Prm1 Functions as a Disulfide-linked Complex in Yeast Mating

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Prm1 is a pheromone-induced membrane glycoprotein that promotes plasma membrane fusion in yeast mating pairs. HA-Prm1 migrates at twice its expected molecular weight on non-reducing SDS-PAGE gels and coprecipitates with Prm1-TAP, indicating that Prm1 is a disulfide-linked homodimer. The N terminus of a plasma membrane-localized GFP-Prm1 endocytic mutant projects into the cytoplasm, where it is protected from low pH quenching in live cells and from external protease in spheroplasts. In a revised topological map, Prm1 has four transmembrane domains and two large extracellular loops. Mutation of all four cysteines in the extracellular loops blocked disulfide bond formation and destabilized the Prm1 homodimer without preventing Prm1 transport to contact sites in mating pairs. Cys120 in loop 1 and Cys545 in loop 2 form disulfide cross-links in the Prm1 homodimer and are required for fusion activity. Cys120 lies between a hydrophobic segment formerly thought to be a transmembrane domain and an amphipathic helix. An interaction between either of these regions and the opposing membrane could promote fusion.

Cell fusion is an essential process required for fertilization and for the development, maintenance, and repair of syncytial tissues, including muscle, placenta, and the eye (1). Mating in the budding yeast Saccharomyces cerevisiae provides a genetically accessible model system to investigate cell fusion (2). Prm1 is a pheromone-regulated membrane glycoprotein with 14 potential N-glycosylation sites that promotes the plasma membrane fusion step of mating (3, 4). It is targeted to its activity in membrane fusion.

Despite these dramatic phenotypes, the molecular function of Prm1 is not known. Prm1 is not absolutely essential for plasma membrane fusion because a small percentage ofprm1Δ mating pairs are able to fuse (3). Mating is critical for genetic recombination and evolution, so there could be a second, Prm1-independent, fusion pathway. Alternatively, Prm1 could be an accessory factor for an as yet unknown fusion protein.

Several genetic enhancers of the prm1Δ mating defect have been identified. One enhancer is KEX2, which encodes an endoprotease in the Golgi with many uncharacterized substrates (5). A second class of enhancers includes the ERG2, ERG3, and ERG6 genes, which encode enzymes for ergosterol biosynthesis (6). The third prm1Δ enhancer is FIG1, which encodes another pheromone-regulated membrane protein (7). Fig1 regulates a Ca2+ influx that protects cells from stresses, including treatment with high doses of mating pheromones (8, 9). Although this signaling pathway is not directly involved in plasma membrane fusion, Ca2+ was found to promote fusion and inhibit lysis of prm1Δ mating pairs (7). One possibility is that Ca2+ activates a membrane repair pathway that provides prm1Δ mating pairs with a second opportunity to fuse (7).

A more detailed understanding of the Prm1 protein could provide valuable insights into its role in promoting cell fusion. In the present study, Prm1 was found to assemble within the ER to a disulfide-linked homodimer. The identification of four evolutionarily conserved cysteines in the amino acid sequence of Prm1 led us to re-evaluate its transmembrane topology and to discover that Cys120 and Cys545 form disulfide bonds that cross-link the Prm1 homodimer and are essential for its activity in membrane fusion.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The strains, plasmids, and PCR primers used in this study are listed in supplemental Tables S1–S3.

All yeast strains are isogenic to BY4741. The prm1Δ, fus1Δ, fus2Δ, and trp1Δ strains are available from Invitrogen. The Prm1-TAP strain was constructed by amplifying the TAP tag from pBS1173 using primers 10.1 and 10.2, which have 5’-ends with homology to the C terminus of PRM1, and then transforming the PCR product into MATα trp1Δ (10). The correct integration site was verified by PCR from yeast genomic DNA. The fusion activity of Prm1-TAP was verified by mating to a MATα prm1Δ partner.

HA-PRM1 was inserted into p415GPD (11), a CEN plasmid with a LEU2 selectable marker and a strong, constitutive GPD promoter, as previously described (6). The PRM1 gene was

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1 The online version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3.

2 The abbreviations used are: ER, endoplasmic reticulum; GFP, green fluorescent protein; RFP, red fluorescent protein; DTT, dithiothreitol; β-ME, β-mercaptoethanol; TAF, Tris, azide, and fluoride; PMSF, phenylmethylsulfonyl fluoride; DIC, differential interference contrast; HA, hemagglutinin; Endo H, endoglycosidase H; TAP, tandem affinity purification.
amplified from yeast genomic DNA with primers 48 and 49. Primer 48 inserted an EcoRI site into the 5'-end of the PRM1 coding sequence. The 3×-HA tag was amplified from 3MPY3XHA (12) with primers 60 and 62, and inserted into the EcoRI site. The four conserved cysteines were mutated by nested PCR using the following primers: C55S (107), C120S (106), C277S (105), and C545S (104). For GFP tagging of the PRM1 coding sequence, the 3' end was subcloned into p415GPD. For GFP tagging of the PRM1 expression vector pEG311 (4). The constructed vector was then subcloned as a BamHI-SalI fragment into p415GPD. For GFP tagging of the PRM1 expression vector pEG311 (4). The constructed vector was then subcloned as a BamHI-SalI fragment into p415GPD.

