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# The a-Factor Transporter (*STE6* Gene Product) and Cell Polarity in the Yeast *Saccharomyces cerevisiae*

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**Abstract.** *STE6* gene product is required for secretion of the lipopeptide mating pheromone a-factor by *Saccharomyces cerevisiae* *MAT $\alpha$*  cells. Radiolabeling and immunoprecipitation, either with specific polyclonal antibodies raised against a TrpE-Ste6 fusion protein or with mAbs that recognize c-myc epitopes in fully functional epitope-tagged Ste6 derivatives, demonstrated that Ste6 is a 145-kD phosphoprotein. Subcellular fractionation, various extraction procedures, and immunoblotting showed that Ste6 is an intrinsic plasma membrane-associated protein. The apparent molecular weight of Ste6 was unaffected by tunicamycin treatment, and the radiolabeled protein did not bind to concanavalin A, indicating that Ste6 is not glycosylated and that glycosylation is not required either for its membrane delivery or its function. The amino acid sequence of Ste6 predicts two ATP-binding

folds; correspondingly, Ste6 was photoaffinity-labeled specifically with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ . Indirect immunofluorescence revealed that, in exponentially growing *MAT $\alpha$*  cells, the majority of Ste6 showed a patchy distribution within the plasma membrane, but a significant fraction was found concentrated in a number of vesicle-like bodies subtending the plasma membrane. In contrast, in *MAT $\alpha$*  cells exposed to the mating pheromone  $\alpha$ -factor, which markedly induced Ste6 production, the majority of Ste6 was incorporated into the plasma membrane within the growing tip of the elongating cells. The highly localized insertion of this transporter may establish pronounced anisotropy in a-factor secretion from the *MAT $\alpha$*  cell, and thereby may contribute to the establishment of the cell polarity which restricts partner selection and cell fusion during mating to one *MAT $\alpha$*  cell.

**S***TE6* gene product of *Saccharomyces cerevisiae* (Kuchler et al., 1989; McGrath and Varshavsky, 1989) belongs, based on sequence homology, to a super-family of prokaryotic and eukaryotic ATP-dependent transport proteins (reviewed in Ames et al., 1990; Hyde et al., 1990; Kuchler and Thorner, 1992b). We demonstrated previously that Ste6 is required, and rate limiting, for secretion of the mating pheromone a-factor by *MAT $\alpha$*  cells (Kuchler et al., 1989). We have also shown (Sterne, R. E., and J. Thorner, 1986. *J. Cell Biol.* 103:189 [abst.]; Sterne, R. E., and J. Thorner, 1987. *J. Cell Biol.* 105:80 [abst.]; Sterne, 1989) that mature a-factor, a 12-residue lipopeptide (Anderegg et al., 1988) that is essential for stimulating conjugation between haploid yeast cells (Michaelis and Herskowitz, 1988), is generated intracellularly from a larger precursor that lacks a hydrophobic signal peptide (Brake et al., 1985). Proteo-

lytic processing and other posttranslational covalent modifications are required for this maturation (Schafer et al., 1989, 1990; Sterne-Marr et al., 1990). In agreement with the conclusion that Ste6 actually mediates translocation of mature a-factor across the plasma membrane, examination of temperature-sensitive secretion-defective (*sec*) mutants (Schekman, 1985) demonstrated that the route of a-factor export is independent of the classical secretory pathway (Sterne and Thorner, 1986; McGrath and Varshavsky, 1989; Sterne, 1989). On the basis of these findings we (Kuchler et al., 1989; Kuchler and Thorner, 1990, 1992b) and others (Muesch et al., 1990) proposed that Ste6, and perhaps other members of this super-family in eukaryotes, like Mdr1 ("multiple-drug-resistance") (Endicott and Ling, 1989; Kane et al., 1990), might comprise a novel type of secretory apparatus required for the membrane translocation of peptides and proteins that lack a hydrophobic signal sequence.

A direct role for Ste6 in a-factor transport makes several predictions about the membrane insertion, subcellular localization, topology, and regulation of this protein. In this study, we generated and used specific antibodies and other probes to characterize in more detail the membrane association, biochemical properties, and physiological functions of Ste6. The results presented here are fully consistent with the conclusion that Ste6 is the a-factor transporter.

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## Materials and Methods

### Yeast Strains and Culture Conditions

Yeast strains used throughout this study were W303-1A (*MAT $\alpha$  ura3-52 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*) and its isogenic *ste6 $\Delta$*  derivative, WKK7 (*ste6 $\Delta$ ::HIS3*), constructed as described before (Kuchler et al., 1989). Mating tester strains were RC757 (*MAT $\alpha$  sst2-1 rml1 his6 met1 can1 cyh2*) (Chan and Otte, 1982) and L1543 (*MAT $\alpha$  lys9*) (Trueheart and Fink, 1989). Synthetic medium (SD), supplemented with nutrients appropriate for maintenance of plasmids, or rich medium (YPD), were prepared as described elsewhere (Sherman et al., 1986). Cells were grown routinely at 30°C.

### Recombinant DNA Manipulations

A TrpE-Ste6 fusion protein was constructed by ligating the 415-bp SnaBI/SspI fragment of the *STE6* gene (Kuchler et al., 1989), corresponding to the COOH-terminal 123 amino acids of the coding sequence, into the SmaI site of the *Escherichia coli* expression vector pATH22 (Koerner et al., 1991), to yield plasmid pAK17. To insert epitope tags into the *STE6* gene, two different synthetic oligonucleotide linkers, each encoding an antigenic determinant from the *c-myc* gene (Evan et al., 1985) were ligated in-frame at two different positions within the *STE6* coding sequence, as follows. Plasmid pRE4 (Kuchler et al., 1989; Sterne, 1989) was digested to completion with HindIII and then partially with SpeI, and the 5.5-kb SpeI-HindIII fragment containing the entire *STE6* gene was gel purified and ligated into the corresponding restriction sites of the vector pRS316 (Sikorski and Hieter, 1989), to give plasmid pKAK16. pKAK16 was digested briefly with SpeI, converted to blunt ends using the Klenow fragment of *E. coli* DNA polymerase I, and linker 1 (encoding the amino acid sequence EQKLISEEDL) was inserted into the *STE6* coding sequence at the most NH<sub>2</sub>-terminal of two internal SpeI sites (between residues 70 and 71), which lies within the first predicted external hydrophilic loop (see Fig. 1), to yield the low copy (*CEN*-containing) plasmid pRKS1. To generate a multi-copy plasmid containing the same construction, pRKS1 was digested with SacI and HindIII, and the resulting fragment was inserted into the unique SacI and HindIII sites of the vector YEp352 (Hill et al., 1986), to yield the 2  $\mu$ m DNA-containing plasmid pYKS1. To place a *c-myc* epitope near the extreme COOH-terminus of the *STE6* coding sequence, linker 2 (encoding the amino acid sequence LEEQKLISEEDLLRKRstp, where stp represents a TAA translational termination codon) was ligated into the unique HpaI site of pKAK166, a derivative of pKAK16 in which a unique HpaI restriction site (between residues 1285 and 1286) was introduced by site-directed mutagenesis using an appropriate oligonucleotide primer and a commercial in vitro mutagenesis kit (Amersham Corp., Arlington Heights, IL), to produce the *CEN* plasmid pRKS2. A corresponding multi-copy plasmid, pYKS2, was created by cleaving pRKS2 with SacI and HindIII, and inserting the resulting fragment into the SacI and HindIII sites of YEp352. By combining appropriate restriction fragments, plasmids pRKS3 and pYKS3, which each contain both of the *c-myc* linkers (at positions 70 and 1285, respectively) were generated. All of the plasmids constructed were examined by direct nucleotide sequence analysis to confirm proper orientation of the linkers inserted and preservation of the correct reading frame. Plasmids pKK1, a multi-copy plasmid carrying the *MPaI* gene, and pKK16, a multi-copy plasmid carrying both the *MPaI* and *STE6* genes, were described previously (Kuchler et al., 1989). Standard methods were used for DNA-mediated transformation of yeast (Ito et al., 1983) and *E. coli* (Sambrook et al., 1989).

### Preparation of Antisera and mAbs

*E. coli* strains TG1 or DH5 $\alpha$  carrying plasmid pAK17 were treated with indolacrylic acid to induce expression of the TrpE-Ste6 fusion protein and inclusion bodies containing this antigen were isolated by established procedures (Koerner et al., 1991). Protein in the inclusion bodies was subjected to preparative electrophoresis in a 10% polyacrylamide gel containing SDS, and the band representing the TrpE-Ste6 fusion protein was recovered using an Elutrap<sup>®</sup> elution chamber (Schleicher & Schüll, Keene, NH). The purified antigen was used to raise polyclonal antisera in New Zealand White rabbits following standard immunization regimes (Harlow and Lane, 1988). Serum no. 9384 was the polyclonal anti-Ste6 antibody used for the experiments presented here. Hybridoma cell lines (generously provided by J. M. Bishop, University of California, San Francisco) secreting the mAbs Myc-9E10 and CT14-G4, which recognize the *c-myc* epitope used in this work, were used to produce Ascites fluid that was used as the antibody

source without further purification. Recloning of hybridoma cell lines and induction of Ascites tumors in mice was carried out in the Hybridoma Facility of the Cancer Research Laboratory, University of California, Berkeley, CA. Polyclonal antibodies (serum no. 838) directed against the yeast H<sup>+</sup>-translocating plasma membrane ATPase (*PmaI* gene product) were generously provided by R. Serrano (Polytechnic University, Valencia, Spain) (Serrano et al., 1991). Polyclonal antibodies (pooled sera A, B, and C) directed against the glycolytic enzyme, yeast phosphoglycerate kinase (*Pgk1* gene product) were prepared in this laboratory as described previously (Baum et al., 1978).

