A Cdc24p-Far1p-Gβγ Protein Complex Required for Yeast Orientation during Mating

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Abstract. Oriented cell growth requires the specification of a site for polarized growth and subsequent orientation of the cytoskeleton towards this site. During mating, haploid Saccharomyces cerevisiae cells orient their growth in response to a pheromone gradient overriding an internal landmark for polarized growth, the bud site. This response requires Cdc24p, Far1p, and a heterotrimeric G-protein. Here we show that a two-hybrid interaction between Cdc24p and Gβγ requires Far1p but not pheromone-dependent MAP-kinase signaling, indicating Far1p has a role in regulating the association of Cdc24p and Gβγ. Binding experiments demonstrate that Cdc24p, Far1p, and Gβγ form a complex in which pairwise interactions can occur in the absence of the third protein. Cdc24p localizes to sites of polarized growth suggesting that this complex is localized. In the absence of CDC24-FAR1-mediated chemotropism, a bud site selection protein, Bud1p/Rsr1p, is essential for morphological changes in response to pheromone. These results suggest that formation of a Cdc24p-Far1p-Gβγ complex functions as a landmark for orientation of the cytoskeleton during growth towards an external signal.

Key words: chemotropism • landmark • oriented growth • Ste4p Ste18p • yeast mating

Eukaryotic cells are able to polarize their growth in response to both external and internal signals. Polarization to external signals plays a crucial role in development and tissue formation. During yeast mating, cells of opposite mating type secrete peptide pheromones and respond to pheromone from their mating partner (for review see Sprague and Thorner, 1992; Chenevert, 1994; Leberer et al., 1997a). Mating pheromone binds to specific G-protein-coupled receptors on cells of opposite mating type (Bender and Sprague, 1989; Blumer et al., 1988). Receptor activation results in cell cycle arrest, transcriptional activation, morphological changes, and polarized growth towards a partner cell (Sprague and Thorner, 1992; Chenevert, 1994; Leberer et al., 1997a).

Cells respond to a gradient of mating pheromone by oriented growth along this gradient (Segall, 1993). Such chemotropic growth is essential for efficient mating (Dorer et al., 1995; Valtz et al., 1995; Nern and Arkowitz, 1998). During oriented growth, the actin cytoskeleton and secretory apparatus polarize towards the tip of the mating projection (Baba et al., 1989; Read et al., 1992). As a result, cell wall and plasma membrane material is deposited at the tip of this pear-shaped cell known as a shmoo (Lipke et al., 1976; Tkacz and Mackay, 1979). Pheromone receptors and the heterotrimeric G-protein composed of Gα (GPA1), Gβ (STE4), and Gγ (STE18) are required for chemotropic growth (Jackson et al., 1991; Schrick et al., 1997; Xu and Kurjan, 1997). Certain alleles of the cyclin-dependent kinase inhibitor FAR1, such as far1-H7 (Valtz et al., 1995), and of the GDP-GTP exchange factor for the small GTPase Cdc42p CDC24, such as cdc24-m1 (Nern and Arkowitz, 1998), are specifically defective in chemotropic growth. These mutants are unable to orient in a pheromone gradient and select a site for mating projection growth adjacent to their previous bud site. Similarly, in the presence of saturating uniform concentrations of mating pheromone, shmoo formation occurs next to the previous bud site (Madden and Snyder, 1992; Dorer et al., 1995). The latter process has been referred to as default mating (Dorer et al., 1995).

During vegetative growth, haploid cells bud at a specific site next to their previous bud site, resulting in a characteristic axial budding pattern (Chant and Pringle, 1995). The BUD genes are required for this budding pattern (Chant and Herskowitz, 1991; Drubin and Nelson, 1996). During budding, cells polarize their actin cytoskeleton (A dams and Pringle, 1984; Kilmartin and A dams, 1984) and secretory apparatus towards the bud site (Tkacz and Lampen, 1972; Field and Schekman, 1980). However, this internal signal generated during budding is overridden upon expo-
sure to a mating pheromone gradient, allowing cells to orient growth towards their mating partner (Madden and Snyder, 1992). How cells switch from an internally programmed polarized growth process to a process dictated by an external cue is unknown.

The pheromone receptors and the heterotrimeric G-protein are also required for cell cycle arrest, mitogen-activated protein (MAP) \(^1\)-kinase-mediated gene induction, and cell morphological changes during mating (Sprague and Thorner, 1992; Chenevert, 1994; L eberer et al., 1997a). Genetic studies indicate that G\(\beta\gamma\) activates all these processes (Whiteway et al., 1990) with G\(\alpha\) having a negative regulatory role (Dietzel and K urjan, 1987; Miyajima et al., 1987). By analogy to other G-protein coupled receptors, receptor activation results in dissociation of G\(\alpha\) from G\(\beta\gamma\). G\(\beta\gamma\) is found as a complex at the plasma membrane (Hirschman et al., 1997).

Previously we have shown that an association between G\(\beta\gamma\) and Cdc24p is involved in oriented growth during mating (Nern and A rkowitz, 1998). We now show this Cdc24p-G\(\beta\gamma\) complex also contains Far1p. Genetic studies are consistent with the involvement of Far1p in this complex. Cdc24p localizes to sites of polarized growth in shmooing cells, suggesting that the complex is localized. In the absence of growth orientation mediated by this complex, cells form a mating projection adjacent to the bud site in a manner that is dependent on Budd1, suggesting Budd1 can regulate Cdc24p when chemotroping signals is blocked. Together our results suggest that Cdc24p-Far1p-G\(\beta\gamma\) acts as a landmark for cytoskeleton orientation in response to a pheromone gradient.

Materials and Methods

General Techniques

Standard techniques and media were used for growth and genetic manipulation of yeast (Rose et al., 1991). Unless otherwise indicated, yeast cells were grown at 30°C.

Strains and Plasmids

The yeast strains used in this study are described in Table I. In general, deletion mutants were constructed by PCR-based gene disruption as described (A rkowitz and L owe, 1997; Nern and A rkowitz, 1998). \(\Delta\)Far1 strains were constructed either by PCR-based gene disruption (\(\Delta-1\)) or with a knockout cassette (\(\Delta-2\)). This cassette contained the \(\Delta\)Far1 ORF followed by 100 bp 3' sequence with URA3 filling all but the first 109 codons. Far1-H7, a far1 allele with a truncated COOH terminus (V alitz et al., 1995), was constructed by replacing codons 757–830 of \(\Delta\)Far1 with a stop codon followed either by H1SS5p or URA3 kI. Gene disruptions were confirmed by PCR and expressed proteins of the correct size by immunoblotting using either 12CA5 (anti- \(\beta\)Actin) mAb, B aB tissue culture supernatant at 1:40 dilution, or anti-pheromone factor (A mersham). Strains with tagged proteins mated with wild-type mating efficiencies and arrested growth normally in response to \(\alpha\)-factor.

Cdc24A GFP was constructed by fusing a HA epitope followed by P acl, S plhi, NotI, and SacI restriction sites to the COOH terminus of Cdc24p using PCR and p414cdc24 (Nern and A rkowitz, 1998) as a template. This resulted in p414cdc24A which had the amino acids Y P D Y D P Y D A A A A A S to the COOH terminus. Yeast enhanced green fluorescent protein (GFP; C ormac et al., 1997) followed by the A DH terminator was PCR amplified from pM K109 (a gift from E. Schiebel) with an oligonucleotide that added a P acl site at the 5' end and a N otI site at the 3' end. This PCR product was cloned into p414cdc24 A using P acl and N otI sites resulting in Cdc24p followed by Y P D Y D P Y D A A A A A S fused to GFP followed by the A DH terminator (p414cdc24A G F P). p1466a-H1A Ste18 was constructed by cloning H A ST to pG pl containing the G A L I site at the 5' end. The A DE2 gene from pS F73 de2 cloned by PCR from genomic DNA with oligonucleotides that added a E coRI site at the 3' end and a A xhoI site at the 5' end was released by digestion with E coRI and B srG I followed by blunting. This fragment was cloned into pS F425 in which the LEU2 gene had been removed by digestion with T th111I and N alI followed by blunting resulting in p2UA. TPI-STE18 (triose isomerase promoter) from p416TSte18 (pS F416 with TPI cloned into the SacI1 E agl site and STE18 cloned into B amHI E coRI sites) was cloned into the SacI E coRI sites of p2UA resulting in p2UA T S T E18. A n oligonucleotide encoding the GAL4 LEU2 nucleic localization signal (N LS) M D K A E L I PE P KK K R K V E L followed by an N coI restriction site was cloned into the E agl B amHI sites of p2UA T S T E18 yielding p2UA T N L S H A T S E18. Subsequen- tially, an oligonucleotide encoding an H A epitope tag was cloned into the N colo B amHI sites resulting in the following N LS-H A A sequence.

