Prm1 Targeting to Contact Sites Enhances Fusion during Mating in *Saccharomyces cerevisiae* \(^\dagger\)†

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Prm1 is a pheromone-regulated membrane glycoprotein involved in the plasma membrane fusion event of *Saccharomyces cerevisiae* mating. Although this function suggests that Prm1 should act at contact sites in pairs of mating yeast cells where plasma membrane fusion occurs, only a small percentage of the total Prm1 was actually detected on the plasma membrane. We therefore investigated the intracellular transport of Prm1 and how this transport contributes to cell fusion. Two Prm1 chimeras that were sorted away from the contact site had reduced fusion activity, indicating that Prm1 indeed functions at contact sites. However, most Prm1 is located in endosomes and other cytoplasmic organelles and is targeted to vacuoles for degradation. Mutations in a putative endocytosis signal in a cytoplasmic loop partially stabilized the Prm1 protein and caused it to accumulate on the plasma membrane, but this endocytosis mutant actually had reduced mating activity. When Prm1 was expressed from a galactose-regulated promoter and its synthesis was repressed at the start of mating, vanishingly small amounts of Prm1 protein remained at the time when the plasma membranes came into contact. Nevertheless, this stable pool of Prm1 was retained at polarized sites on the plasma membrane and was sufficient to promote plasma membrane fusion. Thus, the amount of Prm1 expressed in mating yeast is far in excess of the amount required to facilitate fusion.

Membrane fusion has been studied extensively in the context of viral infection and intracellular membrane fusion. These fusion events are mediated by fusases—proteins that mediate membrane fusion. Some of the best-studied fusases are the SNAREs (soluble N-ethylmaleimide-sensitive factors) that mediate fusion of intracellular organelles and the hemagglutinin (HA) protein of influenza virus that mediates fusion of the viral envelope membrane with host endosomes (13). However, little is known about how the plasma membranes of two cells fuse during cell fusion.

Cell fusion is essential for the development of multicellular organisms. Some cell fusion processes involve a single pair of cells, as in sperm-egg fusion. Many other developmental processes require multiple fusion events, as in fusion of myoblasts for muscle formation. However, all fusion events must overcome a common obstacle—maintaining the integrity and selective permeability of the two plasma membranes while fusing the hydrophobic cores of their phospholipid bilayers.

We study cell fusion in mating pairs of the yeast *Saccharomyces cerevisiae*. This organism offers a genetically tractable model amenable to many biochemical and cell biological assays. The mating pathway in yeast is comprised of 5 steps: pheromone signaling, adhesion, degradation of the intervening cell walls, plasma membrane fusion, and karyogamy. *S. cerevisiae* has two haploid mating types: *MATa* and *MATα*. Haploid cells secrete pheromones that bind to G-protein-coupled receptors on the surface of cells of the opposite mating type. Pheromone binding activates a signaling cascade that causes cell cycle arrest, expression of pheromone-inducible genes, and polarized growth to form a mating projection (or shmoo tip). The binding of two cells of opposite mating type to form a mating pair is mediated by complementary agglutinins located on the shmoo tips. Then, the cell walls of the two cells are joined to form a unified wall protecting the mating pair, and the walls between the two cells are degraded. This allows the plasma membranes to come into contact and fuse. The initial fusion pore between cells expands to allow cytoplasmic mixing and, ultimately, karyogamy. After mating is complete, the mitotic cell cycle resumes, and a diploid daughter cell buds from the neck connecting the two parent cells (5, 30).

This work focuses on Prm1, a glycoprotein that promotes the plasma membrane fusion step of mating. *PRM1* was discovered in a bioinformatic screen designed to identify Prm (pheromone-regulated membrane) proteins (11). Prm1 has four transmembrane domains and functions as a disulfide-linked dimer (20). Prm1-deficient mating pairs experience one of three fates: arrest as late prezygotes (unfused mating pairs with no intervening cell walls), lysis once their plasma membranes come into contact, or fusion. Electron microscopy revealed that the two plasma membranes in a late prezygote were only ~8 nm apart but did not fuse. Additional studies showed that ~30% of *prm1Δ* mating pairs lyse after membrane contact (1, 14). However, 50% of *prm1Δ* mating pairs fuse on standard yeast extract-peptone-dextrose (YPD) medium, implying that Prm1 is important, but not required, for fusion. Mating becomes more dependent upon Prm1 activity if Ca\(^{2+}\) or ergosterol is limiting (1, 15).

On the basis of its apparent role in membrane fusion, Prm1...
its native promoter by PCR amplifying 224 nucleotides from the tors with various constitutive promoters (18) and was placed under the control of fusion was subcloned as a 2.7-kb BamHI-SalI fragment into pRS415-based vec-
ADH in place of the GFP-PRM1 constructed by amplifying PRM1 knockouts were created by crossing the MHY153 strain (11) with the genes and rapidly degraded.

**MATERIALS AND METHODS**

**Yeast strains and plasmids.** The yeast strains used in this study are listed in Table 1. Unless otherwise noted, all strains are in the BY4741/BY4742 genetic background and were derived from strains in the yeast knock-out collection, which is available from InViGen. The ups4 mutant expressing genomically tagged Prm1-GFP (Prm1 with green fluorescent protein [GFP] fused to the C terminus of Prm1) was created by crossing the MHY153 strain (11) with the ups4::G418 knockout strain.

Plasmids used in this study are listed in Table 2. The GFP-PRM1 fusion was constructed by amplifying PRM1 from genomic DNA by PCR and inserting the product into the EcoRI and SalI sites of pEG311 (11). The GFP-PRM1 fusion was subcloned as a 2.7-kb BamHI-Sall fragment into pRS415-based vectors with various constitutive promoters (18) and was placed under the control of its native promoter by PCR amplifying 224 nucleotides from the PRM1 5′ untranslated region (5′ UTR) and inserting the product as a SacI-Xbal fragment in place of the ADH promoter in pEG711. The GFP-PRM1 fusion was also subcloned as a 2.7-kb BamHI-PstI fragment into pNB529, a vector with a GAL terminus of Prm1) was created by crossing the MHY153 strain (11) with the genes and rapidly degraded.