For the protease protection assay, 20 optical density units of yeast cells were collected, washed with ice-cold TAF buffer, and resuspended in 150 μl of lysis buffer supplemented with protease inhibitors. 150 μl of acid-washed glass beads (Sigma-Aldrich) were added, and the cells were vortexed at full speed for 2 min. 40 μl of 5× Laemmli sample buffer was added. The cells were vortexed for an additional minute and then boiled for 5 min. When indicated, 5% β-mercaptoethanol (β-ME) was added to the 5× SDS buffer to reduce disulfide bonds. To cleave N-linked glycans, yeast extract in lysis buffer was supplemented with 1% SDS and incubated at 42 °C for 10 min. The denatured extract was brought to pH 5.5 with 50 mM sodium citrate and then treated with 1000 units of endoglycosidase H and/or 10 mM dithiothreitol (DTT) for 1.5 h at 37 °C.

**Topology Determination—MATa cells expressing GFP—PRM1**

Cells were collected by centrifugation, washed with 2 ml of ice-cold TAF buffer, and resuspended in 150 μl of lysis buffer supplemented with protease inhibitors. 150 μl of acid-washed glass beads (Sigma-Aldrich) were added, and the cells were vortexed at full speed for 2 min. 40 μl of 5× Laemmli sample buffer was added. The cells were vortexed for an additional minute and then boiled for 5 min. When indicated, 5% β-mercaptoethanol (β-ME) was added to the 5× SDS buffer to reduce disulfide bonds. To cleave N-linked glycans, yeast extract in lysis buffer was supplemented with 1% SDS and incubated at 42 °C for 10 min. The denatured extract was brought to pH 5.5 with 50 mM sodium citrate and then treated with 1000 units of endoglycosidase H and/or 10 mM dithiothreitol (DTT) for 1.5 h at 37 °C.
**RESULTS**

**Prm1 Forms a Homotypic, Disulfide-linked Complex in the ER**—Prm1 is a membrane protein expressed by both cells of a mating pair. We tested for an interaction between Prm1-TAP expressed in MATa cells and HA-Prm1 expressed in MATa cells (Fig. 1A). Prm1-TAP was constructed by integrating a TAP tag at the C terminus of the native PRM1 gene. HA-Prm1 has an N-terminal 3X-HA epitope tag and was expressed from a centromeric (CEN) plasmid with a GPD promoter, which provides for strong, constitutive expression. Both tagged Prm1 proteins were functional in mating assays. HA-Prm1 coprecipitated with Prm1-TAP from cell lysates prepared after 90 min of mating, and a stronger interaction was detected after 135 min of mating. The time course for assembly of this HA-Prm1/Prm1-TAP complex is consistent with the formation of a trans-complex prior to plasma membrane fusion, but it is also consistent with an interaction between two Prm1 proteins coexpressed in fused zygotes after fusion. To directly test for formation of a cis-complex, HA-Prm1 and Prm1-TAP were coexpressed in MATa cells induced with synthetic Ca2+-free SC plate at 110 min at 30°C. Mating was stopped by transferring the filters to ice-cold TAF buffer. Mating pairs were identified in an unbiased manner from DIC images and then scored for fusion, arrest, or lysis. At least 150 mating pairs were scored for each sample.

**Cell Fusion**—Cell fusion was assayed as previously described (6) except for three significant changes. First, the wild-type and mutant forms of Prm1 were expressed only in MATa prm1Δ cells. All strains used were then mated to the same MATa prm1Δ partner. Second, the mating reactions were incubated on low Ca2+-free yeast nitrogen base (Sunrise Science) and agar washed with 5 mM EDTA. Third, the mating reaction was stained with 0.3 mM methylene blue to facilitate the detection of lysed mating pairs. Briefly, 106 MATa GFP and MATa RFP cells from log phase cultures were mixed and collected on filters. The filters were immersed in 1 mM Ca2+ and then incubated on a nominally Ca2+-free SC plate for 110 min at 30°C. Mating was stopped by transferring the filters to ice-cold TAF buffer. Mating pairs were identified in an unbiased manner from DIC images. Prezygotes were identified as pairs of cells with GFP-Prm1 restricted to one cell and mCherry restricted to the other.