M D K A E L I PE P KK K R K V E L P W M Y P V D P Y D A A A A A S to the COOH terminus of STE18 yielding p2UA T S T E18. An A coRI SacI1 fragment of p2UA T N L S H A T S E18 containing TPI-N L SH A -STE18 was then cloned into pS F413 resulting in p413T N L S H A -STE18. STE18 was removed from this vector by digestion with B amHI and E coRI and replaced with the coding sequence of F AR1 from pG A D 420FAR1 (see below) yielding p413T N L S H A F A R1.

The coding sequences of the entire F AR1 ORF and Far1-H7 were amplified by PCR from genomic DNA and cloned into pG A D 424. SpeI PstI fragments of F AR1 and Far1-H7 from pG A D 424 plasmids were cloned into p416M B C (N ew E ngland Biolas) resulting in pM B Far1 (amino acid resi- dues 133–831) and pM F AR1H7 (amino acid residues 133–756). pM F AR1AN (amino acid residues 638–831) and pM F AR1AC (amino acid residues 133–297) were derived from pM F AR1 by removal of a B amHI and H indI11 fragment, respectively. pM F AR1H7AN (amino acid residues 638–756) was derived from pM F AR1H7 by removal of a B amHI fragment. GST Cdc24 is comprised of the NH2-terminal 472 amino acids of Cdc24p fused to GST as described (Nern and A rkowitz, 1998).

Two-Hybrid

Two-hybrid interactions were tested by growth on SC-leu-trp-as described (Nern and A rkowitz, 1998). Identical results were obtained with at least three transformants.

Expression of a LacZ reporter from Y187 derived two-hybrid strains was quantified by \(\beta\)-galactosidase assays (Miller, 1972). An E coRI site was inserted by oligonucleotide-directed mutagenesis after amino acid 153 of Spa2p (A rkowitz and L owe, 1997). This 153- amino acid Spa2p fragment was then cloned into pG A D 424. P J 69-4A cdc24-m1 (R AY 1449) was constructed by PCR-mediated gene replacement as described (Nern and A rkowitz, 1998) and confirmed by PCR and mating defect phenotype. Three independent PJ 69-4A cdc24-m1 strains were used for two-hybrid analyses. Because T RP1 is used to replace C D4 Cdc24 with cdc24-m1, STE4

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1. Abbreviations used in this paper: GFP, green fluorescent protein; GST, glutathione-S-transferase; HA, hemagglutinin; M AP, mitogen-activated protein; MBP, maltose binding protein; TPI, triose phosphate isomerase; T EV, tobacco etch virus.

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Table I. Yeast Strains Used in This Study

| Strain | Genotype | Source |
|--------|-----------|--------|
| K699   | Matα, ura3-52, his3-11, leu2-3, trp1-901, gAL4Δ, gal80Δ, GAL2-ADH2, MET2, GAL7-lacZ | K. Nasmyth (IMP, Vienna) |
| PJ69-4A| Matα, trp1-901, leu2-3, his3-11, ura3-52, his3-200, gAL4Δ, gal80Δ, GAL2-ADH2, LY22::GAL4::HIS3, met2::GAL7-lacZ, can' | James et al., 1996 |
| SEY6210| Matα, leu2-3, his3-11, ura3-52, his3-200, trp1-901, lys2-801, suc2-Δ9 | S. Emr (University of California, San Diego) |
| SEY6211| Matα, leu2-3, his3-11, ura3-52, his3-200, trp1-901, ade2, suc2-Δ9 | S. Emr |
| SFY526 | Matα, gal4, gal80, his3-200, trp1-901 ade2-101, lys2-801, ura3-52, leu2-3,112, URA3::pGal1-lacZ, can' | Clontech |
| Y187   | MATα, gal4, gal80, his3, trp1-901, ade2-101, ura3-52, leu2-3,112, URA3::pGal1-lacZ | Clontech |
| RAY719 | Same as SEY6210 with bent1Δ::His3 | Arkowitz and Lowe, 1997 |
| RAY899 | Same as SEY6210 with ste20::LoxP HIS5Sp LoxP | This study |
| RAY910 | Same as SEY6211 with ste4::HASTE4 | This study |
| RAY912 | Same as SEY6211 with ste4::HASTE4 cdc24::TRP1 3xmyc CDC24 | This study |
| RAY931 | Same as SEY6211 with cdc24Δ-1::LoxP HIS5Sp LoxP and pEG(KT)CDC24 | Nern and Arkowitz, 1998 |
| RAY1034| Same as SEY6211 with cdc24::TRP1 CDC24 | Nern and Arkowitz, 1998 |
| RAY1035| Same as SEY6211 with cdc24::TRP1 cdc24-m1 | Nern and Arkowitz, 1998 |
| RAY1036| Same as PJ69-4A with ste7Δ-1::URA3KI | This study |
| RAY1041| Same as SEY6210 with cdc24::TRP1 CDC24 | This study |
| RAY1072| Same as PJ69-4A with ste4Δ-1::URA3KI | This study |
| RAY1074| Same as PJ69-4A with ste5Δ-1::URA3KI | This study |
| RAY1086| Same as PJ69-4A with akr1Δ-1::URA3KI | This study |
| RAY1109| Same as RAY1034 with far1Δ-1::URA3KI, far1::FAR1 ProtA HIS5Sp | This study |
| RAY1111| Same as RAY1035 with far1Δ-1::URA3KI | This study |
| RAY1113| Same as RAY1034 with far1Δ-2::URA3KI | This study |
| RAY1114| Same as RAY1034 with ste20Δ-1::URA3KI | This study |
| RAY1121| Same as RAY1034 with kss1Δ-1::URA3KI | This study |
| RAY1123| Same as RAY1034 with ste11Δ-1::URA3KI | This study |
| RAY1135*| Same as SEY6210 except LEU2 | This study |
| RAY1139| Same as RAY1034 with bud1Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1142| Same as RAY1035 with bud1Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1160| Same as RAY1035 with ste20Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1168| Same as RAY1035 with bent1Δ-1::His3 | This study |
| RAY1173| Same as RAY1034 with bent1Δ-1::His3 | This study |
| RAY1179| Same as PJ69-4A with fus3Δ-1::URA3KI | This study |
| RAY1182| Same as Y187 with far1H7::LoxP HIS5Sp LoxP | This study |
| RAY1183| Same as Y187 with far1Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1246| Same as RAY1034 with spo2Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1248| Same as RAY1035 with spo2Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1249| Same as RAY1034 with far1Δ-1::URA3KI, bud1Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1254| Same as K699 with cdc24::TRP1 3xmyc CDC24 | This study |
| RAY1258| Same as K699 with cdc24::TRP1 3xmyc CDC24, far1::FAR1 ProtA HIS5Sp | This study |
| RAY1260| Same as K699 with cdc24::TRP1 3xmyc CDC24-m1, far1::FAR1 ProtA HIS5Sp | This study |
| RAY1271*| Same as RAY1034 with ste20Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1276| Same as SEY6211 with ste4::HASTE4 ProtA HIS5Sp, cdc24::TRP1 3xmyc CDC24 | This study |
| RAY1336| Same as K699 with cdc24::TRP1 3xmyc cdc24, far1::far1H7 ProtA HIS5Sp | This study |
| RAY1342| Same as RAY1034 with sst2Δ-1::URA3KI | This study |
| RAY1350| Same as RAY1034 with sst2Δ-1::URA3KI, bud1Δ-1::LoxP HIS5Sp LoxP | This study |
| RAY1360| Same as RAY931 with p141CdCdc24HAGFP instead of pEG(KT)CDC24 | This study |
| RAY1449| Same as PJ69-4A with cdc24::TRP1 cdc24-m1 | This study |

HIS5Sp refers to HIS5 from S. pombe and URA3KI refers to URA3 from K. lactis.
*Transformed with LEU2 fragment to make LEU2'
1 Strains made by deletion in a haploid, crossing with appropriate haploid followed by sporulation.
2 Made by crossing RAY1035 with RAY899 followed by sporulation.
3 Made by crossing RAY1035 with RAY719 followed by sporulation.
4 Made by crossing RAY1034 with RAY719 followed by sporulation.
5 Made by crossing RAY1034 with RAY899 followed by sporulation.