**Microscopy.** Wide-field fluorescence microscopy was performed with an Axioplan 2 motorized microscope (Zeiss) outfitted with a mercury arc lamp, band pass filters (Chroma), differential interference contrast (DIC) optics, a 100×/1.40 Plan apochromat lens, an Orca ER digital camera (Hamamatsu), and Openlab software (Improvision) for automated image collection and analysis. For quantitative mating assays, random image fields were identified using DIC optics, and sets of DIC, GFP, and mcherry images were collected automatically. The confocal microscopy was done using an LSM 700 microscope (Zeiss) with a 63×/1.4 Plan apochromat lens. Zen software was used to collect a series of optical sections (pinhole size, 1 airy unit) separated by 1 μm, and to assemble them into a maximum-intensity projection.

**Immunofluorescence assays.** Cultures (10 ml) were grown overnight at 25°C in selective SC medium to an optical density at 600 nm (OD600) of 0.8. To fix the cells, 1.3 ml of 37% formaldehyde and 1 ml of 1.0 M KPO4 (pH 6.5) were added to each culture. The cells were incubated on a rocking platform for 30 min at room temperature, pelleted, resuspended in 5 ml of 4% formaldehyde and 0.1 M KPO4 (pH 6.5), and rocked at room temperature for 1.5 h. The fixed cells were washed twice in 5 ml of 100 mM KPO4 (pH 7.5) and once in 5 ml of KS buffer (100 mM KPO4, pH 7.5, 1 M sorbitol). Cell walls were degraded by treatment with 5 μl of β-mercaptoethanol and 45 μl of 5 mg/ml lyticase in 1 ml of KS buffer for 30 min at 30°C. The spheroplasts were washed twice in 3 ml of HS buffer (100 mM HEPES [pH 7.4], 1.0 M sorbitol) and then permeabilized with 0.5% SDS in HS buffer for 5 min at room temperature. After the spheroplasts were washed twice with HS buffer, the permeabilized spheroplasts were resuspended in 1 ml of HS buffer. Spheroplasts (20 μl) were placed on a 0.1% polylysine (Sigma)-coated cover slip in a 10-well microscope slide (Cel-Line; Thermo Scientific). The spheroplasts were blocked with PBT buffer (phosphate-buffered saline [PBS], 1 mg/ml bovine serum albumin [BSA], 0.02% Tween) and then incubated with anti-grown glutinin (anti-HA) (23CA9) monoclonal antibodies overnight at room temperature. The wells were washed and then incubated with rabbit anti-mouse antibodies conjugated to Alexa Fluor 568 for 1 h in the dark. The working concentration for both antibodies was 1:1,000. Mounting medium containing antifade reagents (23) was added between wells to prevent bleaching. Coverslips were mounted over the slide for imaging.

**Surface biotinylation and immunoprecipitation.** Cultures (50 ml) in exponential growth were pelleted, washed twice with PBS, and then incubated in 100 mM NaCl, NaOH (pH 9.4) for 10 min at room temperature to loosen the cell wall. All subsequent steps were performed at 4°C to inhibit membrane transport. The cells were pelleted, washed with PBS, and then incubated in 200 μl of 5 mg/ml sulfo-NHS-LC-biotin (sulfosuccinimidyl-6-[biotin-amido]hexanoate) (Thermo

**TABLE 2. Plasmids used in this study**

| Plasmid | Relevant genotype or description | Reference or source |
|---------|---------------------------------|---------------------|
| pEG697 | pPrm1::GFP-PRM1 CEN LEU2        | This work           |
| pEG520 | pPrm1::GFP-PRM1-RAAA CEN LEU2   | This work           |
| pEG729 | pPrm1::GFP-PRM1 K35SR CEN LEU2  | This work           |
| pEG730 | pPrm1::GFP-PRM1 F358A CEN LEU2  | This work           |
| pEG724 | pPrm1::GFP-PRM1-ISTX CEN LEU2   | This work           |
| pEG711 | pPrm1::GFP-PRM1 CEN LEU2        | This work           |
| pEG726 | pPrm1::GFP-PRM1-K35SR CEN LEU2  | This work           |
| pEG715 | pPrm1::GFP-PRM1 F358A CEN LEU2  | This work           |
| pEG716 | pPrm1::GFP-PRM1 F358A CEN LEU2  | This work           |
| pEG712 | pPrm1::GFP-PRM1 CEN LEU2        | This work           |
| pEG713 | pPrm1::GFP-PRM1-RAAA CEN LEU2   | This work           |
| pEG427 | pPrm1::HA-PRM1 CEN LEU2         | This work           |
| pEG462 | pPrm1::HA-PRM1 integrates at LEU2 | This work           |
| pEG783 | pPrm1::GFP-PRM1 integrates at LEU2 | This work           |
| pEG1387 | pPrm1::GFP-PRM1 integrates at LEU2 | This work           |
| pCB260 | FYVE-GFP CEN LEU2               | Burd and Emr (4a)   |
| pSM1493 | STE6-GFP CEN URA3               | Kelm et al. (17)    |
| pEG3463 | pPrm1::mCherry URA3 integrates 3′ of SS01 | Nolan et al. (19) |
| pEG3599 | pPrm1::GFP-SNC1                 | Lewis et al. (17a)  |
After the cells were pelleted and the supernatant was aspirated, the cells were incubated again in 200 μl of the sulfo-NHS-LC-biotin solution for 20 min. The cells were then washed twice with PBS and incubated twice for 5 min in 50 mM glycine (pH 2.5) to quench the biotinylation reagent. The cells were then washed with TAF buffer, resuspended in 1.5 ml of lysis buffer (20 mM HEPES [pH 7.4], 150 mM potassium acetate [KOA], 1 mM EDTA, 1 mM dihydrothreitol [DTT], 10 mM phenylmethylsulfonyl fluoride [PMSF], 5 μM antipain, 10 μM pepstatin, 15 μM chymostatin, 15 μM leupeptin), and vigorously agitated in a BeadBeater (BioSpec) for 4 min with 0.5-mm zirconia/silica beads (BioSpec). The homogenate was recovered from the beads and treated with 0.8% Triton X-100 for 5 min on ice. The lysate was cleared of unbroken cells and other debris by centrifugation for 5 min at 500 × g and then for 10 min at 15,000 × g. For immunprecipitations, rabbit anti-GFP antibody was cross-linked to protein A beads (Invitrogen) using disuccinimidyl suberate (DSS) (Thermo Scientific). The conjugated beads were washed in 50 mM Tris HCl (pH 7.5) and then quenched with 100 mM glycine (pH 3). Lysate (1 ml) was incubated with 100 μl of conjugated antibody on a rocking platform for 4 h at 4°C.

