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Functional Analysis of the Interaction between Afr1p and the Cdc12p Septin, Two Proteins Involved in Pheromone-induced Morphogenesis

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Saccharomyces cerevisiae mating pheromones induce production of Afr1p, a protein that negatively regulates pheromone receptor signaling and is required for normal formation of the projection of cell growth that becomes the site of cell fusion during conjugation. Afr1p interacts with Cdc12p, which belongs to a family of filament-forming proteins termed septins that have been studied primarily for their role in bud morphogenesis and cytokinesis. The significance of the interaction between Afr1p and Cdc12p was tested in this study by examining the effects of *AFR1* mutations that destroy the Cdc12p-binding domain. The results demonstrate that sequences in the C-terminal half of Afr1p are required for interaction with Cdc12p and for proper localization of Afr1p to the base of the mating projection. However, the Cdc12p-binding domain was not required for regulation of receptor signaling or for mating projection formation. This result was surprising because cells carrying a temperature-sensitive *cdc12-6* mutation were defective in projection formation, indicating a role for Cdc12p in this process. Although the Cdc12p-binding domain was not essential for Afr1p function, this domain did improve the ability of Afr1p to promote morphogenesis, suggesting that the proper localization of Afr1p is important for its function.

INTRODUCTION

The conjugation of the yeast *Saccharomyces cerevisiae* offers opportunities to use genetic approaches to help determine the mechanisms of hormone-induced cell polarization and morphogenesis. Conjugation is initiated when haploid *MAT***a** and *MAT*α cells signal each other with secreted mating pheromones (Marsh *et al.*, 1991; Kurjan, 1992; Sprague and Thorner, 1992). Pheromone signaling arrests cell division in G_1 and then promotes polarized cell growth in the direction of an appropriate mating partner. Polarized morphogenesis results in the formation of an acute projection that becomes the site at which cells fuse together to form a zygote.

The mating pheromone signal is transduced by components that are highly homologous to the hormone signaling pathways present in multicellular organisms. The α-factor and **a**-factor pheromone receptors belong to the large family of G protein-coupled receptors that includes rhodopsin, the adrenergic receptors, and the leukocyte chemoattractant receptors (Dohlman *et al.*, 1991; Murphy, 1994). These G protein-coupled receptors transduce their signal by stimulating the α subunit of a heterotrimeric G protein to bind GTP, causing the Gα subunit to dissociate from the G$\beta\gamma$ subunits (Hepler and Gilman, 1992). The other steps in the signal pathway include Cdc42p, a member of the rho family of small GTP-binding proteins (Simon *et al.*, 1995; Zhao *et al.*, 1995); the Ste20p protein kinase (Leberer *et al.*, 1992); and a protein kinase cascade with similarity to the family of mitogen-activated protein (MAP) kinase cascades (Herskowitz, 1995). Activation of the signaling pathway stimulates the Ste12p transcription factor to induce expression of mating-specific genes (Sprague and Thorner, 1992).

Mating pheromone receptor signaling determines the site of cell polarization in yeast. Cells polarize their

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Table 1. Yeast strains

Strains	Genotypes
JKY25	*MAT***a** *ade2-1° his4-580ᵃ lys2° trp1ᵃ tyr1° leu2 ura3 SUP4-3ᵗˢ bar1-1*
JKY26	*MAT***a** *ade2-1° his4-580ᵃ lys2° trp1ᵃ tyr1° cry1 leu2 ura3 SUP4-3ᵗˢ bar1-1 afr1::URA3*
DGO7B	*MATα leu2 ura3 can1 cdc12-6*
JKY71	*MATα leu2 ura3 his4-580ᵃ trp1ᵃ SUP4-3ᵗˢ cdc12-6*
yLG44-5-3	*MAT***a** *ade2-1° his4-580ᵃ lys2° trp1ᵃ tyr1° leu2 ura3 bar1-1 cdc12-6*
yLG45-1	*MAT***a** *ade2-1° his4-580ᵃ lys2° trp1ᵃ tyr1° leu2 ura3 bar1-1*
yLG75-1	*MAT***a** *ade2-1° his4-580ᵃ lys2 trp1ᵃ tyr1° leu2 ura3 bar1-1 afr1::URA3 YIplac128-AFR1-C6*
yLG76-1	*MAT***a** *ade2-1° his4-580ᵃ lys2° trp1ᵃ tyr1° leu2 ura3 bar1-1 cdc12-6 afr1::URA3 YIplac128-AFR1-C6*
L40	*MAT***a** *his3 leu2 trp1 LYS2::lexA-lacZ URA3::lexA-HIS3*

growth in the direction of a gradient of pheromone emanating from a cell of opposite mating type (Jackson *et al.*, 1991; Segall, 1993). In the absence of a gradient, cells use a default site that is usually adjacent to the previous site of bud formation (Madden and Snyder, 1992; Dorer *et al.*, 1995; Valtz *et al.*, 1995). Ultimately, the pheromone signal promotes changes in the actin cytoskeleton that are required for polarized growth (Read *et al.*, 1992; Chenevert, 1994; Drubin and Nelson, 1996). The activation of Cdc42p by receptor signaling is thought to play a key role in stimulating cell polarization because Cdc42p is also required for polarized growth of vegetative cells (Adams *et al.*, 1990; Ziman *et al.*, 1993).

The intensity of pheromone receptor signaling determines the type of polarized growth that occurs. Cells arrested with a low dose of α-factor pheromone (i.e., $<10^{-8}$ M) form elongated cells, whereas cells arrested at high doses (i.e., $>10^{-7}$ M) form acute projections (Moore, 1983). High levels of signaling may be needed to induce sufficient levels of *AFR1*, a pheromone-induced gene that is required for normal projection formation (Konopka, 1993; Konopka *et al.*, 1995). Genetic analysis indicates that *AFR1* also acts to regulate receptor signaling (Konopka, 1993). Regulation of receptor signaling is important for projection formation because C-terminal truncation mutants of the α-factor receptor that show 10-fold increased sensitivity to pheromone due to a defect in adaptation are also defective in forming projections (Konopka *et al.*, 1988). In addition, mutation of the distal four phosphorylation sites in the receptor C terminus caused increased sensitivity and a defect in projection formation (Chen and Konopka, 1996). Thus, these results suggest that signaling and morphogenesis are coordinately regulated.

The Afr1p protein interacts with Cdc12p (Konopka *et al.*, 1995), a member of a family of homologous proteins known as septins that also includes Cdc3p, Cdc10p, and Cdc11p (Longtine *et al.*, 1996). The septins are filament-forming proteins (Field *et al.*, 1996) that were shown previously to be involved in bud morphogenesis and cytokinesis (Haarer and Pringle, 1987; Hartwell, 1971; Ford and Pringle, 1991; Kim *et al.*, 1991; Sanders and Field, 1994; Chant, 1996). Recently, septins have also been implicated in other processes, including sporulation (Fares *et al.*, 1996). Septin homologues have been found in a number of organisms, including *Drosophila*, mice, and humans (Longtine *et al.*, 1996). Studies on septin homologues in *Drosophila* suggest that the homologues are involved in cytokinesis and in other morphogenetic processes (Neufeld and Rubin, 1994; Fares *et al.*, 1995). Therefore, the function of Afr1p and the septins in pheromone-induced morphogenesis was explored in this study by examining the significance of interaction between Cdc12p and Afr1p.