### Preparation of Cell Extracts, Membranes, and Solubilized Membrane Protein

Yeast cells grown to mid-exponential phase ( $A_{600nm} = 0.5-1$ ) in SD or YPD medium were harvested by brief centrifugation in a microfuge, washed once in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.8, containing 2% 2-mercaptoethanol and 1 mM phenylmethylsulfonylfluoride) and resuspended in the same ice-cold buffer at a concentration of  $A_{600nm} = \sim 5$  per 100  $\mu$ l. All subsequent steps were carried out at 4°C. Prechilled glass-beads (0.45-0.6 mm diameter) were added to the meniscus of the suspension and lysis was achieved by vigorous vortex mixing for three 2-min intervals with 1 min of cooling in between. The resulting homogenate was diluted 5-10-fold with the same buffer, and unbroken cells, glass beads, and large debris were removed by centrifugation at 1,000 rpm for 5 min in a Sorvall SS34 rotor (Sorvall Instruments, Newton, CT). Membranes were collected from the clarified lysate by sedimentation for 1 h at 60,000 rpm in a table top ultracentrifuge (Beckman TL100, TLA-100.3 rotor; Beckman Instruments, Inc., Fullerton, CA). The membrane pellet was washed once by resuspension and recentrifugation in lysis buffer, resuspended in a conical tube using a plastic conical pestle in 10 mM Tris-HCl, 1 mM EDTA, pH 7.8, and frozen in aliquots at -70°C. When membranes were to be used for photoaffinity labeling (see below), cells were grown and lysed exactly as described above, except that 2-mercaptoethanol was omitted from the lysis buffer. If total extracts were to be analyzed by immunoblotting, cultures were grown and harvested as described above, but were resuspended in PAGE sample buffer containing 5% SDS and 10% 2-mercaptoethanol (Dreyfuss et al., 1984) before disruption of the cells by vortex mixing with glass beads. Under these conditions, the proteolytic degradation of Ste6 that was observed occasionally during preparation of membranes was eliminated. Protein concentration was measured by the method of Lowry et al. (1951) in the presence of 1% SDS to solubilize membrane proteins, using BSA as the standard. For immunoblot analysis,  $\sim 50-100$   $\mu$ g of protein were suspended in SDS-PAGE sample buffer, heated at 55°C for 10 min, and subjected to electrophoresis in a 7% SDS-PAGE gel. Transfer of proteins from SDS-PAGE gels (Dreyfuss et al., 1984) to nitrocellulose (BA85, pore size 0.45  $\mu$ m, Schleicher & Schüll) was carried out by standard procedures (Towbin et al., 1979). Proteins on immunoblots were detected using a chemiluminescence detection system (Amersham Corp.) and conditions recommended by this manufacturer.

For subcellular fractionation by differential centrifugation, total cell-free homogenates were prepared as described above and subjected to sedimentation at 12,000 g for 30 min. The supernatant fraction (S1) was removed from the resulting pellet (P1) and subjected to further sedimentation at 100,000 g for 1 h, producing a second pellet (P2) and supernatant fraction (S2). Each fraction was adjusted to an identical final volume and equivalent amounts of each fraction were dissolved in SDS-PAGE sample buffer and subjected to electrophoresis in a 7% gel. The resolved proteins were transferred to nitrocellulose filters as described above and detected with the following antisera: Ste6, 1:10,000 dilution of Ascites fluid containing anti-c-Myc mAb 9E10; PmaI, 1:35,000 dilution of polyclonal antiserum no. 838; and, Pgk1, 1:50,000 dilution of pooled sera A, B, and C. For membrane fractionation by sucrose gradient buoyant density sedimentation, a sample of the P1 material prepared as described above was subjected to centrifugation at 170,000 g for 16 h in a 30-55% sucrose gradient, essentially as described by Bowser and Novick (1991). Fractions (1.2 ml) of the gradient were collected from the top using a peristaltic pump (Gilson Medical Elec. Inc., Middleton, WI) and analyzed by electrophoresis in a 7% SDS-PAGE followed by blotting and immunodetection with anti-Ste6 and anti-PmaI antibodies, as described above.

### Radiolabeling of Cells and Immunoprecipitation

To label total proteins, cells were grown in low sulfate medium (LSM+), prepared according to Julius et al. (1984) and supplemented (Sherman et

al., 1986) with all L-amino acids except cysteine and methionine. Cells were labeled continuously in the presence or absence of tunicamycin at a density of  $A_{600\text{nm}} = 5\text{--}10$  per ml for 45–60 min using 30  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]cysteine and [ $^{35}\text{S}$ ]methionine (Trans- $^{35}\text{S}$ -Label™, 1,097 Ci/mmol, ICN Radiochemicals, Irvine, CA) per  $A_{600\text{nm}}$  of cells. To label phosphoproteins, cultures were grown for 40–60 min at 30°C in low phosphate medium (Blumer et al., 1988) containing 2 mCi of  $\text{H}_3^{32}\text{PO}_4$  (320 TBq/mmol, NEN) per  $A_{600\text{nm}}$  cells in the absence or presence of 5  $\mu\text{M}$   $\alpha$ -factor pheromone. Tunicamycin treatment, glass bead lysis of radiolabeled cells, immunoprecipitation using polyclonal anti-Ste6 antibodies (serum no. 9384) or anti-c-myc mAb (Myc-9E10), and binding to concanavalin A-Sepharose beads were carried out by methods described in detail elsewhere (Franzoso et al., 1991). Fluorography of SDS-PAGE gels used a commercially available amplifier (Amplify™, Amersham Corp.) and conditions recommended by the manufacturer.

### Photoaffinity Labeling

Yeast membranes (4 mg total protein), prepared from a *ste6* $\Delta$  mutant or from cells expressing Ste6 from pYKS3, were suspended in labeling buffer (10 mM Tris-HCl, pH 7.6, 5 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{NaH}_2\text{PO}_4$ , 0.1 mM NaF, 0.1 mM  $\text{Na}_4\text{P}_2\text{O}_7$ ) and incubated with carrier-free 8-azido-( $\alpha$ - $^{32}\text{P}$ )ATP (10 Ci/mmol, ICN Radiochemicals) at a concentration of 2–5  $\mu\text{M}$  on ice for 20 min in a total volume of 100  $\mu\text{l}$ . Alternatively, Ste6 was immunoprecipitated under nondenaturing conditions from membranes solubilized with 1% Triton X-100, and the immunoprecipitates (equivalent to 4 mg membrane protein) were resuspended in labeling buffer. Photolysis and cross-linking was achieved by irradiating the samples with a hand-held short-wave UV-lamp (254 nm, UVG-54, Ultraviolet Products, San Gabriel, California) at 0°C from a distance of 2.5 cm (lamp output 100  $\mu\text{A}$ , equivalent to 77 erg/ $\text{mm}^2/\text{s}$ ) for two 1-min intervals with 1 min of cooling in between. When added as competitor, nonradioactive 8-azido-ATP was present at 100  $\mu\text{M}$  final concentration. Reactions were quenched by addition of DTT to a final concentration of 2 mM, diluted with SDS-PAGE gel sample buffer, heated at 55°C for 10 min, and analyzed by SDS-PAGE and autoradiography.

### Mating Pheromone Induction and Quantitative Mating

Cells expressing Ste6 from the *CEN*-plasmid pRKS3 were grown in SD medium overnight at 30°C and then diluted into prewarmed YPD medium to  $A_{600\text{nm}} = 0.5$ . After growing the cells for 2 h at 30°C, the culture was split into two equal portions and total extracts were prepared from one sample by glass bead lysis. To the second portion, synthetic  $\alpha$ -factor mating pheromone (Peninsula Laboratories, Burlingame, California) was added to a final concentration of 5  $\mu\text{M}$  and incubation was continued at 30°C. Aliquots (5 ml) were taken at various times after pheromone addition, and total extracts were prepared and used for immunoblot analysis using mAb Myc-9E10.

Quantitative mating experiments were carried out on YPD plates, according to the procedure of Elion et al. (1990). Semiquantitative matings were carried out essentially as described by Trueheart et al. (1987). Production of  $\alpha$ -factor pheromone was measured by an agar diffusion (“halo”) bioassay, as described before (Kuchler et al., 1989).

### Indirect Immunofluorescence

Immunofluorescence microscopy was carried out as described in detail elsewhere (Pringle et al., 1991) with the following modifications. After fixation of the yeast, digestion of the cell wall was performed using Zymolyase 100K (Kirin Brewery) at 20  $\mu\text{g}/\text{ml}$  for 15 min at 30°C. The digested fixed cells were suspended in wash buffer (1.2 M sorbitol, 50 mM Tris-HCl, 2 mM EDTA, pH 7.6) containing 1% SDS and, after a 1-min incubation, were washed by resuspension and recentrifugation three times with wash buffer lacking detergent. After two additional washes with PBS, the permeabilized fixed cells were suspended in PBS at a density of  $A_{600\text{nm}} = 2$  per ml. When stored at 4°C, samples prepared in this way remained suitable for antibody decoration for at least 10 d. Ascites fluid containing the mAbs Myc-9E10 or CT14-G4 was used at dilutions of 1:50, 1:250, and 1:1000. To prepare pheromone-treated cells for analysis by immunofluorescence, exponentially growing cells ( $A_{600\text{nm}} = 0.75\text{--}1$ ) were exposed to  $\alpha$ -factor (final concentration 7  $\mu\text{M}$ ) for 120 min before fixation with 5% formaldehyde. Secondary antibodies were 1:100-diluted affinity-purified FITC-conjugated sheep anti-mouse immunoglobulin (Boehringer Mannheim Corp., Indianapolis, IN). Slides were mounted in Citifluor after brief treatment with 1 mg/ml 4,6-diamino-2-phenylindole (DAPI). A Nikon Optiphot fluorescence mi-

croscope was used to view the cells at a magnification of 400–1,000. Images were recorded on Kodak T-MAX 400 or T-MAX 3200 black and white print film, or alternatively, Kodak Ektachrome P800/1600 color reversal film using a Nikon FX-35WA camera (exposure time typically 2–6 s) attached to a AFX-IIa automatic shutter controller.

