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The yeast cell fusion protein FUS1 is O-glycosylated and spans the plasma membrane

(protease protection/*sec* mutant/hybrid protein/conjugation)

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ABSTRACT **Previous work has shown that efficient cell fusion during conjugation in *Saccharomyces cerevisiae* requires a pheromone-induced surface protein encoded by *FUS1*. We show that the FUS1 protein migrates on SDS/polyacrylamide gels with an apparent molecular mass of 80 kDa, although the mass is predicted to be 58 kDa from the gene coding capacity. This discrepancy results from the presence of O-linked mannose oligosaccharides attached to the clustered serines and threonines at the amino terminus of the protein. The addition of mannose is completely abolished in the early secretory mutant *sec53*, attenuated in the late-endoplasmic reticulum-blocked *sec18*, and unaffected in *sec7*, which is blocked late in the Golgi phase of secretion. Membrane fractionation and protease protection experiments indicate that FUS1 spans the plasma membrane, with its glycosylated amino terminus projecting into the periplasmic space.**

Mating in the yeast *Saccharomyces cerevisiae* involves the fusion of haploid cells of opposite mating type followed by the fusion of nuclei to form a diploid. Formation of the zygote requires the coordination of two processes—cell fusion and nuclear fusion. Cells stimulated by the appropriate mating pheromone augment their expression of surface agglutinins (1, 2), arrest their cell-cycle at the G_1 stage, and elongate to form a discernible tip (a process dubbed "shmooing") (3, 4; for review, see ref. 5). When the appropriate partners have achieved contact, presumably at the shmoo tip, the cells rapidly fuse, a process that requires the degradation and/or reorganization of the cell wall and the fusion of the two plasma membranes.

Two pheromone-inducible genes, *FUS1* and *FUS2*, are required specifically for cytoplasmic fusion during conjugation. In addition, a FUS1–LacZ protein fusion localizes to the cell surface, specifically to that region of membrane growth in the arrested cell known as the shmoo tip, a region thought to correspond to the fusion site (6). These observations support the notion that the *FUS1* gene product participates directly in cell fusion. In this report we demonstrate the surface localization of the native FUS1 protein and establish its membrane orientation in the plasma membrane. In addition, we find that FUS1 is O-glycosylated via the secretory pathway (7).

MATERIALS AND METHODS

Media, Strains, and Plasmids. Yeast extract/peptone (YEP) medium and SC-ura medium (synthetic complete medium lacking uracil) have been described (8). Yeast strains are listed in Table 1 and were grown at 30°C, unless indicated otherwise. Pheromone induction involved the addition of 0.01 vol of 0.5 mM synthetic α factor (Sigma) in methanol to exponentially growing cells (A_{600} = 0.5) and subsequent incubation at 30°C for 2 hr. Cells growing in SC-ura medium were centrifuged and resuspended in YEP medium just before pheromone addition.

For galactose induction experiments cells were grown to early logarithmic phase (A_{600} = 0.2) at 22°C in SC-ura medium/0.1% glucose, pelleted and resuspended at the same density in SC-ura medium/2% galactose, and incubated for 6 hr at 36°C or 11 hr at 22°C. (Cells grown at 36°C were shifted to the high temperature for 20 min before galactose addition.)

Plasmids used in this study are listed in Table 1. All protein fusions contain the amino-terminal 254 amino acids of the FUS1 protein. The *FUS1–SUC2* fusion lacks the invertase signal sequence (9). pSB249 contains the *Escherichia coli trpE* gene fused to the 1.4-kilobase (kb) *FUS1 Alu* I fragment, encoding the carboxyl-terminal 402 amino acids of FUS1 and is derived from pATH1 (gift of T. Koerner and A. Tzagoloff, Columbia University). All plasmids were routinely propagated in *Escherichia coli* HB101. The lithium acetate procedure (10) was used to transform yeast to uracil prototrophy.

