A Role for a Protease in Morphogenic Responses during Yeast Cell Fusion

Lisa Elia and Lorraine Marsh

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Abstract. Cell fusion during yeast mating provides a model for signaling-controlled changes at the cell surface. We identified the AXL1 gene in a screen for genes required for cell fusion in both mating types during mating. AXL1 is a pheromone-inducible gene required for axial bud site selection in haploid yeast and for proteolytic maturation of α-factor. Two other bud site selection genes, RSRI, encoding a small GTPase, and BUD3, were also required for efficient cell fusion. Based on double mutant analysis, AXL1 in a MATα strain acted genetically in the same pathway with FUS2, a fusion-dedicated gene. Electron microscopy of axl1, rsr1, and fus2 prezygotes revealed similar defects in nuclear migration, vesicle accumulation, cell wall degradation, and membrane fusion during cell fusion. The axl1 and rsr1 mutants exhibited defects in pheromone-induced morphogenesis. AXL1 protease function was required in MATα strains for fusion during mating. The ability of the Rsr1p GTPase to cycle was required for efficient cell fusion, as it is for bud site selection. During conjugation, vegetative functions may be reemployed under the control of pheromone signaling for mating purposes. Since Rsr1p has been reported to physically associate with Cdc24p and Bem1p components of the pheromone response pathway, we suggest that the bud site selection genes Rsr1p and Ax1p may act to mediate pheromone control of Fus2p-based fusion events during mating.

Key words: yeast • mating • morphogenesis • cell fusion • metalloprotease • GTPase

Cell fusion occurs in biological processes such as fertilization, viral entry into cells, myogenesis, and yeast mating (56). While the cell biology of fusion has been well described, very little is known about the molecular components and signaling events involved. Recent studies on myoblast fusion have begun to identify components that are required for this cell fusion event, which include a small GTPase, Drac1, a Drosophila member of the rho family of GTPases that mediate cytoskeleton polarization in many organisms (28). Metalloproteases, as well as proteins of unknown function, are also involved. (15, 21, 33).

Studies of the mating reaction in the yeast Saccharomyces cerevisiae have begun to identify components required for cell fusion in this system. When two haploid cells of opposite mating type encounter one another, they secrete peptide pheromones that bind to G-protein-coupled receptors on the cell surface and activate a MAP kinase signal transduction pathway (for review see reference 36). MATα cells release α-factor and respond to α-factor, and MATα cells release α-factor and respond to α-factor. The haploid cell response to pheromone is threefold. Cells stop their progression through the cell cycle. Transcription of mating-specific genes is stimulated. Cells polarize toward each other by redirecting actin cytoskeleton assembly and secretion toward a site on the cell surface defined by external pheromone produced by the partner cell. Eventually, cell–cell contact and cell fusion occur to give rise to a diploid cell.

Cell fusion in yeast involves processes that lead to degradation of a small portion of the cell wall at the cell–cell contact region where fusion will occur (17, 36). Often, vesicles are observed poised on opposite sides of the cell–cell contact region in each cell (417). In myoblast fusion, vesicles similarly align on each side of the plasma membrane in a region where localized membrane and cytoplasmic fusions will occur (15). Several yeast genes, including the fusion-specific genes FUS1 (37, 54) and FUS2 (19, 54), have been identified that when mutated lead to an accumulation of cell pairs blocked in cell fusion.

The components that define the site of cell fusion in response to mating pheromones have not been determined. The polarity establishment proteins, Bem1p, Cdc24p, and Cdc42p, which are required for bud formation during vegetative growth (29), may play a role in establishing the fusion site on the cell surface during mating since they are...
known to play additional roles in the pheromone signaling pathway (12, 13, 58). During budding, these proteins direct cytoskeleton polarization in response to positional information provided by a complex of bud site selection proteins that identify and decode a cortical landmark site on the cell surface. During mating, this internal budding polarization is redirected toward the mating site, which is proposed to be marked in part by the activated pheromone receptors (11, 41).

Previously, we reported a screen to isolate cell fusion–defective yeast mutants (17). We report here that the haploid-specific budding site selection gene, AXL1, is required for morphogenesis in response to mating pheromone and cell fusion during mating. Two other haploid bud site selection genes, RSR1, a small GTPase, and BUD3, are also required. Rsr1p has known physical associations with two components of the pheromone response pathway (31, 43, 51, 58). It is possible that AXL1, RSR1, and BUD3 may function in a pathway for coupling pheromone responses to cell fusion.

**Materials and Methods**

**Reagents, Media, and Yeast Strains**

Yeast strains and plasmids are listed in Table I. Yeast rich medium (YPD), synthetic minimal medium (SD), and synthetic drop-out medium are standard media (17). 4,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co. (St. Louis, MO). Affinity-purified anti-Cdc42p antibodies were a kind gift from D. Johnson (University of Vermont, Burlington, VT).

**Cloning of the CEF3 Fusion Gene**

CEF3 was cloned by complementation of the mating defect of the cef3 strain, LE1B3. A plasmid (p7-17e) was isolated from a low-copy CEN-based Ref. 2.

### Table I. Yeast Strains and Plasmids Used in This Study

| Strain                  | Genotype                                      | Source       |
|-------------------------|-----------------------------------------------|--------------|
| LM23-3az (parental)     | MATa bar1 [FUS1–lacZ::URA3] his4 leu2 met1 trp1 ura3–52 | Ref. 35      |
| SRM5                    | MATa axl1 (cef3) -1 bar1 [FUS1–lacZ::URA3] his4 leu2 met1 ura3–52 | This study  |
| LE3-1B                  | MATa axl1 (cef3) -1 bar1 leu2 his4 met1 trp1 | This study   |
| LE1B3                   | MATa axl1 (cef3) -1 bar1 met1 ura3–52 | This study   |
| LE6B3                   | MATa axl1 (cef3) -1 bar1 met1 ura3–52 | This study   |
| LM104                   | Isogenic to LM23-3az but cured of [FUS1–lacZ::URA3] | This study   |
| LE74x                   | Isogenic to LM104 but axl1 -Δ1 | This study   |
| LEB1                    | Isogenic to LM104 but rsr1 Δ:URA3 | This study   |
| LEB3                    | Isogenic to LM104 but bud3 Δ:URA3 | This study   |
| LE281                   | Isogenic to LM104 but fus1 -Δ1 | This study   |
| EYL36                   | MATa his4–34 trp1 -Δ1 ura3–52 fus2-Δ3 | Ref. 19      |
| LM110                   | Isogenic to LM104 but Δste6::URA3 | Ref. 17      |
| IH7101                  | MATa ade6 his2 | Ref. 17      |
| FC139                   | MATa bar1 lys5 met1 ura3–52 | Ref. 17      |
| LE49a                   | Isogenic to FC139 but axl1 -Δ1 | This study   |
| LE17a                   | Isogenic to FC139 but axl1 -Δ2 | This study   |
| LEB1                    | Isogenic to FC139 but rsr1 Δ:URA3 | This study   |
| LEB3                    | Isogenic to FC139 but bud3 Δ:URA3 | This study   |
| LE49h1                  | Isogenic to FC139 but rsr1 Δ:URA3 | This study   |
| LE14                    | Isogenic to FC139 but fus1 Δ:URA3–1 | This study   |
| LE272                   | Isogenic to FC139 but fus2-Δ3 | This study   |
| LE49h1-1                | Isogenic to LE49a but fus1 Δ:URA3–1 | This study   |
| LE49f2-1                | Isogenic to LE49a but fus2 Δ:URA3–1 | This study   |
| RC757                   | MATa ss2-1 his6 met1 can1 cyh2 rme | Ref. 6       |

| Plasmid               | Description                                      | Source       |
|-----------------------|--------------------------------------------------|--------------|
| p7-17e                | YCp50 genomic library clone | This study   |
| p7-17eΔ1              | Identical to p7-17e but axl1-Δ1 | This study   |
| YCp17e1               | SacI 5.2-kb fragment containing an AXL1 allele in YCp50 | This study   |
| pLEA7                 | Ylp5 containing axl1-Δ1 allele | This study   |
| pPB181                | rsr1 Δ:URA3 | Ref. 3      |
| pBUD3Δ9d2             | bud3 Δ:URA3 | Ref. 9      |
| pL32-GFP              | RPL32 fused to GFP in pRS316 | J. Warner    |
| pΔBA1                 | axl1-Δ(848–1,142) allele in YCp50 | This study   |
| p129                  | AXL1 allele in pRS316 | Ref. 1       |
| p126                  | axl1-H68A allele in pRS316 | Ref. 1       |
| p138                  | axl1-E71A allele in pRS316 | Ref. 1       |
| YEps (RSR1)           | RSR1 LEU2-2-μm based | Ref. 2       |
| YEps (rsr1<sup>val12</sup>) | rsr1<sup>val12</sup> LEU2-2-μm based | Ref. 2       |
| pLE131                | ste6 (cef1-1) in YEps13 | Ref. 17      |
based *S. cerevisiae* genomic library (47) that when reintroduced rescued the mating and fusion defects of LE1B3. The complementing plasmid (p7-17) contained an insert of 10.7 kb. Partial sequencing data obtained from p7-17 revealed that the insert DNA corresponded to a region of Ch.XVI that contained several open reading frames (ORFs), including *AXL1*.