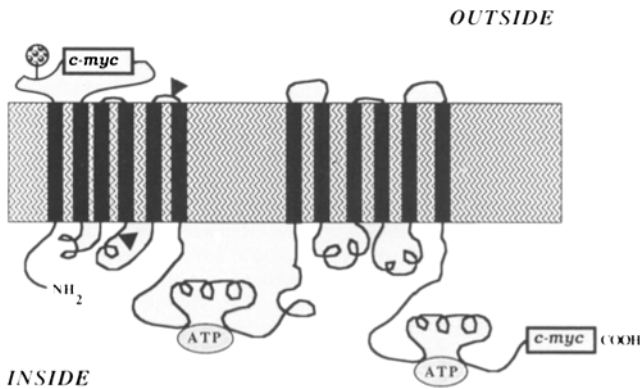
For higher resolution analysis of the distribution of Ste6 by indirect immunofluorescence, strain FC180 (*MATa/MATa trp1/+ his4/+ cyh2/+ can1/can1 ura3/ura3 lys2/lys2 cryll/cryll SUP4<sup>+</sup>/SUP4<sup>+</sup> hml $\alpha$ ::LEU2/hml $\alpha$ ::LEU2*) (gift of F. Cross, Rockefeller University, New York) was transformed with either pYKS3 producing Ste6-Myc or the corresponding vector, YEp352. Cultures of the transformants were grown at 30°C to mid-exponential phase in SC-Ura medium, split into two equal portions, one of which was treated with  $\alpha$ -factor (5  $\mu\text{M}$  final concentration) for 3 h. The cultures were then adjusted to a final concentration of 5% formaldehyde as recommended by Pringle et al. (1991), harvested at room temperature by centrifugation, washed twice with an equal volume of PBS and twice with a small volume of SK buffer (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5). The fixed cells were resuspended in 1 ml of SK containing 25 mM 2-mercaptoethanol at an  $A_{600\text{nm}} = 0.5$  and digested with 20  $\mu\text{g}/\text{ml}$  Zymolyase 100T at 30°C for 30 min. The digested fixed cells were washed once with an equal volume of SK, twice with an equal volume of PBS, and resuspended in 200  $\mu\text{l}$  SK. The washed cells were then allowed to settle at room temperature onto polylysine-coated glass slides for 30 min, which were then rinsed extensively with PBS. The slides were preblocked with 10 mg/ml BSA in PBS for 20 min, incubated with primary antibody (1/100 dilution of anti-c-Myc mAb 9E10) in the same buffer for 1 h, rinsed extensively with the buffer, incubated in the dark with FITC-labeled second antibody, rinsed, and mounted as described above. The cells were examined under a Nikon fluorescence microscope equipped for either standard or confocal viewing, and images were recorded either photographically or via computer-based digital methods.

## Results

### Immunodetection of the STE6 Gene Product

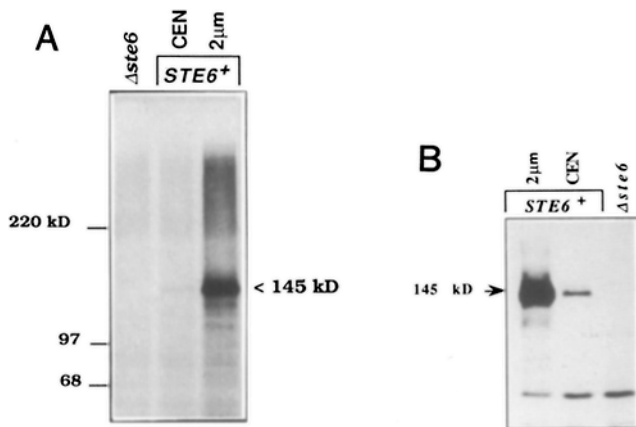
The *STE6* DNA sequence predicts an open reading frame of 1,290 amino acids (calculated molecular weight of 144,744) (Kuchler et al., 1989; McGrath and Varshavsky, 1989). To determine if yeast cells actually produce a corresponding polypeptide, and to examine the relationship between the primary translation product and the native functional protein, polyclonal antibodies directed against a TrpE-Ste6 fusion protein were raised in rabbits. When *MATa* cells were radiolabeled with  $^{35}\text{S}$ -Cys and  $^{35}\text{S}$ -Met, one of the sera raised (no. 9384), but not the corresponding pre-immune serum, specifically and efficiently immunoprecipitated a protein with an apparent molecular weight of 145,000, in excellent agreement with the size of Ste6 deduced from its gene sequence (see Fig. 2 A). This 145-kD species was absent in extracts of a *MATa ste6* $\Delta$  mutant, and markedly overproduced in *MATa* cells carrying the *STE6* gene on a multicopy plasmid (see Fig. 2 A), confirming that the 145-kD protein is indeed the product of the *STE6* gene. The polyclonal antibodies failed, however, to detect Ste6 on immunoblots. Rather than attempt to generate additional polyclonal sera against other domains of Ste6, an alternative strategy was used for detection of Ste6 by immunoblotting.

Synthetic oligonucleotides encoding an antigenic determinant from the *c-myc* proto-oncogene, for which mAbs are available (Evan et al., 1985), were inserted in-frame into the coding sequence of the *STE6* gene at two different positions (Fig. 1). In this way, three “epitope-tagged” versions of Ste6 were generated: one with 10 residues from c-Myc inserted between amino acids 70 and 71; one with 16 residues of c-Myc attached, as the COOH-terminal sequence, following residue 1,285; and, a doubly tagged version containing both Myc epitopes. In a similar way, a linker encoding the so-

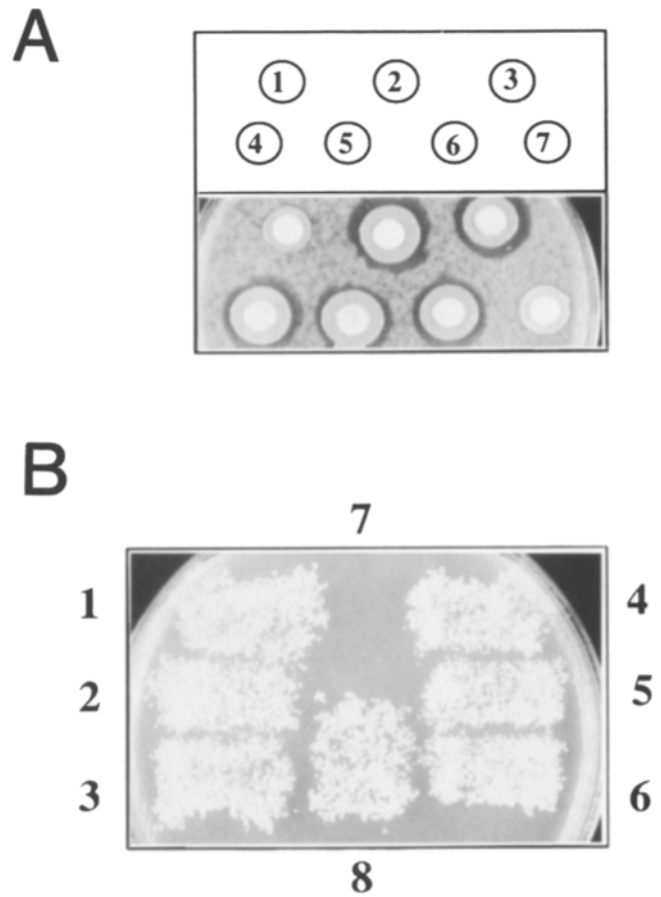


**Figure 1.** Predicted topology of Ste6 in the yeast plasma membrane. (—) polypeptide chain; (■) transmembrane helices; (●) consensus ATP-binding motifs; (⊙) the potential *N*-glycosylation site analogous to that found in mammalian P-glycoprotein. The approximate positions of insertion of the epitope tags, c-Myc (□) and FLAG (▲) are indicated schematically.

called “FLAG” epitope (DYKDDDDK) (Hopp et al., 1988) was inserted into *STE6* either at a unique *EcoRI* site (between residues 205 and 206), which lies within the second predicted internal hydrophilic loop, or at a unique *BclI* site (between residues 282 and 283), which lies within the third predicted external hydrophilic loop (Fig. 1). To determine if the physiological function of Ste6 was preserved in these constructions despite the presence of the small insertions of additional amino acids, the ability of the epitope-tagged derivatives to complement a *ste6Δ* mutation was examined by two independent assays. Both of the Ste6 derivatives contain-

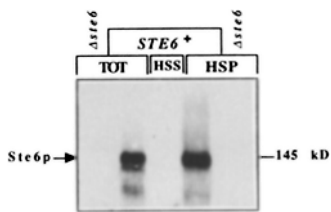


**Figure 2.** Immunological detection of Ste6 protein. (A) Immunoprecipitation. Cultures of WKK7 (*MATa ste6Δ*) and the same strain expressing the normal *STE6* gene either from a low copy plasmid, pKAK16 (*CEN*), or from a multi-copy plasmid, pKK16 (2  $\mu$ m), were metabolically labeled with  $^{35}$ S-Met and  $^{35}$ S-Cys, extracted with detergent, subjected to immunoprecipitation with polyclonal rabbit anti-Ste6 antibodies (serum no. 9384), and analyzed by SDS-PAGE, as described in detail in Materials and Methods. (B) Immunoblotting. Cultures of WKK7 (*ste6Δ*) carrying the vector pRS316 and the same strain expressing a c-Myc epitope-tagged version of Ste6 either from plasmid pRKS1 (*CEN*) or from plasmid pYKS1 (2  $\mu$ m), were extracted, fractionated by SDS-PAGE, transferred to filter paper, and probed with mAb Myc-9E10, as described in detail in Materials and Methods.