Antibodies. FUS1 antigen was prepared from *E. coli* HB101 harboring pSB249 that was starved for tryptophan by adding indole acrylic acid to logarithmic cultures (11). Nonidet P-40-insoluble extracts were prepared essentially as described (12) and run on a 7.5% preparative polyacrylamide gel (13). The band corresponding to the TrpE–FUS1 fusion protein was cut from the gel, equilibrated with Dulbecco's phosphate-buffered saline (PBS) overnight, macerated with a Poly-Tron homogenizer, emulsified with an equal volume of Freund's adjuvant, and injected peritoneally into 4-week-old mice (≈5 μg of antigen per animal). Booster injections were performed every 3 to 4 weeks, with serum collected at these times from tail bleeds (diluted with an equal volume of PBS). All experiments shown used serum collected from a single mouse 3 months after the first injection. Preimmune serum contained no antibodies cross-reactive to yeast proteins on an immunoblot (data not shown).

Preparation of Extracts and Immunoblotting. Yeast extracts used for immunoblotting were prepared by glass bead disruption in the presence of trichloroacetic acid (14). SDS/PAGE (7.5% acrylamide) was done according to the method of Laemmli (13). In general, each lane contained the trichloroacetic acid-precipitable material from 0.6 A_{600} units of cells dissolved in 25 μl of sample buffer. Subsequent transfer of protein to nitrocellulose was by established procedures (15). Immunoblotting was performed in 50 mM Tris·Cl, pH 7.5/150 mM NaCl (TBS) plus 5% dry nonfat milk. Anti-FUS1 protein (diluted 1/500 in TBS-milk), mouse monoclonal anti-β-galactosidase (1/20) (gift of T. Mason, University of Massachusetts, Amherst), a rabbit antiinvertase (1/1000) (gift of R. Schekman, University of California, Berkeley), or rabbit

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Abbreviations: SC-ura, synthetic complete medium lacking uracil; YPD, yeast extract/peptone.

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Table 1. Strains and plasmids used

	Genotype
Yeast strains	
9399-7B	a *ura3-52 his4Δ29 GAL*$^+$
JY132	a *ura3-52 trp1Δ1 lys2-801 his4-34*
DBY1701	a *ura3-52 lys2 his4 suc2Δ9**
JY447	a *ura3-52 sec7 GAL*$^+$ [pSB251]
JY449	a *ura3-52 sec18 cyh2 GAL*$^+$ [pSB251]
JY450	a *ura3 leu2 sec53-6 GAL*$^+$ [pSB251]
JY505	a *ura3 leu2 sec53-6 GAL*$^+$ [pSB255]
Plasmids	
pSB273	2 μm, *URA3, FUS1*
pSB234	2 μm, *URA3, FUS1–lacZ*
pSB236	*CEN4, URA3, FUS1–SUC2*
pSB251	2 μm, *URA3, GAL–FUS1*
pSB255	2 μm, *URA3, GAL–FUS1–lacZ*

*From the collection of D. Botstein (Genentech).

antihexokinase (1/100,000) (gift of H. Ma, Cold Spring Harbor Laboratory) antiserum was added for 1–3 hr. The nitrocellulose was then washed three times with TBS plus 0.1% Nonidet P-40 and 0.05% SDS, twice with TBS, and incubated with ≈5 × 10^6 cpm of ^{125}I-labeled sheep anti-mouse immunoglobulin (for the first two primary antibodies) or ^{125}I-labeled protein A (for the latter two antibodies) (Amersham). After incubation for 1–2 hr, the blots were washed as before and exposed at −70°C to Kodak XAR autoradiographic film with an intensifying screen.

Radioactive Labeling. Cells were grown to midlogarithmic phase in SC-ura medium/0.5% sucrose. Two A_{600} units of cells were pelleted and resuspended in 4 ml of YEP/0.5% sucrose, and α factor dissolved in methanol or methanol alone was added. After 15 min at 30°C, 0.4 mCi of [2-^{3}H]mannose (gift of P. Robbins, Massachusetts Institute of Technology) was added to both tubes, and incubation was continued for 2 more hr. In preparing labeled FUS1 protein for β-elimination, 2 ml of cells and 2 mCi of label were used; for the protease protection experiment in Fig. 4, 3 ml of cells and 1 mCi of label were used.