To determine if *AXL1* was sufficient to rescue the mating and fusion defects of our cef3 strains (LE1B3 and LE6B3), a 5.5-kb SalI fragment containing *AXL1* was subcloned into YCP50 to create YCP17e1. Introduction of YCP17e1 into LE1B3 or LE6B3 was sufficient to rescue the mating and fusion defects (data not shown). *AXL1* was deleted from the complementing plasmid (p7-17) by digesting with SnaBI to release a 4.7-kb fragment containing the *AXL1* ORF plus 1.0 kb of upstream and 0.08 kb of downstream flanking genomic DNA. The linearized plasmid was relegate to yield plasmid p7-17Δ1. p7-17Δ1 failed to restore efficient mating and cell fusion to LE1B3 or LE6B3.

**Deletion of Genomic AXL1, RSR1, and BUD3**

*AXL1* was deleted using a pop-in, pop-out strategy. A 3.0-kb SphI fragment containing the *AXL1* genomic region deletion (described above) was isolated from p7-17Δ1 and cloned into the Yip5 integration vector. The resulting plasmid, pLEA7, was linearized with Xhol to target integration at the *AXL1* locus and transformed into FC139 or LM104. Transformants were cured of the URA3 marker on 5-fluoro-orotic acid (5-FOA) plates and screened for deletion of *AXL1* by scoring for an altered bud site selection pattern. *RSR1* and *BUD3* were disrupted using plasmids pPB181 and pUBU3Δ2α, respectively (3, 9).

**Construction of an AXL1 Internal Domain Deletion Allele**

An *axl* allele that contained an internal deletion encompassing a domain conserved in related proteases was generated (25). YCP17e1 was digested with Bsu36I and AflII, which cut at sites 848 and 1142 within the coding region. The linearized DNA was treated with SI nuclease to create blunt ends and reanimated to generate pBQA1, which contained an in-frame deletion from codons 848–1142. The structure of the deletion in pBQA1 was confirmed by DNA sequencing.

**Quantitative Mating Assays**

Quantitative matings were performed to determine the efficiency of diploid formation. The frequency of diploid formation was calculated as the number of cell pairs blocked in fusion per total number of viable input cells.

**Quantitative Cell Fusion Assay**

Filter matings were performed as described above, except that 2 × 106 cells of *MATα* and *MATα* cells were used. Cells were washed from the filters, sonicated to break up clumps, and viewed under the microscope using phase contrast optics. The frequency of prezygote accumulation was determined as the number of cell pairs blocked in fusion per total number of cell pairs counted (prezygotes + zygotes). Cell pairs blocked in cell fusion were identified by the appearance of an intact septum/fusion bridge between the cells. Fused cell pairs (zygotes) either completely lack the septum/fusion bridge or have a partial septum/fusion bridge with an identifiable region of cytoplasmic continuity. At least 200 cell pairs were observed for each mating. The prezygote accumulation assay is very reproducible, with ~10% variance due to environmental conditions during the mating reaction (4, 17). To visualize the mutant cell partner in mating pairs, various strains were transformed with a plasmid encoding a fusion of the L32 promoter with the gene encoding green fluorescent protein (pLS2-GFP, a kind gift from J. Warner, Albert Einstein College of Medicine). Mating cell pairs were set up so that only one partner carried or lacked a GFP plasmid.

**Pheromone-induced Morphological Changes**

Cultures (3 ml) were grown to log phase in YPD medium at 30°C. α-Factor was added to a final concentration of 40 nM or 400 nM. After incubation for 6 h at 30°C, 16% EM-grade paraformaldehyde (Electron Microscopy Sciences, Warrington, PA) was added directly to the cultures to a final concentration of 4%. Cultures were incubated for 1 h at 25°C and then washed with PBS. Cell morphology was examined by light microscopy.

**Analysis of Budding Pattern**

Cells were grown to log phase in rich medium at 30°C. Budding patterns were scored as described by Chant and Herskowitz (8). Single cells were plated onto YPD medium and incubated at 30°C for approximately two cell divisions. Microcolonies at the four-cell stage were scored for an axial, bipolar, or random budding pattern.

**Pheromone Response Assays**

To determine sensitivity to cell cycle arrest by pheromone, ~1 × 10^6 of exponentially growing cells were spread onto YPD agar, and 0.1 μg of α-factor (Sigma Chemical Co.) was spotted in duplicate onto the plates that were incubated for ~24 h at 30°C. Zones of growth inhibition were quantitated. To measure gene induction, a plasmid (pSB234) carrying the pheromone-inducible *FUS1::lacZ* reporter gene fusion (54), was transformed into strains. Different concentrations of α-factor were added to cultures of exponentially growing strains, which were then incubated for 1 h at 30°C and processed to determine β-galactosidase levels (48).

**FUS1 and FUS2 Disruptions**

FUS1 and FUS2 disruption constructs were created by PCR. For the *FUS1* disruption construct, 1.04 kb of *FUS1* coding region was replaced with the *URA3* gene, leaving 0.25 and 0.24 kb of flanking *FUS1* region DNA. For the *FUS2* disruption construct, 1.97 kb of *FUS2* coding region was replaced with the *URA3* gene, leaving 0.23 and 0.24 kb of flanking *FUS2* genomic DNA. The fus1Δ::URA3 construct was transformed into FC139 to create FC141 and into LE49a to create LE49f1-1. The fus2Δ::URA3 construct was transformed into LE49a to create LE49f2-1. Presence of the disruption alleles in FC141, LE49f1-1, and LE49f2-1 was confirmed by PCR.

**Immunofluorescence Microscopy**

Logarithmically growing cells were treated with 5 × 10^{-7} M α-factor until >80% of the cells had a single mating projection (~2.75 h at 30°C) and then fixed with 3.7% formaldehyde and prepared for staining with affinity-purified anti-Cdc42p antibodies as described by Ziman (60).

**Electron Microscopy**

Strains were mated on nitrocellulose filters as described above for 3.5–4 h. Cells were fixed for electron microscopy as described in reference 57. Cells were washed from the filters and diluted with SD. Dilutions were plated onto the appropriate selective medium to determine the number of diploids formed or onto YPD medium to meter the total number of viable input cells.

**Results**

**The cef3 Mutation, Complemented by AXL1, Causes Cell Fusion Defects in Two Cell Types**

Previously, we reported a screen to identify mutants (Cef-) specifically defective for the cell fusion step of mating (17). Strains able to initiate mating but unable to complete cell fusion were isolated using microscopic observation of mating cell pairs as the final step of the screen. A strain, SRM5, bearing a mutation provisionally designated cef3, was chosen for further characterization.

Segregation analysis and scoring for a fusion defect revealed that 50% of the SRM5 progeny (14 fusion defec-
ative/28 total cells scored) failed to fuse when mated to a wild-type tester strain (FC139). Thus, the cef3 mutation segregated 2:2 as a single gene trait. Among the mutant progeny tested, we found that an equal number of MATα and MATα strains displayed a fusion defect (8/14 MATα cef3; 6/14 MATα cef3). These results suggested that the mutation could act in both haploid cell types.

A genomic clone (p7-17) complementing the cef3 mating defect was isolated from a yeast centromeric library (47). Partial sequence data was obtained from the complementing clone, and the chromosomal region to which the yeast insert DNA corresponded was identified. The insert contained several ORFs, including a yeast insert DNA revealed that a 5.3-kb SalI fragment containing Axl1p. Subcloning and deletion analysis of the complementing insert revealed that a 5.3-kb SalI fragment containing AXL1 coding sequence (YCp17e1) was sufficient to rescue the mating and cell fusion defects of the cef3 strains (LE1B3 and LE6B3).