**Disulfide Bonds in Prm1 Are Required for Cell Fusion**

**FIGURE 1. Prm1 forms a homotypic complex.** A, kinetics of HA-Prm1/Prm1-TAP complex assembly in mating pairs. HA-Prm1, with an N-terminal HA epitope tag, was cloned into a CEN plasmid and expressed from the strong, constitutive GPD promoter. A TAP tag was integrated at the C terminus of the native PRM1 gene. MATa PRM1-TAP cells were mated to MATa HA-Prm1 cells for the indicated times. The cells were lysed, and Prm1-TAP was collected on IgG-coated beads. Coprecipitation of HA-Prm1 with Prm1-TAP indicates assembly of a homotypic complex. Western blots of the lysates show that Prm1-TAP expression from the native PRM1 promoter is induced during mating, while HA-Prm1 is constitutively expressed. B, the homotypic Prm1 complex assemblies in the ER. Dominant C-terminal targeting signals for ER retention (KK...) induce during mating, while HA-Prm1 is constitutively expressed. Flow cytometry analysis of HA-Prm1 and Prm1-TAP was performed using a Becton Dickinson FACSCalibur and analyzed using FlowJo software.
To investigate the site of Prm1 complex assembly, the -KKXX ER localization signal of Wbp1 was fused to the C terminus of HA-Prm1. This -KKXX signal causes heterologous proteins including Prm1 to be retrieved from the cis-Golgi back to the ER (14). HA-Prm1-KKKKK coprecipitated with Prm1-TAP, indicating that the homotypic Prm1 complex must form in the ER or cis-Golgi (Fig. 1B, lane 2).

Ist2 has an unusual signal at its C terminus that drives transport directly from the ER to the plasma membrane, bypassing the normal secretory pathway (15). An HA-Prm1-Ist2 fusion protein coprecipitated with Prm1-TAP, indicating that transit through the cis-Golgi is not essential for Prm1 complex assembly (Fig. 1B, lane 3). In conclusion, the Prm1 complex assembles in the ER.

Because disulfide bonds are formed and rearranged by protein disulfide isomerases in the ER, we investigated whether disulfide bonds might contribute to the assembly or stability of the Prm1 complex by comparing the SDS-PAGE migration of HA-Prm1 from yeast cell lysates prepared with or without the reducing agent β-mercaptoethanol (β-ME). In the non-reduced sample, there were three bands: a major band migrating at >225 kDa, a minor band migrating at 100 kDa, and a faint band at 75 kDa (Fig. 2, lane 1). In the reduced sample, the two lower bands were the same, but the upper band was missing, and a new series of bands appeared which migrated from 100 to 150 kDa (Fig. 2, lane 2).

The lyses were treated with endoglycosidase H (Endo H) to investigate the contribution of N-linked glycosylation to these migration patterns. Endo H cleaves N-linked glycans from denatured proteins and works most efficiently under reducing conditions. After Endo H treatment with 10 mM DTT as a reducing agent, there was a single band migrating at 75 kDa, which corresponds to the molecular weight predicted from the amino acid sequence of Prm1 (Fig. 2, lane 4). If DTT was omitted from the Endo H reaction buffer, most of the >225-kDa form of HA-Prm1 shifted to 150 kDa (Fig. 2, lane 3).

We hypothesize that the >225-kDa band is a homodimer of mature HA-Prm1, the 150-kDa band is a homodimer of deglycosylated HA-Prm1, the bands migrating from 102 to 150 kDa are HA-Prm1 monomers with mature N-linked glycans, the 102-kDa band is an immature HA-Prm1 monomer that was core glycosylated in the ER, and the 75-kDa band is an unglycosylated monomer. The mature glycosylated dimer ran as a relatively sharp band when compared with the glycosylated monomer because proteins larger than 225 kDa are not well resolved on a 7% polyacrylamide gel. On a 4–12% gradient gel, the glycosylated dimer ran as a broader band than the glycosylated monomer (data not shown). Based upon the shift from homodimer to monomer after treatment with either DTT or β-ME, we conclude that Prm1 is cross-linked by one or more disulfide bonds.

The coprecipitation and non-reducing gel assays both indicate that Prm1 assembles into an oligomeric complex. However, the two assays gave apparently different estimates of the percentage of Prm1 proteins associated with the complex. At least 80% of the HA-Prm1 migrated at >225 kDa on non-reducing gels, but <10% coprecipitated with Prm1-TAP. An explanation for this difference is that HA-Prm1, which was expressed from a GPD promoter, was present at significantly higher levels than Prm1-TAP, which was expressed from the native PRM1 promoter by treating MATα cells with α-factor. Thus, homotypic HA-Prm1/HA-Prm1 complexes are more abundant than the mixed HA-Prm1/Prm1-TAP complexes detected in the coprecipitation assay.