β Elimination and Paper Chromatography. Immunoprecipitated [^{3}H]mannose-labeled FUS1 was cut from a 7.5% acrylamide gel and electroeluted in an Isco 1750 electrophoretic concentrator. Salt and detergent were removed from the eluate by multiple centrifugations with distilled water in a Centricon 30 filter (Amicon) with 50 μg of bovine serum albumin as carrier. After a final resuspension in 70 μl of water, sodium hydroxide was added to 0.1 M, and the reaction proceeded overnight at room temperature. The solution was neutralized with equimolar acetic acid, dried down to 10 μl, spotted on chromatography paper (Whatman 1), and chromatographed overnight in ethyl acetate/1-butanol/acetic acid/water (6:8:5:8) with raffinose, maltose, and mannose (subsequently silver-stained) as size standards.

Protease Protection. Pheromone-induced yeast cells were washed and resuspended in lyticase buffer (1.4 M sorbitol/25 mM Tris·HCl, pH 7.5/2 mM magnesium chloride/0.05% sodium azide/0.5% 2-mercaptoethanol) at a concentration of 22 A_{600} units per ml. Lyticase (Enzogenetics, Corvallis, OR) was added to a concentration of 12 μg/ml and incubated for 30 min at 30°C. Triton X-100 was added (or not) to 0.1%, and proteinase K (Boehringer) was added to 500 μg per ml and incubated for 10 min at room temperature. Trichloroacetic acid was added to a final concentration of 17% to stop the reaction, and the samples were frozen and processed as described (14) for trichloroacetic acid extraction, except that glass beads were omitted for the cells that had been lysed with detergent.

RESULTS

Identification of the FUS1 Gene Product. Antiserum raised against a TrpE–FUS1 fusion protein (containing the carboxyl-terminal 402 amino acids of FUS1) recognizes an 80-kDa protein specific to cells induced with mating pheromone (Fig. 1). This 80-kDa signal is considerably enhanced in pheromone-induced cells harboring the *FUS1* gene on a