Deletion of the AXL1 Gene Affects Mating Ability of a MATα Strain

Many of the effects on mating caused by an axl1 mutation when present in a MATα strain can be explained by the defect in a-factor processing (1, 4). It has been shown that reduced pheromone production produces a cell fusion defect (4). Segregation analysis suggested that AXL1 might be required for efficient cell fusion during mating in MATα strains as well as in MATα strains. To avoid concerns that the MATα phenotypes we observed were due to tightly linked mutations in the original cef3 strain, and to create an isogenic series of strains, we deleted AXL1 in the MATα strain FC139 by gene replacement to create a strain LE49a. Because analysis of mating effects caused by an axl1 mutation is complex in a MATα strain, most of the studies reported here concern effects in MATα strains. The Axl1p protease has been shown to play no role in processing or secretion of the α-factor pheromone in MATα strains (4, 10). Deletion of AXL1 also had no effect on production of the α-factor pheromone in our MATα strain background (data not shown). The AXL1 deletion did cause a shift from an axial to a bipolar bud site selection pattern, as has been reported in other strain backgrounds (Table II) (1, 25).

To confirm that AXL1 was required for cell fusion in MATα cells during mating, we scored fusion by observation of mating forms microscopically. Deletion of AXL1 in the MATα strain FC139 conferred a defect in cell fusion (Fig. 1, c–f). The results of quantitation of the MATα axl1Δ fusion defect are shown in Table II. Since wild-type yeast complete cell fusion rapidly, mating intermediates rarely accumulate in normal matings. Fusion-defective strains (4, 5, 17, 18, 20, 37, 54, 55) either fail to complete mating or take longer to complete fusion steps and so accumulate prezygote forms microscopically. Deletion of AXL1 in the MATα strain FC139 also had no effect on budding or take longer to complete fusion steps and so accumulate prezygote forms microscopically.

**Table II. Role of Bud Site Selection Genes in Mating**

| Relevant genotype | Prezygote accumulation | Mating frequency | Bud site selectiona |
|------------------|------------------------|------------------|--------------------|
| MATα strains     |                         |                  |                    |
| FC139            | Parental               | 5                | 21.0 ± 5.0         | 92 |
| LE49α            | axl1Δ                  | 55               | 6.0 ± 2.0          | 37 |
| LEb1             | rsrlΔ                  | 60               | 4.0 ± 2.0          | 14 |
| LEb3             | bud3Δ                  | 44               | 8.0 ± 0.6          | 29 |
| LE49b1           | axl1Δ rsrl1Δ           | 64               | 5.0 ± 0.6          | 13 |
| LE49b3           | axl1Δ bud3Δ            | 64               | 5.0 ± 0.1          | 16 |
| LE14             | fus1Δ                  | 41               | 14.0 ± 0.9         | —  |
| MATα strains     |                         |                  |                    |
| LE74x            | axl1Δ                  | 51               | 0.3 ± 0.1          | 40 |
| LEab1            | rsrl1Δ                 | 83               | 3.2 ± 0.4          | 17 |
| LEab3            | bud3Δ                  | 64               | 2.6 ± 0.9          | —  |

a Prezygote accumulation was determined by microscopic observation as described in Materials and Methods. More than 500 mating pairs were scored for each strain pair. MATα partner is LM23-3az.

b Mating frequency was measured as the percent of prototrophic diploids formed/total viable cells after limited mating to the MATα partner, LM23-3az, and is the average ± standard deviation of duplicate filter mating determinations.

c The frequency of axial budding (not bipolar or random) was determined. Budding patterns were assayed for >100 microcolonies.

d Prezygote accumulation and mating frequencies determined as above. The MATα partner for both assays was FC139.

---

*Figure 1. Light microscopy of yeast mating pairs. Photomicrographs of typical mating cell pairs using Nomarski optics. (a and b) LM23-3az × FC139 [Wt × Wt]. (c–f) LM23-3az × LE49α [Wt × axl1] (two isolates). (g and h) LM23-3az × LEb1 [Wt × rsrl1Δ]. (i and j) LM23-3az × LEab3 [Wt × bud3Δ].*
cumulate mating intermediates (prezygotes) in which cells have initiated mating, but in which nuclear and cytoplasmic fusion have not occurred. The \textit{axl1} strain, LE49a, accumulated 11-fold more prezygotes than the parental strain FC139 (Table II). As a comparison, a \textit{fus1} deletion mutant (LEf14) accumulated eightfold more prezygotes than the parental strain, FC139. The mating frequency of the \textit{axl1} mutant strain was reduced to 29\% of the wild-type (FC139) level. Thus, \textit{AXL1} and \textit{FUS1} had comparable effects on cell fusion under these conditions.

**Genetic Interaction between AXL1 and FUS2 Suggest They Act in the Same Pathway**

\textit{AXL1} is a haploid-specific, pheromone-induced gene (1, 25). We wanted to determine if \textit{Axl1p} acted in a pathway with either of two other known haploid-specific, pheromone-induced genes, \textit{FUS1} and \textit{FUS2}, that are required only for cell fusion (37, 54). Fus1p and Fus2p are thought to act in parallel pathways leading to cell fusion (19, 37, 54). Double mutant \textit{axl1 fus1} and \textit{axl1 fus2} strains were constructed by gene replacement in the \textit{axl1} strain, LE49a (see Materials and Methods for details). The \textit{axl1 fus1} strain, LE49f1-1, exhibited defects in both cell fusion and mating assays that were greater than the single \textit{axl1} or \textit{fus1} mutant strains (Table III). LE49f1-1 accumulated 87\% prezygotes, whereas prezygote accumulations of 39 and 53\% were determined for the \textit{fus1} (LEf14) and \textit{axl1} (LE49a) single mutant strains, respectively. LE49f1-1 displayed a mating efficiency that was about fourfold lower than the \textit{axl1} strain (LE49a) and about sevenfold lower than the \textit{fus1} strain (LEf14) (Table III).

In contrast, \textit{fus2} and \textit{axl1} did not exhibit an additive defect (Table III). The \textit{axl1 fus2} strain LE49f2-1 accumulated 46\% prezygotes and exhibited an 8.4\% mating efficiency. The \textit{fus2} strain accumulated 38\% prezygotes and mated with an efficiency of 14.8\%. The \textit{axl1} strain LE49a accumulated 53\% prezygotes and mated with an efficiency of 8.0\%. These results suggested that \textit{axl1} might act in the same pathway as \textit{FUS2}.

**Role of Other Haploid Bud Site Selection Genes in Cell Fusion**

\textit{AXL1} was identified as a gene required for axial bud site selection in haploid yeast strains (1, 25). Other genes are known that also are required for this morphogenic program (7, 29). We wanted to determine if other bud site selection components were required for efficient cell fusion during mating. We tested the role of \textit{RSR1} and \textit{BUD3} in cell fusion. \textit{RSR1} encodes a ras-like GTPase that may in turn regulate the GTPase Cdc42p (3, 7, 59). \textit{BUD3} encodes a product that interacts directly or indirectly with septins and appears to act upstream of \textit{Axl1p} and \textit{Rsr1p} (9, 29). We constructed \textit{rsr1} and \textit{bud3} mutant strains by gene disruption in our \textit{MAT\alpha} parental strain, FC139. As shown in Fig. 1, g–j, and in Table II, \textit{rsr1} (LEb1) and \textit{bud3} (LEb3) mutant strains were defective in cell fusion during mating. The \textit{MAT\alpha} strain (LEb1) accumulated 60\% prezygotes, and the \textit{MAT\alpha bud3} strain (LEb3) accumulated 44\% prezygotes. Mating frequencies were reduced in the \textit{rsr1} and \textit{bud3} strains to an extent similar to that of the \textit{axl1} strain (Table II). The \textit{MAT\alpha rsr1} strain (LEb1) and the \textit{MAT\alpha bud3} strain (LEb3) mated to the wild-type tester strain (LM23-3az) with frequencies of 4 and 8\%, respectively.

The \textit{rsr1} and \textit{bud3} strains exhibited the expected defects in bud site selection (Table II). The parental strain FC139 exhibited an axial budding pattern. This pattern was shifted in the \textit{rsr1} (LEb1) and the \textit{bud3} (LEb3) mutant strains. The \textit{rsr1} mutant exhibited a random pattern of bud site selection, whereas the \textit{bud3} mutant exhibited a bipolar pattern as described by others (1, 8, 9, 25). \textit{MAT\alpha rsr1} (LEa1b) and \textit{MAT\alpha bud3} (LEab3) mutants exhibited mating defects similar to the \textit{MAT\alpha} mutant strains, indicating that \textit{RSR1} and \textit{BUD3} play a role in mating in both cell types (Table II).

Since several components required for proper haploid bud site selection appeared to be required for fusion steps in mating, we wanted to determine if \textit{RSR1} and \textit{BUD3} might be acting in the same pathway as \textit{AXL1}. We constructed an \textit{axl1 rsr1} strain (LE49b1) and an \textit{axl1 bud3} strain (LE49b3) and mated them to the tester strain LM23-3az. The strain LE49b1 mated at 24\% of the wild-type level and accumulated 13-fold more prezygotes, similar to what was determined for the single \textit{axl1} and \textit{rsr1} mutants (Table II). The strain LE49b3 also mated at 24\% of the wild-type level and accumulated 13-fold more prezygotes. Thus, it appeared that \textit{RSR1}, \textit{BUD3}, and \textit{AXL1} might act in the same pathway to promote cell fusion during mating as they do to promote bud site selection during vegetative growth, though the results were less clear cut than the interaction of \textit{AXL1} and \textit{FUS2}.