MATERIALS AND METHODS

Strains and Media

Cells were grown in media described by Sherman (1991). Plasmids were transformed into yeast strains using lithium acetate transformation (Schiestl and Gietz, 1989). Yeast strains are described in Table 1. Strain JKY71 was obtained from a cross between *cdc12-6* strain DG07B (Haarer and Pringle, 1987) and JKY25. Strain yLG44-5-3 was obtained from a cross between JKY71 and JKY25. Strain yLG45-1 was derived from yLG44-5-3 by conversion of *cdc12-6* to *CDC12* by gene replacement with use of an *Xba*I/*Pst*I fragment bearing the wild-type *CDC12* allele. Strains yLG75-1 and yLG76-1 were derived from yLG45-1 and yLG44-5-3, respectively, by deleting *AFR1* and integrating the *AFR1-C6* allele at the *AFR1* locus.

Plasmids

AFR1 deletion mutants were constructed by PCR amplification with use of Taq DNA polymerase. All 5′ oligonucleotide primers contained a *Sal*I site followed by a start codon and then a portion of the *AFR1*-coding sequence. All 3′ oligonucleotide primers contained a *Bam*HI site preceded by a stop codon, in some cases an *Xho*I site, and then the *AFR1* sequence. Truncation mutants *AFR1-N1*, *-N2*, *-N3*, *-N4*, *-C1*, *-C6*, and *-C7* were made by use of PCR with the following combinations of oligonucleotides: OLG3/OLG4, OLG8/OLG4, OLG7/OLG4, OLG6/OLG4, OLG1/OLG9, OLG1/OLG17, and OLG1/OLG20. The *Sal*I/*Bam*HI fragments were cloned downstream of the *GAL10* promoter in a modified version of YEp51 (Rose and Broach, 1991) containing a polylinker. Internal deletion mutants were constructed by cloning the appropriate PCR product containing a C-terminal fragment into a vector containing an N-terminal fragment. The PCR primers were designed so that ligation of the *Xho*I site in the N-terminal fragment with the *Sal*I site of the C-

terminal fragment restored the reading frame of *AFR1*. The mutants *AFR1-LDM2*, *-LDM3*, and *-LDM4* are modified only at the ligation site because the two PCR products reconstitute the entire coding sequence. In *AFR1-LDM2*, codons 298–300 were changed from KKK to LDM; in *AFR1-LDM3*, codons 413–415 were changed from QKL to LDM; and in *AFR1-LDM4*, codon 350 was changed from D to L. The combinations of PCR primers used to construct the internal deletion and LDM mutants are indicated in parenthesis as follows: *AFR1-I1* (OLG1/OLG9 + OLG7/OLG4), *AFR1-I4* (OLG1/OLG11 + OLG8/OLG4), *AFR1-I8* (OLG1/OLG17 + OLG7/OLG4), *AFR1-I3* (OLG1/OLG2 + OLG6/OLG4), *AFR1-LDM2* (OLG1/OLG9 + OLG8/OLG4), *AFR1-LDM3* (OLG1/OLG2 + OLG7/OLG4), and *AFR1-LDM4* (OLG1/OLG17 + OLG16/OLG4). For each plasmid construction, three independent clones were transformed into yeast strain JKY26. After galactose-induced expression of the *AFR1* genes, all three clones always gave the same phenotype for effects on morphogenesis and α-factor resistance, so only one was used for subsequent experiments. The oligonucleotides used were as follows: OLG1, GCTGTCGACCATGGAGGGCTCATATCT; OLG2, CGGGATCCTACTCGAGTCGTATTTCTAATTGC; OLG3, GGTCGACATGGTCATACCTATTCCTCC; OLG4, CGGGATCCTAAATATGTATGACGCC; OLG6, GGTCGACATGAGAA GGAATTCGCTGCC; OLG7, GGTCGACATGCACGAGACCTCGCACAAC; OLG8, GGTCGACATGACTATTCTGGGCACTGAG; OLG9, CGGGATCCTACTCGAGTCTATCAAATAGGCCTG; OLG11, CGGGATCCTACTCGAGATTATGAGGCCCCAC; OLG16, GGTCGACATGGTTACGCATTCTGATGG; OLG17, CGGGATCCTACTCGAGGATAGGAATTCTCCTTC; and OLG20, CGGGATCCTACTCGAGATTACTTCCGGAGAACC.

To carry out two-hybrid analysis, *AFR1* alleles made in YEp51 as described above were subcloned into the *LexA* DNA-binding domain plasmid pBTM116 (Bartel *et al.*, 1993). *AFR1* and *lexA* sequences were joined in frame by modifying pBTM116 so that the *Sal*I site was filled-in with the Klenow DNA polymerase, and then a *Sal*I site was reintroduced at the *Eco*RI site with the adaptor AATTGTCGAC to create pLG3 or the adaptor AATTTGTCGACA to create pLG7. *AFR1-C1*, *-C6*, *-C7*, *-I1*, *-I3*, *-I4*, *-I8*, *-LDM2*, *-LDM3*, and *-LDM4* were cloned in pLG3. *AFR1-N1*, *-N2*, *-N3*, and *-N4* were cloned in pLG7. The Gal4p–Cdc12p fusion plasmid contained the entire *CDC12* open reading frame as described previously (Konopka *et al.*, 1995).

The effects of *AFR1* overexpression were examined in strains carrying *AFR1* alleles on the multicopy plasmid vector YEplac181 (Gietz and Sugino, 1988). The genomic *Sal*I/*Bam*HI fragment containing the entire *AFR1* gene, including the promoter, was first cloned in YEplac181 to created pJK52 (Konopka, 1993). The C-terminal truncation genes and the internal deletion genes were cloned into YEplac181 by using the *Avr*II/*Bam*HI fragments from the YEp51-derivative plasmids to replace the corresponding fragment in pJK52. The N-terminal truncation mutants lacked the *Avr*II site, so the *Avr*II site in pJK52 was converted to an *Xho*I site using an adaptor oligonucleotide (CTAGCCTCGAGG). This permitted a *Sal*I/*Bam*HI fragment from N-terminal mutants to be inserted in frame with the ATG initiation codon. Integration of *AFR1* alleles into the genome was carried out with use of the integrative plasmid YIplac128 (Gietz and Sugino, 1988). The *Sal*I/*Bam*HI fragments from the multicopy plasmids were introduced into the polylinker sites of YIplac128. These integrative plasmids were linearized with *Spe*I and then integrated in strain JKY26 at the *AFR1* locus.

A clone of the *CDC12* gene was obtained by transforming the *cdc12-6* strain JKY71 with a multicopy plasmid yeast genomic library, and then plasmids that rescued the temperature sensitivity were recovered in *Escherichia coli*. The identity of the *CDC12* gene was confirmed by restriction mapping and partial DNA sequence analysis. The *Xba*I/*Pst*I fragment was used to convert the *cdc12-6* allele of yLG44-5-3 to create yLG45-1.

