Comparative Topology Studies in Saccharomyces cerevisiae and in Escherichia coli

THE N-TERMINAL HALF OF THE YEAST ABC PROTEIN Ste6*

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Gene fusions have provided a strategy for determining the topology of polytopic membrane proteins in Escherichia coli. To evaluate whether this highly effective approach is applicable to heterologously expressed eukaryotic integral membrane proteins, we have carried out a comparative topological study of the eukaryotic membrane protein Ste6 both in bacteria and in yeast. Ste6, is an ATP binding cassette (ABC) protein, essential for export of the a-factor mating pheromone in Saccharomyces cerevisiae. The topogenic reporters, invertase in S. cerevisiae and alkaline phosphatase in E. coli, were fused to Ste6 at identical sites and the fusions were expressed in yeast and bacteria, respectively. The results obtained in both systems are similar, although more definitive in E. coli, and support the predicted six-transmembrane spans organization of the N-terminal half of Ste6. Thus, the topological determinants for membrane insertion of polytopic proteins in prokaryotic and in eukaryotic systems appear to be highly similar. In this study we also demonstrate that Ste6 does not contain a cleaved signal sequence.

We have recently used a gene fusion approach in Escherichia coli to examine the membrane topology of the eukaryotic multidrug resistance protein, mouse Mdr1 (1, 2). Similar studies have been carried out with other eukaryotic integral membrane proteins expressed as gene fusions in E. coli, for instance the human a2-adrenergic receptor (3) and the cyclic nucleotide-gated ion channels (4). The proposal that it may be valid to carry out structural analysis of eukaryotic membrane proteins in E. coli stems from the assumption that the endoplasmic reticulum (ER) membrane of eukaryotes and the cytoplasmic membrane of prokaryotes exhibit similar properties in regard to protein translocation, and that polytopic membrane proteins are assembled into their final membrane topology in both of these membranes. In order to explore this hypothesis, we have initiated a direct comparative examination of the topology of the N-terminal half of eukaryotic membrane protein Ste6, both in its native system, Saccharomyces cerevisiae, and in the heterologous prokaryotic system, E. coli.

Ste6 is a polytopic integral membrane protein which plays an essential role in secretion of the a-factor mating pheromone in S. cerevisiae (5–7). Ste6 belongs to a superfamily of transporters designated the ATP binding cassette (ABC) superfamily (8) or traffic ATPases (9). Members of the ABC superfamily include the multidrug resistance protein (Mdr) (10, 11), the cystic fibrosis transmembrane conductance regulator (CFTR) (12), the TAP1/TAP2 peptide antigen transporter (13), the bacterial hemolysin exporter (HlyB) (14), and a variety of bacterial periplasmic permeases (15). Proteins in this family are modular in design and contain two homologous halves, each half containing a nucleotide binding domain located on the cytosolic face of the membrane and a membrane spanning domain (MSD) predicted to contain multiple transmembrane segments (TM).

A general secondary structure model for Ste6 can be proposed based on hydrophathy analysis. Such a hydrophathy profile, generated according to the algorithm of Kyte and Doolittle (16) predicts that 6 TMS are present in the MSD of each half of Ste6, analogous to the secondary structure model proposed for the closely related protein, Mdr. While this model has not been experimentally addressed for Ste6, the topology of Mdr has been examined in an in vitro system (17–19), an in vivo heterologous expression system (1, 2), and an in vivo yeast system (20). The experimental data accumulated so far for Mdr indicates that its topology may differ from that predicted by its hydrophathy profile, but notably, answers that emerge from the heterologous bacterial system provide a more clear cut picture of the topology than results obtained in vitro, suggesting the systems used for these analyses must be evaluated further.