**Electron Microscopy of [Wt × Wt] Prezygotes**

To characterize the mating steps at which fusion-specific genes might act on an ultrastructural level, we performed electron microscopy to identify potential mating intermediates (prezygotes) (42). Wild-type (Wt) intermediates were characterized first. Prezygote intermediates of wild-type strains have been observed but are rare and remain poorly characterized (17). We sought to characterize wild-type fusion forms to better define the steps that might be defective in our mutant strains (Fig. 2, A–F). Several features were clear in these wild-type prezygotes. The cell walls of the mating cell pair were knitted to form a smooth, nearly seamless structure resembling the walls ob-

---

Table III. Double Mutant Analysis of AXL1, FUS1, and FUS2

| MAT\alpha strain | Relevant genotype | Prezygote accumulation* | Mating frequency‡ |
|------------------|------------------|-------------------------|------------------|
| FC139            | \textit{AXL1}    | 7                       | 21.2 ± 6.3       |
| LEf14            | \textit{fus1}Δ   | 39                      | 15.9 ± 0.0       |
| LE272            | \textit{fus2}Δ   | 38                      | 14.8 ± 1.1       |
| LE49a            | \textit{axl1}Δ   | 53                      | 8.0 ± 0.1        |
| LE49f1-1         | \textit{axl1}Δ\textit{fus1}Δ | 87                      | 2.4 ± 0.7        |
| LE49f2-1         | \textit{axl1}Δ\textit{fus2}Δ | 46                      | 8.4 ± 0.1        |

*Prezygote accumulation was determined as described in Materials and Methods. More than 200 mating pairs were scored for each strain pair. MAT\alpha partner was LM23-3az.

‡Mating frequency was measured as the percent of prototrophic diploids formed/total viable cells after limited mating to MAT\alpha partner, LM24-3az. Averages ± SD of duplicate filter mating determinations.
served in zygotes, suggesting that outer cell wall fusion may precede septum degradation. Nuclei were oriented generally close to the fusion zone, as previously described (17, 19, 30) (Fig. 2D). In a few of the prezygotes, we observed what appeared to be streaming of electron-dense material across the cell–cell contact region at a localized point between the two cells (Fig. 2D). The [Wt×Wt] prezygotes at some stages had an overall morphological appearance similar to our previously described [ste6cef1]Wt prezygotes, indicating that mutations that block cell fusion do not always disrupt prezygotic structure (17).

Electron Microscopy of [axl1Δ×Wt] Prezygotes

The [axl1Δ×Wt] prezygotes had a very different appearance from the [Wt×Wt] prezygotes (Fig. 2, I–L). In many of the cell pairs observed, we often found that one of the two nuclei was not positioned near the fusion zone and appeared misaligned with respect to the partner nucleus. We observed a similar nuclear positioning defect in [fus2Δ×Wt] prezygotes (Fig. 2G). Nuclear misalignment has been previously reported for fus2 mutant strains (19). Of 18 cell pairs in which nuclear position of both cells could be observed in [axl1Δ×Wt] crosses, 11% had both nuclei properly aligned, as opposed to 100% of nuclei aligned for [Wt×Wt] crosses. In these electron micrographs, we could not unambiguously determine if it was the mutant or wild-type partner that contained a misaligned nucleus. We determined the frequency of nuclear alignment by staining [mutant×mutant] prezygotes with DAPI to visualize the nuclei and counting the number of cell pairs with aligned nuclei vs. misaligned nuclei (Table IV).

Table IV. Quantitation of Nuclear Positioning by DAPI Staining

| Mating pair* | Relevant genotype | Nuclei aligned† |
|--------------|------------------|----------------|
| LE281/FC139  | fus1Δ fus1Δ      | 97             |
| EYL36/FC272  | fus2Δ fus2Δ      | 16             |
| LE74x/LE17a  | axl1Δ fus1Δ      | 8              |
| LEb1/LEb1    | rsr1Δ rsr1Δ      | 22             |

*MATa and MATα cells were mated on filters for 3.5 h at 30°C (refer to Materials and Methods). Mating pairs were: LE281 [MATa fus1Δ::URA3], EYL36 [MATa fus2Δ::URA3], LE74x [MATa axl1Δ::URA3], and LEb1 [MATa rsr1Δ::URA3].

†Mating mixtures were stained with DAPI to visualize nuclei (refer to Materials and Methods). More than 30 mating pairs were scored for each strain combination.
97%. In contrast, \([\text{fus2}\Delta \times \text{fus2}\Delta]\) and \([\text{axl1}\Delta \times \text{axl1}\Delta]\) prezygotes exhibited aligned nuclei with a frequency of 16 and 8%, respectively.

In all of the cell pairs observed by electron microscopy, the cell that harbored the misaligned nucleus exhibited a distorted, swollen appearance. The swollen cell often appeared to bulge over each side of the cell–cell contact region, giving the appearance of engulfing its partner (Fig. 2 I). In addition, we observed pockets of electron-dense material within the cell wall space on either side of the cell–cell contact region in many of the \([\text{axl1}\Delta \times \text{Wt}\)] prezygotes (Fig. 2 L). Based on light microscopy experiments described below (Fig. 3), we believe that the swollen partner cells are the \text{axl1} mutants. In \([\text{Wt} \times \text{Wt}\)] matings, a portion of the cell wall separating the mating pair underwent breakdown without apparent alterations of other portions of the cell wall, which might indicate that \text{axl1} mutants have a defect in control of cell wall changes in mating.

**Electron Microscopy of Prezygotes of \([\text{rsr1}\Delta \times \text{Wt}\)]**

The overall structure of \([\text{rsr1}\Delta \times \text{Wt}\)] prezygotes was similar to the \([\text{axl1}\Delta \times \text{Wt}\)] prezygotes (Fig. 2, M and N). In cell pairs where nuclei were visible, we often observed that one of the nuclei failed to align with the fusion septum. However, the nuclear misalignment observed in \([\text{rsr1}\Delta \times \text{Wt}\)] prezygotes was not as severe as what we observed in \([\text{axl1}\Delta \times \text{Wt}\)] prezygotes. DAPI staining of \([\text{rsr1}\Delta \times \text{rsr1}\Delta]\) prezygotes revealed that nuclei were aligned with a frequency of 22%, which was greater than the frequencies observed for \([\text{fus2}\Delta \times \text{fus2}\Delta]\) or \([\text{axl1}\Delta \times \text{axl1}\Delta]\) (Table IV) but less than wild-type. As with the \([\text{axl1}\Delta \times \text{Wt}\)] prezygotes, we often observed that one of the cells in the \([\text{rsr1}\Delta \times \text{Wt}\)] prezygotes bulged out over each edge of the cell–cell contact region. The distorted cell in \([\text{rsr1}\Delta \times \text{Wt}\)] pairs often contained the misaligned nucleus. Overall, the \text{rsr1} defects resembled those of the \text{axl1} cells but appeared less severe.

**Accumulation of Vesicles in Prezygotes**

We noticed that vesicles often accumulated in prezygotes formed by the \text{axl1}, \text{rsr1}, and \text{fus2} mutant strains (Fig. 2). In \([\text{Wt} \times \text{Wt}\)] prezygotes, apparent vesicles \(\sim\)100 nm in size were occasionally observed poised on either side of the cell–cell contact region, suggesting that they might play a role in fusion processes (4, 17). In many of the \([\text{axl1}\Delta \times \text{Wt}\)] prezygotes, we observed that vesicles had accumulated to a larger extent in the presumptive (swollen) \text{axl1} partner than in the presumptive wild-type partner or in \([\text{Wt} \times \text{Wt}\)] prezygotes (Fig. 2, A–F). It was interesting that in the partner with fewer vesicles in \([\text{axl1}\Delta \times \text{Wt}\)] prezygotes, we observed what appeared to be vesicles fusing with the plasma membrane. However, in the partner cell with excess vesicles, we never observed a presumptive vesicle fusion event (Fig. 2). A similar vesicle over-accumulation pattern was observed in \([\text{fus2}\Delta \times \text{Wt}\)] and in \([\text{rsr1}\Delta \times \text{Wt}\)] prezygotes.