Pheromone-Response Assays

Cells were grown to midlogarithmic phase in synthetic medium lacking leucine to select for plasmid maintenance. Resistance to α-factor-induced cell division arrest was assayed by spotting approximately 1×10^3 cells on an agar plate containing the indicated concentration of α-factor. Plates were incubated at 30°C for 2 d and then photographed to record the ability of cells to grow in the presence of α-factor. Liquid cultures were induced by the addition of α-factor to 5×10^{-7} M for the indicated time. Immunoblot analysis was carried out essentially as described previously (Konopka *et al.*, 1995). Cells (10^8) were induced with α-factor for 2 h and extracted with lysis buffer (2% SDS, 100 mM Tris, pH 7.5), and then equal amounts of extracts were resolved by electrophoresis on a SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose, and then the blot was probed with rabbit anti-Afr1p antibody (Konopka *et al.*, 1995). Immunoreactive bands were detected with an alkaline phosphatase-conjugated secondary antibody (Bio-Rad, Richmond, CA).

Microscopy

Morphological analysis of mating projections was carried out on cells that were induced with α-factor for 6 h, fixed with formaldehyde, and then examined microscopically with use of differential interference contrast (DIC) optics. The morphology of cells induced with galactose to express *AFR1* was examined essentially as described previously (Konopka *et al.*, 1995). Immunofluoresence analysis was carried out as described previously (Pringle *et al.*, 1991; Konopka *et al.*, 1995). Cells were induced for the indicated time and fixed with formaldehyde, cell walls were digested with glusulase, and then the cells were attached to glass slides. Immunostaining was carried out with affinity-purified rabbit anti-Afr1p antibodies followed by fluorescein-conjugated goat anti-rabbit IgG (Cappell, Durham, NC). The cells were photographed with Tmax film (Kodak, Rochester, NY) on a BH-2 microscope (Olympus, Lake Success, NY). Results presented in the text were observed consistently in at least two independent experiments.

RESULTS

Cdc12p Interacts with the C-Terminal Half of Afr1p

A series of deletion mutations was constructed in the *AFR1* gene to identify the Cdc12p-binding domain. The proteins encoded by these mutated *AFR1* genes were then tested for the ability to interact with Cdc12p in the two-hybrid assay (Fields and Song, 1989). For this analysis, cells were engineered to produce one fusion protein containing a portion of Afr1p fused to the DNA-binding domain of *LexA* and another fusion protein containing the entire Cdc12p protein fused to the Gal4p activation domain as described previously (Konopka *et al.*, 1995). Interaction between LexA–Afr1p and Gal4–Cdc12p was detected in this assay because the interaction of the two fusion proteins reconstitutes a transcription factor that activates the expression of a *lacZ* reporter gene.

Analysis of N-terminal deletion mutants showed that residues 1–300 of the 620-residue Afr1p were not required for interaction with Cdc12p because Afr1–N2p (Δ1–300) gave a positive signal in the two-hybrid assay (Figure 1 and summarized in Table 2). The C-terminal 147 residues were also not required because Afr1–C7p (Δ474–620) also gave a positive signal. In contrast, mutations within the domain identified by these boundaries abolished the interaction with Cdc12p. Afr1–I8p (Δ350–415) and Afr1–I3p (Δ413–

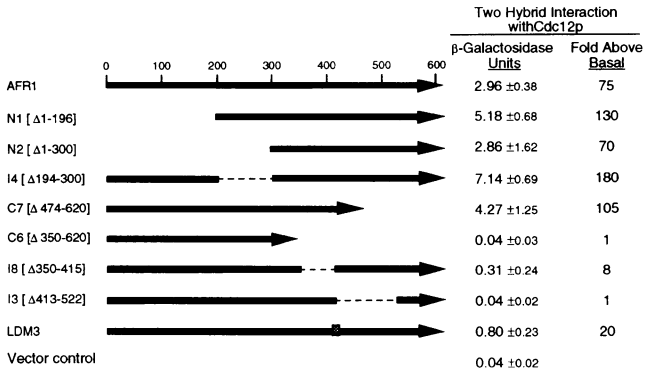

Figure 1. Interaction between mutant Afr1p and Cdc12p detected by two-hybrid analysis. Two-hybrid analyses were carried out with strain L40 carrying one plasmid that contains the *lexA* DNA-binding domain fused to *AFR1* and another plasmid containing the *GAL4* activation domain fused to *CDC12*. Cells carrying either a plasmid control or the indicated *lexA-AFR1* plasmid were assayed for β-galactosidase as described previously (Bartel *et al.*, 1993). Induction of β-galactosidase indicates activation of a *lacZ* reporter gene due to interaction between Afr1p and Cdc12p. None of these *AFR1* mutant alleles activated the reporter gene in the absence of the *GAL4-CDC12* plasmid.

522) did not interact significantly with Cdc12p. Furthermore, we discovered that the Afr1–LDM3p protein, in which codons 413–415 were mutated from QKL to LDM (see MATERIALS AND METHODS), was partially defective in interacting with Cdc12p. To confirm that the failure of these constructs to generate a signal in the two-hybrid assay was not due to effects on protein stability, the fusion proteins were assayed by Western immunoblot analysis with anti-Afr1p antibody and found to be stably produced (our unpublished results).

The results of two-hybrid assays are often confirmed by determining whether the two proteins coimmunoprecipitate, but we were unable to carry out this analysis because Afr1p was found in the insoluble fraction after cell lysis. Instead, we tested the ability of the mutant Afr1p proteins to interfere with Cdc12p function in vivo. Constitutive expression of wild-type *AFR1* during vegetative growth causes cells to form elongated buds that are phenotypically similar to the elongated buds formed by $cdc12^{ts}$ mutants at the restrictive temperature (Haarer and Pringle, 1987; Konopka *et al.*, 1995). (The *AFR1* gene is normally expressed at low basal levels but is highly induced by mating pheromones.) Constitutively produced Afr1p is thought to bind Cdc12p and prevent it from carrying out its normal function during bud formation. Mutants that retain the ability to bind Cdc12p are also expected to promote the formation of elongated buds. To examine this, cells carrying the mutant *AFR1* genes under the control of the galactose-inducible *GAL10* promoter were induced with galactose, and then the bud morphology was examined microscopically (Figure 2). All of the *AFR1* mutants that contained the Cdc12p-binding domain as determined in the two-hybrid assay (*AFR1-N1*, *-N2*, *-C7*, and *-I4*) formed elongated buds after galactose induction. Conversely, mutants defective in Cdc12p-binding (*AFR1-C6*, *-I3*,

Table 2. Summary of *AFR1* mutants

AFR1 allele	Codons mutated	Cdc12p interaction	α-Factor resistance	Projection formation	Immuno-localization
AFR1		+	+	+	Neck
afr1Δ	Δ1–620	–	–	–	–
N1	Δ1–196	+	+	+	Neck
N2	Δ1–300	+	–	–	ND
N3	Δ1–415	–	–	–	ND
N4	Δ1–522	–	–	–	ND
C1	Δ298–620	–	–	–	ND
C6	Δ350–620	–	+	+	Apical
C7	Δ474–620	+	–	+	Neck
I4	Δ194–300	+	–	–	Nonlocal
I1	Δ298–415	b	–	ND	ND
I8	Δ350–415	–	+	–	Apical
I3	Δ413–522	–	–	–	Apical
LDM2	298–300[a]	+	+	+	ND
LDM3	413–415[a]	+/–	+	+	Neck
LDM4	350[a]	+	+	+	ND

ND, not determined.