Cloned into the 2μ URA3 GAL4 DBD vector pGBDU-C1 (James et al., 1996) was used in this strain. Diploid two-hybrid strains were constructed by transformation of either DDB fusions or AD fusions along with p2μA T and p413T plasmids into SFY526 or Y187 and crossing these strains. After two-hybrid assays, phenotypes (diploid state and sterility) of diploid and haploid deletion two-hybrid strains were confirmed. Expression of NLS HA Far1p and NLS HA Ste18p in two-hybrid strains were confirmed by analysis of yeast extracts using SDS-PAGE, immunoblotting, probing with 12CA 5 mAb b, and ECL visualization.

Immunoprecipitation
R A Y 1254, R A Y 1258, R A Y 1260, and R A Y 1336 cells carrying p416GALHA Ste4 were grown to an O D 600 of 0.5 in SC-ura with 2% (wt/vol) raffi-
nose, galactose was added to a final concentration of 2% (wt/vol) and the cultures grown for 4 h. All subsequent steps were carried out at 4°C. Cells were harvested by centrifugation and lysed by agitation with glass beads in buffer A (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMFS, 40 μg/ml each of leupeptin, chymostatin, pepstatin A, aprotinin, and antipain) containing 0.1% Triton X-100. Before use IgG-Sepharose was cross-linked with dimethylpimelimidate (Sigma; Harlow and Lane, 1988). Cell extracts were clarified by two centrifugations (10,000 g for 10 min). Supernatants, which contained the majority of the tagged proteins, were incubated with 20 μl of IgG-Sepharose (Pharmacia) equilibrated in buffer A containing 0.1% Triton X-100 for 1 h. Resin was then washed four times with buffer A containing 0.1% Triton X-100 and Far1-protein A fusions were specifically eluted by incubation with 20 U of TEV-protease (Boehringer Mannheim) for 4 h at 16°C in the same buffer. Eluates were analyzed by SDS-PA PAGE and immunoblotting using polyclonal sera against myc and far1 (a gift from M. Peter) at 1:1,000 dilution followed by visualization with ECL.

Protein Purification

A ll purification steps were carried out at 4°C. MBP and GST fusion proteins were expressed in E. coli with MBP for ar1 and MBP for ar1-H7 bacteria grown at 30°C. Cells were resuspended in buffer B (PBS, 1 mM DTT, 0.1% Triton X-100), frozen in liquid N2 and stored at −70°C. Cells were lysed by sonication in buffer B with 1 mM PMFS. Extracts were clarified by a second centrifugation (10,000 g for 10 min) and fusion proteins were isolated using glutathione-agarose (Sigma) or amylose resin (New England Biolabs). MBP fusion proteins were eluted with 10 mM maltose in buffer B and dialyzed against buffer C (50 mM Tris-HCl pH 7.4, 10 mM Mgc12, 1 mM DTT, 10% (vol/vol) glycerol). Protein concentrations were determined by the Bradford method or by comparing intensities of bands on Coomassie stained SDS-PA GE gels with BSA (Sigma) as a standard. For both MBP for ar1 and MBP for ar1-H7, concentrations used refer to the full-length protein and not proteolytic breakdown products.

HA Ste4(TEV)-protein A was purified from RAY1276 cells using IgG-Sepharose under conditions similar to those described in (Song et al., 1996). Cells were grown in YEPD to an OD600 of ~0.9, harvested by centrifugation, resuspended in 20 mM Tris-HCl pH 7.4 with 50 mM NaCl at ~300 μg/ml, snap frozen in liquid N2, and stored at −70°C. Typically 250 OD600 units of cells were broken in buffer D (buffer A containing 2 mM EDTA and 3 mM Mgc12) by agitation with glass beads. Triton X-100 was added to cells at an final concentration of 1%. A 1 h incubation the extract was centrifuged at 10,000 g for 20 min. The supernatant was incubated overnight with 250 μl IgG-Sepharose equilibrated in buffer D containing 1% Triton X-100. The resin was collected by centrifugation, washed once with buffer D containing 1% Triton X-100 and twice with buffer D containing 0.1% Triton X-100. HA Ste4p was specifically eluted by incubation with 20 U of TEV-protease in 400 μl buffer D containing 0.1% Triton X-100 for 5 h. Comparison of the amounts of total protein and HA Ste4p in yeast extracts (treated with TEV-protease) and eluted HA Ste4p preparations indicated that HA Ste4p was enriched over 1,000-fold in comparison to cell extracts. By immunoblotting both 3×myc-Cdc24 and MBP fusion proteins were eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by immunoblotting using ECL.

Binding Studies

Binding experiments were all carried out at 4°C. For binding of GSTCdc24 and MBP fusion proteins ~10 μg of GSTCdc24 bound to glutathione-agarose was incubated with respective MBP fusion proteins in 100 μl of buffer C overnight. Glutathione-agarose samples were washed twice with 1 ml of buffer C and once with 1 ml of buffer B. Proteins were eluted with SDS-PA GE sample buffer and analyzed by SDS-PA GE followed by Coomassie blue staining or transfer to nitrocellulose, probing with anti-MBP mAb (Sigma) at 1:4,000 dilution and visualized by ECL. For binding experiments with yeast H A Ste4p, the HA Ste4p preparation was diluted 10-fold into buffer C and 100 μl was incubated with either resin bound GST or MBP fusions. MBP fusions were bound to amylose resin by incubation with −5 μg of resin with 20 μl of amylose resin for 1 h. GSTCdc24-MBP for ar1 was prepared by passing a bacterial extract (from 100 ml of cells) containing GSTCdc24 over a column with ~500 μg of MBP for ar1 bound to amylose resin. The column was washed with buffer B and then GSTCdc24-MBP for ar1 was eluted with buffer B containing 10 mM maltose. The eluate was incubated with glutathione-agarose for 30 min which was then washed 3 times with buffer B. Proteins bound to

The resin were analyzed by SDS-PA GE and Coomassie blue staining or used for HA Ste4p binding.

Mating and Pheromone Response Assays

Quantitative matings were carried out as described in (Arkowitz and Lowe, 1997; Nern and Arkowitz, 1998) with M ata cells as indicated and M aly RAY Y 1135 cells. Pheromone induced cell cycle arrest and induction of a Fus3p-Cdc24 reporter were assayed as described (Nern and Arkowitz, 1998). For pheromone treatment ~0.2 OD600 of log-phase cells were collected by centrifugation, resuspended in 2 ml YEPD containing 12 μM α-factor (synthesized by David Owen, MRC LMB) and incubated for 3 h. Cells were fixed with formaldehyde and actin was visualized as described (Nern and Arkowitz, 1998) using rhodamine phallolidin (Molecular Probes). To examine cell morphologies in mating mixtures, M ata cells were stained with 10 μg/ml Calcofluor white (Pringle, 1991) (Sigma) in YEPD for 5 min at rt and subsequently washed extensively with YEPD. A approximately 5 × 105 stained cells were then mixed with unstained M ata (RAY Y 1135) cells and incubated on filters. A 2 h cells were washed from the filters, briefly sonicated, resuspended in PBS and fixed with formaldehyde. Images of cells were taken using a Zeiss A kiosk microscope with either a NA 1.4 × 63 or NA 1.3 × 100 objective and recorded with a Princeton Instrument Micromax CCD camera. Fluorescence and differential interference-contrast (DIC) images were merged to permit identification of M ata cells.