We determined the relative vesicle accumulation in the prezygotes (Table V). To quantitate vesicle accumulation in \([\text{mutant} \times \text{Wt}\)] crosses, we calculated a ratio of the (highest vesicle number)/(lowest vesicle number) for cell pairs. We found that vesicles were present at a ratio of 1.4 in \([\text{Wt} \times \text{Wt}\)] prezygotes. Thus, in \([\text{Wt} \times \text{Wt}\)] prezygotes both cells tended to have a similar number of vesicles in EM slices in which vesicles were visible. A similar ratio of 1.7 was measured for the \([\text{Wt} \times \text{ste6}\text{(cef1)}]\) prezygotes (17), which resembled \([\text{Wt} \times \text{Wt}\)] prezygotes in structure. In contrast, the \([\text{axl1}\Delta \times \text{Wt}\)] prezygotes accumulated vesicles at a ratio of 4.0, and the \([\text{rsr1}\Delta \times \text{Wt}\)] prezygotes accumulated vesicles at a ratio of 4.6. The \([\text{fus2}\Delta \times \text{Wt}\)] prezygotes accumulated vesicles at a ratio of 2.7. Thus,

![Figure 3](image-url)
Table V. Ratio of Vesicle Accumulation Observed by Electron Microscopy

| Cross          | Relevant genotype | Vesicle accumulation differential* |
|----------------|-------------------|-----------------------------------|
| LM23-3az × FC139 | Wt × Wt | 1.4 |
| LM23-3az × LM110p131 | Wt × cef1-1 | 1.7 |
| LM23-3az × FC272 | Wt × fus2-Δ1 | 2.7 |
| LM23-3az × LE17a | Wt × axl1-Δ2 | 4.0 |
| LM23-3az × LEb1  | Wt × rsr1Δ:URA3 | 4.6 |

*Ratio of partner with largest number of vesicles/partner, with smaller number of vesicles. The number of mating cell pairs scored for each cross were: Wt × Wt, 5; Wt × cef1-1, 1; Wt × fus2-Δ1, 4; Wt × axl1-Δ2, 5; Wt × rsr1Δ:URA3, 5.

some but not all of the fusion mutants led to an asymmetry of vesicle accumulation. The cells studied did not represent a large enough pool for us to be conclusive on this point, though the difference did appear striking. Also, an assumption of this type of calculation is that the lowest number of vesicles represents wild-type and the highest number of vesicles represents the mutant. We did not attempt to determine which was wild-type and which was mutant in this analysis. Vesicle quantitation also is made difficult by the fact that not every EM slice contained vesicles.

**Light Microscopy of [axl1Δ × Wt] and [rsr1Δ × Wt] Prezygotes**

Prezygotes of both [axl1Δ × Wt] and [rsr1Δ × Wt] were characterized by light microscopy. To distinguish mutant and wild-type cells in prezygotes, we rendered one of the partner strains fluorescent. A construct that expressed GFP fused to a constitutively expressed L32 ribosomal gene promoter was introduced into one partner. Prezygotes were identified as cell pairs containing only one fluorescent cell (16). Partial fusion, if it occurred, or full fusion could also be identified because the GFP marker is localized to the cytoplasm and thus provided a means to monitor a breach in the septum leading to cytoplasmic mixing and staining of the whole zygote.

The prezygotic structures formed in LE49a/LM104 [axl1Δ × Wt] mating mixtures appeared to be distorted as expected from the ultrastructural analysis (Fig. 3, E–H). The mutant cell tagged with GFP was swollen in size relative to the wild-type partner. Often we observed in [axl1Δ × Wt] prezygotes a bulging of the mutant partner cell on either side of the cell contact region, as observed by electron microscopy. It sometimes appeared that the mutant was “engulfing” the wild-type partner cell. In addition, the cell–cell contact region in [axl1Δ × Wt] prezygotes appeared to be wider than the contact region observed in [ste6(cef1) × Wt] prezygotes (Fig. 3, K and L), which were similar to [Wt × Wt] prezygotes. We observed that the vacuole was swollen in mating axl1 mutant cells but not in wild-type or ste6(cef1) cells. When the L32–GFP fusion protein was expressed in the wild-type partner cells rather than the mutant, the axl1 partner was still larger. Thus, the altered morphology of the GFP-tagged axl1Δ cells was not an artifact due to the presence of GFP. The rsr1 partner (LE1b) in [rsr1Δ × Wt] crosses appeared, like axl1, to be the larger of the two cells in mating pairs as determined by the GFP assay (Fig. 3, I and J).

**An axl1Δ Strain Exhibits a Partial Defect in Morphogenic Response to α-Factor**

Since axl1 cells in mating cell pairs appeared to have an aberrant morphological appearance, we wished to determine if the mutant cells exhibited morphological defects when treated with pheromone in the absence of a partner. We found that differing conditions of α-factor treatment led to the formation of cells with widely differing morphologies. Under some of the conditions used, we observed that axl1 cells formed mating projections that were similar to projections formed by the wild-type cells. However, treatment of either axl1 (LE1B3) or wild-type (LM23-3az) cells with 40 nM α-factor for 6 h led to a distinct difference in the appearance of mating projections. The axl1 cells formed a heterogeneous mixture of mating projections that appeared overall to be broader than normal (“dumpy”) with a mixture of other morphological forms, including undeformed cells and a small number of cells displaying normal mating projections (Fig. 4 A). Under these treatment conditions, the wild-type cells formed a more uniform population of mating projections, giving cells the classic pear-shaped morphology. At a higher pheromone concentration (400 nM, 4 h), the axl1 morphogenic defect persisted but was more subtle. The axl1 cells formed mating projections that were shorter and had rounder tips than wild-type shmoos (Fig. 4 B).

Using a quantitative growth inhibition assay, we found...
that an axl1 strain had an α-factor sensitivity that was equivalent to that of the isogenic wild-type strain (data not shown). The altered morphology observed for the axl1 strain did not appear to be due to perturbation of the underlying actin cytoskeleton, as visualized by staining actin with rhodamine-conjugated phalloidin (data not shown). Also, the axl1 strain appeared to orient properly in a micropipette assay designed to measure chemotropic responses towards a source of pheromone (data not shown) (50).

An rsr1 strain also appeared to form mating projections that were altered in shape compared with mating projections formed by wild-type cells and appeared more defective than mating projections formed by axl1 cells (Fig. 4 B). The axl1 and rsr1 morphogenic defects appeared to be similar to, but much less extreme than that reported for spa2, pea2, and tpml mutants (27, 32, 55).

**Ax11p Protease Activity Is Required for Cell Fusion and Morphogenesis**

AXL1 encodes a metalloprotease with homology to insulin-degrading enzymes (1, 25). Ax11p protease activity is required for a-factor pheromone processing but appears dispensable or required at a lower level for axial bud site selection functions (1). The role of the Ax11p protease in cell fusion during mating was determined. We created a novel protease-defective allele of Ax11p by an in-frame deletion spanning codon 848 to codon 1142. The deletion encompassed a region that lies outside of the presumed metal-binding domain, but which has high homology to mammalian and Drosophila insulinases (1, 25). We first tested the deletion allele for in vivo protease function by assaying for the ability to promote a-factor pheromone processing in MATα axl1 cells. The construct expressing the protease deletion allele, pBA1, failed to rescue the a-factor production defect of the MATα axl1 strain (LE1B3; data not shown). When axl1Δ (848–1142) was expressed in either the MATα strain, LE1B3 (not shown), or in the MATα axl1Δ strain, LE17α, axial bud site selection was restored to a level similar to that provided by the wild-type AXL1 gene (Table VI). By these assays, we could not distinguish a complete protease defect from a severe but partial defect. As a comparison, we tested previously characterized point substitution mutations axl1-H68A and axl1-E71A of AXL1 that inactivate a metal-binding site required for protease activity (1). The axl1 metal-binding site mutants complemented the bud site selection defect conferred by axl1 mutations in our strain background as reported by others. These substituted proteins have been shown to accumulate at steady-state levels that were indistinguishable from wild-type levels in the cell (1).

We tested the AXL1 mutant alleles with apparent protease deficiencies for the ability to promote cell fusion in the MATα axl1Δ strain, LE17α. The axl1Δ (848–1142), axl1-H68A, or axl1-E71A protease-defective alleles failed to rescue the cell fusion defect. The cell fusion defect of LE17α was rescued by wild-type AXL1 expressed from a CEN-based plasmid (Table VI).

The ability of protease-defective alleles of axl1 to complement the morphogenic defects of an axl1 strain was determined. Mutant axl1 strains expressing the protease-defective alleles (LE1B3/pBA1 [not shown] or p126 [axl1-H68A]; Fig. 5 B) were exposed to 40 nM α-factor for 6 h. The protease-defective alleles of AXL1 failed to promote normal morphogenesis in response to α-factor. Thus, Ax11p protease activity appears to be required for efficient morphogenesis and cell fusion during mating.