[a]No codons deleted, but in *LDM2*, codons 298–300 were changed from KKK to LDM; in *LDM3*, codons 413–415 were changed from QKL to LDM; and in *LDM4*, codon 350 was changed from D to L.

[b]This mutant caused a high basal activation of the reporter gene in the two-hybrid assay in the absence of Gal4–Cdc12p.

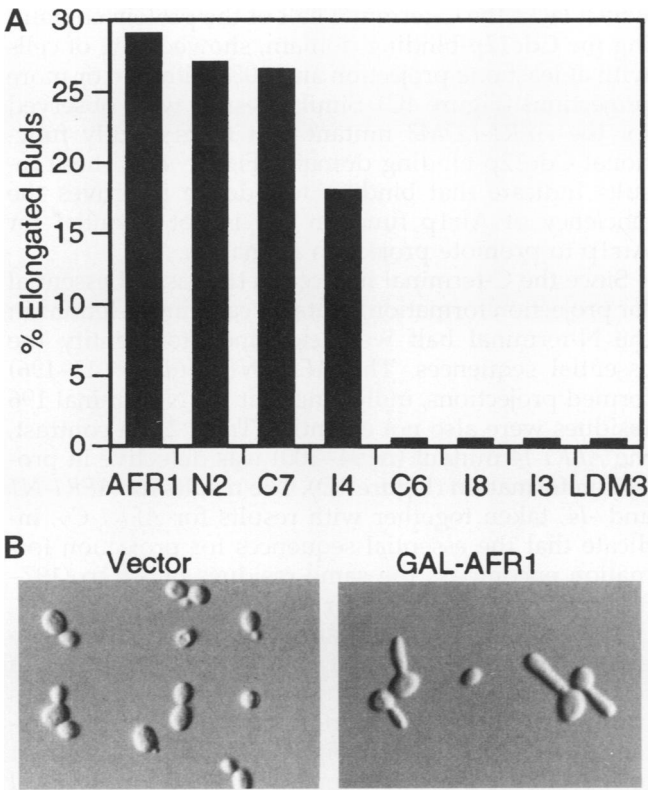

Figure 2. Morphologies of cells induced with galactose to express mutant *AFR1* genes. (A) The bar graph shows the percentage of cells displaying elongated buds for each of the indicated *AFR1* mutants. Plasmids carrying wild-type or mutant *AFR1* genes under control of the galactose-inducible *GAL10* promoter were introduced into strain JKY26 (*afr1::URA3*). Cells were grown to midlogarithmic phase in medium containing the noninducting carbon source raffinose, and then *AFR1* expression was induced with galactose for 6 h. At least 400 cells were examined microscopically for the presence of elongated buds. (B) JKY26 cells carrying the YEp51 vector or YEp51 containing *GAL10-AFR1* were induced as described in A and then photographed.

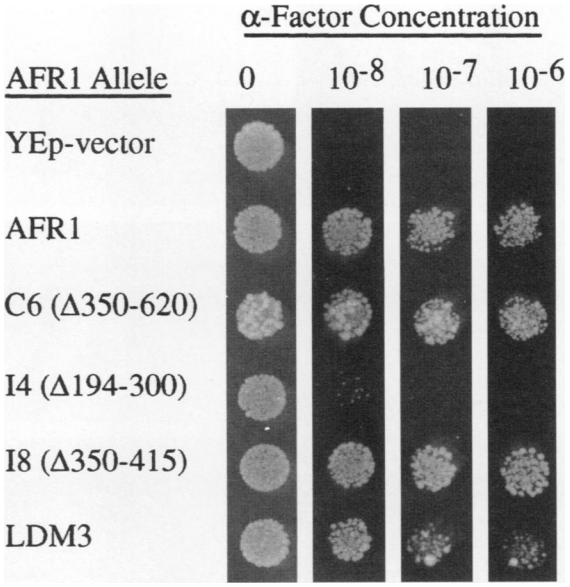

Figure 3. Resistance to α-factor-induced cell division arrest. Strain JKY26 (*afr1::URA3*) carrying the indicated *AFR1* allele on the multicopy plasmid YEplac181 was tested for resistance to α-factor-induced cell division arrest. Approximately 10^3 cells of each type were spotted onto the surface of an agar plate containing the indicated molar concentration of α-factor. The plates were incubated for 2 d at 30°C and then photographed.

-I8, and *-LDM3*) had no discernible effects on bud morphogenesis. Thus, these results are consistent with the observation that residues 301–473 are required for interaction with Cdc12p.

The Cdc12p-binding Domain Is Not Required for Afr1p to Promote Resistance to α-Factor

AFR1 was identified as a regulator of pheromone signaling because its overexpression confers resistance to pheromone (Konopka, 1993). To determine whether the interaction between Afr1p and Cdc12p is important for this process, cells that overexpress the mutant *AFR1* genes were tested for their ability to grow in the presence of α-factor (Figure 3 and Table 2). Overexpression of the *AFR1* mutants was accomplished by inserting the mutant genes into a multicopy plasmid (see MATERIALS AND METHODS), and then the plasmids were introduced into a strain that lacks *AFR1* (JKY26; *afr1::URA3*). As expected, cells carrying a control vector grew in the absence of α-factor but did not grow in the presence of $\geq 10^{-8}$ M α-factor, indicating that they were sensitive to α-factor-induced cell division arrest (Figure 3). In contrast, cells carrying wild-type *AFR1* on a high copy plasmid were able to grow even in the presence of 10^{-6} M α-factor, demonstrating that they were more resistant to the effects of α-factor. Analysis of mutants lacking a functional Cdc12p-binding domain because of C-terminal truncation (*AFR1-C6*, Δ350–620), modification within this region (*AFR1-LDM3*), or internal deletion (*AFR1-I8*, Δ350–415) showed that they still promoted resistance to α-factor. The *AFR1-N1* (Δ1–196) mutant also promoted α-factor resistance (Table 2). Altogether, these results indicate that the domain responsible for resistance lies between residues 197 and 349. Deletion mutants that perturb this region, such as *AFR1-I4* (Δ194–300; Figure 3) and *AFR1-N2* (Δ1–300; Table 2), did not confer resistance to α-factor.