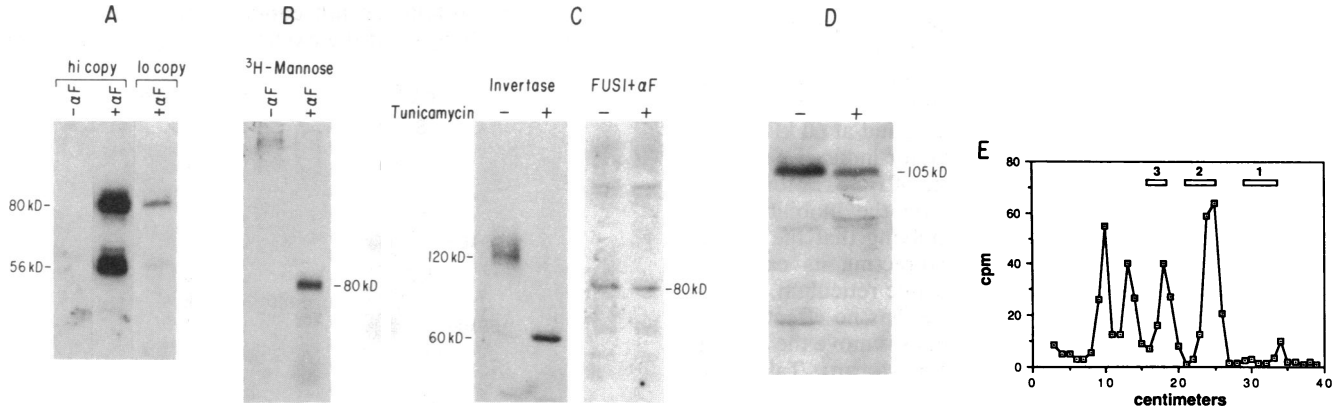

FIG. 1. (*A*) Identification of the *FUS1* gene product. Exponentially growing cells were incubated for 2 hr in the presence of 5 μM α factor (+ αF) or solvent alone (− αF). Total cell protein was extracted, electrophoresed through an SDS/polyacrylamide gel, transferred to nitrocellulose, and probed with anti-FUS1 antibody. Strains: JY132 [pSB273] (high copy); JY132 (low copy). (*B*) Labeling of FUS1 protein with [^{3}H]mannose. Uninduced (− αF) and α-factor-induced (+ αF) 9399-7B [pSB273] cells were incubated with radioactive mannose, pelleted, and resuspended in 1% SDS, disrupted with glass beads, and boiled. Subsequent immunoprecipitation with anti-FUS1 serum (after an initial clearing reaction with preimmune serum) was done as described (16) with 8 μl of anti-FUS1 or preimmune serum and 100 μl of Sepharose conjugated with sheep anti-mouse IgG (Cappel Laboratories). Immunoprecipitates separated by SDS/PAGE were visualized by fluorography. (*C*) Effect of tunicamycin on invertase and FUS1. JY132 cells growing in YEP plus 2% glucose were split into two aliquots, one of which was induced for invertase expression by shifting to YEP/0.1% glucose, and the other induced for FUS1 expression by the addition of α factor. Tunicamycin (10 μg/ml) was added either at the same time as the shift to low glucose for invertase or 20 min before α factor addition (for FUS1). Induction in both cases continued for 2 hr. Cells were subsequently prepared for immunoblotting with the appropriate antiserum. (*D*) Effect of tunicamycin on a FUS1–invertase fusion protein. DBY1701 [pSB236] cells growing in SC-ura medium were shifted to YPD, treated (+) or not treated (−) with tunicamycin and induced with α factor as in *C*. Anti-invertase antibody was used to detect the protein on an immunoblot. (*E*) β-elimination profile of FUS1. Alkali-labile material from gel-purified [^{3}H]mannose-labeled FUS1 was separated by paper chromatography, and radioactivity was counted. Approximately half the counts remained at the origin, as was seen for another solely O-glycosylated protein, chitinase (data not shown). Size markers: 1, mannose; 2, maltose; and 3, raffinose. kD, kDa.

high-copy 2-μm plasmid, an indication that the protein recognized by the antiserum corresponds to the *FUS1* gene product and not another pheromone-induced protein. A strain harboring a deletion of the *FUS1* gene does not accumulate detectable levels of protein (data not shown). A second abundant species, migrating at 56 kDa, is apparent in the cells overproducing FUS1 (and to a lesser extent in the cells bearing only the chromosomal copy). This species varies in its abundance from experiment to experiment and is presumed to be either a degradation product or an unprocessed version of the larger species.

FUS1 Is O-Glycosylated. The observed molecular mass of the FUS1 protein (80 kDa) differs from that predicted by the nucleotide sequence of the gene (58 kDa). The appearance of FUS1–β-galactosidase on the surface of conjugating cells (6) implicates extensive glycosylation during the transit of FUS1 through the secretory pathway as a possible explanation for this discrepancy. The sequence of the gene suggests two possible modes of glycosylation, N-linked and O-linked. The amino terminus is rich in serines and threonines (33 of 71 amino acids); a similar region has been shown to be O-glycosylated in the mammalian low density lipoprotein receptor (17). This domain is followed by a stretch of 25 amino acids the hydrophobic character of which suggests it spans a lipid bilayer (18). The remainder of the polypeptide (416 amino acids) is largely hydrophilic and contains eight potential sites for N-linked glycosylation (Asn-Xaa-Ser/Thr).

That FUS1 is glycosylated was shown by immunoprecipitation of the protein from extracts of cells grown on [^{3}H]mannose. Cells labeled in the presence of mating pheromone yield radioactive FUS1 protein migrating at 80 kDa (Fig. 1*B*). This protein is not present in uninduced cells. Because mannose is present in both N- and O-linked carbohydrate (19), however, this experiment shows only that FUS1 is glycosylated and does not address the nature of this modification.