**Constitutive Activation of RSR1 Inhibits Cell Fusion in Wild-Type Cells**

RSR1 encodes a small GTPase that plays a role in bud site selection. It has been proposed that Rsr1p must cycle between an active and inactive state in order to carry out its role in bud site selection (2). Constitutively activated alleles of RSR1 fail to function in bud site selection. We wanted to determine if Rsr1p functioned in a similar manner during cell fusion, or whether constitutive Rsr1p activation was sufficient for its function in mating. We expressed a constitutively activated allele of RSR1 (rsr1val12) in the MATα rsr1 strain, LEab1 (2). The rsr1val12 strain accumulated 51% prezygotes when mated to a parental tester strain (FC139). LEab1 expressing a wild-type copy of RSR1 accumulated only 7% prezygotes under these conditions (Table VII). This observation indicated that the rsr1val12 allele of RSR1 was unable to provide a Rsr1p function needed in cell fusion. In addition, the activated allele acted in a dominant-negative fashion in a wild-type strain (LM104). The RSR1/rsr1val12 strain LM104(pPB264) accumulated 41% prezygotes when crossed to the wild-type partner strain, FC139. The rsr1val12-activated allele also caused randomization of the bud site selection pattern in the wild-type transformed strain and failed to suppress the budding defect of the rsr1Δ strain, LEab1 (Table VII), as previously reported (2).

**Subcellular Localization of Cdc42p in axl1 and rsr1 Cells**

We wanted to determine if either Ax11p or Rsr1p was involved in localizing Cdc42p during mating. During budding, Cdc42p acts with the bud site selection components, which include Rsr1p, to orient the actin cytoskeleton toward a specific site on the cell surface that will give rise to a bud. During mating, Cdc42p is required to polarize the cytoskeleton in response to pheromone, resulting in pear-shaped cells. Ax11p or Rsr1p might be involved in recruit-
ing Cdc42p to the site on the cell surface where cell fusion would occur. We looked at the subcellular localization of Cdc42p in MATa axl1 (LE74x) and MATa rsr1 (LEab1) mutant strains by indirect immunofluorescence microscopy using anti-Cdc42p antibodies (60). Cdc42p was localized to the mating projection tip of α-factor–treated axl1 and rsr1 mutant cells (Fig. 6). Localization of Cdc42p in the axl1 and rsr1 mutant strains was equivalent to that observed in the parent cells (LM23-3az) and also to what had been described previously by Ziman et al. (60). Occasionally, we observed that the Cdc42p cap staining patterns were slightly offset in the axl1 and rsr1 mutant strains, but the significance, if any, is unclear. Axl1p and Rsr1p appear to be not required for the proper polarization of Cdc42p to the mating projection tip formed in response to pheromone.

**Discussion**

We have found that Axl1p, Rsr1p, and Bud3p components of haploid bud site selection processes in yeast are required for efficient cell morphogenesis and fusion during mating. Axl1p protease activity was required for mediating morphological responses during mating and cell fusion. Rsr1p might mediate a connection to pheromone signaling since it physically interacts with Cdc24p and Bem1p, which in turn interact with the β subunit of G protein coupled to pheromone receptors and the Ste20p (PAK) kinase controlling polarity responses, respectively (31, 43, 51, 58). The group of products that function together for bud site selection in vegetative cells could serve, under pheromone receptor control, to mediate a cell fusion program during mating. AXL1 genetically acted in the same pathway as FUS2, and an axl1 defect produced many of the same ultrastructural defects during mating observed in fus2 strains. We propose that AXL1 and other bud site selection genes may act in conjunction with components of the pheromone response pathway and FUS2 to establish a fusion zone at the cell surface.

Our work may point to control of cell fusion by pheromone signaling at the site of cell–cell contact. Previous speculation on control of cell fusion by the pheromone response has focused solely on transcriptional control of expression of genes like FUS1 and FUS2 (19, 54). Additional control of fusion at the cell surface is likely. The haploid bud site selection machinery appears to be associated with the cell fusion site (as described below). Bud site selection proteins in turn associate with components of the pheromone pathway and may be capable of transmitting signals that control fusion processes. It makes sense that spatial definition of the point of cell–cell contact transmitted by

**Table VII. Ability of the Activated Allele of RSR1, rsr1val12, to Function in Cell Fusion and Bud Site Selection**

| MATa strain/plasmid | RSR1 allele | Prezygote accumulation* | Bud site selection‡ |
|---------------------|-------------|-------------------------|---------------------|
|                     |             | Axial | Bipolar | Random |
| LEab1/pPB161        | wt          | 7.2  | 84.0   | 7.0    | 9.0    |
| LEab1/pPB264        | rsr1val12   | 51.4 | 15.6   | 12.8   | 71.6   |
| LM104/pPB161        | wt, wt      | 3.8  | 88.3   | 9.8    | 1.9    |
| LM104/pPB264        | rsr1val12   | 41.0 | 24.4   | 46.5   | 29.1   |

*Prezygote accumulation was determined by microscopic observation as described in Materials and Methods. More than 200 mating pairs were scored for each strain pair. The MATa partner was FC139.

‡Budding patterns were observed for 100 microcolonies.

**Figure 5.** Pheromone-induced morphologies of a strain defective for Axl1p protease activity. LE1B3 [axl1Δ] with various plasmids derived from p129 treated with α-factor (40 nM) for 6 h. (A) LE1B3/p129 (pAXL1); (B) LE1B3/p126 (paxl1-H68A); (C) LE1B3/pRS316 (pVector).

**Figure 6.** Localization of Cdc42p. MATa cells were treated with α-factor for 2.75 h. Cdc42p was visualized by indirect immunofluorescence. Isogenic strains were (top) LM23-3az [Wt], (middle) LE74x [axl1Δ], (bottom) LEab1 [rsr1].
cell surface pheromone receptors might act to control the activity of the fusion machinery. We envison that in addition to transcriptional processes, localized pheromone-activated signals control regulated secretion, cell wall changes, and cytoskeletal changes needed for cell fusion. Future studies will be required to determine whether the connection between Fus2p function and localized activation of receptors is as simple as this model suggests. The reason that strains underexpressingpheromones exhibit a mating fusion defect could be that the local activation of fusion processes that we propose requires a higher level of receptor occupancy than is required for initiation of early mating responses (39).

Analysis of axl1 fused double mutant strains indicated that AXL1 functioned in the same cell fusion pathway as Fus2 but not Fus1. Fus2 encodes a novel protein that, along with Fus1, is dedicated to cell fusion during mating. Fus1 and Fus2 appear to act in distinct cell fusion pathways with some overlapping aspects (19, 37, 54). Both Fus1 and Fus2 mutant strains are defective in completing cell wall degradation before membrane fusion (37, 54). The identification of additional components acting in one of these established pathways will greatly help the elucidation of the mechanism of fusion since many genes involved in fusion appear to act independently of one another (26). Localization studies of the Fus2p indicated that it may be associated with the cytoskeleton, the contact region between mating cells, and with vesicles localized to the mating projection tip (19). In addition, Fus2p is required for proper nuclear alignment during mating (19). Our electron microscopy analysis revealed that axl1 conferred an overall phenotype that was similar to a fus2 mutant strain. We found that like a fus2 mutant strain, our axl1 mutant strain displayed a defect in nuclear alignment. In addition, both axl1 and fus2 mutant strains often accumulated vesicles at the presumptive fusion site, suggesting a defect in vesicle fusion. In wild-type prezygotes, vesicles were localized to, but not grossly accumulated at, a spatially restricted site within the cell--cell contact region. It may be that AXL1, RSRI, and Fus2 are involved in a process that includes marking the fusion site for microtubule capture and for vesicle fusion.

The vesicle accumulation and cell wall defects observed in the axl1 and fus2 mutants are reminiscent of defects observed for some of the sec mutants. A mutation in SEC3 led to vesicle accumulation in vegetative cells (23). In addition, sec3 mutant cells often accumulated electron dense material in the mother--bud septum region, suggesting that faulty secretion may lead to cell wall defects (23). We observed in several of the [axl1Δ × Wt] prezygotes an accumulation of electron-dense material within the cell wall at the periphery of the cell--cell contact region. In addition, in the light micrographs of [axl1Δ × Wt] prezygotes, the presumed axl1 cell appeared to be “engulfing” the partner cell, which might reflect a change in cell wall plasticity. We believe that these cell wall phenotypes could be the result of misdirected vesicles carrying cell wall degradative enzymes. Our wild-type prezygotes arrested with a smoothly fused, contiguous cell wall and appeared to restrict degradation of the cell wall to one point within the cell--cell contact zone. Whether Axl1p is required for properly localized secretion during mating remains to be tested. It is possible that, like Sec3p, Axl1p plays a role in defining a site on the cell surface where vesicle fusion is directed. Axl1p plays several, apparently independent roles in the cell and hence may not be localized solely to the cell surface (1, 7, 10). The NH2-terminal half of Fus2, like SEC2, contains homology to myosin (19, 40), and Fus2p appears partly to be localized on vesicles. Perhaps an alternative secretory pathway is activated during mating.