Although the Cdc12p-binding domain was not required to confer resistance to α-factor, the results for other *AFR1* mutants indicate that the C-terminal half can influence the function of the N-terminal half. In particular, overexpression of *AFR1-I3* (Δ413–522) and *AFR1-C7* (Δ474–620) did not promote resistance (Table 2), although both mutant proteins contain the N-terminal residues 1–349 that were identified above as

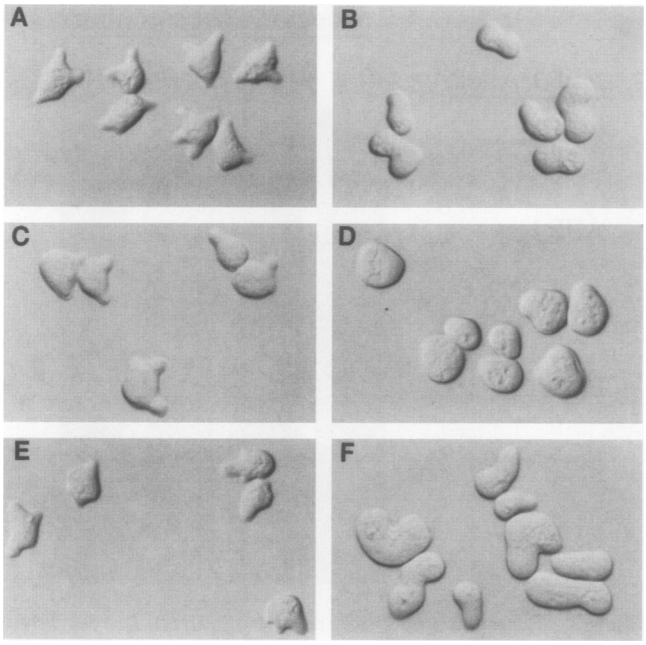

Figure 4. Pheromone-induced morphology of wild-type and *AFR1* mutants strains. (A) Wild-type *AFR1*; (B) *afr1::URA3*; (C) *AFR1-C6* (Δ350–620); (D) *AFR1-I4* (Δ194–300); (E) *AFR1-LDM3*; and (F) *AFR1-I8* (Δ350–415). All strains were incubated with α-factor (5×10^{-7} M) for 6 h, and then cells were fixed with formaldehyde and photographed. The *AFR1* strain was JKY25; the *afr1::URA3* strain was JKY26. Mutant alleles of *AFR1* were integrated into JKY26 with use of the YIplac128 plasmid as described in MATERIALS AND METHODS.

sufficient to promote resistance. The failure of these mutants to function was not obviously due to effects on protein stability because the mutant proteins were found to be stably overproduced after a 90-min induction with α-factor (our unpublished results). The ability of the C-terminal half of Afr1p to affect the functions of the N-terminal half will be discussed below.

The Cdc12p-binding Domain Is Not Required for Afr1p to Promote Projection Formation

To determine the role of the Cdc12p-binding domain in mating projection formation, cells carrying the mutant alleles of *AFR1* integrated into the genome (in *afr1::URA3* strain JKY26) were tested for their ability to form projections (Figure 4 and Table 2). After a 6-h induction with α-factor, nearly all of the wild-type cells produced at least one projection, and 81% produced two or more projections (Figure 4A). Projections are produced sequentially, so these results demonstrate that the wild-type cells formed projections very efficiently under these conditions. In contrast, the *afr1::URA3* deletion strain showed only 13% of cells with one projection and none with two or more (Figure 4B). The truncation mutant *AFR1-C6* (Δ350–620), which lacks the C-terminal half of the protein containing the Cdc12p-binding domain, showed 55% of cells with at least one projection and 30% with two or more projections (Figure 4C). Similar results were observed for the *AFR1-LDM3* mutant that lacks a fully functional Cdc12p-binding domain (Figure 4E). These results indicate that binding to Cdc12p improves the efficiency of Afr1p function but is not essential for Afr1p to promote projection formation.

Since the C-terminal half of Afr1p was not essential for projection formation, mutants carrying deletions in the N-terminal half were examined to identify the essential sequences. The *AFR1-N1* mutant (Δ1–196) formed projections, indicating that the N-terminal 196 residues were also not essential (Table 2). In contrast, the *AFR1-I4* mutant (Δ194–300) was defective in projection formation (Figure 4D). The results for *AFR1-N1* and *-I4*, taken together with results for *AFR1-C6*, indicate that the essential sequences for projection formation encompass the same residues of Afr1p (197–349) that function to confer resistance to α-factor.

Interestingly, some mutants carrying internal deletions in the C-terminal half of Afr1p were defective in forming projections, although the essential sequences in the N-terminal half were intact. In particular, mutant *AFR1-I8* (Δ350–415; Figure 4F), which lacks part of the Cdc12p-binding domain, failed to form typical projections. *AFR1-I8* cells exposed to α-factor were large and usually peanut shaped. Similar results were obtained with the mutant *AFR1-I3* (Δ413–522; Table 2), which also lacks a part of the Cdc12p-binding domain. The failure of Afr1–I8p and Afr1–I3p proteins to function was not due to effects on their stability because Western blot analysis showed that the mutant Afr1p proteins were induced by α-factor and produced at similar levels (Figure 5). The *AFR1-I3* mutant was defective for all of the *AFR1* phenotypes and may, therefore, encode a nonfunctional protein. However, the *AFR1-I8* mutant cannot be discounted as producing a nonfunctional protein because overexpression of this mutant *AFR1* gene conferred resistance to α-factor (Figure 3). Thus, sequences in the C-terminal half can alter the ability of the N-terminal half of Afr1p to promote morphogenesis.

The Cdc12p-binding Domain Restricts Afr1p Localization

Immunolocalization studies show that Afr1p and the septin proteins localize to similar sites in vivo (Konopka *et al.*, 1995; Longtine *et al.*, 1996). Therefore, immunolocalization studies were carried out to determine how mutation of the Cdc12p-binding domain affects the localization of the Afr1p protein. In the case of wild-type *AFR1* and mutants *AFR1-C6*, *-C7*, and *-I8*, we were able to observe detectable immunofluorescence signals in cells carrying a single copy of the

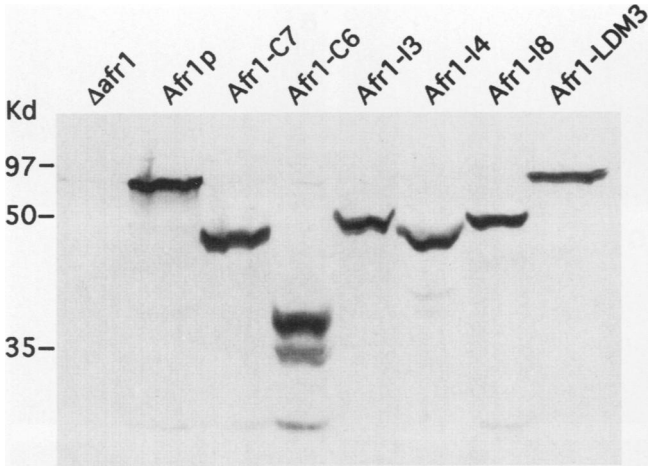

Figure 5. Western immunoblot analysis of Afr1p proteins. JKY26 (*afr1::URA3*) cells carrying an integrated copy of the indicated *AFR1* allele were incubated with α-factor (5×10^{-7} M) for 2 h. Equivalent amounts of cell extract were resolved by electrophoresis on an SDS-PAGE gel and then transferred to nitrocellulose. The blot was probed with rabbit anti-Afr1p antibody, and then immunoreactive bands were detected with an alkaline phosphatase-conjugated secondary antibody.