For Western blot analysis, samples were run on a 7% SDS-polyacrylamide gel (SDS-PAG) and transferred to nitrocellulose membrane (Bio-Rad). For GFP and Sso1/Sso2 detection, the blots were blocked in 5% milk dissolved in PBST (PBS, 0.5% Tween 20) for 30 min, then incubated with anti-GFP at 1:1,000 dilution or anti-Sso at 1:5,000 dilution in 5% milk dissolved in PBST, and treated with goat anti-rabbit secondary antibody coupled to horseradish peroxidase. To detect biotinylated proteins, the blots were incubated with streptavidin coupled to horseradish peroxidase (Jackson ImmunoResearch) at 1:1,000. All blots were developed using an enhanced chemiluminescence kit (Thermo Scientific).

**Galactose-regulated PRM1 expression.** For the fluorescence studies, 10 ml of MATA cells expressing GFP-PRM1 from a GAL promoter were grown overnight to an OD600 of 0.2 in selective medium containing 2% raffinose as the carbon source. The cells were pelleted, resuspended in selective medium containing 2% galactose, and grown for 2 h. After the cells were washed and resuspended in selective medium containing 2% glucose, the cells were split into two aliquots. One aliquot was treated with 5 μM α-factor at the beginning of the experiment and then treated with 5 μM more α-factor 1 h later. For both aliquots, 1 ml of cells was collected every half hour. The cells were spun down and washed in TAF buffer prior to imaging.

For the carbon shift mating assays, MATA cells expressing HA-PRM1 from the GAL promoter were grown overnight in YP medium (yeast extract-peptone) containing 2% galactose (YPGal). At an OD600 of 0.4, the cells were mixed with MATA prm1Δ cells and allowed to mate on SC medium without Ca2+ for 4°C. To collect protein pellets, samples were spun down at 16,000 ×g for 5 min in a 4°C microcentrifuge. Pellets were washed twice for 5 min in 50 mM glycine (pH 2.5) to quench the biotinylation reagent. After the cells were pelleted and the supernatant was aspirated, the cells were incubated again in 200 μl of the sulfo-NHS-LC-biotin solution for 20 min. The cells were then washed without leucine were pelleted, washed, and then lysed with glass beads in 800 μl of lysis buffer. The cells were lysed with glass beads for 10 min in a vortex at full speed, solubilized by adding 25 μl of 5× SDS loading buffer to 100 μl of lysate, and then boiled for 4 min. The lysates were run on a 7% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane, which was probed as previously described.

**Sucrose gradient.** Log-phase cells (3.5 × 10^8) grown overnight in SC medium without leucine were pelleted, washed, and then lysed with glass beads in 800 μl of STE buffer (15% sucrose, 10 mM Tris [pH 7.5], 10 mM EDTA, 10 mM PMSF, 5 μM antipain, 10 μM pepstatin, 15 μM chymostatin, 15 μM leupeptin). After a 5-min microcentrifuge spin at 500 × g to pellet unbroken cells and other debris, the supernatant was loaded onto a 30 to 60% sucrose gradient and spun down for 20 h (4°C, 150,000 ×g, SW50.1 rotor) in a Beckman L-8-70 M ultracentrifuge. Nine fractions (400 μl) were collected from the bottom of the gradient. Proteins in each gradient fraction were concentrated by trichloroacetic acid (TCA) precipitation. The gradient fractions were incubated with 2 volumes (800 μl) of 12% TCA and 0.1 mM of deoxycholate for 1 h at 4°C. To collect protein pellets, samples were spun down at 16,000 ×g for 30 min in a 4°C microcentrifuge. Pellets were washed twice in cold acetone and dried in a hood. The pellets were resuspended in 1× SDS loading buffer and dissolved at 55°C.

**Stability studies with cycloheximide.** Cultures grown overnight to early log phase (OD600 of 0.4) were treated with 100 μg/ml cycloheximide (MP Biomedicals, LLC). At the indicated times, a 5-ml aliquot of the culture was mixed with 45 ml of ice-cold TAF buffer. The cells (107) were pelleted and resuspended in 200 μl of TAF buffer. Protein extracts were prepared by glass bead lysis. Samples were resolved by 7% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-HA antibody as previously described.

**Protease protection assay.** MATA prm1Δ cells expressing GFP-Prm1 (GFP fused to the N terminus of Prm1) (pEG712) or GFP-Prm1-RAAAA (pEG713) were treated with 60 μl of lyticase and/or 125 μg/ml proteinase K for 90 min at 37°C, as previously described (20).

**RESULTS**

**Prm1 functions at contact sites in yeast mating pairs.** The plasma membrane fusion arrest and contact-dependent lysis phenotypes of Prm1-deficient mating pairs suggest that Prm1 should be localized on the plasma membrane at the contact site between the two cells of a mating pair. To confirm this prediction, MATA prm1Δ cells expressing GFP-Prm1 were mated to MATA fus1Δ fus2Δ cells. The fus1Δ and fus2Δ mutations in the mating partner slow down cell wall remodeling, resulting in an accumulation of prezygotes (unfused mating pairs) with an extended zone of cell-cell contact (14, 26). GFP-Prm1 was concentrated at the contact site in these prezygotes (Fig. 1A), as previously described (11, 15).