Axl1p is a member of a large family of metalloendoproteases that include insulinase and N-arginine dibasic convertase (1, 25). We found that an Axl1p protease function is required for morphogenesis and cell fusion during mating. It is interesting to note that N-arginine dibasic convertase has been proposed to be involved in spermatid morphogenesis (14). Proteases of this family usually process small peptides or clip the NH2 terminus of larger proteins (10, 24, 44). It is possible that Axl1p is required to proteolytically process the NH2 terminus of a larger protein involved in morphogenesis or cell fusion. One possible fusion candidate is Fus2p. Fus2p contains a potential site for Ax1p cleavage between residues 12 and 13, a region with homology to myosin (Elia, L., and L. Marsh, unpublished observation). Two-hybrid analysis suggests that Ax1p could be part of a larger complex that includes Fus2p (Boone, C., personal communication). Members of the PAK family of kinases that include Ste20p and Cla4p, which are involved in pheromone signaling and cytoskeletal control, can be activated by proteolysis and could be candidate morphogenesis targets (45).

AXL1 expression is limited to haploid, mating-competent cells and is induced in response to mating pheromones in both MATa and MATα cells (1, 25) (Boone, C., personal communication). These features are commonly found in genes involved in mating (19, 20, 54). Axl1p was previously shown to be required for efficient mating of MATα cells via its role in production of the α-factor mating pheromone (4). The role of Axl1 in MATα cells appears to be limited to pheromone response rather than pheromone production. We, like others, found that Ax1p activity is not required for α-factor processing or secretion (4). Though the axl1 strains exhibited morphogenic defects in mating, the wild-type and axl1 strains appeared equally responsive to the growth arrest activity of α-factor, suggesting that Ax1p does not affect mating via effects on Fus3p (MAP kinase) activation. The appearance of axl1 strains in the absence of pheromone was normal. In addition to AXL1, we found a requirement for two other bud site selection genes, RSRI and BUD3, in cell fusion during mating. These genes are believed to act together with AXL1 during vegetative growth, and they may play mechanistically related roles during cell fusion.

Prior evidence suggested that the bud site selection gene products remain functional during mating. First, cells exposed to pheromone and then allowed to resume vegetative growth have been observed to site the first bud at the mating projection tip, suggesting that bud site selection components can be diverted away from the previous bud site and redirected toward the site of concentrated pheromone signaling (53). Second, it has been shown that mutations in RSRI, BUD2, BUD3, and BUD4 cause cells to alter the site of projection formation from an axial to a

Elia and Marsh Morpogenesis and Cell Fusion in Yeast 1483
random placement in response to a uniform concentration of pheromone, suggesting that they have the ability to influence the direction of pheromone-induced cytoskeletal polarization (34). Third, mutations in the diploid bud site selection genes, BNII (22), SPA2 (27), PEA2 (13, 55), and BUD6/AIP3 (22), also have been shown to influence projection formation and mating. Finally, special alleles of CDC24 have been isolated that cause defects in mating projection formation and mating cell orientation but not in budding during vegetative growth (13, 41). When present in mating cells, these CDC24 alleles appear to disconnect pheromone responses from cell polarization, causing the cells to form mating projections next to the bud site (default locus) rather than toward the source of pheromone. Thus, in normal mating cells, bud site selection products may be recruited to the site of cell fusion. Down regulation of two bud site selection components, Bud4p and Axl2p, during mating has been previously reported (46, 49). These observations may indicate that bud site selection is replaced by a distinct but mechanistically related process during mating. It is intriguing to speculate that pheromone-induced proteins such as Fus2p or Fus1p might re-appear under these conditions.

The Journal of Cell Biology, Volume 142, 1998 1484

We are grateful to Alan Bender, John Chant, Charlie Boone, Jonathan Warner, Elaine Elion, Joshua Trueheart, Ira Herskowitz, and Fred Chang for strains and plasmids; Doug Johnson for anti-Cdc42 antibodies; Neil Adames and Charlie Boone for communication of results before publication; Jeffrey Segall for help with cell orientation assays; Frank Macaluso and Leslie Gunther for expert technical assistance with electron microscopy; and Anjani Shah for helpful comments on the manuscript.

This work was supported by the Cancer Core Support Grant P30CA13330 from the National Cancer Institute.

Received for publication 13 May 1998 and in revised form 17 July 1998.

References

1. Adames, N., K. Blundell, M.N. Ashby, and C. Boone. 1995. Role of yeast insulin-degrading enzyme homologs in propheromone processing and bud site selection. Science. 270:464–467.
2. Bender, A. 1993. Genetic evidence for the roles of the bud-site-selection genes BUD3 and BUD2 in control of the Rsr1p (Bud1p) GTPase in yeast. Proc. Natl. Acad. Sci. USA. 90:9926–9929.
3. Bender, A., and J.R. Pringle. 1989. Multicopy suppression of the CDC24 budding defect in yeast by CDC42 and three newly identified genes including the res-related gene RS1. Proc. Natl. Acad. Sci. USA. 86:9976–9980.
4. Brizzio, V., A.E. Gammie, G. Nijbroek, S. Michaelis, and M.D. Rose. 1996. Cell fusion during yeast mating requires high levels of a-factor mating pheromone. J. Cell Biol. 135:1727–1739.
5. Brizzio, V., A.E. Gammie, and M.D. Rose. 1998. Rs161p interacts with Fus2p to promote cell fusion in Saccharomyces cerevisiae. J. Cell Biol. 141:567–584.
6. Chan, K.K., and C.A. Otte. 1982. Isolation and genetic analysis of Saccharomyces cerevisiae mutants supersensitive to G1 arrest by a factor and a factor pheromones. Mol. Cell. Biol. 2:11–20.
7. Chant, J. 1996. Generation of cell polarity in yeast. Curr. Opin. Cell Biol. 8:557–565.
8. Chant, J., and I. Herskowitz. 1991. Genetic control of bud site selection in yeast by a set of gene products that constitute a morphogenetic pathway. Cell. 65:1203–1212.
9. Chant, J., M. Mischke, E. Mitchell, I. Herskowitz, and J.R. Pringle. 1995. Role of Bud3 in producing the axial budding pattern of yeast. J. Cell Biol. 129:767–778.
10. Chen, P., S.K. Sapperstein, J.D. Choi, and S. Michaelis. 1997. Biogenesis of the Saccharomyces cerevisiae mating pheromone a-factor. J. Cell Biol. 136:251–269.
11. Chenevert, J. 1994. Cell polarization directed by extracellular cues in yeast. Mol. Biol. Cell. 5:1169–1175.
12. Chenevert, J., K. Corrado, A. Bender, J. Pringle, and I. Herskowitz. 1992. A yeast gene (BEM1) necessary for cell polarization whose product contains two SH3 domains. Nature. 356:77–79.
13. Chenevert, J., N. Valz, and I. Herskowitz. 1994. Identification of genes required for normal pheromone-induced cell polarization in Saccharomyces cerevisiae. Genetics. 136:1287–1297.
14. Chesneau, V., A. Prat, D. Secretan, V. Hospital, A. Dupax, T. Foulong, B. Jegou, and P. Cohen. 1996. NRD convertase: a putative processing endoprotease associated with the axoneme and the manchette in late spermatids. J. Cell Sci. 109:2737–2745.
15. Dobberstein, S.K., R.D. Fetter, A.Y. Mehta, and C.S. Goodman. 1997. Genetic analysis of myoblast fusion: blown fuse is required for progression beyond the prefusion complex. J. Cell Biol. 136:1249–1261.
16. Dorer, R., C. Boone, T. Kimbrough, J. Kim, and L.H. Hartwell. 1997. Genetic analysis of default mating behavior in Saccharomyces cerevisiae. Genetics. 146:39–55.
17. Elia, L., and L. Marsh. 1996. Role of the ABC transporter Ste6 in cell fusion during yeast conjugation. J. Cell Biol. 135:741–751.
18. Elion, E., P.L. Grisafi, and G.R. Fink. 1990. Fus3 encodes a cdc2/cdc28-related kinase required for the transition from mitosis into conjugation. Cell. 60:649–664.
19. Elion, E.A., J. Trueheart, and G.R. Fink. 1995. Fus2 localizes near the site of cell fusion and is required for both cell fusion and nuclear alignment during zygote formation. J. Cell Biol. 130:1283–1296.
20. Erdman, S., L. Lin, M. Malczynski, and M. Snyder. 1998. Pheromone-regulated genes required for yeast mating differentiation. J. Cell Biol. 140:461–483.
21. Erickson, M.R.S., B.J. Galletta, and S.M. Abmayr. 1997. Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. J. Cell Biol. 138:589–603.
22. Evangelista, M., K. Blundell, M.S. Lontine, C.J. Chow, N. Adames, J.R. Pringle, M. Peter, and C. Boone. 1997. Bnl1p, a yeast formin linking cdc42p and the actin cytoskeleton during polarized morphogenesis. Science. 276:118–122.
23. Finger, E.P., T.E. Hughes, and P. Novick. 1998. Sec3p is a spatial landmark

The Journal of Cell Biology, Volume 142, 1998 1484
for polarized secretion in budding yeast. Cell. 92:559–571.