Localization of Cdc24p

Cdc24H A G FP (p414Cdc24H A G FP) was transformed into R AY Y 931 which is deleted for Cdc24 and kept alive by the rescuing plasmid pEG(T)-Cdc24 (Nern and Arkowitz, 1998). This strain was able to lose the rescuing plasmid (both by extensive growth in SC-trp media or counter-selection on 5-FOA) as determined by markers and PCR, resulting in RAY Y 1360, indicating that Cdc24H A G FP was functional. RAY Y 1360 grew normally at 22°C, 30°C, and 37°C on YEPD plates. Budding patterns were determined as described (Arkowitz and Lowe, 1997) and mating efficiency was determined as described above. Cdc24H A G FP expression and size was verified by SDS-PA GE, immunoblotting, probing with 12CA 5 mAb and ECL visualization. Confocal microscopy was carried out as described (Arkowitz and Lowe, 1997) except cells were grown in SC supplemented with 55 μg/ml adenosine to reduce fluorescence due to ad26. Pheromone treatment was with 140 μM α-factor. Cells were imaged after 1 h in order to observe early localization. For latrunculin A treatment of budding cells 2 μl of either 10 μM latrunculin A (Molecular Probes) in DM SO or DM SO was added to 200 μl of log-phase cells (final concentration latrunculin A 0.1 μM) and cells were incubated for 3 h (A yscough et al., 1997). For latrunculin A treatment of shmoo, cells were incubated with 140 μM α-factor for 1 h and then 0.1 mM latrunculin A or DM SO was added to cells which were incubated for 2 h. A 2 h observation by confocal microscopy, actin depolymerization was confirmed by staining fixed cells with rhodamine phallolidin as described above.

Results

The Cdc24p-Gβγ Interaction Requires FAR1 but Not Pheromone-dependent Signaling

Cdc24-m alleles are defective in growth orientation along a pheromone gradient, yet do not affect pheromone-dependent MAP-kinase pathway signaling (Nern and Arkowitz, 1998). These mutants are also unable to interact with G β (Ste4p) in two-hybrid assays. In Cdc24p-Gβ two-hybrid assays G β was overexpressed, which has been shown to activate the MAP-kinase pathway (Whiteway et al., 1990). Hence it was possible that MAP-kinase signaling is required for this interaction. T wo-hybrid experiments revealed that while Cdc24p and G β interact in a haploid strain, no detectable interaction was observed in a diploid (compare Fig. 1, A and B), in which several mating specific proteins, including G γ (Ste18p), are not expressed.
Gγ is required for this interaction in a haploid (Nern and Arkowitz, 1998). Surprisingly, overexpression of Gγ in a diploid did not restore the Cdc24p–Gb interaction, whereas overexpression of Gγ in a Δste18 haploid restored this interaction (Table II). This result is consistent with the notion that either a haploid specific component and/or pheromone-dependent signaling is required for the Cdc24p–Gb interaction.

To examine the role of the pheromone-dependent MAP-kinase pathway in the Cdc24p–Gb interaction, two-hybrid strains were constructed in which each component of this pathway was deleted. The MAP-kinase scaffolding protein Ste5p, the PAK kinase Ste20p which phosphorylates Ste11p, the MAPKK Ste4p, the MAPKKK Ste11p, the MAPK Fus3p or Kss1p, and the transcription factor Ste12p were each individually disrupted in a two-hybrid strain. In addition, several of these proteins including Ste5p, Fus3p, and Far1p are only expressed in haploids (Sprague and Thorner, 1992) and thus are candidates for haploid specific components required for the Cdc24p–Gb interaction.

Deletion of SKM1, BEM1, AKR1, or BUD6 had no effect on the Cdc24p–Gb interaction. Deletion of SKM1, BEM1, AKR1, or BUD6 had no effect on the Cdc24p–Gb interaction. Deletion of SKM1, BEM1, AKR1, or BUD6 had no effect on the Cdc24p–Gb interaction. Deletion of SKM1, BEM1, AKR1, or BUD6 had no effect on the Cdc24p–Gb interaction. Deletion of SKM1, BEM1, AKR1, or BUD6 had no effect on the Cdc24p–Gb interaction. Deletion of SKM1, BEM1, AKR1, or BUD6 had no effect on the Cdc24p–Gb interaction. Deletion of SKM1, BEM1, AKR1, or BUD6 had no effect on the Cdc24p–Gb interaction.

(From Figure 1. Two-hybrid interaction between Cdc24p and Gb requires Gγ and Far1p. (A) Overexpression of Gγ and Far1p enhances the Cdc24p–Gb interaction. Assays were carried out in a Y187 strain grown in SC-leu-trp-ade or SC-leu-trp-ade his. LacZ values are the average of three to five determinations with bars showing standard deviation. As indicated, SPA2 (NH2-terminal 153 amino acids), CDC24, STE4, STE18, or FAR1 (entire ORF) were fused either to GAL4 activation domain (AD) or GAL4 DNA binding domain (DBD). Spa2 serves as a DBD Cdc24 negative control and Cdc24-m1 serves as an AD Ste4 negative control. For overexpression, NLSHA-STE18 and NLSHA-FAR1 in plasmids p2μATPI and p413TPI were used. (B) Optimal interaction between Cdc24p and Gb in a diploid requires Gγ and Far1p. Assays were carried out in a diploid strain made by crossing SFY526 and Y187 as described above. (C) Cdc24p–Gb interaction requires FAR1 orientation function. Assays were carried out in a Y187 strain in which FAR1 was either deleted (RAY1183) or replaced by far1-H7 (RAY1182).}

(Sprague and Thorner, 1992). Gγ is required for this interaction in a haploid (Nern and Arkowitz, 1998). Surprisingly, overexpression of Gγ in a diploid did not restore the Cdc24p–Gb interaction, whereas overexpression of Gγ in a Δste18 haploid restored this interaction (Table II). This result is consistent with the notion that either a haploid specific component and/or pheromone-dependent signaling is required for the Cdc24p–Gb interaction.
in these strains. Table II shows that overexpression of Gγ partially restored the Cdc24p-Gβ interaction in Δste5, Δste11, and Δste7 strains and to a lesser extent in Δste20 and Δste12 strains. These results indicate that signaling through the pheromone-dependent MAP kinase cascade per se is not required for the Cdc24p-Gβγ interaction. However, deletion of FAR1 resulted in a loss of the Cdc24p-Gβ interaction which was not restored upon overexpression of Gγ (Table II and Fig. 1C), suggesting that Far1p may be essential for this interaction.

The requirement for Gγ and Far1p in the Cdc24p-Gβ two-hybrid interaction suggested an explanation for the low level of LacZ reporter activity observed in the haploid Y 187 two-hybrid strain and the absence of an interaction in the diploid two-hybrid strain, namely that these two proteins were limiting in haploids and absent in diploids. To test this possibility, we overexpressed Gγ and Far1p individually and together in the Y 187 haploid two-hybrid strain. Fig. 1A shows that overexpression of Gγ in the presence of pA S1Cdc24 and pGA D424Ste4 resulted in an approximately twofold increase in LacZ activity, whereas the additional overexpression of Far1p resulted in a further increase in LacZ activity by ∼3.5-fold. In diploids, overexpression of Gγ did not result in a Cdc24p-Gβ interaction. However overexpression of Far1p resulted in LacZ reporter activity (Fig. 1B) and this was further increased by additional overexpression of Gγ, suggesting that in the absence of pheromone-dependent signaling Far1p is sufficient for restoring the Cdc24p-Gβγ interaction.

FAR1 is necessary for both pheromone-dependent growth arrest and oriented growth towards a pheromone gradient (Chang and Herskowitz, 1990; Valtz et al., 1995). These two functions of FAR1 can be separated, with the Far1p NH2 terminus necessary for cell cycle arrest and the COOH terminus necessary for growth orientation. Our two-hybrid results indicate FAR1 is necessary for the Cdc24p-Gβ interaction, yet it is unclear which function of FAR1 this corresponds to. Because cdc24-m and far1-s appear phenotypically identical and both exhibit orientation defects (Valtz et al., 1995; Nern and Arkowitz, 1998), we examined the effect of the far1-s allele far1-H7 on this interaction. This far1 mutation results in a COOH-terminal 75-amino acid deletion and despite its orientation defect is normal for cell cycle arrest. Fig. 1C shows that a far1-H7 mutation prevents the Cdc24p-Gβ interaction. These results suggest that the FAR1 orientation function is required for the Cdc24p-Gβ association, consistent with the role of this interaction in growth orientation.