We used tunicamycin, an inhibitor of N-linked glycosylation (20, 21), to ask whether FUS1 is modified by the addition of N-linked carbohydrate. A single culture was divided in half; one part was induced for invertase and the other for FUS1. Fig. 1*C* shows the effect of tunicamycin treatment on these proteins. Invertase is subject to extensive glycosylation in untreated cells, resulting in a characteristic fuzzy pattern in the 120-kDa range, but in tunicamycin-treated cells the protein appears as a carbohydrate-free band at 60 kDa (22). In contrast, parallel treatment of α factor-induced cells has virtually no effect on the apparent size of FUS1. Moreover, tunicamycin likewise has no effect on the mobility of a FUS1–invertase fusion (Fig. 1*D*), implying that the amino terminus of FUS1 is incapable of directing its carboxyl terminus to the lumen of the endoplasmic reticulum. Treatment of FUS1 with endoglycosidase H has no effect on its electrophoretic mobility, although it does remove the bulk of the invertase polysaccharide (data not shown). Taken together these experiments show that FUS1 is not modified by asparagine-linked glycosylation.

To determine whether the mannose in FUS1 is O-linked, we subjected gel-purified FUS1, previously labeled with [^{3}H]mannose and immunoprecipitated, to mild alkali treatment to release O-linked carbohydrate. The oligosaccharides were then separated by paper chromatography, and the radioactivity was counted, revealing the pattern shown in Fig. 1*E*. Such a pattern is typical of that obtained from β elimination of bulk yeast mannoprotein, with mannobiose as the predominant species along with mannose, mannotriose, mannotetraose, and mannopentaose (23, 24). From these data we conclude that FUS1 is glycosylated at serine and threonine residues and not at asparagine residues.

Membrane Orientation of FUS1. Although previous experiments with cell fractionation (25) and immunofluorescence (6) suggested that FUS1–LacZ fusions are localized to the plasma membrane (specifically to the shmoo tip), these studies did not examine the localization of the native protein. To this end a crude membrane fraction was prepared from pheromone-induced cells, and the distribution of FUS1 in membranes was compared with that of the supernatant. All the detectable FUS1 and none of the hexokinase, a soluble protein, is found in the membrane fraction (Fig. 2).

The orientation of FUS1 relative to the plasma membrane was determined by protease protection experiments. If the amino terminus of FUS1 faces the cell exterior, it should be sensitive to exogenously added protease, whereas the bulk of the protein should be protected. Upon removal of the cell wall and lysis and detergent extraction of the plasma membrane, however, the entire protein should become accessible to protease. That it is the amino terminus that is exposed to protease is demonstrated by the analysis of two FUS1 hybrid proteins that have in common the first 254 amino acids of FUS1 fused to either β-galactosidase or invertase.

Analysis of the protease protection experiment in Fig. 3*A* indicates partial degradation of FUS1 in the absence of cell lysis and complete degradation upon cell lysis. Hexokinase remains inaccessible to protease in the absence of detergent (Fig. 3*B*), although it is substantially susceptible when available for proteolysis. Upon digestion with external proteinase K, both FUS1 hybrid proteins lose an equivalent amount ($\approx$20 kDa) of peptide and sugar as does the native FUS1 (Fig. 3*C*). Therefore, the sensitive region of FUS1 maps to its amino terminus, which we assume also to be the target for glycosylation.

To confirm that the bulk of FUS1 oligosaccharide resides on the external domain of the protein, we demonstrate the loss of radioactive mannose from a FUS1–LacZ fusion protein upon external protease digestion of intact yeast cells. Pheromone-treated cells were labeled with [^{3}H]mannose and subsequently digested with proteinase K. The immunoblot (Fig. 4*A*) shows that the proteinase K digestion produces a significant population of truncated proteins, but based on the absence of an augmented radioactive band (Fig. 4*B*, +PK), the truncated proteins do not contain significant mannose. Therefore, proteolysis of the external domain of the protein