24. Fricke, B., R. Betz, and S. Friebe. 1995. A periplasmic insulin-cleaving proteinase (ICP) from Actinobacter calcoaceticus-sharing properties with protease III from Escherichia coli and IDE from eucaryotes. J. Basic Microbiol. 35:21–31.

25. Fujita, A., C. Oka, Y. Arikawa, T. Katagai, A. Tonouchi, S. Kuhara, and Y. Misumi. 1994. A yeast gene necessary for bud-site selection encodes a protein similar to insulin-degrading enzyme. Nature. 372:567–570.

26. Gammie, A.E., V. Brizzio, and M.D. Rose. 1998. Distinct morphological phenotypes of cell fusion mutants. Mol. Biol. Cell. 9:1395–1410.

27. Gehring, S., and M. Snyder. 1990. The SPA2 gene of Saccharomyces cerevisiae is important for pheromone-induced morphogenesis and efficient mating. J. Cell Biol. 111:1451–1464.

28. Hall, A. 1994. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu. Rev. Cell Biol. 10:31–54.

29. Herskowitz, I., H.O. Park, S.L. Sanders, N. Valtz, and M. Peter. 1995. Programming of cell polarity in budding yeast by endogenous and exogenous signals. Cold Spring Harbor Symp. Quant. Biol. 60:717–727.

30. Kurihara, L.J., C.T. Beh, M. Laiterich, R. Schekman, and M.D. Rose. 1994. Nuclear congression and membrane fusion: two distinct events in the yeast karyogamy pathway. J. Cell Biol. 126:911–924.

31. Leeu, T., A. Fourastie-Lieuvin, C. Wu, T. Chennevert, K. Clark, M. White-way, D.Y. Thomas, and E. Lebèrer. 1995. Pheromone response in yeast: association of Bemlp with proteins of the MAP kinase cascade and actin. Science. 270:1210–1213.

32. Liu, H., and A. Bretscher. 1992. Characterization of a coiled-coil domain essential for vesicular transport and a dispensable carboxy-terminal domain. J. Biol. Chem. 267:630–636.

33. Luo, L., Y. Liao, L. Jan, and Y. Jan. 1994. Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev. 8:1395–1410.

34. Marsh, L. 1992. Substitutions in the hydrophobic core of the a-factor pheromone of Saccharomyces cerevisiae. Changes in the fine structure during the mating reaction. Arch. Microbiol. 97:27–38.

35. Park, H.O., E. Bi, J.R. Pringle, and I. Herskowitz. 1997. Two active states of the Ras-related Bud1/Ras1 protein bind to different effectors to determine yeast cell polarity. Proc. Natl. Acad. Sci. USA. 94:4463–4468.

36. Marsh, L., and M.D. Rose. 1997. The pathway of cell and nuclear fusion. Mol. Biol. Cell. 8:1395–1410.

37. McCaffrey, G., F. Clay, K. Kelsey, and G.F. Sprague, Jr. 1987. Identification and regulation of a gene required for cell fusion during mating of the yeast Saccharomyces cerevisiae. Mol. Cell. Biol. 7:2680–2690.

38. Michalis, S., and I. Herskowitz. 1988. The a-factor pheromone of Saccharomyces cerevisiae is essential for mating. Mol. Cell. Biol. 8:1309–1318.

39. Moore, S.A. 1983. Comparison of dose-response curves for a factor-induced cell division arrest, agglutination, and projection formation of yeast cells. J. Biol. Chem. 258:13849–13856.

40. Nair, J., H. Muller, M. Peterson, and P. Novick. 1990. Sec2 protein contains a coiled-coil domain essential for vesicular transport and a dispensible carboxy-terminal domain. J. Cell Biol. 110:1897–1909.

41. Nern, A., and R.A. Arkowitz. 1998. A GTP-exchange factor required for cell orientation. Nature. 391:195–198.

42. Osumi, M., C. Shimoda, and N. Yanagishima. 1974. Mating reaction in Saccharomyces cerevisiae. Changes in the fine structure during the mating reaction. Arch. Microbiol. 97:27–38.

43. Park, H.O., E. Bi, J.R. Pringle, and I. Herskowitz. 1997. Two active states of the Ras-related Bud1/Ras1 protein bind to different effectors to determine yeast cell polarity. Proc. Natl. Acad. Sci. USA. 94:4463–4468.

44. Pierotti, A.R., A. Prat, V. Chesneau, F. Gaudoux, A.M. Leseney, T. Fou-lon, and P. Cohen. 1994. N-arginine dibasic convertase, a metalloendoproteinase as a prototype of a class of processing enzymes. Proc. Natl. Acad. Sci. USA. 91:6078–6082.

45. Ramos, E., R. Wysolmerski, and R. Masaracchia. 1997. Myosin phosphorylation by human cdc42-dependent S6/H4 kinase/gammaPAK from placa-centa and lymphoid cells. Recept. Signal Transduct. 7:99–110.

46. Roeper, T., K. Madden, J. Chang, and M. Snyder. 1996. Selection of axial growth sites in yeast requires Ax1p, a novel plasma membrane glycoprotein. Genes Dev. 10:777–793.

47. Rose, M.D., P. Novick, J.H. Thomas, D. Botstein, and G.R. Fink. 1987. A Saccharomyces cerevisiae genomic plasmid bank based on a centromere-containing shuttle vector. Gene. 60:237–243.

48. Rose, M.D., F. Winston, and P. Hieter. 1990. Methods in Yeast Genetics: A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 198 pp.

49. Sanders, S.L., and I. Herskowitz. 1996. The BUD4 protein of yeast, required for axial budding, is localized to the mother/BUD neck in a cell cycle-dependent manner. J. Cell Biol. 134:413–427.

50. Segall, J.E. 1993. Polarization of yeast cells in spatial gradients of a mating factor. Proc. Natl. Acad. Sci. USA. 90:8332–8336.

51. Simon, M.N., C. De Virgilio, B. Souza, J.R. Pringle, A. Abo, and S.I. Reed. 1995. Role for the Rho-family GTPase Cdc42 in yeast mating-pheromone signal pathway. Nature. 376:702–705.

52. Sprague, G.F., Jr. 1991. Assay of yeast mating reaction. Methods Enzymol. 194:77–93.

53. Tkacz, J.S., and V.L. MacKay. 1979. Sexual conjugation in yeast: cell surface changes in response to the action of mating hormones. J. Cell Biol. 80:326–333.

54. Truchheart, J., J.D. Boeke, and G.R. Fink. 1987. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. Mol. Cell. Biol. 7:2316–2328.

55. Valtz, N., and I. Herskowitz. 1996. Pea2 protein of yeast is localized to sites of polarized growth and is required for efficient mating and bipolar budding. J. Cell Biol. 135:725–739.

56. White, J.M. 1992. Membrane fusion. Science. 258:917–924.

57. Wright, R., M. Basson, L. D’Ari, and J. Rine. 1988. Increased amounts of HMG-CoA reductase induce “karmellae”: a proliferation of stacked membrane pads surrounding the yeast nucleus. J. Cell Biol. 107:101–114.

58. Zhao, Z.S., T. Leung, E. Manser, and L. Lim. 1995. Pheromone signalling in Saccharomyces cerevisiae requires the small GTP-binding protein Cdc42p and its activator CDC24. Mol. Cell. Biol. 15:5246–5257.

59. Zheng, Y., A. Bender, and R.A. Cerione. 1995. Interactions among proteins involved in bud-site selection and bud-site assembly in Saccharomyces cerevisiae. J. Biol. Chem. 270:626–630.

60. Ziman, M., D. Preuss, J. Mulholland, J.M. O’Brien, D. Botstein, and D.J. Johnson. 1993. Subcellular localization of Cdc42p, a Saccharomyces cerevisiae GTP-binding protein involved in the control of cell polarity. Mol. Biol. Cell. 4:1307–1316.