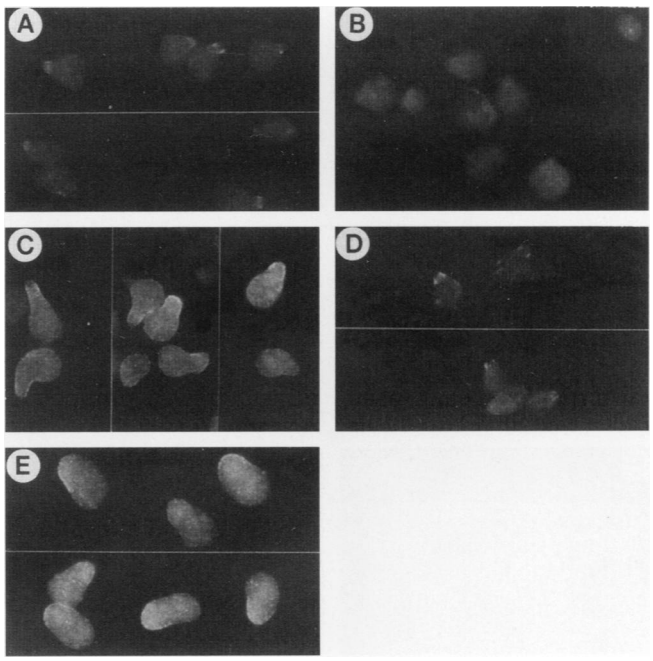

Figure 6. Immunolocalization of Afr1p proteins in strains carrying mutant *AFR1* genes integrated in the chromosome. Strains carrying the indicated allele of *AFR1* integrated in the chromosome were induced with α-factor (5×10^{-7} M) for 3 h. (A) Wild-type *AFR1*; (B) *afr1::URA3*; (C) *AFR1-C6* (Δ350–620); (D) *AFR1-C7* (Δ474–620); and (E) *AFR1-I8* (Δ350–415). Cells were fixed with formaldehyde and then analyzed for immunofluorescence with anti-Afr1p antibodies as described in MATERIALS AND METHODS. The wild-type *AFR1* strain was JKY25. The mutant *AFR1* alleles were integrated into *afr1::URA3* strain JKY26.

corresponding *AFR1* gene (Figure 6). To obtain significant immunofluorescence signals for *AFR1-I4*, *-N1*, *-LDM3*, and *-I3*, the mutant *AFR1* genes were introduced into cells on multicopy plasmids so that the Afr1p proteins were overproduced and easier to detect (Figure 7). Overproduction was probably necessary to observe a detectable signal for some of the mutant Afr1p proteins either because they do not display a restricted localization or because they react poorly with the anti-Afr1p antibody because they lack a portion of the N-terminal sequences against which the antibody was raised (Konopka *et al.*, 1995).

After induction of the cells with α-factor, wild-type Afr1p was detected as a patch at the site of polarization in cells that have not yet made a projection. In cells with a projection, Afr1p was detected at the base of the projection (Figures 6A and 7A) at the same site expected for Cdc12p (Konopka *et al.*, 1995; Longtine *et al.*, 1996). Comparison of different focal planes suggested that Afr1p was present in association with the plasma membrane and may form a ring around the neck of the projection. The C-terminal half of Afr1p was required for this restricted localization because Afr1–C6p (Δ350–620) showed staining throughout the polarized end of the cell (Figure 6C). In contrast, Afr1–C7p (Δ474–620), which contains the sequences present in Afr1–C6p plus a functional Cdc12p-binding domain, was detected at the neck of projections (Figure 6D). Similarly, Afr1–N1p (Δ1–196), which contains a functional Cdc12p-binding domain, was detected at the neck of projections (Figure 7C). These results indicate that the sequences required to restrict Afr1p localization to the neck of the projection overlap with the sequences required for interaction with Cdc12p. Deletion of the Cdc12p-binding domain in Afr1–C6p correlated with a failure of this protein to show a restricted localization even though the *AFR1-C6* cells were still capable of forming projections. Altogether, the subcellular localization of these mutant Afr1p proteins indicates that the Cdc12p-binding domain functions to localize Afr1p to the base of projections.

Analysis of Afr1p localization in *AFR1* mutant strains that were defective in forming projections was consistent with the Cdc12p-binding domain playing an important role in the subcellular localization of Afr1p. However, because these mutants fail to form projections it is also possible that their altered protein localization is an indirect consequence of the altered cell morphology. Afr1–I8p (Δ350–415; Figure 6E) and Afr1–I3 (Δ413–522; Figure 7F), which contain internal deletions within the C-terminal half that inactivate Cdc12p binding, were detected throughout the polarized end of the cell. Afr1–LDM3p (Figure 7E), which contains a partially defective Cdc12p-binding domain, was difficult to detect at the neck unless it was overproduced, in which case the projections were slightly

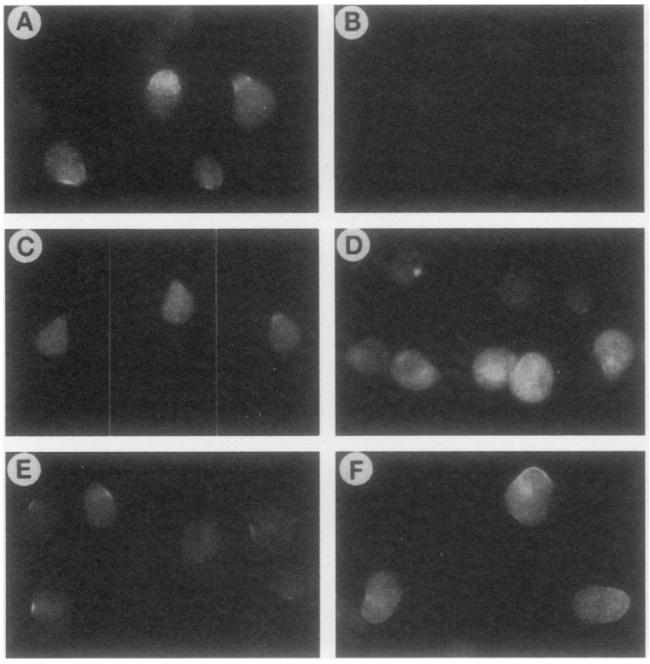

Figure 7. Immunolocalization of Afr1p proteins in strains carrying *AFR1* genes on multicopy plasmids. Strain JKY26 (*afr1::URA3*) carrying the indicated allele of *AFR1* on a multicopy plasmid was induced with α-factor (5×10^{-7} M) for 2 h. (A) Wild-type *AFR1*; (B) YEplac181 vector; (C) *AFR1-N1* (Δ1–196); (D) *AFR1-I4* (Δ194–300); (E) *AFR1-LDM3*; and (F) *AFR1-I3* (Δ413–522). Cells were fixed with formaldehyde and then analyzed for immunofluorescence with anti-Afr1p antibodies as described in MATERIALS AND METHODS.