**Figure 3.** Ste6 containing epitope insertions retains biological function. (A) Measurement of a-factor secretion by agar diffusion bioassay. A *MATa ste6Δ* mutant (WKK7) was transformed with the following plasmids: pKK1, expressing the *MFaI* gene (1); pKK16, expressing both the *STE6* gene and the *MFaI* gene (2); pRKS1, expressing Ste6 containing a single c-Myc epitope in its  $NH_2$ -terminal domain (4); pRKS2, expressing Ste6 containing a single c-Myc epitope at its COOH terminus (5); pRKS3, expressing Ste6 containing both of the c-Myc epitopes (6); and, pRS316, an empty vector (7). As an additional control (3), an otherwise isogenic *MATa STE6+* strain (W303-1A) transformed with YEp352, an empty vector, was also examined. Secretion of a-factor gives a clear zone (“halo”) in the lawn of *MATa sst2* indicator cells, as described in Materials and Methods. (B) Measurement of a-factor production by a semiquantitative mating assay. A *MATa ste6Δ* strain (WKK7) was transformed with *CEN*-plasmids expressing c-Myc epitope-tagged Ste6, pRKS1 (1), pRKS2 (2), and pRKS3 (3), multi-copy plasmids expressing the same epitope-tagged Ste6 derivatives, pYKS1 (4), pYKS2 (5), and pYKS3 (6), an empty vector, pRS316 (7), and a control plasmid, pKAK16 (8), and the ability of these plasmids to restore mating competence was tested, as described in Materials and Methods. Successful mating is indicated by the appearance of the patch of prototrophic diploid colonies.

ing a single c-Myc epitope, and even the doubly tagged version, were able to fully restore a-factor secretion when introduced into a *MATa ste6Δ* strain, even on low-copy (*CEN*) plasmids (Fig. 3 A). Similarly, the Ste6 derivative containing the FLAG epitope in the third external loop was also able to complement the *ste6Δ* mutation for a-factor secretion; in contrast, the Ste6 derivative carrying the FLAG insertion within the second internal loop was completely non-functional (data not shown). In agreement with these conclu-



**Figure 4.** Ste6 is found exclusively in the membrane fraction. Total extracts (TOT), prepared from a *MATa ste6Δ* strain, WKK7, carrying either plasmid pYKS3 (*STE6*<sup>+</sup>) or the vector YEp352 (*Δste6*), were clarified by low speed centrifugation, then fractionated

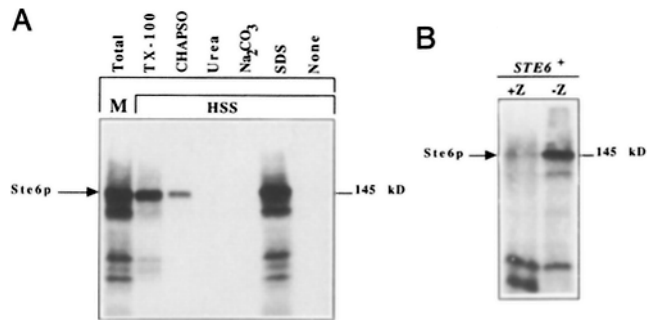
by ultracentrifugation at 200,000 *g* into a high speed pellet (HSP) and a high speed supernate (HSS), and analyzed by immunoblotting using the anti-c-Myc mAb 9E10.

sions, the *MATa ste6Δ* mutant strain carrying any of the three c-Myc epitope-tagged versions of Ste6, on either low-copy (*CEN*) or high copy (2  $\mu$ m DNA) plasmids, was capable of mating with an appropriate *MATa* partner, a process which requires production of extracellular  $\alpha$ -factor (Fig. 3 B). Correspondingly, the Ste6 derivative carrying the FLAG tag in the putative third external loop complemented the *ste6Δ* mutation for mating, whereas the derivative carrying the FLAG tag in the putative second internal loop did not (data not shown). These findings demonstrated that the presence of the c-Myc epitopes, and one of the FLAG insertions, did not disrupt the proper folding, localization, or function of Ste6.

Insertion of a single 10-residue c-Myc epitope was sufficient to permit detection of Ste6 on immunoblots, using either of the anti-Myc mAbs: 9E10 (Fig. 2 B) or CT14-G (data not shown). Using the chemiluminescence detection system (ECL, Amersham), the amount of Ste6 expressed from a *CEN* plasmid was readily detectable in 50  $\mu$ g of protein from a total cell extract separated by gel electrophoresis. In agreement with observations on authentic Ste6 (Fig. 2 A), production of the epitope-tagged Ste6 derivatives was increased 20–30-fold when expressed from a multi-copy plasmid (Fig. 2 B). The c-Myc epitope-tagged versions were also efficiently immunoprecipitated by mAb 9E10 (see Figs. 6 A and 7), and essentially comigrated with authentic Ste6 immunoprecipitated with the polyclonal rabbit serum (data not shown). For either immunoprecipitation or immunoblotting, it was critical that samples solubilized with SDS were not heated above 55°C. When boiled in the presence of SDS, Ste6 aggregated and did not enter polyacrylamide gels.

### Ste6 Is an Integral Membrane Protein

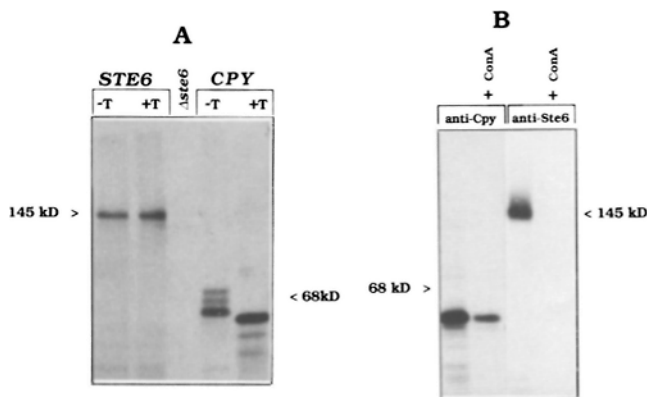
Hydropathy analysis of the deduced Ste6 polypeptide predicts 12 highly hydrophobic, potentially  $\alpha$ -helical, putatively membrane-spanning segments (Fig. 1) (Kuchler et al., 1989; McGrath and Varshavsky, 1989). To determine whether Ste6 is indeed a membrane-associated protein, subcellular fractionation by differential centrifugation of total cell-free extracts was performed and analyzed by immunoblotting. Ste6 was found exclusively in the membrane fraction (HSP) (Fig. 4). No immunoreactivity was found in the high speed supernatant fraction (HSS), or in any subcellular fractions prepared from a *ste6Δ* strain. To determine the nature of this membrane association, a variety of extraction conditions were used as a means to solubilize the Ste6 protein from the membrane fraction. Total membranes were suspended in different solutions and resedimented at 200,000 *g* in an ultracentrifuge, and equivalent portions of the resulting supernatant solutions were analyzed by immunoblotting (Fig. 5 A). The nonionic deter-



**Figure 5.** Ste6 is an integral membrane protein and exposed at the cell surface. (A) Solubilization of Ste6 from the membrane fraction. Total membranes (Total) were isolated from WKK7 carrying plasmid pYKS3, resuspended in 10% glycerol, 1 mM EDTA, 50 mM Tris-HCl, pH 7.8, 10 mM MgCl<sub>2</sub>, and a portion (M) was withdrawn. Identical samples of the remaining membrane material (equivalent to 50  $\mu$ g membrane protein) were treated under the various conditions indicated for 1 h on ice, subjected to sedimentation for 1 h at 200,000 *g* in a table top ultracentrifuge, and equivalent samples of the resulting high speed supernatant fractions (HSS) were analyzed by SDS-PAGE and immunoblotting with the 9E10 anti-c-Myc mAb. TX-100, 1% Triton X-100; CHAPSO, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate; Urea, 2 M; Na<sub>2</sub>CO<sub>3</sub>, 0.1 M, pH 11; SDS, 1%; and None, no treatment. (B) Ste6 is accessible to proteolysis in intact cells. Exponentially growing WKK7 cells (equivalent to 5 A<sub>600nm</sub> U) harboring plasmid pYKS3 (*STE6*<sup>+</sup>) were harvested, resuspended in spheroplasting buffer (1.2 M sorbitol, 60 mM EDTA, 100 mM citrate, pH 6.8), and incubated in the absence (-Z) and presence (+Z) of 100  $\mu$ g/ml Zymolyase 100K (Miles Inc., Kankakee, IL). After treatment, samples (equivalent to 1 A<sub>600nm</sub> U) of each were lysed in 2% SDS at 55°C, homogenized with glass beads, and analyzed by SDS-PAGE and immunoblotting using the anti-c-Myc antibody 9E10.

gent Triton X-100 was nearly as efficient in solubilizing Ste6 from total membranes as the strong anionic detergent SDS. CHAPSO, a zwitterionic detergent frequently used for solubilization of intrinsic membrane proteins, was less effective under the same circumstances. Two treatments that normally release only peripherally bound proteins from membranes (0.1 M Na<sub>2</sub>CO<sub>3</sub> at pH 11 and 2 M urea) did not release detectable amounts of Ste6, as expected if Ste6 is indeed an intrinsic membrane protein. In all cases, the proportion of the total Ste6 remaining in the particulate material was inversely proportional to the amount of Ste6 solubilized by a given treatment (data not shown), even though some degradation of Ste6 occurred during the time required for these manipulations, as observed by immunoblotting (Fig. 5 A).