To investigate the functional significance of this localization...
two different dominant targeting signals were appended to the C terminus of GFP-Prm1 to direct it away from the contact site. The C-terminal domain of Ist2 targets membrane proteins directly from the endoplasmic reticulum (ER) to the plasma membrane (16). Proteins with this Ist2 domain bypass the conventional secretory pathway and are therefore not delivered to bud tips or contact sites. The KKXX sequence from the C terminus of Wbp1 acts as an ER retention signal (12). In contrast to wild-type GFP-Prm1, GFP-Prm1-Ist2 was found at peripheral sites on the cell surface, and GFP-Prm1-KKXX had an ER localization pattern. Neither of these chimeras was detected at contact sites (Fig. 1A).

To assay for mating function, MATα prm1Δ cells expressing the Prm1 chimeras were mated to MATα prm1Δ cells expressing cytoplasmic mCherry. Plasma membrane fusion was detected by observing mCherry diffusion into the MATα cell (19).

Expression of wild-type GFP-Prm1 in only one cell of each mating pair promoted fusion under stringent low-Ca²⁺ conditions. The Ist2 and KKXX targeting signals reduced the mating activity of GFP-Prm1 by >90% (Fig. 1B). Prm1 chimeras with GFP, HA, TAP, or HBH fused to the C terminus had normal activity (data not shown), so the C-terminal Ist2 and KKXX extensions are unlikely to have inactivated Prm1 by occluding an active site or inhibiting protein folding. In conclusion, Prm1 is inactive when it is targeted away from the contact site.

Intracellular localization of Prm1 in shmoos and budding cells. A previous study reported that Prm1-GFP was targeted to shmoo tips (11). Although we observed a similar localization pattern in some cells, we also noted that many cells did not accumulate Prm1-GFP in shmoo tips (Fig. 2A). In contrast to the small amount of Prm1-GFP in shmoo tips, most Prm1-GFP was localized in punctate spots scattered throughout the cyto-
plasm, as confirmed by confocal microscopy (see Fig. S1 in the supplemental material). Prm1-GFP was also detected in the ER and/or in vacuoles in some cells.

An N-terminally GFP-tagged Prm1 fusion protein was used to extend this finding. When expressed from the PRM1 promoter in shmoo cells, GFP-Prm1 had a localization pattern very similar to that of Prm1-GFP (Fig. 2A). In mitotic cells, GFP-Prm1 was expressed from the ADH1 promoter because the PRM1 promoter requires mating pheromones for transcriptional activity. The ADH1 promoter provides a modest level of Prm1 expression that is unlikely to saturate normal intracellular transport pathways (15). Constitutively expressed GFP-Prm1 accumulated in vacuoles both in mitotic cells and in shmoo cells. Some mitotic cells also had GFP-Prm1 on the surface of their bud (Fig. 2A). However, the resolution of these fluorescent images is not sufficient to distinguish between the plasma membrane and cortical ER underlying the plasma membrane.

A polarity index comparing the mean fluorescent intensity of Prm1 in the shmoo tip to that of the entire cell was used to quantify the relative amount of Prm1 in shmoo tips (see Fig. S2 in the supplemental material). Results from 25 cells confirmed that Prm1-GFP is not highly concentrated in shmoo tips (Fig. 2C). These observations were originally made with a wide-field microscope, but substantially identical results were obtained by confocal microscopy (see Fig. S3 in the supplemental material). Furthermore, Prm1-GFP had the same localization pattern at pheromone concentrations ranging from 1.5 to 48 μM and at times ranging from 75 to 105 min after pheromone addition, a range which corresponds to the peak fusion time during mating (see Fig. S4 in the supplemental material). Prm1-GFP was more concentrated in shmoo tips at an earlier time point (60 min), suggesting that newly synthesized Prm1-GFP may pass through the plasma membrane on its way to the vacuole.

To investigate whether mating pair assembly enhances the localization of Prm1 to polarized sites on the cell surface, the Prm1 polarity index was measured in prezygotes assembled from MATα prmlΔΔ cells expressing GFP-tagged Prm1 and MATα fus1Δ fus2Δ cells expressing mCherry. Prm1-GFP was concentrated at the contact site in 60 to 75% of the prezygotes and had a significantly higher polarity index in mating pairs than in shmoo cells. Similar results were found with GFP-Prm1 expressed from the PRM1 and ADH1 promoters (Fig. 2B and C).

Cell fractionation was utilized as an orthogonal method to determine whether a substantial amount of Prm1 is on the plasma membrane. Lysates from mitotic cells expressing HA-Prm1 from the strong constitutive GPD promoter were centrifuged into a 30 to 60% sucrose gradient. Two forms of HA-Prm1 were detected on a Western blot. The mature form runs as an N-glycosylated smear with a molecular mass ranging from 100 to 150 kDa (11, 20). The sharp band at 100 kDa has immature N-linked glycans and is likely to reside in the endoplasmic reticulum. Essentially no mature HA-Prm1 was detected in the plasma membrane peak, which is marked by Pma1 (Fig. 2D).

In summary, Prm1 accumulates at contact sites in mating pairs, where it promotes plasma membrane fusion, but it is primarily localized in punctate cytoplasmic structures in mating cells, mitotic cells, and α-factor-treated cells. These results suggest that Prm1 is preferentially targeted to and/or retained at the plasma membrane in the late stages of mating.

**Prm1 is targeted to vacuoles for degradation.** We hypothesized that the punctate GFP-Prm1-containing structures were small vesicular organelles, such as endosomes or the Golgi complex. To identify these punctate structures, the localization of HA-Prm1 was probed by immunofluorescence microscopy in mitotic cells that also expressed GFP-tagged organelle markers (Fig. 3A). HA-Prm1 partially colocalized with two endosome markers, GFP-FYVE, a domain that binds to membranes enriched with phosphatidylinositol 3-phosphate (PI3P) (26), and Ste6-GFP, the yeast a-factor exporter which was previously reported to be a reliable endosome marker (17). In contrast, HA-Prm1 did not colocalize with Sec7-GFP, a marker of the trans-Golgi (24). Additionally, Prm1-GFP was concentrated in the enlarged endosome (class E compartment) that accumulates in the vps4 mutant, which is defective in transport to vacuoles (2, 22) (Fig. 3B).