We next investigated whether Far1p interacted with Cdc24p and Gβ. Fig. 1 and Table III show that in two-hybrid assays Far1p can interact with both Cdc24p and Gβ. The Cdc24p–Far1p interaction was observed in strains deleted for STE4, STE18, FUS3, or STE12, indicating that it does not require Gβγ nor pheromone-dependent MAP kinase signaling (including Fus3p-dependent phosphorylation of Far1p). Similarly, the Far1p–Gβ interaction did not require STE18, FUS3, or STE12. The Far1p–Gβ interaction also did not require the CDC24 orientation function as we observed this interaction in a cdc24-m1 two-hybrid strain. In addition we examined Cdc24p-m1p, which we had previously shown does not interact with Gβ, and found that Cdc24p-m1p also did not interact with Far1p (data not shown). Together these results suggest that Far1p and Cdc24p can associate and this association is independent of pheromone signaling.

**Far1p Binds Cdc24p**

To further investigate these interactions epitope-tagged versions of Far1p and Cdc24p were constructed. Myc and protein A domains were fused to Cdc24p and Far1p, respectively and these fusions were used to replace wild-type genes. Far1-protein A fusions had a tobacco etch virus (TEV) protease cleavage site between Far1p and protein A to allow specific elution. These strains grew normally and exhibited normal vegetative morphology. Furthermore, both fusions mated with similar efficiencies as a wild-type strain when crossed to a wild-type tester or to an enfeebled tester. Far1-protein A fusions promoted normal cell cycle arrest and cells carrying this fusion formed shmoos that appeared normal upon exposure to mating pheromone. Together these results indicated that the fusion proteins were functional. Fig. 2 shows that when Far1-protein A was isolated with IgG-Sepharose, myc-tagged Cdc24p was bound (compare lanes 1 and 2 with 3 and 4). When Cdc24p or Far1p orientation mutants were used, a substantial decrease in the amount of Cdc24p bound to Far1p was observed in both cases (lanes 5–8). These results reveal the molecular basis for the similar phenotypes

| Strain | Cdc24p Ste4p interaction | + Ste18p |
|--------|-------------------------|---------|
| Δste4  | ++                      | ND      |
| Δste18 | -                       | ++      |
| Δste5  | -                       | +       |
| Δste20 | -                       | ±       |
| Δste11 | +                       |         |
| Δste7  | -                       |         |
| Δks1   | +                       | ND      |
| Δfas3  | ++                      | ND      |
| Δste12 | -                       | ±       |
| Δfar1  | -                       |         |
| Δhem1* | ++                      | ND      |
| Δahr1  | ++                      | ND      |
| Δhsl6  | ++                      | ND      |
| Δskml  | ++                      | ND      |

Two-hybrid assays were carried out in strain PJ69-4A with the indicated gene deletions. Identical results were obtained with at least three transformants. ** Denotes clear growth on selective plates lacking histidine. * Plates incubated at 25°C.

| Strain | Far1 Cdc24 interaction | Far1 Ste4 interaction |
|--------|------------------------|-----------------------|
| Wild-type | ++                      | +                     |
| Δste4   | ++                      | +                     |
| Δste18  | ++                      | +                     |
| Δfas3   | ++                      | +                     |
| Δste12  | ++                      | +                     |
| cdc24-m1| ND                      | +                     |

Two-hybrid assays were carried out in strain PJ69-4A with the indicated gene deletions. Identical results were obtained with at least three transformants. ** Denotes growth on SC-leu-trp-his or in the case of cdc24-m1 SC-leu-ura-his.
of cdc24-m1 and far1-H7 mutants. Although two-hybrid results indicated a Far1p–Gβ association, this was apparently not stable enough to observe by immunoprecipitation.

To address whether these protein interactions were direct, binding experiments were carried out using purified proteins. Far1p and the NH2-terminal half of Cdc24p (amino acids 1–472; Nern and Arkowitz, 1998) were purified from bacteria as fusions to maltose binding protein (MBP) and glutathione-S-transferase (GST), respectively. Fig. 3 A shows that MBPFar1 bound GSTCdc24 but not GST alone. MBPFar1-H7 (Fig. 4 A) does not significantly bind GSTCdc24p, consistent with immunoprecipitation results (see above). In these binding experiments an excess of GSTCdc24 is used and an increase in MBPFar1 binding occurs as its concentration in the binding reaction is increased (Fig. 3 B). These results demonstrate that Far1p can bind Cdc24p directly in the absence of other proteins.

These binding studies demonstrated that the COOH terminus of Far1p is necessary for GSTCdc24 binding, hence we examined if this region was also sufficient for binding. Fig. 4 B shows that a 200–amino acid Far1p COOH-terminal fragment (lane 10) is not sufficient for GSTCdc24 binding and furthermore an NH2-terminal Far1p fragment did not bind GSTCdc24 (lane 9). MBPFar1-H7 and MBPFar1ΔC, which do not bind GSTCdc24 are unlikely to be grossly misfolded as they retain the ability to bind Gβ (see below). These results indicate that although the COOH terminus of Far1p is necessary for binding Cdc24p it is not sufficient. A n immunoblot of the MBPFar1 bound to GSTCdc24 (Fig. 4 B, lane 7) revealed that proteolytic fragments of Far1 with as little as 25 kD of the NH2-terminus (approximately residues 133–350) are coreplicated with MBPFar1 and GSTCdc24. This region of Far1p, which includes a Lim domain (Sanchezgarcia and Rabbitts, 1994), does not bind Cdc24p directly (Fig. 4 B, lane 9), suggesting that this region may mediate Far1p multimerization.

**Far1p Binds Gβ**

Hemagglutinin (HA)-tagged Gβ (Ste4p) was purified from yeast in order to examine its binding to MBPFar1 (Fig. 4 C). For this purpose a strain in which the wild-type copy of STE4 was replaced with HA Ste4-(TEV)-protein A was used. This fusion was functional for mating and cell cycle arrest. The Ste4p fusion was isolated with IgG-Sepharose and eluted by specific cleavage between Ste4p and the protein A domains using TEV protease, yielding HA Ste4p which was over 1,000-fold enriched compared with cell ex-
with MBP fusions (\(z\) GST alone did not bind any MBP fusions. (C) The NH2 terminus ing 5% of added MBP fusions and lanes 7–12 show resin eluates. Anti-MBP mAb and ECL. Lanes 1–6 show standards represent-
lyzed by 10% SDS-PAGE, followed by immunoblotting with
amino acid residues 133–831 for Far1, residues 133–756 for Far1-
D, residues 638–756 for Far1-H7, residues 133–297 for Far1
and residues 638–831 for Far1-C. (D) Indicated MBP fu-
Binding does not appear to be cooperative and is more likely to be the sum of contributions from
Cdc24p and Far1p. Because both Far1p and Cdc24p can
individually bind each other or G\(b\), it is likely that in a trimeric complex each protein contacts the other two pro-
tines. These binding studies together with the two-hybrid
results suggest that Far1p binding to Cdc24p does not dis-
place G\(b\), and are consistent with the formation of a com-
plex of all three proteins.

To directly test whether a complex of all three proteins
could form we determined whether a stoichiometric com-
plex of Cdc24p–Far1p could bind G\(b\) (Ste4p). GSTCdc24–
MBPFar1 was isolated by sequential purification using
amylose and glutathione resin. Fig. 5 B shows that
GSTCdc24–MBPFar1 contained roughly equal amounts of
these two fusion proteins. Purified HASte4p was then
incubated either with this complex or GSTCdc24 alone.
Densitometric quantification showed that twofold more
HA Ste4p bound to Cdc24p–Far1p (Fig. 5 B compare lanes
1 and 2) than to a similar amount of Cdc24p alone, demon-
strating that trimeric Cdc24p–Far1p–G\(b\) can form. This
increase in G\(b\) binding does not appear to be cooperative and
is more likely to be the sum of contributions from Cdc24p and Far1p. Because both Far1p and Cdc24p can
individually bind each other or G\(b\) it is likely that in a tri-
meric complex each protein contacts the other two pro-
tines. These binding studies together with the two-hybrid
results suggest that Cdc24p–Far1p–G\(b\) is necessary for mat-
ing projection orientation.