Although total membranes were isolated in the presence of a serine protease inhibitor and EDTA, these precautions were not sufficient to completely prevent Ste6 proteolysis during the time required for isolation of total membranes from glass-bead lysates (Fig. 5 A). When whole cells were suspended in SDS-containing sample buffer prior to lysis with glass beads, proteolytic degradation of Ste6 was virtually eliminated. Much more severe non-specific breakdown of Ste6 was observed when cells were first converted to spheroplasts by digestion of the cell wall with Zymolyase at 30°C for 30 min before membrane solubilization with SDS (Fig. 5 B). When total cell homogenates were incubated for 30 min at 30°C, the majority (60–70%) of the Ste6 recovered in the membrane fraction (HSP) was intact, as judged by



**Figure 6.** Ste6 is not a glycoprotein. (A) Cultures of strain WKK7 carrying either plasmid pYKS1 (*STE6*) or the vector YEp352 ( $\Delta$ *ste6*) were labeled with  $^{35}\text{S}$ -Met and  $^{35}\text{S}$ -Cys in the absence ( $-T$ ) or presence ( $+T$ ) of tunicamycin, as described in Materials and Methods, and total extracts were prepared by glass-bead lysis. Aliquots of the clarified lysates (equivalent to 2  $A_{600\text{nm}}$  U) were subjected to immunoprecipitation using anti-c-Myc antibody 9E10 (*STE6* and  $\Delta$ *ste6*) or rabbit polyclonal anti-carboxypeptidase Y antibodies (*CPY*), and analyzed by SDS-PAGE and fluorography. (B) Strain WKK7 harboring plasmid pKK16 was labeled with  $^{35}\text{S}$ -Met and  $^{35}\text{S}$ -Cys and a total extract was prepared as described in Materials and Methods. The clarified lysate (equivalent to 3  $A_{600\text{nm}}$  U) was solubilized by treatment with 1% Triton X-100, divided into two equal portions, and subjected to immunoprecipitation with either rabbit polyclonal anti-carboxypeptidase Y antibodies (anti-Cpy) or rabbit polyclonal anti-Ste6 serum no. 9384 (anti-Ste6). The immunoprecipitates, collected using protein A-Sepharose beads, were dissociated by treatment with a small volume of 2% SDS, subjected to sedimentation in a microfuge, and the resulting supernatant fractions were split into two equal portions. One portion was dissolved immediately in SDS-PAGE sample buffer; the other sample was adjusted to 1% Triton X-100 and reprecipitated using concanavalin A-Sepharose beads ( $+ConA$ ). After sedimentation and washing of the beads, the bound material was extracted using SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE in a 7% gel, followed by fluorography.

SDS-PAGE and immunoblotting (data not shown). Hence, nonspecific proteolysis of Ste6 by lysis of a small fraction of the cells during spheroplast formation could not account for the dramatic loss of Ste6 observed (Fig. 5 B). It appeared, therefore, that the bulk of the Ste6 in intact spheroplasts was susceptible to the proteases known to contaminate commercial preparations of Zymolyase (Scott and Schekman, 1980), suggesting that Ste6 is located in the plasma membrane (see below) and that at least a portion of the Ste6 polypeptide is exposed at the exocellular surface.

### Ste6 Is Not a Glycoprotein

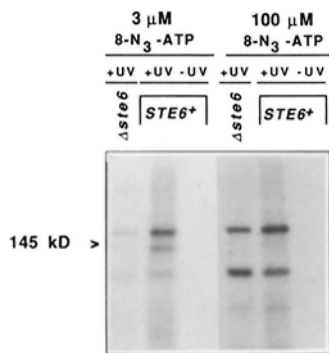
The most homologous counterpart to Ste6 in metazoans, Mdr1, is a glycoprotein in situ (termed "P-glycoprotein") and carries a single N-linked mannose-rich oligosaccharide (Endicott and Ling, 1989; Kane et al., 1990). The presumed transmembrane topology of Ste6 (Kuchler et al., 1989) closely resembles that of Mdr1 and places one of the 14 consensus sites for addition of Asn-linked carbohydrate present in Ste6 within the first putative external hydrophilic loop (Fig. 1) and, hence, exposed to the lumen of the compartments of the secretory pathway. The single Asn-linked chain

in Mdr1 is attached to a site in a nearly identical position in this mammalian protein (Endicott and Ling, 1989).

To determine if Ste6 is glycosylated, cells expressing an epitope-tagged version of Ste6 were metabolically labeled with  $^{35}\text{S}$ -Cys and  $^{35}\text{S}$ -Met in the absence and presence of tunicamycin, an inhibitor of N-linked glycosylation. Ste6 was immunoprecipitated from lysates of the labeled cells and its apparent molecular weight was examined by SDS-PAGE. As a control, a known yeast glycoprotein, carboxypeptidase Y (*CPY*) (Schekman, 1985; Franzusoff et al., 1991), from the same cells was also examined. Treatment with tunicamycin had no detectable effect on the apparent mobility of Ste6, suggesting that Ste6 does not contain any N-linked oligosaccharide; whereas, tunicamycin efficiently prevented glycosylation of *CPY* (Fig. 6 A). The Ste6 derivative used had a c-Myc epitope situated (following amino acid 70) in rather close proximity to the position of the potential glycosylation site (Asn at amino acid 61) in the first external loop (Fig. 1). Hence, it seemed possible that the inserted epitope may have perturbed the structure of Ste6 in this region sufficiently to interfere with carbohydrate addition. To rule out this explanation for the apparent absence of N-glycosylation in Ste6, cells expressing authentic Ste6 were radiolabeled. After isolation of Ste6 from Triton X-100-solubilized membranes by immunoprecipitation with the rabbit polyclonal antibodies, the labeled protein was released from the immunocomplexes by incubation in SDS-containing buffer and its ability to bind to concanavalin A, a lectin that recognizes both the O- and N-linked sugars (almost exclusively mannose) on all yeast glycoproteins (Kukuruzinska et al., 1987), was examined. No detectable amount of Ste6 was adsorbed to concanavalin A-agarose beads; whereas, almost all of the control glycoprotein, *CPY*, was bound to the lectin beads under the same conditions (Fig. 6 B). Thus, native Ste6 appears not to carry either O- or N-linked oligosaccharide chains, and therefore glycosylation is not required for membrane delivery or function of Ste6.

### Ste6 Is an ATP-Binding Protein

More than 40 eukaryotic members of the super-family of Ste6-like transporters have been described to date. Like the other members of the family that are above 100,000 kD, Ste6 is predicted from its sequence to have two discrete nucleotide-binding folds (Kuchler et al., 1989) that are similar in structure to those found in other transport-related ATPases (Futai et al., 1989). Indeed, hydrolysis of ATP has been shown to energize the actual transport process mediated by the highly related transporter, Mdr1 (Hamada and Tsuruo, 1988), and by other members of this super-family (Kuchler and Thorner, 1992b). To determine whether Ste6 is, in fact, an ATP-binding protein, affinity labeling of Ste6 by the photoactivatable ATP analog, 8-azido-ATP, was used. Photo-affinity labeling is typically an inefficient process and generally requires large amounts of purified protein or extraordinary high levels of expression of the protein of interest. In addition, yeast plasma membrane is very rich in a highly active  $\text{H}^+$ -translocating ATPase (Serrano et al., 1991) and contains other ATP-binding proteins, which compete with Ste6 for the substrate analog. Not surprisingly, therefore, several attempts to photolabel Ste6 in total membranes gave unsatisfactory results and required extremely



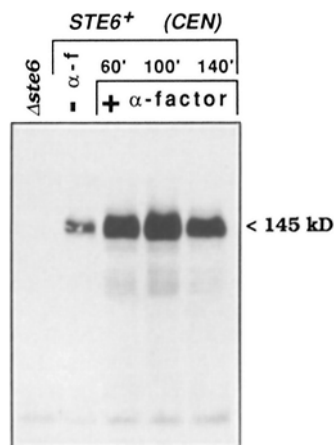
**Figure 7.** Ste6 is an ATP-binding protein. Membranes (equivalent to 5 mg total membrane protein) were prepared from strain WKK7 carrying either plasmid pYKS3 (*STE6*<sup>+</sup>) or the corresponding vector YEp352 ( $\Delta$ *ste6*), solubilized with 1% Triton X-100, and subjected to immunoprecipitation with monoclonal antibody 9E10. The immunoprecipitates were incubated on ice with carrier-free 8-azido-

[ $\alpha$ -<sup>32</sup>P]-ATP alone (3  $\mu$ M 8-N<sub>3</sub>-ATP) or in the presence of an excess of unlabeled nucleotide (100  $\mu$ M 8-N<sub>3</sub>-ATP); equal portions of each mixture were either subjected to photolysis and cross-linking (+UV) or left unirradiated (-UV), as described in Materials and Methods, resuspended in SDS-PAGE sample buffer, heated to 55°C for 10 min, and analyzed by SDS-PAGE and autoradiography.

long exposure times. To circumvent the problems outlined above, Ste6 was solubilized from membranes with Triton X-100 (see Fig. 5 A) and then partially purified by immunoprecipitation under nondenaturing conditions (in the absence of SDS). Ste6 in the immunoprecipitate (still bound to protein A-agarose beads) was then used for the affinity labeling, and the products examined by SDS-PAGE and autoradiography. Using this procedure, a band of 145 kD was labeled with 8-azido-[ $\alpha$ -<sup>32</sup>P]ATP (Fig. 7). This cross-linking was specific to Ste6 because no labeled species was detectable in cells not expressing Ste6, because appearance of the photolabeled 145-kD product required UV-irradiation, and because labeling was effectively competed by the presence of excess unlabeled nucleotide. The two most prominent photolabeled species present were derived from the Ascites fluid used as the antibody source for immunoprecipitation of Ste6, as determined in separate experiments (data not shown).

### Level of Ste6 Is Under Hormonal Control

Expression of the *STE6* gene is *MATa*-cell specific (Wilson and Herskowitz, 1984), and transcription of *STE6* is significantly induced when *MATa* cells are exposed to  $\alpha$ -factor pheromone (Kuchler, K., S. Van Arsdell, R. Freedman, and J. Thorner, unpublished results). Transcriptional activation requires *cis*-acting elements found upstream of the promoter for *STE6* (Wilson and Herskowitz, 1986) and the promoters for other pheromone-responsive genes (Van Arsdell et al., 1987; Van Arsdell and Thorner, 1987). To determine if mating pheromone induction leads to a concomitant elevation of the level of Ste6 protein, cells expressing epitope-tagged Ste6 from the *CEN*-plasmid pRKS3 were treated with  $\alpha$ -factor and total extracts were prepared at various times and analyzed by immunoblotting. Treatment of *MATa* cells with  $\alpha$ -factor increased the expression of Ste6 by nearly an order of magnitude within 100 min after addition of pheromone (Fig. 8); in contrast, the cellular level of either phosphoglycerate kinase or calmodulin was not detectably altered during the same time course, as determined in separate experiments (data not shown). Thereafter, however, the level of Ste6 declined somewhat, possibly due to adaptation of the cells or to intrinsic instability of Ste6. Metabolic insta-



**Figure 8.** Expression of Ste6 is under hormonal control. Total extracts were prepared from strain WKK7 harboring either plasmid pRKS3 (*STE6*<sup>+</sup> *CEN*) or the vector pRS316 ( $\Delta$ *ste6*) that had been treated in the absence (- $\alpha$ -f) or in the presence of 5  $\mu$ M  $\alpha$ -factor mating pheromone (+ $\alpha$ -factor), as described in Materials and Methods. Samples of equal volume were withdrawn at the times indicated in min (') and analyzed by immunoblotting using anti-c-Myc antibody 9E10.