The fluorescence data suggested that Prm1 is transported through the endocytic pathway en route to vacuoles for degradation. To confirm this model, the stability of HA-Prm1 was measured after treating cells with cycloheximide. HA-Prm1 was rapidly degraded in mitotic wild-type cells and was stabilized in the pep4 mutant, which is deficient in vacuolar proteolysis (Fig. 3C). In conclusion, newly synthesized Prm1 is targeted via endosomes to the vacuole for degradation.

**Prm1 contains a putative endocytosis signal.** Prm1 could be transported through the plasma membrane on its way to the vacuole. To investigate this possibility, GFP-Prm1 was expressed in the end4 mutant. This mutant is deficient in endocytosis, so proteins that normally reside only transiently on the plasma membrane will accumulate on the surfaces of end4 cells (21). For example, the secretory vesicle v-SNARE Snc1, which normally cycles between the Golgi complex, the plasma membrane, and endosomes, accumulated on the surfaces of end4 cells (see Fig. S6 in the supplemental material), as previously shown (21). When GFP-Prm1 was expressed in end4 cells from its native promoter, only a small percentage of the cells responded to mating pheromones by expressing Prm1 and extending a mating projection. GFP-Prm1 accumulated on the surfaces of shmoo tips in these cells (Fig. 4A), indicating that some GFP-Prm1 is transported to the plasma membrane before being internalized and degraded in vacuoles. However, when GFP-Prm1 was expressed from the constitutive ADH1 promoter in mitotic cells, it accumulated both on the plasma membrane and in vacuoles (see Fig. S6 in the supplemental material). These results suggest that Prm1 exits the Golgi complex in two different types of transport vesicles, one directed to the plasma membrane and the other directed directly to endosomes or vacuoles, and that transport to the plasma membrane may predominate in cells responding to pheromone.

Accumulation of GFP-Prm1 on the plasma membrane of end4 cells prompted an inspection of the PRM1 sequence for potential endocytosis signals. The Kluyveromyces lactis PRM1 gene contains a previously characterized endocytosis signal, NPFxFD, located in the cytoplasmic loop between the second and third transmembrane domains (27). In a multiple-sequence alignment, the PRM1 genes of *S. cerevisiae*, *Vanderwalttozyma polyspora*, and *Ashbya gossypii* all contain a similar
sequence, DPFXD (Fig. 4B). Interestingly, a conserved lysine was found immediately upstream of this signal in all four sequences. To test whether this conserved sequence functions as an endocytosis signal, we mutated the lysine to arginine and the other amino acids to alanine to construct the “RAAAA” mutant. GFP-Prm1-RAAAA accumulated on the surfaces of both α-factor-treated cells and mitotic cells, in stark contrast to the punctate cytoplasmic localization of wild-type GFP-Prm1 (Fig. 4C).

Mutation of phenylalanine to alanine in the NPFXD signal is sufficient to disrupt endocytosis (27). Similarly, a phenylalanine to alanine mutation (F358A) in the DPFXD signal caused GFP-Prm1 to accumulate on the plasma membrane when expressed from the PRM1 promoter (see Fig. S5 in the supplemental material). However, unlike the RAAAA mutant, which has 5 point mutations, GFP-Prm1-F358A was found in vacuoles as well as on the cell surface when constitutively expressed from the ADH1 promoter, suggesting that the F358A mutant reduces but does not completely eliminate Prm1 endocytosis. We also constructed a K355R mutation, which had no effect on Prm1 localization (see Fig. S5 in the supplemental material). The RAAAA mutant was used for subsequent experiments because it had the most severe localization defect.

Since mutant membrane proteins are often trapped in the cortical ER, which can be difficult to distinguish from the plasma membrane by fluorescence microscopy, two biochemical methods were used to confirm that GFP-Prm1-RAAAA accumulates on the plasma membrane. First, we took advantage of our recent finding that the N terminus of Prm1 is located on the cytoplasmic side of the membrane (20). Cells expressing GFP-Prm1-RAAAA were treated with lyticase to remove the cell wall and with proteinase K to degrade extracellular regions of plasma membrane proteins. If GFP-Prm1-RAAAA is on the surface, an 110-kDa N-terminal fragment should be protected from degradation. The predicted fragment was readily detected in the RAAAA mutant and was much less abundant in the wild-type control (Fig. 4D). In summary, the fluorescence microscopy, protease protection, and cell surface bio-

![Figure 3](image-url)
tinylation assays all indicate that the Prm1-RAAAA mutant accumulates on the plasma membrane and thereby support the hypothesis that the DPFXD sequence is an endocytosis signal. **Prm1 degradation is delayed by the RAAAA mutation.** If Prm1 must be transported through the plasma membrane before it is targeted to the vacuole for degradation, inhibiting endocytosis with the RAAAA mutation should stabilize Prm1. To test this hypothesis, we compared the stability of GFP-Prm1 and GFP-Prm1-RAAAA. As expected, the RAAAA mutant had a longer half-life than wild-type Prm1 did and was expressed at higher levels due to this delayed degradation (Fig. 5). However, the RAAAA mutant was still almost completely degraded after 60 min. These results indicate that accumulation of Prm1 on the plasma membrane is not sufficient to prevent Prm1 degradation. One potential explanation is that the mutations reduce but do not eliminate the interaction of Prm1 with endocytic adaptors. An alternative possibility is that most newly synthesized Prm1 is targeted directly from the