The Luminal/Extracellular Domain of Prm1 Has Four Conserved Cysteines—Disulfide bonds form between cysteines in the lumen of the ER. Prm1 has 12 cysteines. Four of these (Cys55, Cys120, Cys277, and Cys545) are 100% conserved among the six closest PRM1 homologs and highly conserved in all 46 PRM1 homologs (all from fungi) identified in an NCBI BLAST search. The other eight cysteines are poorly conserved. In their original description of Prm1, Heiman and Walter proposed a transmembrane topology model based upon 5 hydrophobic sequences identified by the SOSUI algorithm and 14 potential N-glycosylation sites (3). In this model, Cys277 and Cys545 are accessible to protein disulfide isomerase in the lumen of the ER, but Cys55 is in the first cytoplasmic loop and Cys120 is in the second transmembrane domain (Fig. 3A). In contrast, an alternative topological model calculated using the HMMTOP algorithm places Cys55 and Cys120 in a large luminal/extracellular loop. In the HMMTOP model, a hydrophobic sequence proposed by Heiman and Walter to be the second transmembrane domain is instead found in a large extracellular loop, and the orientation of the first transmembrane domain is flipped, placing the N terminus in the cytoplasm.

We investigated the orientation of the N terminus of Prm1 to distinguish between the two topological models and determine whether Cys55 and Cys120 could form disulfide bonds in the ER. To facilitate this analysis, GFP was fused to the N terminus, and an endocytosis signal in the cytoplasmic loop was mutated to trap GFP-Prm1 on the plasma membrane (Fig. 3B). The plasma membrane-localized GFP-Prm1end fusion protein was functional in a mating assay when expressed from a CENT plasmid with a GPD promoter, indicating that the alterations have not altered its transmembrane topology.

3 V. N. Olmo and E. Grote, submitted for publication.
To determine the orientation of the N terminus, we first assayed for quenching of the GFP fluorescence by protonation. GFP-Prm1end- fluorescence was quenched at pH 3 in a solution containing the metabolic poisons NaN₃ and NaF but was not quenched in SC growth media buffered to pH 3 (Fig. 3C). Identical results were obtained with yeast expressing a control plasma membrane protein, Sso2-GFP, known to have a cytoplasmically oriented GFP. Because metabolically active yeast are able to maintain a neutral cytoplasmic pH while growing in low pH media, these results suggest that the N terminus of Prm1 is located in the cytoplasm, as predicted by HMMTOP.

The cytoplasmic orientation of the N terminus was confirmed using a more conventional protease protection assay (Fig. 3D). In this assay, protein domains displayed on the outer surface of the plasma membrane are cleaved by an extracellular protease, but cytoplasmic and transmembrane domains are protected because the protease cannot cross or enter the plasma membrane. Yeast expressing GFP-Prm1end- were treated with lyticase to remove the cell wall and with the indicated concentrations of Proteinase K (µg/ml) to degrade proteins on the extracellular surface of the plasma membrane. One sample was lysed with glass beads during the protease treatment to allow Proteinase K to degrade cytoplasmic proteins. After 90 min at 37 °C, PMSF was added to inhibit Proteinase K, and a total cell lysate was prepared. The Western blot was probed with anti-GFP serum. The arrow indicates a 31-kDa protected fragment representing GFP fused to the N-terminal cytoplasmic domain and first transmembrane domain of Prm1.

FIGURE 3. Prm1 topology. A, comparison of the original Prm1 topology map with the topology predicted by HMTMM. The positions of the 12 cysteines are shown, with conserved cysteines highlighted in boldface type. GFP is fused to the N terminus (NT). The membrane is depicted by two gray bars with cytoplasmic (Cyt) and extracytoplasmic (Ex) surfaces. B, Prm1end- is concentrated on the plasma membrane. GFP-PRM1end- and GFP-Sso2 were cloned behind a TEF1 promoter and integrated into the 3’ untranslated repeat of the SSO1 locus in MATa prm1Δ cells. The cells were observed by epifluorescence and DIC microscopy.

C, low pH quenching of GFP fluorescence. Cells expressing GFP-Sso2 and the endocytosis-deficient GFP-Prm1 mutant were pelleted and resuspended in either normal growth medium (SC) or buffer containing the metabolic poisons NaN₃ and NaF (N3/F) at the indicated pH. GFP fluorescence was measured in a fluorometer. After subtracting background from non-fluorescent control cells, the data were normalized by setting the fluorescence in SC medium at pH 7–100 to compensate for the 7-fold higher fluorescent intensity of GFP-Sso2. D, MATa prm1Δ cells expressing GFP-PRM1end- were treated with lyticase to remove the cell wall and with the indicated concentrations of Proteinase K (µg/ml) to degrade proteins on the extracellular surface of the plasma membrane. One sample was lysed with glass beads during the protease treatment to allow Proteinase K to degrade cytoplasmic proteins. After 90 min at 37 °C, PMSF was added to inhibit Proteinase K, and a total cell lysate was prepared. The Western blot was probed with anti-GFP serum. The arrow indicates a 31-kDa protected fragment.
mic side of the membrane, supporting a topological model that places the four conserved cysteines in the lumen of the ER, where they can form disulfide bonds.