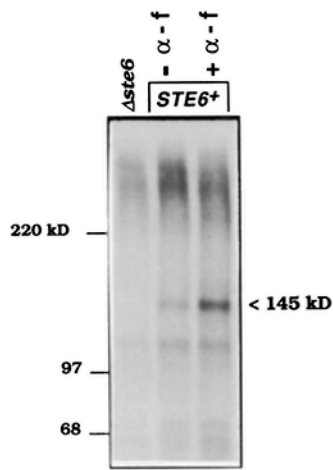
bility of Ste6 has also been noted by others (Berkower and Michaelis, 1991). Increased expression of Ste6 in pheromone-treated cells also resulted in elevated  $\alpha$ -factor secretion (data not shown), confirming our earlier findings that Ste6 is rate-limiting for  $\alpha$ -factor export (Kuchler et al., 1989).

Several of the gene products involved in the mating signal transduction pathway, including Ste2 (Reneke et al., 1988), Ste5 (Freedman, R., M. Hasson, and J. Thorner, unpublished results), and Ste12 (Song et al., 1991), are phosphoproteins. All of these proteins are phosphorylated at a basal level in the absence of pheromone, but display a marked elevation in phosphorylation after exposure of cells to pheromone. Furthermore, the reaction mechanism of some transport ATPases involves a covalent phosphoenzyme intermediate (Carafoli and Scarpa, 1982). To examine whether Ste6 is a phosphoprotein, and if its phosphorylation is stimulated by  $\alpha$ -factor, cells overexpressing authentic Ste6 were radiolabeled with <sup>32</sup>PO<sub>4</sub><sup>2-</sup> at steady state in the absence or presence of  $\alpha$ -factor, and Ste6 was immunoprecipitated with the rabbit polyclonal anti-Ste6 serum (Fig. 9). Even in the absence of pheromone treatment, Ste6 was detectably phosphorylated. In the presence of  $\alpha$ -factor, the radioactivity incorporated into Ste6 was significantly increased (Fig. 9); however, the increase in phosphate incorporation mirrored the increase in total Ste6 protein observed over the same time period (Fig. 8). Therefore, it is likely that increased expression of Ste6 after pheromone induction, rather than increased phosphorylation of pre-existing Ste6 molecules, accounts for the observed apparent increase in phosphorylation upon  $\alpha$ -factor treatment.

### Ste6 Is a Plasma Membrane Protein

Because Ste6 appeared to be an integral membrane protein (Figs. 4 and 5), and because other mammalian and bacterial ATP-dependent transporters are located in the cytoplasmic membrane, it seems reasonable to presume that Ste6 is localized to the plasma membrane (Kuchler et al., 1989). To test this prediction rigorously, the intracellular distribution of Ste6 was analyzed by two independent methods for subcellular fractionation of membranes. First, the partitioning of Ste6 by differential centrifugation was compared with that of known marker proteins. Just like the plasma membrane-associated H<sup>+</sup>-translocating ATPase (*PMA1* gene product), the bulk of the total cellular content of Ste6 sedimented at



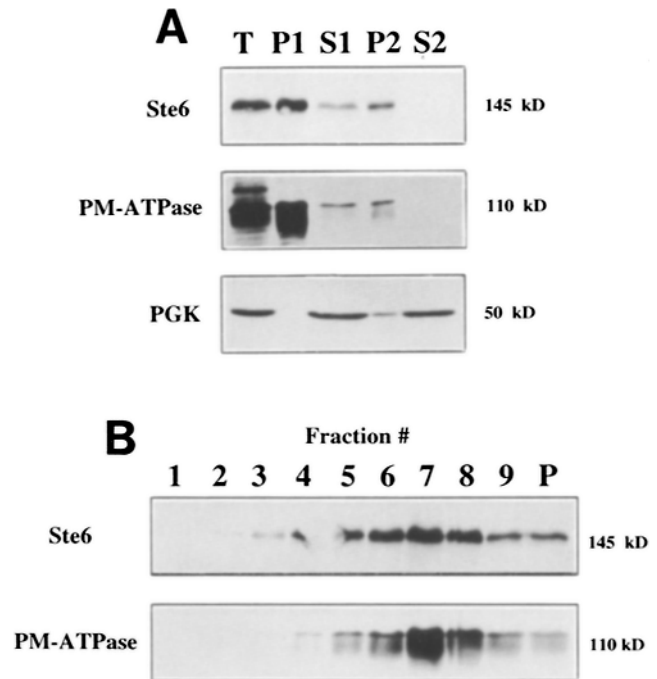


**Figure 9.** Ste6 is a phosphoprotein. Exponentially growing cells of strain WKK7 carrying either plasmid pKK16 (*STE6*<sup>+</sup>) or the vector YEp352 ( $\Delta$ *ste6*) were labeled with 2 mCi of  $H_3^{32}PO_4$  in low-phosphate medium at 30°C. After 40 min, the cultures were split in half; one portion was withdrawn ( $-\alpha$ -f) and the other portion was incubated in the presence of 5  $\mu$ M  $\alpha$ -factor ( $+\alpha$ -f) for an additional 40 min. Lysates were prepared from these samples (equivalent to 2  $A_{600nm}$  U) by glass bead breakage, precleared with pre-immune serum, subjected to

immunoprecipitation with rabbit polyclonal anti-Ste6 antiserum no. 9384, and analyzed by SDS-PAGE and autoradiography.

12,000 g; and, like Pmal, the remainder of the total Ste6 sedimented at 100,000 g (Fig. 10 A). In marked contrast, none of the phosphoglycerate kinase sedimented at 12,000 g, whereas the majority of this protein remained in the supernatant fraction even after sedimentation at 100,000 g (Fig. 10 A). To demonstrate that the Ste6 found in the 12,000 g pellet fraction was membrane-associated rather than simply aggregated or associated with some other particulate material, flotation in a sucrose buoyant density gradient used (Fig. 10 B). For this purpose, the material in the 12,000 g pellet was suspended in 55% sucrose, overlaid with sucrose solutions of decreasing density, and subjected to prolonged high speed centrifugation (Bowser and Novick, 1991). As expected for an authentic membrane-associated protein, Ste6 moved from the dense 55% sucrose cushion to the lighter densities characteristic of membranes. Most significantly, the distribution of Ste6 was identical, fraction-for-fraction, to that observed for the plasma membrane ATPase (Fig. 10 B).

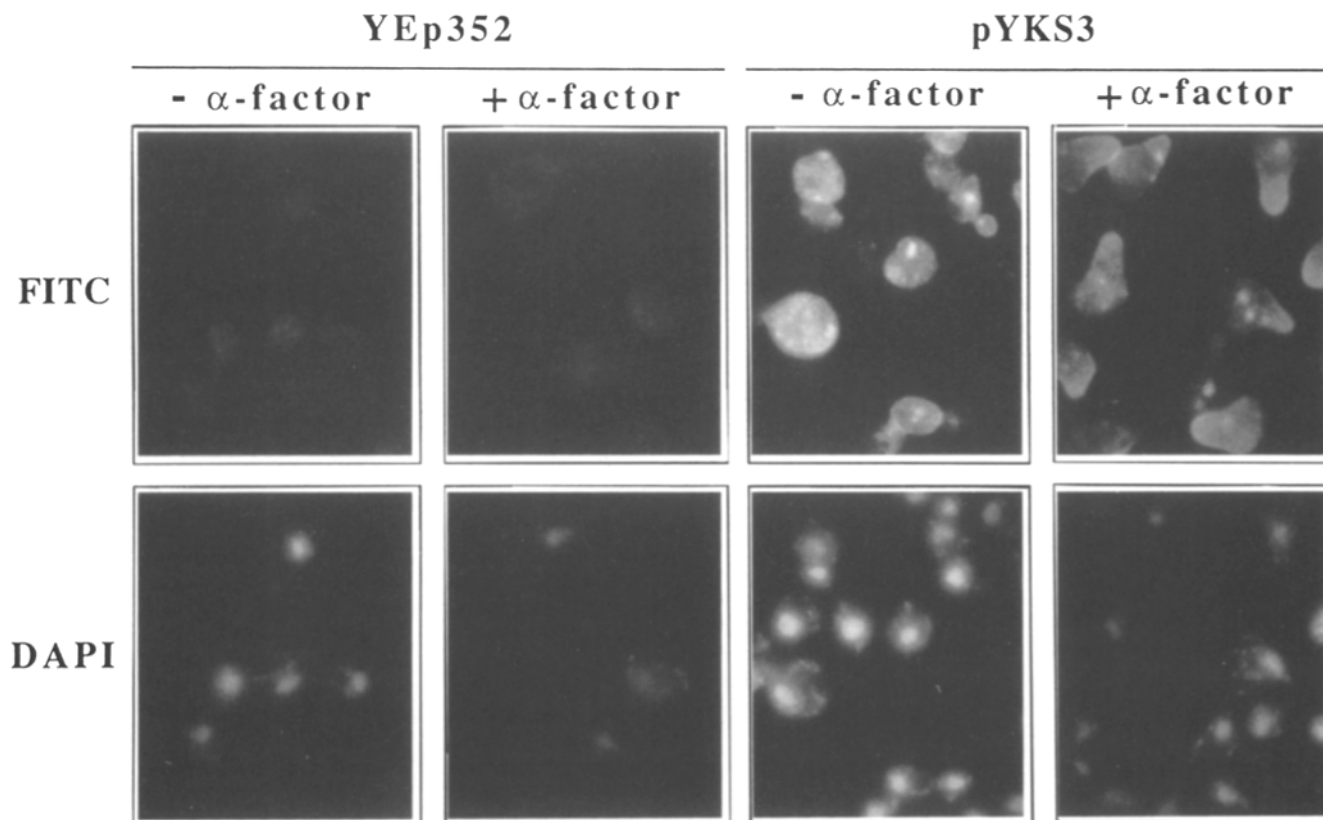
As yet another means to determine its intracellular location, the distribution of Ste6 was examined by indirect immunofluorescence. In *MAT $\alpha$*  haploid cells expressing epitope-tagged Ste6 from a multi-copy plasmid, Ste6 was found predominantly in the plasma membrane in a patchy, rather nonuniform pattern, as indicated by the uneven apparent surface staining (Fig. 11). In addition, fluorescence was consistently observed in vesicle-like bodies that seemed to subtend the inner surface of the plasma membrane. To increase the resolution of this technique, *MAT $\alpha$ /MAT $\alpha$*  diploid cells expressing the epitope-tagged Ste6 were examined. When viewed by setting the focal plane in the center of these large diploid cells, the patchy rim-like staining and plasma membrane-associated vesicular bodies were more obvious (Fig. 12). Essentially the same pattern of plasma membrane and vesicle-like staining, although less intense, was observed in cells expressing Ste6 from a low copy *CEN*-vector, or when optical sections (0.5  $\mu$ m) of the *MAT $\alpha$ /MAT $\alpha$*  diploids were observed by confocal microscopy (data not shown). The staining in vesicle-like bodies could reflect newly synthesized Ste6 within the compartments of the secretory pathway en route to the cell surface. Alternatively, these structures could represent a special pool of secretory vesicles