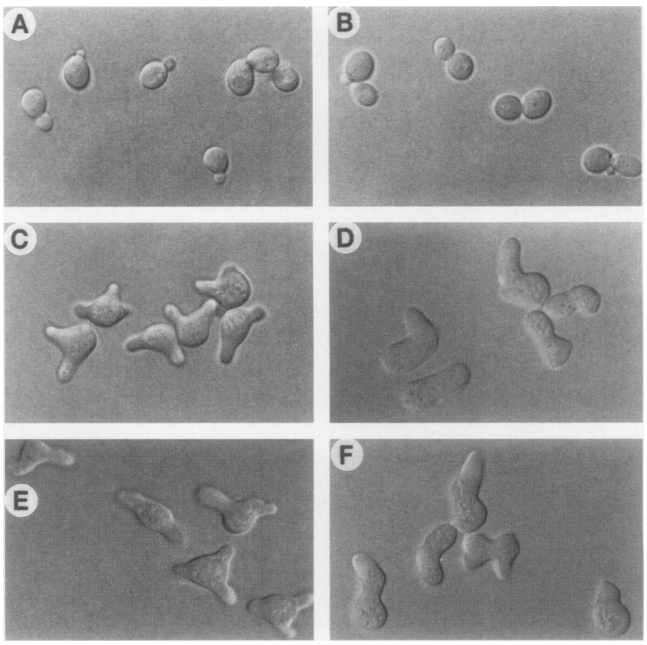

Figure 8. Pheromone-induced morphology of *cdc12–6* cells. Wild-type (A) and *cdc12–6* (B) cells incubated in the absence of α-factor. Wild-type (C), *cdc12–6* (D), *AFR1-C6* (E), and *cdc12–6 AFR1-C6* double mutant (F) cells incubated in the presence of α-factor. Cells were grown to midlogarithmic phase at 24°C, incubated in the absence (A and B) or presence (C–F; 5×10^{-7} M final concentration) of α-factor for 6 h, and then fixed with formaldehyde and photographed. The wild-type strain was yLG45-1, the *cdc12-6* strain was yLG44-5-3, the *AFR1-C6* strain was yLG75–1, and the *AFR1-C6 cdc12-6* double mutant strain was yLG76-1.

abnormal. Afr1–LDM3p was detected in a restricted pattern that often seemed to be unevenly distributed across the neck although this could be due to the abnormal morphology of the projections. Sequences in the N-terminal half of Afr1p that were not required for interaction with Cdc12p were also important because Afr1–I4p (Δ194–300) did not display a restricted staining pattern and appeared to be present throughout the cell (Figure 7D). Inspection of the residues missing in Afr1–I4p suggests that a cluster of 24 basic residues (K + R) found between residues 270 and 350 may direct Afr1p to the membrane. Perhaps membrane localization may facilitate interaction with Cdc12p. Thus, sequences in both the N-terminal and the C-terminal halves of Afr1p are important for its proper subcellular localization.

CDC12 Is Required for Pheromone-induced Morphogenesis

CDC12 is known to be required for bud morphogenesis and cytokinesis (Haarer and Pringle, 1987; Hartwell, 1971). The surprising observation that the Cdc12p-binding domain on Afr1p was helpful but not essential for mating projection formation raised the question as to whether *CDC12* is required for this process. Therefore, we examined the ability of cells carrying the temperature-sensitive *cdc12-6* mutation to induce projection formation. Interestingly, *cdc12-6* cells showed a defect in projection formation even at the permissive temperature for bud morphogenesis (24°C). The *cdc12-6* cells treated with α-factor at 24°C appeared as large elongated cells (Figure 8D). In contrast, nearly all of the wild-type cells showed at least one projection, and 69% produced two or more projections (Figure 8C). The morphogenesis defect of the *cdc12-6* cells was not an indirect consequence of the budding defect of these cells because *cdc12-6* cells showed normal morphology at permissive temperature (24°C) in the absence of α-factor (Figure 8B).

Cdc12p is predicted to play a role in restricting Afr1p to the neck of the projection, so the location of Afr1p in *cdc12-6* mutant cells was examined. Immunofluoresence analysis of *cdc12-6* cells that were induced with α-factor showed Afr1p staining throughout the cell (Figure 9). Thus, Afr1p was not restricted to a particular region in *cdc12-6* cells.

The similar defects in pheromone-induced morphogenesis observed for *afr1::URA3* cells and *cdc12-6* cells

suggested that the projection formation defect of *cdc12-6* cells may be due to the failure to localize Afr1p properly. Alternatively, *cdc12-6* cells may also be defective in other aspects of morphogenesis. To distinguish between these possibilities, we examined the ability of *cdc12-6* cells carrying the *AFR1-C6* mutation to produce projections because this Afr1p mutant protein can induce projection formation even though it lacks the Cdc12p-binding domain. However, the *cdc12-6 AFR1-C6* double mutant was still defective in projection formation (Figure 8F). The failure of the *AFR1-C6* mutation to suppress the defect of the *cdc12-6* mutation indicates that *CDC12* has other functions in addition to binding Afr1p during pheromone-induced morphogenesis.

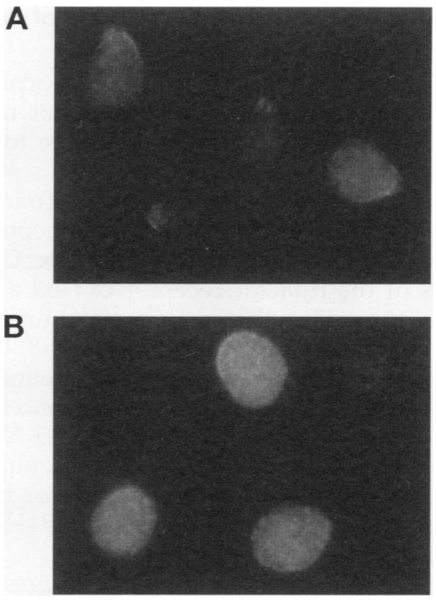

Figure 9. Afr1p localization in *cdc12-6* cells. Immunofluorescent staining of Afr1p in *CDC12* strain yLG45-1 (A) and *cdc12-6* strain yLG44-5-3 (B) carrying *AFR1* on YEplac181. Cells were induced with α-factor (5×10^{-7} M) for 3 h at 36°C and then analyzed by immunofluorescence with affinity-purified anti-Afr1p antibody.

DISCUSSION

Cdc12p Concentrates Afr1p at the Neck of the Projections

During bud formation, the Cdc3p, Cdc10p, Cdc11p, and Cdc12p septins are thought to form a filamentous ring on the inner surface of the plasma membrane at the neck between the mother cell and its bud (Byers and Goetsch, 1976). Interestingly, the localization of the septins may be regulated by Ste20p-like kinases (Cvrckova *et al.*, 1995). Several observations suggest that the septins function to localize specific components at the bud neck. For example, septins are required for the localization to the bud neck of the Bud3p (Chant *et al.*, 1995), Bud4p (Sanders and Herskowitz, 1996), and Axl2p (Roemer *et al.*, 1996) proteins that are involved in bud site selection. The septins may also function to localize chitin synthesis to the bud neck and to localize actin to the site of cytokinesis at the bud neck (Adams and Pringle, 1984; Mulholland *et al.*, 1994; Longtine *et al.*, 1996).