**Identification of Cysteines That Form Intermolecular Disulfide Bonds in the Prm1 Complex**—To investigate the structural and functional consequences of disulfide bond formation, the four conserved cysteines in HA-Prm1 were mutated individually and in combination to serine, an amino acid of similar size and charge that cannot form disulfide bonds. The 4X-Ser mutant (HA-Prm1-C55S/C120S/C277S/C545S) comigrated on a polyacrylamide gel with the reduced form of wild-type HA-Prm1 (Fig. 2, lanes 2 and 5). The gel migration of 4X-Ser was not affected by reducing agents (DTT or β-ME) (Fig. 2, lane 6). Nevertheless, 4X-Ser remained sensitive to Endo H (Fig. 2, lane 7) and had the same proportion of partially and fully glycosylated forms as wild-type HA-Prm1 (Fig. 2, lanes 2 and 6). We conclude that the four conserved extracellular cysteines are required to form a disulfide-linked HA-Prm1 homodimer.

The complete collection of HA-Prm1 cysteine to serine mutants was expressed in the PRM1-TAP strain and assayed for Prm1 complex assembly. Mutation of all four extracytoplasmic cysteines strongly reduced, but did not completely eliminate, the coprecipitation of HA-Prm1 with wild-type Prm1-TAP (Fig. 4A, gel 1, lane 8). In addition, coexpression of the 4X-Ser-HA-Prm1 mutant with wild-type Prm1-TAP inhibited disulfide bond formation by Prm1-TAP, causing some Prm1-TAP to migrate as a fully glycosylated monomer (Fig. 4A, gel 2, lane 8). In combination, these results suggest that 4X-Ser-HA-Prm1 can bind to Prm1-TAP in vivo, competing with the assembly of Prm1-TAP into a disulfide-linked Prm1-TAP/Prm1-TAP homodimer. However, the 4X-Ser-HA-Prm1/Prm1-TAP heterodimer is unstable because it lacks disulfide cross-links, and it falls apart after cell lysis in the coprecipitation assay.

The single Cys mutant HA-Prm1 proteins all coprecipitated with Prm1-TAP to some extent, and the coprecipitated proteins migrated predominantly as disulfide-linked dimers (Fig. 4A, gel 1, lanes 4–7). Because no individual cysteine is essential to form a disulfide-linked complex, the native complex is likely to contain more than one disulfide cross-link.

Cys120 and Cys545 both contribute to the formation of disulfide cross-links in the Prm1 dimer. In total cell lysates a significant fraction of the C120S and C545S mutant proteins migrated as glycosylated monomers (Fig. 4A, gel 1, lanes 5 and 7), indicating that Cys120 and Cys545 contribute to dimerization. Furthermore, Cys120 and Cys545 are both sufficient for disulfide cross-linking because triple Cys mutants altering all cysteines except Cys120 or Cys545 coprecipitated with Prm1-TAP as disulfide-linked dimers (Fig. 4C, gel 1, lanes 4 and 6). Interestingly, the disulfide-linked form of these triple Cys mutants was highly enriched by coprecipitation and was not detected in the non-reduced lysates (Fig. 4C, gel 3, lanes 4 and 6).
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The C277S mutant had reduced glycosylation manifested by a reduction in the intensity of the heterogeneously glycosylated monomer bands on the immunoblot of reduced lysates (Fig. 4A, gel 4, lane 6). This glycosylation defect suggests that Cys\textsuperscript{277} is required for Prm1 folding and transport to the Golgi, where N-linked glycans are extended. Consequently, the C277S mutant had significantly reduced dimerization in the non-reduced lysate (Fig. 4A, gel 3, lane 6) and reduced binding to Prm1-TAP (Fig. 4A, gel 1, lane 6). Double and triple mutants in which C277S was combined with the C55S and C120S mutations also had reduced glycosylation (Fig. 4B, gel 4, lanes 5 and 7, and Fig. 4C, gel 4, lane 6). In contrast, combining the C545S mutation with C277S restored normal glycosylation (Fig. 4B, gel 4, lane 9, and Fig. 4C, gel 4, lanes 3 and 4), suggesting that the folding and glycosylation defects result from an improper disulfide bond formed by Cys\textsuperscript{545} only when Cys\textsuperscript{277} is mutated.