**Figure 10.** Ste6 cofractionates with an authentic plasma membrane marker protein. (A) Whole cell homogenates were prepared, fractionated by differential centrifugation, and the content of Ste6,  $H^+$ -translocating plasma membrane ATPase (PM-ATPase), and phosphoglycerate kinase (PGK), was determined by SDS-PAGE and immunoblotting, as described in detail in Materials and Methods. The apparent molecular weights of these three species, based on their mobilities relative to a set of standard proteins, are indicated. T, total extract; P1 and S1, pellet and supernatant fraction, respectively, of 12,000 g sedimentation of T; and P2 and S2, pellet and supernatant fraction, respectively, of 100,000 g sedimentation of S1. (B) A P1 fraction was prepared from a whole cell homogenate, subjected to flotation in a sucrose buoyant density gradient according to the method of Bowser and Novick (1991), and the content of Ste6 and PM-ATPase in each fraction was determined by SDS-PAGE and immunoblotting, as described in Materials and Methods. Fraction no. 1, top of the gradient (30% sucrose); Fraction no. 9, bottom of the gradient (55% sucrose); and, P, insoluble material in the pellet at the bottom of the tube.

(Holcomb et al., 1988a) containing stored Ste6, providing a reservoir from which Ste6 could be delivered to the plasma membrane in a rapid (and perhaps regulated) manner.

Cytoskeletal elements and other intracellular structures undergo rather pronounced changes in organization when haploid cells are exposed to pheromone (Baba et al., 1989; Drubin, 1991). As a result, budding of *MAT $\alpha$*  and *MAT $\alpha$*  cells is suppressed during mating and, instead, the cell wall and plasma membrane grow asymmetrically to produce a prominent projection. The elongated mating cells fuse at the tips of their respective projections (reviewed in Cross et al., 1988; Fields, 1990). Given the striking increase in Ste6 level that occurred after pheromone stimulation (Fig. 8), it was of interest to explore whether localization of Ste6 also was altered in  $\alpha$ -factor-treated cells. Indeed, a dramatic change in the surface distribution was found upon pheromone treatment; after exposure to  $\alpha$ -factor, plasma membrane staining of Ste6 either in haploid *MAT $\alpha$*  cells (Fig. 11) or in diploid *MAT $\alpha$ /MAT $\alpha$*  cells (Fig. 12) was observed primarily along the



**Figure 11.** Ste6 localizes to the tip of the projection in pheromone-treated cells. Exponentially growing cultures of a *MATa* haploid strain (WKK7) carrying either plasmid pYKS3 or vector YEp352 were split into two portions; one sample ( $-\alpha$ -factor) was fixed with formaldehyde immediately and the other sample ( $+\alpha$ -factor) was treated with  $7 \mu\text{M}$   $\alpha$ -factor for 120 min before formaldehyde fixation. After permeabilization, the fixed cells were applied to cover slips, decorated with anti-c-Myc antibody 9E10 followed by FITC-conjugated sheep anti-mouse immunoglobulin (FITC), stained with 4,6-diamino-2-phenyl-indole to reveal nuclear DNA (DAPI), and viewed in a fluorescence microscope, all by procedures described in detail in Materials and Methods.

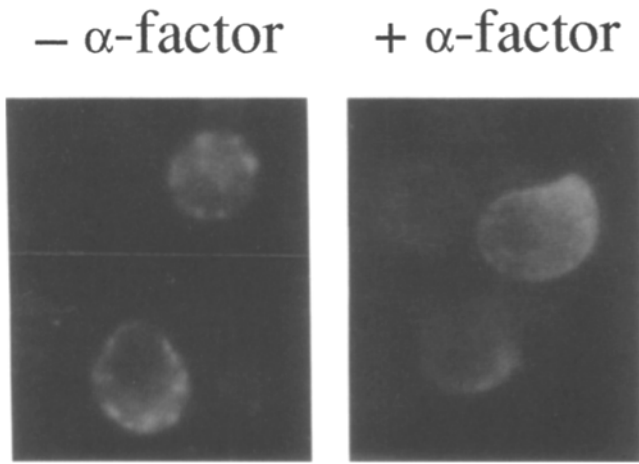
surface of the projection, and most predominantly at its tip. The intracellular vesicle-like structures were present in the pheromone-treated cells, although the number and intensity of staining of these bodies appeared to be reduced.

### Discussion

Based on the essential role of Ste6 in  $\alpha$ -factor export (Kuchler et al., 1989) and on its homology to known transport proteins (Kuchler et al., 1989; McGrath and Varshavsky, 1989), we proposed previously that Ste6 is a plasma membrane-bound transporter that is responsible for secretion of  $\alpha$ -factor by *MATa* cells (Kuchler et al., 1989). In the study described here, we used both polyclonal antibodies raised against a TrpE-Ste6 fusion protein (Fig. 2 A), and mAbs that recognize a c-Myc epitope inserted into the Ste6 protein (Fig. 2 B), to determine the subcellular localization of Ste6 and to examine various biochemical characteristics of this protein. By analogy to Mdr1 (Endicott and Ling, 1989) and to certain other polytopic membrane proteins (Hartmann et al., 1989), we suggested previously (Kuchler et al., 1989) that Ste6 would be an integral plasma membrane protein containing twelve membrane-spanning segments positioned with both its amino and carboxyl termini on the cytosolic face of the plasma membrane. The effects of insertion of the epitope tags we used provides some indirect support for this topolog-

ical arrangement (Fig. 1). Neither of two different epitopes (c-Myc and FLAG) inserted in regions of the protein predicted to be on the external surface of the cell, nor a c-Myc epitope at the extreme COOH terminus, detectably perturbed Ste6 function, as judged by two independent assays for  $\alpha$ -factor secretion (Fig. 3), whereas one of the same epitopes (FLAG) inserted into a region of the protein predicted to face the cytosol was incompatible with Ste6 function. That a portion of the Ste6 polypeptide is exposed to the exterior of the cell was further supported by the observation that Ste6 is accessible to attack by the protease(s) present in the enzyme preparation standardly used for the generation of yeast spheroplasts (Fig. 5 B). The application of various extraction procedures demonstrated that Ste6 is an intrinsic membrane protein (Figs. 4 and 5 A) with an apparent molecular weight (145 kD) in excellent agreement with the size of the molecule deduced from its predicted amino acid sequence. Furthermore, subcellular fractionation (Fig. 10, A and B) indicated that Ste6 indeed resides in the plasma membrane. Immunofluorescence microscopy confirmed that Ste6 is located in the plasma membrane (Figs. 11 and 12). To our knowledge, Ste6 is the first example of the use of the epitope tag method to localize a yeast integral membrane protein by indirect immunofluorescence.

The use of epitope-tagged derivatives of Ste6 was especially advantageous because the highly specific anti-c-Myc mAbs used in this work recognized the antigenic site inserted



**Figure 12.** Redistribution of Ste6 upon pheromone treatment. Exponentially growing cultures of a *MATa/MATa* homozygous diploid strain (FC180) carrying either pYKS3 or vector YEp352 were split into two portions; one sample ( $-\alpha$ -factor) was fixed with formaldehyde immediately and the other sample ( $+\alpha$ -factor) was treated with  $5\ \mu\text{M}$   $\alpha$ -factor for 180 min before formaldehyde fixation. After permeabilization, the fixed cells were examined as described in the legend to Fig. 11, except that only the FITC fluorescence is shown here. As before (Fig. 11), control cells containing the vector alone displayed no detectable staining (data not shown).

into Ste6 under both denaturing and nondenaturing conditions. This fact allowed us to use immunoprecipitation as a method to partially purify Ste6 from membranes solubilized by treatment with Triton X-100 detergent and to demonstrate by photoaffinity labeling that, as predicted by its primary sequence (Kuchler et al., 1989), Ste6 is an ATP-binding protein (Fig. 7). This latter result suggests that binding of ATP, and most likely ATP hydrolysis, are required for translocation of  $\alpha$ -factor across the plasma membrane of *MATa* cells. This conclusion is further supported by a recent finding that mutations that fall within the predicted nucleotide-binding folds of Ste6 also abolish  $\alpha$ -factor transport by Ste6 (Berkower and Michaelis, 1991); however, it is not yet known whether these mutations actually prevent ATP binding and/or hydrolysis by Ste6. Mammalian counterparts of Ste6, like P-glycoprotein (Mdr1) (Hamada and Tsuruo, 1988) and the cystic fibrosis transmembrane conductance regulator (CFTR) (Anderson et al., 1991), appear to energize their specific transport function by consumption of ATP. As in Ste6, point mutations within the nucleotide-binding folds of Mdr1 debilitate its function in vivo (Azzaria et al., 1989). ATP appears to regulate the function of CFTR at two different levels. First, to be competent to act as a  $\text{Cl}^-$  channel, CFTR must be phosphorylated by cAMP-dependent protein kinase (Cheng et al., 1991) or  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (Wagner et al., 1991); second, to open the channel itself, ATP binding is required (Anderson et al., 1991). Like CFTR and Mdr1, Ste6 also appears to be a phosphoprotein (Fig. 9); however, at present, it is not known whether phosphorylation of Ste6 is required either for its intracellular transport and delivery to the plasma membrane or for its function. Triton X-100 efficiently extracted Ste6 from membranes (Fig. 5A) and preserved its ability to bind ATP (Fig. 7), even when complexed to the anti-c-Myc anti-

bodies. Thus, immunoaffinity purification of larger amounts of functional Ste6 might enable detailed characterization of the mechanism of the peptide transport process, for example, by reconstitution of  $\alpha$ -factor transport in proteoliposomes in vitro.