**Cdc24p and Far1p Function in the Same Shmoo Orientation Process**

To examine if CDC24 and FAR1 function in the same pro-
cess we compared the mating efficiencies of both single
and double \(\Deltafar1\) and cdc24-m1 mutants. Fig. 6 shows that the presence of a cdc24-m1 mutation in a \(\Deltafar1\) back-
ground did not result in a further decrease in mating effi-
ciency, suggesting that FAR1 and CDC24 function in the same orientation process. The mating defect of the double
mutant is closer to that of the \(\Deltafar1\) mutant that in addi-
tion to a chemotropism defect does not arrest growth in
response to mating pheromone. If cdc24-m1 affected che-
motropism similarly to \(\Deltafar1\), then a double mutant with
\(\Deltaspa2\), a gene required for the default mating pathway
(Dorer et al., 1995), should have a mating defect greater
than the product of the individual mating defects, a phenomenon known as synthetic sterility (Dorer et al., 1995). Fig. 6 shows that \( \Delta \)spa2 cdc24-m1 mutants exhibited synthetic sterility.

We also examined genetic interactions between cdc24-m1 and \( \Delta \)bem1 or \( \Delta \)ste20, two genes involved in polarized growth and mating. Bem1p is associated with the cytoskeleton (Leeuw et al., 1995), binds Cdc24p (Peterson et al., 1994; Zheng et al., 1995), and Far1p (Lyons et al., 1996). Bem1 mutants are unable to form shmoos and instead form round cells in the presence of mating pheromone (Chenevert et al., 1992). \( \Delta \)bem1 cdc24-m1 mutants showed similar temperature sensitive growth and morphological defects (large round cells) as \( \Delta \)bem1 cells, providing further evidence that cdc24-m1 has no effect on vegetative growth. Even in cells lacking BEM1 which cannot form shmoos, cdc24-m1 resulted in a substantial decrease in mating efficiency (Fig. 6), i.e., synthetic sterility. Because deletion of the PAK kinase STE20 in our strain background did not result in complete sterility, we were able to examine the mating defect of \( \Delta \)ste20 cells in the presence and absence cdc24-m1. In the absence of STE20, cdc24-m1 resulted in a further decrease in mating efficiency. Together these results suggest that in \( \Delta \)bem1 and \( \Delta \)ste20 mutants, which are unable to form shmoos, polarization may still be necessary for mating perhaps for the localization of proteins necessary for cell fusion. Furthermore, because BEM1 and STE20 are not required for default mating (Dorer et al., 1997), such synthetic mating defects with cdc24-m1 are consistent with a genetic linkage between shmoo formation and orientation.

**Cdc24p Localization**

If Cdc24p transmits signals from bud site selection proteins or G\( \beta \)\( \gamma \), it might be localized to regions of polarized growth. We therefore examined the localization of a Cdc24p green fluorescent protein (GFP) fusion. Cdc24-HAGFP expressed from its own promoter on a CEN plasmid complemented \( \Delta \)cdc24 as determined by growth at different temperatures, budding patterns, and mating efficiencies (data not shown). Fig. 7 A shows the localization of Cdc24HAGFP in living cells at different stages in the cell cycle. In unbudded cells Cdc24p localized as a tight patch at the membrane, and in cells with small buds at the growing end. In larger buds, this localization became more spread out. Finally, during cytokinesis Cdc24p generally localized to the mother–bud neck. Curiously, a preliminary report showed that an overexpressed GSTCdc24 fusion protein had a circumcellular distribution in budding cells (Pringle et al., 1995). Cdc24HAGFP also localized to sites of polarized growth after \( \alpha \)-factor treatment. Fig. 7 B shows different shmoos in which Cdc24HAGFP is observed as a patch at the tip of the mating projection. Cdc24HAGFP was localized similarly in mating mixtures (data not shown). Furthermore, as the sole copy of Cdc24p in a \( \Delta \)cdc24 strain, Cdc24-m1HAGFP also localized to sites of polarized growth in budding and mating cells (data not shown).

![Figure 6. CDC24 and FAR1 function in same shmoo orientation pathway.](image)

Figure 6. CDC24 and FAR1 function in same shmoo orientation pathway. Quantitative matings were carried out with a wild-type tester (RAY 1135) and mating efficiencies (number of diploid cells divided by total number of cells) are the average of three to five determinations with wild-type mating efficiency (42%) set to 100%. Strains RAY 1034, RAY 1035, RAY 1109, RAY 1111, RAY 1246, RAY 1248, RAY 1271, RAY 1160, RAY 1173, and RAY 1168 were used. Bars indicate standard deviation.
not shown), indicating that this mutant is not defective in its localization to sites of polarized growth. These data demonstrate that Cdc24p localizes to sites of polarized growth.

The early localization of Cdc24p in the cell cycle and its localization to the shmoo tip are consistent with its function in polarity establishment. The localization of Cdc24p is similar to that of its substrate Cdc42p (Ziman et al., 1993). To determine whether the actin cytoskeleton was necessary for polarized Cdc24p localization, budding and shmooing cells were treated with the actin depolymerizing drug latrunculin A (Ayscough et al., 1997). Fig. 8 A shows that even in the absence of actin polymerization, Cdc24p is localized to sites of polarized growth in budding cells. In contrast, latrunculin A treatment of shmooing cells resulted in a substantial decrease in Cdc24p localization (Fig. 8 B). Upon latrunculin A treatment the number of cells with Cdc24p localized to the shmoo tip decreased by fivefold (n = 100) and in cells that exhibited localized Cdc24p, there ap-
In the Absence of CDC24- or FAR1-mediated Chemotropism, the Bud Site Selection Machinery Is Essential for Shmoo Formation

During mating, a pheromone gradient serves as the external cue for growth orientation. This external signal allows haploid cells to orient growth in a pheromone gradient emanating from any direction (Madden and Snyder, 1992), whereas the site for bud formation in haploids is fixed adjacent to the previous bud site (Chant and Pringle, 1995). The selection of a site for the mating projection must over ride the fixed location of the bud. If Cdc24p acts as a switch between internal signals during budding and external signals during mating (Nern and Arkowitz, 1998), we would predict that bud site selection proteins become important for cell mating when the capacity for shmoo orientation is lost in mutants such as cdc24-m1.

The ras related small G-protein Bud1p/Rsr1p is essential for bud site selection, yet is not required for chemotropism or default mating in saturating pheromone (Roemer et al., 1996; Dorer et al., 1997). However, Bud1p can directly associate with Cdc24p (Zheng et al., 1995; Park et al., 1997) and this association is likely to functionally important (Bender and Pringle, 1989; Michelitch and Chant, 1996). We therefore examined the phenotype of Δbud1 cdc24-m1 double mutants to determine if the loss of CDC24-mediated chemotropism caused a BUD1-dependent mating defect. Both Δbud1 and Δbud1 cdc24-m1 cells grew normally, were not temperature sensitive for growth, and had the expected random budding pattern (data not shown). Surprisingly, the Δbud1 cdc24-m1 double mutant showed a stronger mating defect (an eightfold further decrease in mating efficiency) than cdc24-m1 alone (Fig. 9 A). In contrast, Δbud1 alone had no effect on mating efficiency in agreement with previous studies (Chant and Herskowitz, 1991; Dorer et al., 1997). Microscopic observation of Δbud1 cdc24-m1 double mutants treated with a high concentration of mating pheromone (Fig. 9 B) or exposed to pheromone gradients in mating mixtures (Fig. 9 C) revealed that these cells were defective in shmoo formation. Instead of forming typical pear-shaped shmoos, most cells were enlarged and round. On closer inspection a small protrusion was occasionally observed on these cells. Furthermore, the actin cytoskeleton in the double mutants was depolarized, with actin cortical patches and cables disorganized (Fig. 9 D). In contrast, both Δbud1 and cdc24-m1 single mutants formed shmoos. Otherwise, Δbud1 cdc24-m1 double mutants responded normally to pheromone by undergoing cell cycle arrest and pheromone-dependent gene induction (data not shown). These results suggest that in the absence of chemotropism, BUD1 and perhaps the bud site selection machinery becomes essential for shmoo formation. Surprisingly, in saturating uniform concentrations of mating pheromone, Δbud1 does not result in a mating defect (Dorer et al., 1997), raising the possibility that this novel role of BUD1 is revealed specifically when signaling from Gβγ to Cdc24p is blocked.