Intracellular Localization of the Cys to Ser Mutant Prm1 Proteins—In yeast, as in mammalian cells, disulfide-bonded proteins must be properly folded and cross-linked before they can exit the ER (16). Prm1 is no exception to this rule because GFP-Prm1\textsuperscript{end} was retained in the ER in yeast cells grown in media containing DTT (Fig. 5A). DTT inhibited cell fusion of both wild-type and prm1Δ mating pairs, indicating that Prm1 is not the only disulfide-bonded protein required for mating.

The localization of wild-type and mutant GFP-Prm1 proteins was investigated to identify cysteines required for intracellular Prm1 transport. Prm1 is transported through the secretory pathway and localizes to contact sites in mating pairs (3). However, most Prm1 proteins are targeted to vacuoles and degraded.\textsuperscript{3} To monitor Prm1 localization, \textit{MATa prm1Δ} cells expressing GFP-tagged mutant Prm1 proteins were mated to \textit{MATa fus1 fus2} mCherry cells to accumulate prezygotes. Wild-type GFP-Prm1 was located at contact sites (arrow) and in vacuoles (arrowhead). GFP-Prm1-KXX was retained in the ER surrounding the nucleus (arrow) and at cortical sites (arrowhead).

![FIGURE 5. GFP-Prm1 localization. A, DTT inhibits Prm1 transport. \textit{MATa prm1Δ} cells expressing GFP-Prm1\textsuperscript{end} from a \textit{CEN} plasmid under control of a galactose-regulated promoter were grown to log phase in 2% raffinose media and then transferred to 2% galactose media to induce GFP-Prm1\textsuperscript{end} expression. After 30 min at 30 °C, 2% glucose was added to repress GFP-Prm1\textsuperscript{end} synthesis, and the cells were grown for 2 additional hours in the presence or absence of 9 mM DTT. B, \textit{MATa prm1Δ} cells expressing wild-type or mutant forms of Prm1 from \textit{CEN} plasmids with GFP promoters were mated to \textit{MATa fus1 fus2} mCherry cells to accumulate prezygotes. Wild-type GFP-Prm1 was located at contact sites (arrow) and in vacuoles (arrowhead). GFP-Prm1-KXX was retained in the ER surrounding the nucleus (arrow) and at cortical sites (arrowhead).](https://example.com/figure5.png)

TABLE 1
Localization and cell fusion activity of the cysteine to serine mutant forms of Prm1

| Mutation | GFP-Prm1 localization | Fusion activity |
|----------|-----------------------|----------------|
| C55S     | Contact site          | +              |
| C120S    | ER                    | 0              |
| C277S    | Vacuole               | +              |
| C545S    | ER                    | +              |

Prezygotes from matings as described in the legend for Fig. 5B were scored according to the following scale: 0, no localization detected; 1, unambiguous localization in 1–20% of prezygotes or faint localization in 20–80% of prezygotes; 2, unambiguous localization in >80% of prezygotes; 3, unambiguous localization in >80% of prezygotes; 4, unambiguous localization in >80% of prezygotes; 5, unambiguous localization in >80% of prezygotes; 6, no localization detected. Cell fusion activity in matings as described in the legend for Fig. 6 were scored according to the following scale. +, within 10% of wild-type HA-Prm1; +/−, 70–90% of wild-type activity; 0, less than 20% of wild-type activity.

\textsuperscript{3} To monitor Prm1 localization, \textit{MATa prm1Δ} cells expressing GFP-tagged mutant Prm1 proteins were mated to \textit{MATa fus1 fus2} partner expressing cytoplasmic mCherry (Fig. 5B and Table 1). The \textit{fus1} and \textit{fus2} mutations delay cell wall remodeling, resulting in an accumulation of prezygotes (unfused mating pairs) with mCherry restricted to the \textit{MATa} partner. Wild-type GFP-Prm1 is concentrated at contact sites in these prezygotes, consistent with its proposed function in plasma membrane fusion, but can also be seen in endosomes, vacuoles, and the ER.\textsuperscript{3} In yeast, endosomes are small punctuate spots in the cytoplasm, vacuoles are large round structures that appear as indentations in DIC images, and the ER can be recognized as a ring surrounding the nucleus connected by thin strands to cortical ER underlying the plasma membrane. The Prm1-KXX fusion protein with a C-terminal ER retention signal had an ER localization pattern, as expected. In contrast, the 4X-Ser-GFP-Prm1 mutant was targeted to contact sites, although it had more prominent ER fluorescence when compared with wild-type GFP-Prm1. This result is supported by the detection of mature glycosylated 4X-Ser-HA-Prm1 on a Western blot (Figs. 2 and 4B). Thus, the absence of disulfide bonds does not prevent the folding or ER exit of a Prm1 protein that lacks free sulfhydryls.