Unlike both mammalian P-glycoprotein (Endicott and Ling, 1989; Kane et al., 1990) and CFTR (Riordan et al., 1989), yeast Ste6 does not appear to carry any oligosaccharide moieties (Fig. 6), even though Ste6 has a consensus site for addition of N-linked carbohydrate at a position precisely analogous to that found in mammalian Mdr1 (Fig. 1). This observation indicates that glycosylation is not required either for Ste6 function or for its intracellular transport. In this regard, it is noteworthy that other polytopic plasma membrane proteins of yeast, such as Pma1, the  $\text{H}^+$ -translocating ATPase (Serrano et al., 1991), and Fur4, the uracil permease (Silve et al., 1991), are not N-glycosylated, despite the presence of potential N-glycosylation sites. Likewise, Mdr1 is not glycosylated when it is expressed in yeast in functional form (Kuchler and Thorner, 1992a; Raymond et al., 1992); and, correspondingly, glycosylation is apparently also not essential for Mdr1 function in animal cells (Beck and Cirtain, 1982; Ling et al., 1983). In contrast, oligosaccharide addition appears to be important for the proper targeting of CFTR because certain mutations that result in incompletely glycosylated CFTR also lead to degradation of the protein in the endoplasmic reticulum (Cheng et al., 1990).

Although the majority of Ste6 protein was localized to the cell surface, indirect immunofluorescent staining also detected Ste6 in vesicle-like structures at or near the inner side of the plasma membrane (Figs. 11 and 12). The spatial distribution of *STE2* gene product, the receptor for  $\alpha$ -factor mating pheromone on the surface of *MATa* cells (Blumer et al., 1988; Reneke et al., 1988), displays almost an identical pattern when examined by indirect immunofluorescence microscopy (Marsh and Herskowitz, 1988; Jackson et al., 1991). The apparent vesicular staining for Ste6 could be an artifact of overexpression; however, this conclusion seems unwarranted because a similar staining pattern, albeit much weaker, was observed when Ste6 was expressed from a *CEN* plasmid. The observed vesicle-like bodies could arise from some perturbation of Ste6 structure caused by the presence of the c-Myc epitopes, resulting in its mislocalization or aggregation within the secretory compartment. This explanation also seems unlikely because the epitope-tagged Ste6 derivatives examined immunocytologically were fully functional physiologically (Fig. 3). Moreover, our laboratory has used c-Myc-epitope tagging to successfully localize several other yeast proteins, including Sst2 (Dohlman, H., unpublished results) and Mot1 (Davis et al., 1992), without detecting any vesicle-like staining whatsoever. Therefore, the vesicular bodies most likely represent newly-synthesized Ste6 being delivered to the plasma membrane. In fact, many nascent plasma membrane proteins in yeast can also be found in intracellular vesicles isolated by biochemical fractionation (Holcomb et al., 1988b). On the other hand, it has been reported that a significant fraction of yeast Pma1 ATPase exists in an intracellular pool of membrane vesicles in which the ATPase is fully functional (Serrano et al., 1991). Similarly, the vesicles that contain Ste6 may not simply represent waystations in its intracellular transport. The Ste6-containing vesicles could function as an intracellular reser-

voir of this transport protein, which, in response to an appropriate stimulus, may be mobilized for rapid insertion into the plasma membrane. A precedent for such a regulatory mechanism exists in mammalian adipocytes in the case of insulin-stimulated exocytosis of pre-existing vesicles that contain a particular glucose transporter isotype (James et al., 1989). In fact, the regulated exocytotic insertion of plasma membrane transporters and channels appears to be a more general phenomenon (Al-Awqati, 1989).

The mating pheromones ( $\alpha$ -factor and  $\alpha$ -factor) trigger a signal transduction cascade that induces a complex series of biochemical and morphological changes in their corresponding target cells (*MAT $\alpha$*  and *MAT $\beta$*  haploids, respectively). The responses induced result in a massive rearrangement of the cytoskeleton leading to asymmetric growth and formation of a projection. The tip of this elongated projection is the actual site of conjugation where the two haploid cell types fuse to form a zygote. We found that both the level (Fig. 8) and the localization (Figs. 11 and 12) of Ste6 undergo a dramatic change when yeast cells are exposed to mating pheromone. As we have shown previously, elevated levels of Ste6 result in a dramatic increase in the production of extracellular  $\alpha$ -factor (Kuchler et al., 1989). Moreover, both of the genes that encode the  $\alpha$ -factor precursors, *MF $\alpha$ 1* and *MF $\alpha$ 2*, are also pheromone inducible (Brake et al., 1985; Dolan et al., 1989). Hence, in response to receipt of an  $\alpha$ -factor signal, *MAT $\beta$*  cells will be provoked into emitting a greatly elevated  $\alpha$ -factor signal. Furthermore, because we found that the majority of Ste6 in  $\alpha$ -factor-treated *MAT $\beta$*  cells localizes primarily to the tip of the growing projection, most of the  $\alpha$ -factor secreted will presumably be released from the tip of this projection, resulting in a pronounced anisotropy in the gradient of  $\alpha$ -factor surrounding the *MAT $\beta$*  cell. The ability of yeast cells to respond to pheromones, and to increase pheromone production in response to a stimulus, has been referred to as "courtship" and has been shown to be a prerequisite for efficient mating between haploid cells (Jackson and Hartwell, 1990*a,b*). A given haploid cell selects for mating only the haploid cell of opposite mating type that produces the highest level of extracellular pheromone, and that is able, in turn, to respond to pheromone by formation of its own projection, a process referred to as "partner discrimination" (Jackson and Hartwell, 1990*a, b*). Thus, both the elevated production and the highly polarized localization of Ste6 in an  $\alpha$ -factor-induced *MAT $\beta$*  cell help to provide a molecular explanation for the phenomena of "courtship" and "partner discrimination."

Our results suggest that, in response to pheromone, a *MAT $\beta$*  haploid will release a high level of  $\alpha$ -factor at a highly localized site on its cell surface, the projection tip. Furthermore, because  $\alpha$ -factor is a lipopeptide (Anderegg et al., 1988), its hydrophobicity and its poor diffusibility (Schäfer et al., 1989; Sterne, 1989; Sterne-Marr et al., 1990) will help to maintain a steep concentration gradient. Presumably only the *MAT $\alpha$*  cell nearest the projection tip of the *MAT $\beta$*  cell will be exposed to a concentration of  $\alpha$ -factor sufficiently high to elicit full mating competence, projection formation, and construction of a complementary conjugation site. Thus, we propose that Ste6 may be essential for efficient mating for two distinct reasons: Ste6 is required for  $\alpha$ -factor secretion per se (Kuchler et al., 1989) and, in addition, may be critical for establishment and/or maintenance of the polarity in

$\alpha$ -factor secretion that is required for effective courtship and proper partner selection. This latter role for Ste6 may explain why exogenously added  $\alpha$ -factor does not rescue efficiently the mating defect of *MAT $\beta$  mfa1 mfa2* mutants (Marcus et al., 1991; Michaelis and Herskowitz, 1988).

The asymmetric localization of Ste6 at the tip of the projection on  $\alpha$ -factor-treated *MAT $\beta$*  cells is almost identical to the pattern seen for Fus1, another plasma membrane-associated transmembrane protein that is highly expressed only in pheromone-treated cells and that is required for efficient cell fusion and zygote formation (Trueheart et al., 1987; Trueheart and Fink, 1989). The pheromone receptors, Ste2 and Ste3, are also pheromone-inducible integral membrane proteins, localize to the projection tip (Marsh and Herskowitz, 1988; Jackson et al., 1991), and appear to be critical determinants of cell type recognition during mating (Bender and Sprague, 1989). In addition, gene products required for the formation of the projection in pheromone-induced cells, like Spa2 (Gehring and Snyder, 1990) and Bem1 (Chenevert et al., 1992), have been identified. Whether localization of Ste6 to the projection tip requires the function of any of these other gene products or, conversely, whether the spatial distribution of any of these other proteins depends on the polarized deposition of Ste6, is unknown.

Finally, during mammalian development, there are often circumstances where one cell induces a differentiative event in a neighboring cell, or a tissue induces developmental changes that are confined to a single layer of overlying epithelial cells. Some of the agents that appear to be responsible for such events, for example interleukin-1 $\alpha$  and -1 $\beta$  and acidic and basic fibroblast growth factors, do not appear to be secreted by the classical secretory pathway (reviewed in Kuchler and Thorner, 1990). Given that Ste6 is highly homologous to mammalian Mdr proteins and given that Mdr proteins are, under some circumstances, capable of transporting peptides (Raymond et al., 1992; Sharma et al., 1992), perhaps the transporter-mediated release of a differentiative peptide is a device for the highly localized delivery of a developmental signal that has been evolutionarily conserved from yeast to mammals.

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