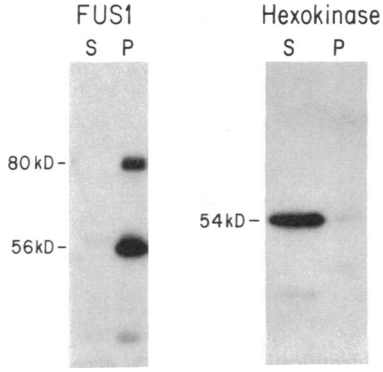

FIG. 2. Membrane localization of FUS1. A membrane-rich pellet (P) and its cytosolic supernatant (S) were prepared from 9399-7B [pSB273] cells and probed for FUS1 and for hexokinase on immunoblots. Thirty A_{600} units of pheromone-induced yeast cells were washed and resuspended in 0.2 ml of water. Cells were disrupted, and crude membranes were prepared as described in ref. 26, except that 20% (vol/vol) glycerol was substituted for 6% (wt/vol) sorbitol, and membranes were pelleted at 75,000 rpm (200,000 $\times$ *g*) for 15 min in a Beckman TL100 tabletop ultracentrifuge equipped with a TLA 100.2 rotor. The high-speed supernatant (0.85 ml) was removed, and the membrane pellet was rinsed with glycerol buffer and solubilized by boiling in 0.1 ml of 1% SDS/1% 2-mercaptoethanol/10 mM Tris·Cl, pH 8.0. Twenty microliters of the supernatant and 2.5 μl of the solubilized pellet were used for immunoblotting. kD, kDa.

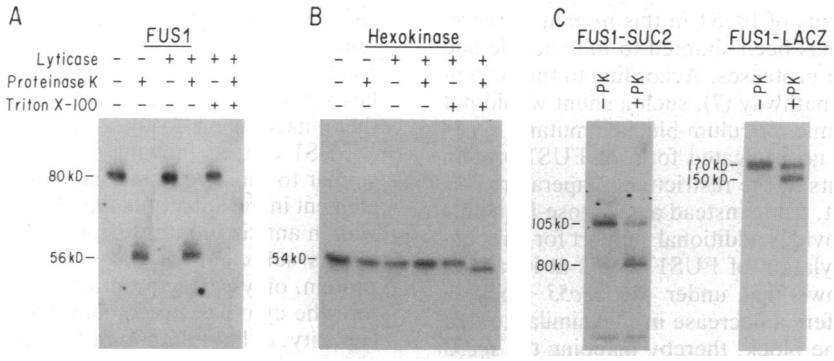

FIG. 3. Protease sensitivity of FUS1 (*A*) and hexokinase (*B*) in intact cells and spheroplasts. 9399-7B [pSB273] cells were induced with α factor and prepared for proteinase K digestion either as intact cells (no lyticase), as spheroplasts (plus lyticase), or as lysates (plus lyticase and Triton). Nitrocellulose-bound protein was probed with anti-FUS1 serum or with anti-hexokinase serum. It has been reported that limited proteolysis of hexokinase degrades only the amino-terminal 11 amino acids of the polypeptide (27). (*C*) Protease protection of FUS1–invertase and FUS1–β-galactosidase fusions. DBY1701 [pSB236] (FUS1–SUC2) and 9399-7B [pSB234] (FUS1–lacZ) were induced with α factor and prepared for proteinase K digestion as intact cells. Immunoblots were probed with anti-invertase serum or with anti-β-galactosidase monoclonal antibody. kD, kDa.

results in the concomitant loss of mannose residues. We conclude that most, if not all, of the O-linked mannose in FUS1 is found on its external domain, which we show in the previous experiment to correspond to the serine- and threonine-rich amino terminus.

Progression Through the Secretory Pathway. The yeast secretory pathway is defined by conditional mutants (called *sec*) that block the secretion of certain yeast-surface proteins, such as invertase and acid phosphatase (7). Asparagine-linked oligosaccharides are added as they travel from the endoplasmic reticulum into the Golgi apparatus, and different *sec* mutants arrest the process at discrete steps in the pathway. Such an analysis has not been reported for a specific O-glycosylated protein. The progression of the O-glycosylation pathway can be monitored by immunoblot analysis of FUS1 protein extracted from these secretory mutants; the absence of N-glycosylation simplifies the interpretation of such an experiment. To avoid the problem that the induction of FUS1 protein was poor in several *sec* mutants at their restrictive temperature, we used a *GAL–FUS1* promoter fusion so that we could induce FUS1 with galactose in the *sec* strains. This overexpression produces two major FUS1 bands (80 kDa and 56 kDa), as well as some minor bands. For the purposes of the following experiment we compare only the slowest species appearing in each lane to monitor the addition of O-linked mannose through the secretion pathway.