Many of the GFP-Prm1 Cys to Ser mutants had an intracellular localization pattern similar to that of wild-type GFP-Prm1 (Fig. 5B and Table 1). In contrast, mutants such as C277S, which displayed immature glycosylation on Western blots, were...
enriched in the ER. Nevertheless, a small amount of GFP-Prm1-C277S was visible at contact sites in some mating pairs.

The C120S and C545S Mutations Inhibit the Fusion Activity of Prm1—Membrane fusion activity of the Prm1 mutants was assayed by mating MATa prm1Δ cells expressing mutant HA-Prm1 proteins to a MATα prm1Δ tester strain (Fig. 6 and Table 1). Fusion was detected by monitoring the exchange of cytoplasmic GFP expressed in the MATa cells and cytoplasmic mCherry expressed in the MATα cells (4, 17). All single and combinatorial mutants containing C120S and/or C545S had a severe reduction in mating activity, but these mutants still retained some residual function when compared with the empty vector control. The C277S mutant, although largely retained in the ER, had 80% of the mating activity of wild-type HA-Prm1. This counterintuitive result is consistent with previous observations that the amount of Prm1 protein expressed from the GPD promoter is in significant excess over the threshold required for membrane fusion activity (6). When expressed at a lower level from the CYC promoter, the C277S mutation inhibited mating by >90% compared with a wild-type control HA-Prm1 protein also expressed from the CYC promoter.

Prm1 Forms a Homotypic Complex in Vivo—If Prm1 forms a homotypic complex in vivo, it should be possible to alter the intracellular localization of GFP-Prm1 by expressing mutant HA-Prm1 proteins that are either retained in the ER (Prm1-KKXX) or targeted directly from the ER to the plasma membrane (Prm1-Ist2). Indeed, high level expression of HA-Prm1-KKXX caused wild-type GFP-Prm1 to accumulate in the ER, whereas expression of HA-Prm1-Ist2 redirected GFP-Prm1 to the plasma membrane (Fig. 7).

**DISCUSSION**

Prm1 assembles within the ER into a stable complex maintained by intermolecular disulfide bonds. Disulfide bonds contribute to the folding and maturation of Prm1, but they are not absolutely essential for Prm1 to exit from the ER or for Prm1 transport to contact sites in mating pairs. Nevertheless, mutations in either of the two cysteines (Cys120 and Cys545) that form intermolecular disulfide bonds strongly reduce the plasma membrane fusion activity of Prm1.
Disulfide Bonds in Prm1 Are Required for Cell Fusion

between the second and third transmembrane domains contains an endocytosis signal, and mutations in this signal trap Prm1 on the cell surface. An endocytosis signal must be on the cytoplasmic side of the membrane to interact with a cytoplasmic adapter protein. Prm1 has the same topology as CD9, a tetraspanin protein essential for sperm-egg fusion, but it lacks the CCG and PXXCX motifs that distinguish tetraspanins from other four-span membrane proteins (18). Other proteins with four transmembrane domains and two extracellular loops include those in Fig1, another yeast cell fusion protein (7, 8, 19), and Spe-38, a Caenorhabditis elegans sperm protein essential for fertilization (20).

In the model, two Prm1 proteins are linked by Cys^{120}–Cys^{120} and Cys^{545}–Cys^{545} disulfide bonds. Two cross-links are likely because none of the single Prm1 Cys/Ser mutations prevented formation of a disulfide-linked complex. In addition, if there was only a single cross-link, one of the extracellular cysteines would have a free sulfhydryl group. Cys^{120} and Cys^{545} must be able to form intermolecular cross-links because a disulfide-linked Prm1-TAP/Prm1-HA complex was detected with triple HA-Prm1 Cys/Ser mutants in which either Cys^{120} or Cys^{545} were the only extracellular cysteines, but not with a quadruple HA-Prm1 Cys/Ser mutant that had no extracellular cysteines. Homotypic Cys^{120}–Cys^{120} and Cys^{545}–Cys^{545} bonds rather than a pair of heterotypic Cys^{120}–Cys^{545} bonds are proposed because a small fraction of the HA-Prm1-C120S and HA-Prm1-C545S mutants migrated as dimers on a non-reducing gel when expressed in the absence of Prm1-TAP. However, the C120S and C545S mutants were inactive and could have non-native disulfide bonds. Thus, wild-type Prm1 could be a symmetrical dimer with two Cys^{120}–Cys^{120} bonds.