The observations that Afr1p interacts with Cdc12p and that they localize to similar sites in vivo suggested that Cdc12p functions to localize Afr1p. Immunolocalization studies showed that the Afr1p sequences that were needed for interaction with Cdc12p in the two-hybrid assay (residues 301–473) were required to restrict Afr1p to the neck of projections (summarized in Table 2). Mutant Afr1p proteins that failed to interact with Cdc12p also failed to localize properly in vivo. In addition, wild-type Afr1p was mislocalized in a *cdc12-6* mutant. Sequences in the N-terminal half of Afr1p also appear to contribute to Afr1p localization because Afr1–I4p (Δ194–300) interacted with Cdc12p but did not display a restricted localization in vivo.

The role of Cdc12p in localizing Afr1p in mating projections is consistent with the role of Cdc12p and the other septins in localizing proteins at the bud neck. However, examination of the amino acid sequence of the Cdc12p-binding domain in Afr1p did not reveal any obvious similarity to Bud3p, Bud4p, Axl2p, or other known proteins. Thus, the ability of septins to localize proteins is apparently not mediated by a highly conserved protein sequence motif.

Binding to Cdc12p Is Not Required for Afr1p Function

Although both Afr1p and Cdc12p are required for projection formation, analysis of *AFR1* mutants demonstrated that interaction with Cdc12p was not required for Afr1p to promote the formation of mating projections. This was shown most clearly for the *AFR1-C6* mutant, which formed projections although it lacks most of the C-terminal half of Afr1p, including sequences required to bind Cdc12p. In addition, the Cdc12p-binding domain was not required for overexpressed Afr1p to promote resistance to α-factor. There was no detectable difference in the degree to which overexpression of wild-type *AFR1* or *AFR1-C6* was able to promote resistance to pheromone. However, it cannot be concluded that the localization of Afr1p is unimportant for its function. Immunofluorescence analysis showed that the distribution of Afr1-C6p overlapped with the expected location of wild-type Afr1p at the polarized end of the cell. The more restricted localization of wild-type Afr1p may account for the improved efficiency with which wild-type cells can form projections. Thus, the proper localization of Afr1p may be significant for its function in promoting projection formation.

Relationship between Morphogenesis and Signaling Functions of Afr1p

The ability of Afr1p to promote both morphogenesis and adaptation could be due to different manifestations of a single activity or could be due to multiple independent activities of Afr1p. Previous studies on the α-factor receptors have suggested a functional relationship between the regulation of receptor signaling and morphogenesis. Mutations in the C-terminal sequences of the α-factor receptor caused a defect in projection formation that was proportional to their defect in adaptation (Konopka *et al.*, 1988; Chen and Konopka, 1996). Consistent with this, deletion analysis of *AFR1* indicates that the essential sequences for morphogenesis and adaptation overlap. The N-terminal 349 residues present in Afr1-C6p were sufficient to promote both morphogenesis and α-factor resistance; a deletion in this domain (*AFR1-I4*, Δ194–300) abolished both functions.

Analysis of additional *AFR1* mutants suggests that the morphogenesis and adaptation functions could also be due to separate activities of Afr1p. The observation that some mutants selectively lost the ability to promote adaptation or morphogenesis indicates that these functions are genetically separable and that Afr1p may carry out independent activities. In particular, *AFR1-I8* (Δ350–415) promotes α-factor resistance but not projection formation; *AFR1-C7* (Δ474–620) fails to induce α-factor resistance but promotes projection formation. However, both *AFR1-I8* and *AFR1-C7* mutants contain the N-terminal sequences (1–349) that were sufficient to promote morphogenesis and α-factor resistance in *AFR1-C6*. Since these mutants are altered by deletion of distinct subdomains in the C-terminal half of the protein, we cannot rule out the possibility that there is a single activity of Afr1p that can be differentially affected by sequences in the C-terminal half. One possibility is that, because of the strong alterations made in these *AFR1* deletion mutants, some of the resulting proteins have abnormal properties because of effects on protein folding. This could account for the *AFR1-I3* (Δ413–522) mutant, which contains the essential domain but is defective for all Afr1p properties. Alternatively, because the sequences deleted in Afr1-I3p remove a portion of the Cdc12p-binding domain, as is true for Afr1-I8p, and also remove a portion of the sequences deleted in Afr1-C7p, it is possible that Afr1-I3p combines the defects of Afr1-I8p and Afr1-C7p. Perhaps these mutants are revealing a physiological role for the C-terminal half of Afr1p in regulating the N-terminal half.

Septins Carry Out Multiple Roles in Cell Growth and Development

Septins have been studied in *S. cerevisiae* primarily for their role in cytokinesis and bud formation. More recently, they have been implicated in a number of other cellular processes (reviewed in Longtine *et al.*, 1996) such as bud site selection (Flescher *et al.*, 1993; Chant *et al.*, 1995; Sanders and Herskowitz, 1996), pseudohyphal growth (Blacketer *et al.*, 1995), and sporulation (Fares *et al.*, 1996). The results of this study show that septins are also important for the morphological differentiation induced by mating pheromones because *cdc12-6* cells were defective in projection formation. It was also interesting that the projection formation defect of *cdc12-6* cells was detected at the permissive temperature for cytokinesis and bud formation. This suggests that there may be different requirements for septin function in budding cells and mating cells. One function of Cdc12p that is unique to projection formation is to promote the localization of Afr1p. However, this is probably not the essential function of Cdc12p because *AFR1-C6* cells formed projections even though Afr1-C6p failed to localize properly and was defective in binding Cdc12p. *CDC12* was still required in *AFR1-C6* cells because *AFR1-C6 cdc12-6* cells were defective in projection formation. These experiments indicate that Cdc12p has additional function(s) other than restricting Afr1p to the neck of the projection.

Analysis of septin homologues in *Drosophila melanogaster* suggests that septins in this organism play a role in organizing proteins at the cell cortex in a wide range of embryonic and differentiated cell types (Neufeld and Rubin, 1994; Fares *et al.*, 1995). In particular, it is interesting that mutations in a septin homologue called *pnut* affect photoreceptor cell development. The pnut protein was concentrated at the apex of developing photoreceptor cells at the position expected for cell surface signaling molecules that control development as well as the G protein-coupled photoreceptors (Neufeld and Rubin, 1994). Perhaps septins play a conserved role in organizing signaling proteins because Cdc12p influences the localization of Afr1p that regulates the signaling activity of the G protein-coupled pheromone receptors. Further analysis will be required to dissect the function of septins, but the distribution of septins in different organisms indicates that they are likely to be playing multiple roles in organizing proteins at the plasma membrane.

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