When *sec53* cells [deficient in phosphomannomutase (27)] harboring a *GAL–FUS1* fusion are shifted to nonpermissive temperature and subsequently induced by the addition of galactose, they express a smaller, presumably unmodified FUS1 product that migrates at 58 kDa (Fig. 5*A*). This species is presumed to be the unglycosylated form because its apparent molecular mass agrees with that predicted from the sequence. Invertase, which is solely N-glycosylated, encounters a similar block in *sec53*, accumulating in the endoplasmic reticulum with little or no carbohydrate addition (28, 29).

sec18 cells, which accumulate core-glycosylated invertase in the endoplasmic reticulum at the high temperature, are apparently able to add some, but not all, of the proper mannose residues to FUS1 at 36°C (Fig. 5*A*). The difference in the migration between the *sec18* and the *sec53* products suggests that 15 kDa has been added, which, if composed entirely of mannose, would correspond to ≈75 residues (see *Discussion*).

Analysis of FUS1 protein in *sec7* reveals that the protein has received its full complement of mannose additions before this late Golgi block (30). We interpret the appearance of the

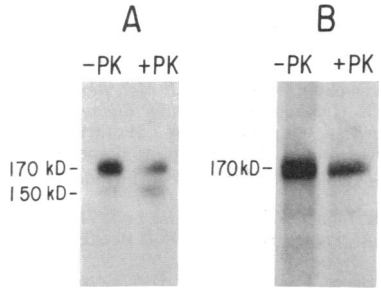

FIG. 4. Protease sensitivity of [^{3}H]mannose-labeled FUS1–β-galactosidase. 9399-7B [pSB234] cells were induced with α factor, labeled with [^{3}H]mannose, and incubated with proteinase K (PK) as described. The cells were broken, and protein was extracted by the trichloroacetic acid method (17). Immunoprecipitation conditions followed those described in ref. 16, with 100 μl of monoclonal anti-β-galactosidase, 20 μl of anti-mouse IgG Sepharose, and 1 mM diisopropylfluorophosphate. Immunoprecipitates were divided in two, separated by SDS/PAGE, and immunoblotted with anti-β-galactosidase antibody (*A*) or visualized by fluorography (*B*). kD, kDa.

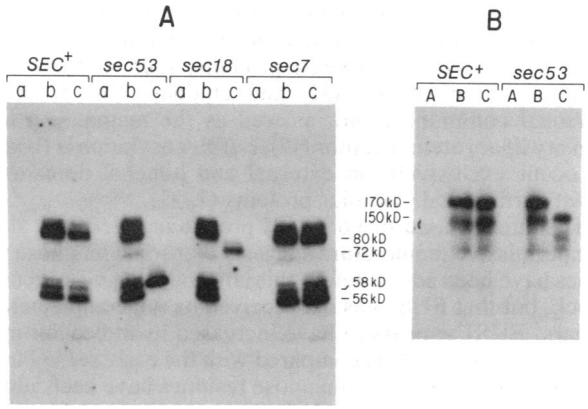

FIG. 5. (*A*) FUS1 intermediates in *sec* mutants. Cells were pregrown at 22°C in SC-ura medium plus 0.1% glucose (lanes a), shifted to 2% (wt/vol) galactose at 22°C for 11 hr (lanes b) or to 2% galactose at 36°C for 6 hr (lanes c). Strains: 9399-7B [pSB251] (*SEC*$^+$); JY450 (*sec53*); JY449 (*sec18*); JY447 (*sec7*). (*B*) FUS1–β-galactosidase in *SEC*$^+$ and *sec53* cells. Uninduced (lanes a), 22°C galactose-induced (lanes b), and 36°C galactose-induced (lanes c) cells were grown as in *A*. Strains: 9399-7B [pSB255] (*SEC*$^+$); JY505 (*sec53*). kD, kDa.

low molecular mass forms of FUS1 in this mutant to represent a population that has been shunted to the vacuole and subjected to its resident proteases. According to the current model of the secretory pathway (7), such a shunt would not be seen in the endoplasmic reticulum-blocked mutants *sec53* and *sec18*, and indeed no truncated form of FUS1 protein appears in those mutants at the restrictive temperature.

