mutant combinations, including N39S G60S, N39S G12S, and G12H, N39S D65G, N39S Y64H, and Y64H P69T, were capable of enhancing the two-hybrid association between Cdc42p and the Ste20 CRIB domain containing the H345D substitution. Although the initial N39S P69T allele did not influence residues implicated in the GTPase activity of Cdc42p, residues equivalent to G12 and Y64 have been found to reduce the GTPase activity of Cdc42 homologs in other systems (Nur *et al.* 1992; Best *et al.* 1996; Murray and Johnson 2001). Thus it is possible that these latter changes prolong the time the protein spends in the active, CRIB-binding conformation and that this increase is sufficient to allow detection of the interaction in the two-hybrid assay. Direct assessment of the roles that the mutant residues play in protein structure and function will be necessary to test these models.

The reduced mating and pheromone inducibility of the H345D and H348D substitutions contrasts with what was observed for the deletion of the CRIB domains of Ste20p (Peter *et al.* 1996; Leberer *et al.* 1997). This is consistent with the removal of the CRIB domain region deleting not only the Cdc42p-binding sequence, but also an inhibitory function. The point mutant constructs appear to influence only the CRIB-binding function and to leave the autoinhibitory function intact. Intriguingly, the reduced mating seen in the point mutants and not in the deletion was correlated with the ability to localize to shmoo tips. GFP versions of the point mutants did not localize to either the bud or the shmoo tips, while the deletion mutant protein was able to associate with the shmoo tip but not with the bud tip (Leberer *et al.* 1997). It has been speculated that the bud tip localization of Ste20p is controlled by Cdc42p binding, while the association with the shmoo tip may be regulated by the interaction with Ste4p (Leberer *et al.* 1997; Leeuw *et al.* 1998). We show here that CRIB domain point mutants that prevent Cdc42p interaction also block the shmoo tip localization of Ste20p. This may imply that a conformational change in Ste20p caused by Cdc42p binding is necessary for Gβ binding and thus for subsequent localization to sites of polarized growth in mating cells. Alternately, the ability of the CRIB-deleted Ste20p to localize to shmoo tips during mating (Peter *et al.* 1996; Leberer *et al.* 1997) may be related to the abnormal activity of this protein (Lamson *et al.* 2002).

The improved function exhibited by the H345D and H348D strains in the presence of the N39S P69T version of Cdc42p was limited to improving mating and pheromone responsiveness. The modified Cdc42p did not rescue the inviability of cells containing the histidine substitutions in *STE20* when they were coupled to the *cla4* mutation, and the modified Cdc42p did not dramatically improve the localization of the histidine substitution mutants of the Ste20-GFP fusion protein. The two-hybrid association of the H345D and H348D alleles of Ste20p with the N39S P69T allele of Cdc42p was considerably less than the association measured for the wild-type proteins. Thus the improvement in some Ste20p functions and not in others may be attributed to the strength of the associations; weak improvements in function or localization may improve mating without allowing the rescue of the missing *cla4* function.

Previous studies provided conflicting data on the requirement for Cdc42p in Ste20p function in the mating pathway (Simon *et al.* 1995; Peter *et al.* 1996; Leberer *et al.* 1997; Moskow *et al.* 2000). The present work shows that the interaction between the kinase and the GTPase plays a significant role in mating, and this interpretation has been independently shown by work that analyzed related but distinct CRIB domain mutations (Lamson *et al.* 2002). This association appears critical for proper localization of the kinase to sites of polarized growth in both vegetative and shmooing cells, but not for kinase catalytic activity measured *in vitro*. Further work will be required to determine the structural role of Cdc42p in Ste20p action.

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