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**FIG. 4.** Cell surface accumulation of the RAAAA mutant. (A) GFP-Prm1 expressed from its native promoter accumulates on the surfaces of shmoo tips in the *end4* mutant. (B) Multiple-sequence alignment of Prm1 homologues using ClustalW reveals a putative endocytosis signal (boldface letters). PRM1 sequences from *Kluyveromyces lactis*, *Ashbya gossypii*, *Vanderwaltozyma polyspora*, *S. cerevisiae*, and the RAAAA mutant are shown. (C) GFP-Prm1-RAAAA accumulates over the entire cell surface in shmooes (PRM1 promoter) and mitotic cells (ADH1 promoter). (D) Protease protection assay. Cells expressing wild-type or RAAAA mutant GFP-Prm1 were treated (+) with lyticase and/or proteinase K as indicated. The 33-kDa protected fragment predominant in the RAAAA mutant contains GFP (27 kDa) fused to the N-terminal cytoplasmic domain and the first transmembrane domain of Prm1 (5 kDa). The black arrow denotes the position of the protected fragment. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gels. (E) Cell surface biotinylation. Phoromone-treated (+) and untreated (−) cells expressing GFP-Prm1 or the RAAAA mutant were treated with sulfo-NHS-biotin to biotinylate extracellular lysines. GFP-tagged Prm1 proteins were immunoprecipitated and treated with endo-β-N-acetylglucosaminidase H (EndoH) to deglycosylate Prm1 (102 kDa). Cells expressing GFP-Hmg1, a resident ER protein, were included as a negative control.

**FIG. 5.** Delayed proteolytic degradation of GFP-Prm1-RAAAA. Cells expressing GFP-Prm1 or GFP-Prm1-RAAAA were collected after the indicated times (in minutes) in cycloheximide (CHX). Cell extracts were treated with EndoH to collapse all glycosylated forms into a single band. The lower half of the blot was probed for Sso1/Sso2 as a loading control. Films were exposed for 1 min (short exposure) or 15 min (long exposure).
Golgi complex to the vacuole without ever being transported to the plasma membrane.

**Pmr1-RAAAA accumulation on the plasma membrane does not promote fusion.** If Pmr1 must be localized on the plasma membrane to promote cell fusion, Pmr1-RAAAA might have enhanced activity because it accumulates on the plasma membrane. Pmr1-RAAAA fully complemented the cell fusion phenotype of the prm1Δ mutant, indicating that the RAAAA mutant is functional (Fig. 6A). To quantify fusion activity, the wild-type and RAAAA mutant GFP-Pmr1 proteins were expressed from the ADH1 promoter in a prm1Δ erg6Δ double mutant. Deletion of ERG6 alters the sterol composition of the plasma membrane, thereby increasing the dependence of fusion upon high-level Pmr1 expression without altering GFP-Pmr1 localization (15). Pmr1 expressed at moderate levels from the ADH1 promoter in prm1Δ erg6Δ double mutants has 50% of the mating activity of Pmr1 expressed at higher levels from the PRM1 or GPD promoters (15). Under these sensitized conditions, the RAAAA mutant had a subtle, but statistically significant decrease in fusion activity compared to wild-type Pmr1 (Fig. 6B). Thus, accumulating Pmr1 on the plasma membrane inhibits rather than enhances fusion.

**GFP-Pmr1 is retained at polarized sites on the plasma membrane.** GFP-Pmr1 was expressed from the galactose-regulated GAL promoter in order to follow the intracellular transport of a cohort of newly synthesized protein. To verify that GFP-Pmr1 was targeted to vacuoles for degradation as previously shown for HA-Pmr1, GFP-Pmr1 synthesis was induced by growth on galactose-containing medium. The cells were then shifted to glucose-containing medium to repress further GFP-Pmr1 synthesis. The preexisting pool of GFP-Pmr1 was rapidly degraded (Fig. 7A), consistent with the rapid turnover of HA-Pmr1 found after blocking protein synthesis with cycloheximide (Fig. 3C).

By fluorescence microscopy, GFP-Pmr1 was localized primarily in the ER after 2 h of induction with galactose (Fig. 7B). Glucose was then added to repress GFP-Pmr1 synthesis. Thirty minutes later, GFP-Pmr1 had translocated to cytoplasmic punctae and to the plasma membrane. After 60 min, GFP-Pmr1 was primarily localized in vacuoles. Surprisingly, a small amount of GFP-Pmr1 was detected on the cell surface at bud tips and shmoo tips 60 min after the cells were shifted to glucose-containing medium (Fig. 7B), a time at which the mature protein was barely detectable by Western blotting (Fig. 7A).

To investigate the effects of mating pheromones on GFP-Pmr1 turnover and localization, P_{GAL}-GFP-PRM1 cells were grown for 2 h in galactose-containing medium and then shifted to glucose-containing medium supplemented with α-factor. The pheromone had no effect on the rate of GFP-Pmr1 turnover (Fig. 7A). Shmoos formed after 60 min, and they had GFP-Pmr1 localized at their tips. Interestingly, retention at the plasma membrane was more robust in mating projections than in buds, suggesting that other pheromone-regulated proteins contribute to GFP-Pmr1 retention (Fig. 7C).

**The stable pool of Pmr1 at contact sites is sufficient to promote fusion.** To determine whether the residual pool of stable Pmr1 localized on the surface of mating projections is functional, MATa P_{GAL-HA-PRM1} cells were grown in galactose-containing medium to induce HA-Pmr1 expression and were then allowed to mate on glucose-containing medium to MATa prm1Δ cells expressing cytoplasmic mCherry. A Western blot confirmed that the major pool of HA-Pmr1 was rapidly degraded in these mating cells but that a tiny amount of mature HA-Pmr1 could still be detected even after 120 min (Fig. 8A). After 120 min, 50% of the mating pairs had fused, indicating that HA-Pmr1 proteins that had been synthesized before the MATa and MATa cells were mixed to initiate mating remained active for fusion after the preliminary processes of pheromone signaling, mating pair assembly, and cell wall remodeling were completed (Fig. 8B). In the mating conditions used for this experiment (glucose-containing medium at 30°C), most fusion events occur during the interval from 70 to 110 min after the start of mating, and no fusions were detected during the first 60 min (data not shown). Thus, fusion occurred at a time when only a vanishingly small fraction of the initial pool of HA-Pmr1 remained. In a parallel mating, MATa P_{GAL-HA-PRM1} cells grown overnight on glucose-containing medium had a cell fusion defect similar to that of prm1Δ cells, confirming that glucose completely repressed HA-PRM1 synthesis. There are two possible explanations for these results: Pmr1 either acts early during mating to establish a stable state that favors later fusion, or the small amount of stable Pmr1 remaining when the two plasma membranes come into contact is sufficient for fusion. We favor the second model, because the stable Pmr1 is concentrated at the fusion site.