The same experiment, using instead a galactose-inducible FUS1–LacZ fusion, provides additional support for our contention that the glycosylation of FUS1 occurs at its amino terminus. Fig. 5*B* shows that under the *sec53* block, a FUS1–LacZ fusion suffers a decrease in size similar to that of FUS1 under the same block, thereby mapping the *sec53* effect to the amino terminus. Because the *sec53* effect amounts to an abolition of glycosylation, the FUS1 amino terminus contains the region that is O-glycosylated, bolstering the arguments to that effect based on protease protection.

DISCUSSION

In this report we present a structural analysis of a specific membrane protein required for efficient cell fusion. At the amino terminus is a preponderance of serines and threonines attached to short oligosaccharides consisting of one to five mannose residues. This domain faces the periplasm, beyond which lies the rigid cell well. This rather small glycopeptide (71 amino acids) is followed by 25 hydrophobic residues that span the plasma membrane and lead to the cytoplasmic bulk of the polypeptide.

Several lines of evidence support our contention that the amino terminus of the FUS1 protein is glycosylated and faces the cell exterior. The latter point is established by the observation that the extent of protease sensitivity in intact cells remains constant for three proteins (FUS1, FUS1–invertase, and FUS1–β-galactosidase), which share only amino-terminal sequences. The following evidence indicates that mannose oligosaccharides are added to this domain: (*i*) the observed size of the two FUS1 hybrid proteins is consistent with a saccharide content of 20–25 kDa; (*ii*) a FUS1–β-galactosidase protein exhibits an ≈20-kDa reduction in size in a *sec53* mutant, where glycosylation is completely abolished; (*iii*) tritiated mannose can be incorporated into FUS1–β-galactosidase and then removed by external proteolysis; (*iv*) external proteolysis of native FUS1 results in a molecular mass shift (24 kDa) inconsistent with 71 unmodified external amino acids; (*v*) the amino terminus contains clustered serines and threonines, which are the putative sites of O-linked glycosylation in yeast proteins, such as the lysine-arginine endopeptidase (32) and chitinase (M. Kuranda, personal communication), as well as the mammalian low-density lipoprotein receptor (17); (*vi*) glycosylation is thought to occur exclusively on external and lumenal domains of yeast surface and vacuolar proteins (7, 31).

The intermediate size of FUS1 protein in *sec18* cells at the nonpermissive temperature suggests that some mannose residues have been added before this late endoplasmic reticulum block, but that FUS1 has not received its full complement of sugars. FUS1 appears to have increased its molecular mass by ≈15 kDa in *sec18* as compared with the early *sec53* block, which suggests that ≈75 mannose residues have been added. Because only 33 O-glycosylation targets are available in the FUS1 protein, we infer either that glycosylated FUS1 runs aberrantly on SDS/PAGE or that more than one mannose has been added per serine or threonine residue. The latter possibility is contradicted by an earlier report that only one O-linked mannose residue per target site is added to bulk mannoprotein before the *sec18* block (24).

None of the eight potential sites for asparagine-linked glycosylation in FUS1 is glycosylated. Likewise, the many sites that are glycosylated in wild-type secreted invertase remain unmodified in a FUS1–invertase fusion. Our explanation for this lack of N-glycosylation is that the target sites lie exclusively in the cytosolic domain of the protein, making them inaccessible to the glycosylation machinery.

FUS1 can be thought of as a membrane fusion protein similar to those found in enveloped animal viruses or as an element in the apparatus that degrades or reorganizes the cell wall in anticipation of fusion. Although pheromone stimulation is sufficient for the synthesis and localization of the protein, only partner contact can initiate cell fusion. Therefore the cytoplasmic domain might function in a regulatory capacity, either inhibiting FUS1 activity before partner contact or stimulating its activity once partner contact has been achieved. Alternatively, the FUS1 protein could serve a cognitive role, with its external domain "sensing" the imminence and position of a partner and transmitting this information to the cytoplasm, triggering rapid, concerted exocytosis of "fusion activity."

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