To evaluate the use of E. coli for topology studies of eukaryotic polytopic membrane proteins and to specifically study the topology of Ste6, we have carried out a detailed comparative study using gene fusions. In E. coli we use the phoA-encoded enzyme alkaline phosphatase (AP) which is enzymatically active only when it is extracellular and thus can act as a topology sensor for the protein sequence to which it is attached (21, 22). In S. cerevisiae we have used the Suc2-encoded enzyme, invertase ( Inv). The extracellular form (but not the cytoplasmic form) of Inv is heavily glycosylated and only extracellular Inv is able to support growth of yeast on sucrose. The results of our topology study confirm the predicted six TM topology in the N-terminal half of Ste6 and importantly, demonstrate that Ste6 exhibits a similar pattern of topology when expressed in
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either S. cerevisiae or E. coli. In addition, we show in this study that contrary to a previous suggestion (23), Ste6 does not contain a cleavable signal peptide.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Yeast Strains, and Growth Conditions—E. coli Genetic Stock Center at Yale University and used for the expression of Ste6-AP hybrids. E. coli HB101 (ompT, lacY ) (Strain number 7092) was obtained from the E. coli Genetic Stock Center at Yale University and used for the expression of Ste6-AP hybrids. E. coli HB101 (ompT, lacY ) (Strain number 7092) was obtained from the E. coli Genetic Stock Center at Yale University and used for the expression of Ste6-AP hybrids.

Plasmid pSM192 (STE6-AP containing in-frame at the C terminus of the used epitope-tagged STE6 gene in pTE/SUC2 was digested with BamHI and SalI, and then digested with HindIII (52). This plasmid was then ligated to pT7-5/lacY (29) that had been digested with BamHI and SalI. After digestion with these two restriction enzymes, PCR products were ligated into the vector pTE/SUC2 (53) from pSM321, a modified version of the shuttle vector pRS/PGK (54). The mouse monoclonal antibodies against the HA epitope were from Babco. Antibodies to AP were from Sigma—Aldrich. Horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Jackson ImmunoResearch.

Western Blot and Endoglycosidase H Treatment—For Western blot analysis, saturated cultures were diluted 1:20 and grown in SD medium. Cells were harvested at stationary growth phase, washed twice with phosphate/citrate buffer, pH 5.0, and resuspended in the same buffer. Aliquots (20 μl) of cells diluted 1:50 in the same buffer were spotted on sucrose plates, which contain 0.5% sucrose, Bacto-yeast nitrogen base without amino acids, and all amino acids except for those needed for selection. Plates were photographed after 24 h at 30°C.

Antibodies—Invertase from S. cerevisiae (U. S. Biochemical Corp.) was used to raise antibodies. Rabbit anti-Inv antibodies were prepared by injecting rabbits with the recombinant human Ste6-Inv (48). The Klenow-treated (in only the 5' end after partial digestion) 4.87-kilobase STE6-containing HindIII fragment from pSM579 (28) was ligated into pT7-5/lacY (29) that had been digested with BamHI, treated with Klenow, and then digested with HindIII. Subsequently, this construct was further modified by PCR-directed mutagenesis to create a Nhel site just 5' to the STE6 stop codon and a BamHI site just 5' of the start codon of STE6. For STE6-SUC2 fusions, the full-length STE6 gene was subcloned from pT7/STE6 into pTESUC2, replacing the Inv signal sequence coding gene. The resulting plasmid, pT7/STE6, contains a full-length STE6-SUC2 fusion that was purified by preparative SDS-PAGE. To generate the parental plasmid for STE6-SUC2 fusions, the full-length STE6 gene fusions were ligated into the vector pTE/SUC2 (53) from pSM321, a modified version of the shuttle vector pRS/PGK (54).

The membrane was incubated with preadsorbed anti-Inv antibodies. Horseradish peroxidase-conjugated goat anti-rabbit antibodies were used to detect the Inv-containing signal.