The Prm1 complex is likely to be a homodimer rather than a higher order oligomer because the >225-kDa band on non-reducing gels can be partially converted by Endo H to 150 kDa, the expected size of an HA-Prm1 dimer. Because the conversion to 150 kDa was incomplete, it is possible that some Prm1 proteins are incorporated into complexes with three or more subunits in a ring linked by Cys^{120}–Cys^{120} bonds. It is unlikely that an unknown protein is disulfide-bonded to Prm1 dimers because no protein detectable by silver staining copurified with Prm1-TAP in stoichiometric amounts.

In addition to the two intermolecular disulfide bonds, an intramolecular bond is depicted between Cys^{545} and Cys^{777}. Partial ER retention of the C277S mutant suggests that this disulfide bond is important for Prm1 folding. However, because the C55S mutant was not retained in the ER, the Cys^{55}–Cys^{777} bond itself cannot be required for ER exit. Instead, we propose that formation of a Cys^{55}–Cys^{777} bond prevents an interaction between Cys^{55} and Cys^{545} that would otherwise interfere with the folding of Prm1 into an export competent conformation. This model explains why the C277S/C545S double mutant is not retained in the ER. Although Fig. 8 presents the best disulfide connectivity model that can be constructed based upon current data, complementary methods using partial reduction and alkylation coupled with mass spectroscopy can more accurately map the disulfide connectivity of native proteins (21).

Protein Complexes and Membrane Fusion—Why is the assembly and stability of a Prm1 complex essential for its function? This is a difficult issue to address without a more detailed understanding of the mechanism whereby Prm1 promotes plasma membrane fusion. Disulfide bonds are found in a wide variety of membrane proteins with diverse functions and are crucial for the activity of most viral fusion proteins. The HA fusion protein of influenza virus has disulfide bonds linking the HA1 and HA2 subunits of each homotrimer. These disulfide bonds are essential for HA unfolding, trimerization, and exit from the ER (22). The gp64 fusion protein of baculovirus has intramolecular disulfide bonds linking the three subunits of a trimer. Reduction of these bonds with DTT inhibits membrane fusion and prevents gp64 from assembling into a higher order fusion complex (23). Disulfide bonds within the human immunodeficiency virus fusion protein must be rearranged by protein disulfide isomerase isoforms present on the cell surface to permit a conformational shift required for membrane merger (24–26). Disulfide bonds are also likely to be critical for the function of non-viral fusogens because extracellular cysteine-rich domains are present in proteins required for both developmental (EFF-1 and AFF-1) and germ line (SPE-38) fusions in C. elegans (20, 27).

Our finding that Prm1 forms disulfide-linked dimers provides important molecular insights and suggests a number of potential mechanisms by which Prm1 may facilitate cell fusion. We previously proposed that Prm1 coordinates the function of multiple fusion proteins to ensure that membrane merger proceeds to complete fusion rather than lysis (4, 6). Dimerization could facilitate this function by allowing the Prm1 complex to interact simultaneously with two binding partners via identical binding sites. Coordinated activity by multiple fusion proteins is a common theme shared by both viral fusion proteins and the SNARE proteins that mediate intracellular membrane fusion (28–32). Multiple fusion proteins are required because the conformational shift of a single viral fusion protein or the assembly of a single SNARE complex does not release sufficient energy to fuse two lipid bilayers (33). In addition, laterally associated fusion proteins might form a ring-like arrangement surrounding the pore and/or attract fusogenic lipids to the fusion site. Experiments with cells expressing different amounts of influenza HA revealed that HA aggregates are most important at later stages of the fusion process, including fusion of the distal phospholipid monolayers and fusion pore expansion (34, 35).

One intriguing feature of our revised topological model is that the hydrophobic region from Leu^{99} to Val^{123}, which was previously thought to be a transmembrane domain, is instead found in the first extracellular loop. Cys^{120}, which forms an intramolecular disulfide bond, lies near the C terminus of this hydrophobic sequence. On the other side of Cys^{120} is an amphipathic sequence from Thr^{137} to Ile^{170} that is predicted to form a parallel coiled-coil related to the coiled domains found in activated viral fusion proteins (36). Cys^{120} must be at the interface between subunits to form a disulfide cross-link, so the two adjacent domains likely form part of the dimer interface. One interesting possibility is that a conformational shift in Prm1 allows an extracellular hydrophobic domain to insert into the lipid bilayer of the opposing plasma membrane to tether the two membranes together, assemble a proteolipid fence surrounding the fusion pore, or induce membrane stresses that
favor fusion and inhibit lysis. The Cys\textsuperscript{120} disulfide bond might have to be reduced to facilitate exposure of the hydrophobic domain. Reduction of disulfide bonds is required to facilitate conformational shifts in several viral fusion proteins and is also essential for mammalian fertilization (37, 38). The proposed interaction between hydrophobic regions of Prm1 in one cell and membrane lipids in the opposing plasma membrane can explain why Prm1 only needs to be expressed on one cell of a mating pair to promote fusion (3).

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