**DISCUSSION**

Pmr1 functions during the plasma membrane fusion step of mating (9, 14). On the basis of this function, Pmr1 was expected to be targeted to the contact site where the plasma membranes of two cells fuse. In mating pairs, only a small amount of Pmr1 was visible at the contact site, but mutant forms of Pmr1 that were sorted away from the contact site were inactive, confirming that Pmr1 must be at the contact site to promote plasma membrane fusion. In contrast to expectations, most Pmr1 was found in endosomes and in the ER and/or vacuoles. Consistent with this localization pattern, Pmr1 expressed in mitotic cells was not detected in the plasma membrane peak on a sucrose gradient, and most newly synthesized Pmr1 was rapidly degraded in the vacuoles of mitotic cells.
α-factor-treated cells, and mating cells. Despite this rapid turnover of most newly synthesized Prm1, a small subpopulation of Prm1 was resistant to degradation. The stable pool of Prm1 was preferentially retained at polarized sites on the plasma membrane and was functional for fusion.

**Prm1 functions at contact sites.** Most Prm1 in both mating and nonmating cells was found in punctate cytoplasmic structures, including endosomes, whereas only a small amount of Prm1 was detected at contact sites. This localization pattern is conserved in the *Neurospora crassa* Prm1 ortholog, which participates in at least 4 fusion events, including germling and hyphal fusion during vegetative growth and fertilization and crozier fusion during sexual development (8). Even though only a small fraction of the Prm1 in a cell was at contact sites, exclusion of Prm1 from the contact sites (Prm1-Ist2) or retention in the ER (Prm1-KKXX) inhibited Prm1 mating activity. The Prm1-Ist2 and Prm1-KKXX proteins both had a low level of fusion activity compared to the vector control. However, very low PRM1 expression is sufficient to fully complement the fusion defect of prm1Δ mating pairs (15), indicating that mistargeting of a small amount of the Prm1-Ist2 and Prm1-KKXX chimeras to contact sites could explain their residual fusion activity.

The Prm1-RAAAA mutant also had reduced fusion activity, but this more subtle defect could only be detected only in the sensitized background of erg6Δ mutant mating pairs. Prm1-RAAAA has five mutations in a putative endocytosis signal. Although there was no direct measurement of an endocytosis defect, the increased stability of Prm1-RAAAA and its localization over the entire surface of the plasma membrane both suggest that Prm1-RAAAA accumulates on the plasma membrane because it cannot be concentrated into endocytic vesicles. One possible explanation for the reduced fusion activity is that Prm1-RAAAA interacts with other proteins involved in membrane fusion and recruits them away from the fusion site. Alternatively, recycling between the plasma membrane, endosomes, and secretory vesicles could potentiate Prm1 activity by maintaining a higher local concentration at exocytic sites ad-

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**FIG. 7.** A small amount of GFP-Prm1 is retained at shmoo tips. (A) Rapid turnover of GFP-Prm1. Cells expressing GFP-Prm1 from the *GAL* promoter were grown to log phase in galactose-containing medium. Prm1 synthesis was repressed by transferring the cells to glucose (Glu)-containing medium, and aliquots were collected at the indicated times (in minutes). For α-factor-treated cells, 6 μM α-factor was added to the glucose-containing medium at time zero (t = 0). At 60 min, 6 μM α-factor was added to replenish the pheromone degraded by Bar1 protease secreted into the medium. (B) GFP-Prm1 localization in mitotic cells and shmoos after repressing GFP-Prm1 synthesis. The *P_{GAL}-GFP-PRM1* cells were grown overnight in glucose-containing medium, incubated in galactose-containing medium for 2 h to induce GFP-Prm1 expression, and then transferred to glucose-containing medium (mitotic cells) or glucose-containing medium with α-factor (shmoos) for the indicated times. Immediately after the cells were returned to glucose-containing medium (0 min), GFP-Prm1 had an ER localization pattern characterized by fluorescence surrounding the nucleus (nuclear envelope) and adjacent to the cell surface (peripheral ER). Shmoos formed after 60 min in α-factor. Bud tips and shmoo tips with polarized GFP-Prm1 (arrows) and without polarized GFP-Prm1 (arrowheads) are indicated. (C) Quantification of GFP-Prm1 polarization. The percentage of bud tips and shmoo tips with polarized GFP-Prm1 was quantified at the indicated time points. More than 200 bud tips and shmoo tips were counted.
Prm1 and other fusion factors.

Adjacent to the fusion site or by facilitating interactions between Glgo vesicles to vacuoles is also possible because GFP-Prm1 was found in vacuoles in some endocytosis-deficient end4 mutant cells. Ubiquitination is commonly used to signal for endocytosis and vacuole targeting. Our preliminary studies indicate that Prm1 appears to be ubiquitinated and is stabilized in a specific rsp5 ubiquitin-ligase mutant (V. Olmo). Regulating the ubiquitin status of Prm1 could allow a stable subpopulation to accumulate at contact sites.

Once Prm1 reaches the plasma membrane, it is concentrated at polarized sites, including the shmoo tip and the contact site. One explanation for this localization pattern is that Prm1 is delivered to polarized sites via the secretory pathway and removed from the plasma membrane by endocytosis once it diffuses away (29). This model is supported by the depolarized localization of the Prm1-RAAAA endocytosis mutant. Another possibility is that Prm1 interacts with free sterols, which are concentrated at shmoo tips and contact sites (3, 15). Polarized lipids may contribute to Prm1 localization, but they cannot be essential, since lipid polarization is reduced in the erg6 ergosterol biosynthesis mutant, which does not inhibit Prm1 targeting to contact sites (15).