Metabolic Labeling in S. cerevisiae and Immunoprecipitation—For analysis of Ste6-Inv hybrids, overnight cultures propagated in SD medium lacking methionine were diluted one-tenth with fresh medium and grown for 4 h at 30°C. Cells (typically 5-ml cultures) were harvested, resuspended in 0.5 ml of fresh medium, and split into 2 aliquots. Tunicamycin (10 μg/ml) was added to one aliquot; both samples were incubated at 30°C for 20 min to establish the tunicamycin block, and subsequently cells were labeled with 15 μCi of [35S]methionine (1000 Ci/mmol) for 15 min. The labeled cells were mixed with lysis buffer (final concentrations: 0.2 M NaOH, 1% β-mercaptoethanol, 1 mM PMSF, and 0.2 μl Trasylol) and proteins were precipitated by addition of ice-cold 0.2 M Tris-acetate buffer (pH 8.0) containing 5% (v/v) Triton X-100. The precipitates were washed twice with acetone and dried at room temperature. The washed precipitates were resuspended in SDS buffer (10 mM Tris-HCl, pH 8.0, 1% SDS, 5 mM EDTA, 1 mM PMSF, and 0.2 μl Trasylol) and placed on ice for 5 min. Ice-cold KI buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2% Triton X-100, 5 mM EDTA, 1 mM PMSF, and 0.2 μl Trasylol) was added, and the samples were incubated for 30 min at 4°C. The insoluble material was pelleted by centrifugation at 4°C. 400 μl were withdrawn and mixed with 300 μl of ice-cold KI buffer. Samples were incubated for 30 min at 4°C with 15 μl of pre-equilibrated protein A suspension to adsorb nonspecific labeled material. After centrifugation (2 min, 13,000 rpm), the pellet was discarded and the supernatant was assayed for total protein content. The insoluble material was then washed twice with high salt buffer (50 mM Tris-HCl, pH 8.1, 1 M NaCl, 2% SDS, 0.5 M EDTA). The second wash was resuspended in 0.5 ml of cold 0.5 M Tris-HCl, pH 8.0, 1% SDS, 5 mM EDTA, 1 mM PMSF, and 0.2 μl Trasylol and placed on ice for 5 min. The cold 0.1 M Tris-HCl, pH 8.0, 1% SDS, 5 mM EDTA, 1 mM PMSF, and 0.2 μl Trasylol was added, and the samples were incubated for 30 min at 4°C with 15 μl of pre-equilibrated protein A suspension to adsorb nonspecific labeled material. After centrifugation (2 min, 13,000 rpm), the pellet was discarded and the supernatant was assayed for total protein content. The insoluble material was then washed twice with high salt buffer (50 mM Tris-HCl, pH 8.1, 1 M NaCl, 2% SDS, 0.5 M EDTA). The second wash was resuspended in 0.5 ml of cold 0.5 M Tris-HCl, pH 8.0, 1% SDS, 5 mM EDTA, 1 mM PMSF, and 0.2 μl Trasylol and placed on ice for 5 min.
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RESULTS

Construction of STE6-SUC2 and STE6-phoA Gene Fusions—To determine the topology of the N-terminal half of Ste6 expressed in yeast and to compare it to the topology of the N-terminal half of Ste6 expressed in E. coli, a series of fusion constructs were created (Fig. 1A) in which varying portions of the N-terminal half of Ste6 are joined to the mature coding sequence of the yeast SUC2 gene (STE6-SUC2) or to the bacterial phoA gene (STE6-phoA). The hybrid proteins encoded by these fusions are referred to as Ste6-Inv, detected as a glycosylated species in certain Ste6-Inv hybrids, which lie in the region preceding putative TM1 of Ste6 and two hybrids, Ala60 and Val72, whose junctions lie in the first putative extracellular loop (L1) of Ste6 by Western blot analysis (Fig. 2A).