Our results indicate that Far1p is required for signaling from Gβγ to Cdc24p. If the shmoo formation defect of Δbud1 cdc24-m1 cells is due to a defect in this signaling, a Δbud1 Δfar1 double mutant should show an analogous decrease in mating efficiency. Δbud1 Δfar1 cells had a stronger mating defect (an eightfold decrease in mating efficiency) than Δfar1 cells. A same control effect the Δbud1 was examined in a Δsst2 strain. Δsst2 cells are supersensitive to mating pheromone as SST2 negatively regulates the heterotrimeric G-protein (Dohlman et al., 1996). Therefore, Δsst2 cells mate as though they are saturated with mating pheromone, mating by the default pathway (Dorer et al., 1997). Δbud1 Δsst2 cells had a similar mating defect as Δsst2 alone, indicating that the absence of chemotropism by itself is not sufficient to reveal BUD1 function in mating. These synthetic mating defects of Δbud1 with cdc24-m1 or far1 show that Bud1p, which normally functions in bud site selection, can play a role in shmoo formation, presumably by regulating Cdc24p.

Discussion

During mating yeast cells grow in a polarized fashion towards their mating partner (Segall, 1993; Dorer et al., 1995; Valtz et al., 1995; Nern and Arkowitz, 1998). Yeast cells are able to sense pheromone gradients and orient their actin cytoskeleton and secretion towards such a gradient. Here we show that a complex comprised of Cdc24p, Far1p, and Gβγ can form and is likely to be required for orientation towards a mating partner. The formation of this complex does not directly require signaling via the pheromone-dependent MAP-kinase pathway. Analyses of mating defects of double mutants indicate that FAR1 and CDC24 both function in the same cell orientation process. Cdc24p localizes to sites of polarized growth suggesting that Cdc24p-Far1p-Gβγ is localized. Cdc24p localization does not depend on the actin cytoskeleton during budding but does depend on the actin cytoskeleton during shmooing. In the absence of signaling from Gβγ to Cdc24p, the bud site selection protein Bud1p is required for shmoo formation, demonstrating a molecular link between growth site selection in mating and budding. Together these results suggest that binding of Gβγ to Far1p and Cdc24p creates an internal landmark for growth towards an external signal.

A Complex Comprised of Cdc24p, Far1p, and Gβγ Links External Signals to Cytoskeleton Orientation

Detection of a pheromone gradient and orientation of growth in such a gradient is a process central to yeast mating and is analogous to Dictyostelium chemotaxis and nerve cell chemotropism (Arkowitz, 1999). A leyle of both far1 (Valtz et al., 1995) and cdc24 (Nern and Arkowitz, 1998) are specifically defective in orientation towards a pheromone gradient. Cells mutant for the α-factor pheromone receptor (Ste2p) or the heterotrimeric G-protein, discriminate poorly between pheromone signaling and nonsignaling mating partners suggesting that these components are also required for chemotropism (Jackson et al., 1995; Valtz et al., 1995; Nern and Arkowitz, 1998). Yeast cells are able to sense pheromone gradients and orient their actin cytoskeleton and secretion towards such a gradient. Here we show that a complex comprised of Cdc24p, Far1p, and Gβγ can form and is likely to be required for orientation towards a mating partner. The formation of this complex does not directly require signaling via the pheromone-dependent MAP-kinase pathway. Analyses of mating defects of double mutants indicate that FAR1 and CDC24 both function in the same cell orientation process. Cdc24p localizes to sites of polarized growth suggesting that Cdc24p-Far1p-Gβγ is localized. Cdc24p localization does not depend on the actin cytoskeleton during budding but does depend on the actin cytoskeleton during shmooing. In the absence of signaling from Gβγ to Cdc24p, the bud site selection protein Bud1p is required for shmoo formation, demonstrating a molecular link between growth site selection in mating and budding. Together these results suggest that binding of Gβγ to Far1p and Cdc24p creates an internal landmark for growth towards an external signal.
Cdc24-m mutants are unable to interact with the G β subunit of the heterotrimeric G-protein (Nern and Arkowitz, 1998). These results led to a model in which G βγ locally activates or recruits Cdc24p, which could then activate Cdc42p and other downstream targets required for cytoskeleton orientation. We conclude from two-hybrid, binding, and genetic data that Far1p is involved in signaling from G βγ to Cdc24p by forming a complex with these proteins.

Our two-hybrid results suggest that the Far1p–G β interaction does not require the CDC24 orientation function, yet Far1p is essential for the Cdc24p–G β interaction. In contrast, in vitro binding experiments show that Cdc24p is able to bind to G β purified from bacteria (Nern and Arkowitz, 1998) and yeast in the absence of Far1p. We attribute this difference between two-hybrid and in vitro binding results to the different methods used. For example, in the two-hybrid experiments interactions occur in the nucleus and on the other hand the in vitro binding studies are carried out with high concentrations of purified proteins. We suggest that although Far1p is important for the Cdc24p–G β interaction it is not absolutely essential, whereas Cdc24p is not necessary for the Far1p–G β interaction. We have demonstrated that a triple complex comprised of Cdc24p-Far1p-G βγ can form using purified proteins and believe that in vivo this complex links receptor activation to cytoskeleton organization (Fig. 10). These results show at a molecular level the role of Far1p in growth orientation. Consistent with the specific phenotype of far1 and cdc24 orientation alleles, we find that pheromone-dependent MAP-kinase cascade signaling is not necessary for the association of this complex. This result indicates...
that the MAP-kinase cascade is not directly required for chemotropism growth, in agreement with recent mating partner discrimination studies (Schrick et al., 1997). Furthermore, the formation of this complex does not require Fus3, which normally phosphorylates Far1p in a pheromone-dependent fashion (Chang and Herskowitz, 1992; E. lion et al., 1993). This phosphorylation of Far1p is necessary for cell cycle arrest, indicating that the cell cycle arrest function of Far1p is not required for interactions between Cdc24p, Far1p, and Gβγ.

How is the formation of this protein complex regulated by pheromone activation of the receptor? Pheromone binding to the receptor is believed to trigger dissociation of Gα from Gβγ. Recent studies suggest Gα binds the pheromone receptor (Kallal and Kurjan, 1997; Medici et al., 1997) and that Gβγ must be membrane associated in order to function (Pryciak and Huntress, 1998). GFP fused to Gγ is localized preferentially to the plasma membrane of the mating projection after pheromone treatment (Nern and Arkowitz, unpublished observation). Upon pheromone stimulation, Far1p levels increase (Chang and Herskowitz, 1992; Valtz et al., 1995) resulting in increased levels of Cdc24p–Far1p (Nern and Arkowitz, unpublished observation). As Far1p localizes to the nucleus during vegetative growth (Henchoz et al., 1997), it would appear likely that Far1p must exit the nucleus in order to carry out its mating orientation function. We envision that released Gβγ recruits Cdc24p–Far1p to the vicinity of activated receptors and Cdc24p–Far1p–Gβγ ultimately directs the cytoskeleton towards this internal landmark. Such a mechanism provides a means of translating local activation of pheromone receptors to cytoskeletal orientation. We would predict that Far1p, like Cdc24p, localizes to the tip of the mating projection in pheromone treated cells.

While this work was being reviewed a paper examining the role of Far1p in polarized growth during mating was published (Butty et al., 1998). In general, our results agree with the findings of this work. The authors postulate that Far1p functions as an adaptor or linker between Gβγ and polarity establishment proteins including Cdc24p. Our in vitro binding results indicate that even in the absence of Far1p, Gβγ can still bind Cdc24p, suggesting that perhaps Far1p is not simply a physical adaptor but may have more complex functions. Overexpressed GFP Far1p was shown to relocate from the nucleus to the cytoplasm upon treatment with a saturating uniform concentration of pheromone for two hours. In these conditions GFP Far1p does not appear to accumulate at shmoo tips. It will be interesting to determine whether wild-type levels of Far1p localizes similarly in cells exposed to a pheromone gradient for various times.

How are these protein interactions involved in transmitting spatial information? Previous studies have indicated that in a pheromone gradient, shmoo orientation improves as a function of time (Segall, 1993). This appears to be due to reorientation of the shmoo tip as it grows (Segall, 1993; Nern and Arkowitz, unpublished observation), indicating that shmoo orientation is a continuous process unlike bud site selection. Perhaps Cdc24p–Far1p–Gβγ dissociates reasonably fast such that this complex is continually dissociating and forming. Such a dynamic process would provide a means for continuous reorientation during mating and could play a central role in translating initial small differences in receptor occupancy into oriented growth.