Enhanced Prm1 localization in mating pairs. Mating pairs had significantly more Prm1 at polarized sites on the plasma membrane than shmoos did. Cell-cell contact is likely to be required for this enhanced accumulation, but the pheromone concentration gradients present in genuine mating pairs could also contribute. Cell contact could have a specific role in Prm1 localization but might also act indirectly by enhancing the overall polarity of the two cells in a mating pair. Actin polarization is unusually sensitive to perturbations in shmoos, which lack the septin ring that restricts protein diffusion between buds and mothers in mitotic cells (25). Thus, if cell-cell contact provides external cues that compensate for the lack of septins, this could enhance actin cable polarization and thereby promote polarized Prm1 delivery to contact sites.

Protein-protein interactions contribute to the localization of cell fusion-associated proteins in other systems. The Eff-1 protein, which mediates cell fusion in the Caenorhabditis elegans hypodermis, is retained at contact sites by a homotypic interaction with Eff-1 proteins expressed on the opposing cell (6). Similarly, the Duf and Sns proteins, which mediate adhesion in Drosophila myoblasts, are retained at contact sites by a heterotypic interaction (9). Trans interactions like those described above cannot be essential for Prm1 localization, because Prm1 is targeted to contact sites in PRM1 × prm1Δ mating pairs, which do not express Prm1 on the opposing cell, and in fus1Δ fus2Δ mating pairs, where the two plasma membranes are separated by cell walls (10, 28). Thus, if protein-protein interactions are important for Prm1 targeting or retention at contact sites, they are likely to be cis interactions.

Mysteries of Prm1 expression and function. Prm1 is among the most highly pheromone-regulated genes in S. cerevisiae (11). It is highly expressed in mating cells and pheromone-treated cells but cannot be detected in mitotic cells (11). One

FIG. 8. The stable pool of Prm1 has fusion activity. (A) Rapid degradation of HA-Prm1 in mating pairs. MATa prm1 Δ cells expressing GFP-Prm1 from the GAL promoter were grown overnight in galactose-containing medium and then mated to MATa prm1 Δ mCherry cells on glucose-containing medium for the indicated times. In the long exposure, a small amount of mature HA-Prm1 was detected 120 min after repressing HA-Prm1 synthesis. Films were exposed for 5 s (short exposure) or 1 h (long exposure). (B) Fusion activity of stable HA-Prm1 or GFP-Prm1 chimeras, so these N-terminal extensions could be responsible for the rapid degradation. However, the tagged and untagged Prm1 proteins had comparable fusion activity, even in a sensitized prm1Δ erg6Δ mating, so the tags are unlikely to have deleterious effects (unpublished observations).

The Prm1-RAAAA endocytosis mutant accumulates on the plasma membrane and is partially stabilized, indicating that at least some Prm1 passes through the plasma membrane before it is delivered to vacuoles. However, a direct pathway from Golgi vesicles to vacuoles is also possible because GFP-Prm1 was found in vacuoles in some endocytosis-deficient end4 mutant cells. Ubiquitination is commonly used to signal for endocytosis and vacuole targeting. Our preliminary studies indicate that Prm1 appears to be ubiquitinated and is stabilized in a specific rsp5 ubiquitin-ligase mutant (V. Olmo). Regulating the ubiquitin status of Prm1 could allow a stable subpopulation to accumulate at contact sites.

Placing PRM1 under the control of a GAL promoter led to the discovery of a minor subpopulation of Prm1 that was relatively stable after repressing PRM1 synthesis. Accumulation of this stable pool of Prm1 at shmoo tips and contact sites lends further support to the conclusion that Prm1 functions on the plasma membrane at sites of cell-cell contact.

Potential mechanisms of Prm1 targeting to and retention at polarized sites. One unexpected finding of these studies is that the most newly synthesized Prm1 is targeted to vacuoles and degraded. All of our stability measurements were made with HA-Prm1 or GFP-Prm1 chimeras, so these N-terminal extensions could be responsible for the rapid degradation. However, the tagged and untagged Prm1 proteins had comparable fusion activity, even in a sensitized prm1Δ erg6Δ mating, so the tags are unlikely to have deleterious effects (unpublished observations).

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might imagine that a protein with this expression pattern would be toxic if expressed in nonmating cells. However, high-level Prm1 expression had no effect on the growth or viability of nonmating cells (data not shown). The Prm1-RAAAA mutant accumulated on the plasma membrane, so this lack of toxicity is not simply due to rapid degradation. Thus, Prm1 may be expressed only in mating cells simply because its only function is to promote cell fusion. Conversely, the high level of Prm1 expression in mating cells suggested that a large number of Prm1 proteins might be required to promote cell fusion. In contrast to this prediction, low levels of Prm1 expression are sufficient to fully complement the mating defect in prm1Δ mutant mating pairs, and fusion was also promoted by the very small pool of stable Prm1 remaining at the time of fusion when Prm1 synthesis was repressed at the start of a mating interaction. Even though excessive Prm1 is superfluous in optimized mating conditions, higher levels of Prm1 expression are required for fusion under more-stressful conditions, such as a Ca^{2+}-free environment.

What can we learn about the function of Prm1 from the ability of a small number of Prm1 proteins to promote fusion? One possibility is that Prm1 acts as a catalyst that can activate many fusion proteins. Another possibility is that it functions as a channel with a high-capacity for Ca^{2+} or other ions. We previously proposed that Prm1 coordinates the activity of multiple fusion proteins to ensure that they cooperate to promote fusion rather than lyse (14). The number of Prm1 proteins required for this task could be quite small because only 8 SNARE complexes or influenza virus HA trimers are required to open a fusion pore (4, 7).

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