Another difference between bud and shmoo formation is that in budding the polarity establishment proteins Cdc42p and Bem1p localize independent of the actin cytoskeleton (Yscough et al., 1997) whereas in the latter process the actin cytoskeleton is necessary for the efficient localization of these proteins (Yscough and Rubin, 1998). During shmoo formation the actin cytoskeleton requirement for localization of Cdc24p and these other polarity establishment proteins appears to be similar. Why is the actin requirement for localization of this group of proteins different in budding and shooming cells? Perhaps the continuous nature of the shooming process compared with the committed directional growth required for budding underlies this different dependence on the actin cytoskeleton. It will be important to examine the role of the actin cytoskeleton in cells responding to a pheromone gradient.

Coordination of Different Pheromone Responses

Pheromone stimulation results in gene induction, cell cycle arrest, and morphological changes (Sprague and Thorner, 1992; Chenewert, 1994; Leberer et al., 1997a). The timing and coordination of these different responses is important for efficient mating. Our genetic studies are consistent with Cdc24p and Far1p being part of the same protein complex functioning in growth orientation and we examined two additional genes that might have a role in coordinating various pheromone responses.

The PAK kinase Ste20p is important for MAP-kinase signaling during mating. It interacts with Bem1p, Ste4p, Ste5p, and Cdc42p (Leeuw et al., 1995; Zhao et al., 1995; Peter et al., 1996; Leberer et al., 1997b; Leeuw et al., 1998).
Recent mating partner discrimination studies (Schrick et al., 1997) suggest that STE 20 may not be required for chemotropism. Our two-hybrid results suggest that STE 20 has some effect on the Cdc24p-Gβ interaction, however cdc24-m1 results in a further mating defect in ste20 cells. While Ste20p binds Gβ, it is unclear how this association relates to the Far1p, Cdc24p, Gβγ interaction. Further studies will be necessary to elucidate the roles of STE 20 in various aspects of mating.

Bem1p is required for polarized growth both during mating and budding (Bender and Pringle, 1991; Cheneyvert et al., 1992). Bem1p cells are defective in shmoo formation, mating pheromone-dependent cell cycle arrest and efficient signaling via the MAP-kinase cascade (Cheneyvert et al., 1992; Lyons et al., 1996). At the molecular level Bem1p interacts with many components required for polarized growth such as the G-protein Bud1p (Zheng et al., 1995; Park et al., 1997), Cdc24p (Peterson et al., 1994; Zheng et al., 1995), Far1p (Lyons et al., 1996), actin (Leeuw et al., 1995), Ste5p (Leeuw et al., 1995; Lyons et al., 1996), and Ste20p (Leeuw et al., 1995). Although Bem1p binds both Cdc24p and Far1p, Bem1p is not required for the formation of Cdc24p-Far1p-Gβγ. Results from Bem1p cdc24-m1 mutants suggest that even cells unable to form shmoo, polarization is important. Perhaps this is because the molecules necessary for cell fusion must be correctly localized. What is the molecular function of Bem1p in mating? We favor the idea that Bem1p acts as a scaffolding component linking pheromone-dependent MAP-kinase signaling, shmoo formation, and shmoo orientation.

**Cdc24p as a Switch between Growth Site Selection in Mating and Budding**

An attractive model (Fig. 10) is that Cdc24p acts as a selector switch that responds to inputs from bud site selection (Sloat et al., 1981) and mating projection orientation (Nern and Arkowitz, 1998). We envision that the localization and activation of Cdc24p is essential for its function in both bud site selection and mating projection orientation. During budding it is likely that local activation of the G-protein Bud1p marks the site for bud formation (Chant et al., 1991; Michielitch and Chant, 1996). The GTP bound form of Bud1p binds Cdc24p (Zheng et al., 1995; Park et al., 1997) and this interaction may be required for Cdc24p localization to the bud site. Interactions of Cdc24p with the bud site selection machinery dictate the site of mating projection growth in the absence of local activation of Cdc24p by Gβγ, such as in the case of far1 (Valtz et al., 1995) or cdc24 (Nern and Arkowitz, 1998) mutants or in the presence of saturating mating pheromone (Madden and Snyder, 1992; Dorer et al., 1997), wherein the mating projection forms adjacent to the previous bud. We show Bud1p becomes essential for shmoo formation specifically in the absence of signaling from Gβγ to Cdc24p. This demonstrates that the bud site selection machinery can function in shmoo formation. It is surprising that under these conditions, BUD1 functions in shmoo formation, while during budding it appears only to function in bud site selection and not bud formation. Interestingly, a specific role for BUD2 in bud formation has been observed in triple mutant combinations with cln1 and cln2 (Benton et al., 1993; Cvrcova and Nasmyth, 1993). A possible explanation for these different functions of BUD1 is that mating projection orientation is a continuous process, in contrast to bud site selection in which once a site for growth is chosen, subsequent directed growth is fixed to this site and there may no longer be a requirement for BUD genes. We attribute the role of BUD1 in shmoo formation to a synthetic effect with cdc24/far1 suggesting this function of BUD1 is normally redundant yet revealed in the absence of Gβγ-mediated chemotropism. Recently it has been proposed that BUD1 is involved in cell fusion (Elia and Marsh, 1998), yet the effects of bud1-m1 we observe in cdc24-m1 mutants, i.e., the inability to form a shmoo, are unlikely to be a result of its role in fusion as we observe this morphological defect in response to mating pheromone without a mating partner. Furthermore, in contrast to the results of Elia and Marsh (1998) but in agreement with previous studies (Dorer et al., 1997), bud1-m1 does not result in a mating defect in our strain background. In addition, bud1-m1 does not affect mating in the presence of saturating uniform mating pheromone concentration (Dorer et al., 1997). Therefore while both mating in the presence of saturating pheromone or mating in a cdc24 or far1 mutant block chemotropic growth, at the molecular level these two situations are not equivalent and this difference is consistent with the suggestion that Cdc24p must be localized or locally activated to function properly (Fig. 10). We can imagine that during mating in saturating uniform pheromone concentrations, the Cdc24p-Far1p-Gβγ linkage is intact, but the external spatial signal is absent. In contrast, in a cdc24-m1 or Far1 mutant while the external signal is present, signaling from BUD1 to Cdc24p is prevented. Furthermore, the early localization of Cdc24p during shmoo and bud formation supports the proposed role of Cdc24p in linking a spatial landmark to polarity establishment.

A simple mechanism for growth site selection during mating and budding is that a threshold level of locally activated Cdc24p is necessary to catalyze the GDP-GTP exchange of Cdc24p. This activation of Cdc24p is presumably generated in part by Bud1p during budding and switched to the region of the cell adjacent to the pheromone source by released Gβγ during mating. In such a mechanism, it would not be necessary to inhibit or erase the incipient bud site during mating as previously suggested (Dorer et al., 1995). It is, however, possible that the binding of Cdc24p to Far1p results not only in an increased level of interaction with Gβγ but also a decrease in the amount of Cdc24p at the bud site, perhaps by decreasing its affinity for Bud1p-GTP. We favor the notion of a balance between Cdc24p activation at the new bud site and at the region of the plasma membrane adjacent to pheromone source. We propose that Far1p serves to bias this equilibrium, i.e., shift the balance, towards the site for shmoo formation.

Cells from a variety of organisms undergo polarized growth in response to external signals. For example, in C. elegans embryonic development it is the sperm entry site that determines antero-posterior axis (Goldstein et al., 1993). In Dictyostelium, cell aggregation occurs via CAMP-mediated chemotaxis (Parent and Devreotes, 1996) and local activation of G-protein signaling events occurs in the absence of cell movement (Parent et al., 1998). Chemo-
taxis is necessary for cell migration responses for example of lymphocytes (Arkowitz, 1999). Chemotropism is also essential for axonal guidance and neuronal growth cone remodeling and extension (Tessier-Lavigne and Goodman, 1996). Such processes are crucial for tissue and organ development. Many of these chemotactic and chemotropes processes appear similar to chemotropism during yeast mating, in that they depend on chemotactant gradients that are recognized and transmitted by a molecular machinery including G-protein coupled receptors, rho-family GT Pases, and their exchange factors. Chemotropin action in yeast is therefore a suitable model for understanding the molecular basis of many different chemotropes and chemotactic processes.

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