Procedures—The Orientation of the N-terminal Transmembrane Segment of Ste6—We initially wished to determine the orientation of the first hydrophobic segment of Ste6 in the membrane and to assess its possible role as a “start transfer” signal. We examined two Ste6-Inv hybrids, Ile20 and Arg21, whose junctions lie in a region preceding putative TM1 of Ste6 and two hybrids, Ala60 and Val72, whose junctions lie in the first putative extracellular loop (L1) of Ste6 by Western blot analysis (Fig. 2A). Samples were separated by SDS-PAGE with or without treatment with Endo H, which removes glycosylated groups from translocated polypeptide products. Wild-type Inv exhibits a dramatic shift in mobility after Endo H treatment, indicative of glycosylation (Fig. 2A, lanes 7 and 8), while the mobility of Ile20 and Arg21 is unaffected by Endo H treatment (Fig. 2A, lanes 3, 4, and 9, 10). Thus, as expected, the Ile20 and Arg21 hybrids are not glycosylated, presumably because they are unable to cross the ER membrane. Indeed, we find that these hybrids are not membrane-associated (not shown). In contrast, hybrids Ala60 and Val72, which lie in the first predicted extracellular loop (L1) of Ste6 exhibit a high M, Endo H-sensitive species (Fig. 2A, lanes 6, 1, and 2), indicating that the Inv moiety portion of these hybrids is glycosylated, and thus must be translocated into the lumen of the ER. Surprisingly, Endo H-treated Ala60 and Val72 migrate similar to Endo H-treated invertase (see below). In addition, we find that hybrids Ala60 and Val72 and all subsequent hybrids examined in this study are membrane-associated (data not shown). Overall, these results suggest that the N-terminal region of Ste6 is cytosolic, that L1 of Ste6 is luminal (i.e. on the extracellular face of the membrane). Thus, TM1 of Ste6 possesses an N_out-C_in orientation, and acts as a start transfer sequence.

An important advantage of using Inv as a reporter is its enzymatic and biological activities. The native homodimeric periplasmic enzyme converts extracellular sucrose into fructose and glucose, thus enabling cells to utilize sucrose as a carbon source for growth. We expect that outwardly exposed Inv, detected as a glycosylated species in certain Ste6-Inv hybrids, will function in the yeast periplasm in a manner analo-
following hybrids Ile20, Arg21, Ala60, or Val72 were split. One-half of the midst that directs the expression of native Inv (pRS/SUC2) reached each of the cerevisiae. Extracts prepared from posed to film for approximately 5 min. was incubated with anti-Inv serum. After incubation with goat anti-rabbit antibodies conjugated to horseradish peroxidase, followed by a short incubation with fluorescent substrate, the nitrocellulose was exposed to film for approximately 5 min. B. Immunoprecipitation of metabolically labeled hybrids from S. cerevisiae treated with tunicamycin. Cells harboring the vector alone (pRS/PGK) or expressing native Inv or various hybrids were incubated with tunicamycin and labeled with [35S]methionine. Extracts were immunoprecipitated with anti-Inv antibodies and the immunoprecipitated material was subjected to SDS-PAGE (7.5%) followed by autoradiography.

Fig. 2. A. Western blots of Ste6-Inv hybrid proteins expressed in S. cerevisiae. Extracts prepared from S. cerevisiae cells containing plasmids that direct the expression of native Inv (pRS/SUC2) or each of the following hybrids Ile20, Arg21, Ala60, or Val72 were split. One-half of the extract was treated with Endo H, the other half was not, as indicated. 50 μg of protein from Endo H treated or untreated samples were subjected to SDS-PAGE (7.5%), electroblotted, and the nitrocellulose was incubated with anti-Inv serum. After incubation with goat anti-rabbit antibodies conjugated to horseradish peroxidase, followed by a short incubation with fluorescent substrate, the nitrocellulose was exposed to film for approximately 5 min. B. Immunoprecipitation of metabolically labeled hybrids from S. cerevisiae treated with tunicamycin. Cells harboring the vector alone (pRS/PGK) or expressing native Inv or various hybrids were incubated with tunicamycin and labeled with [35S]methionine. Extracts were immunoprecipitated with anti-Inv antibodies and the immunoprecipitated material was subjected to SDS-PAGE (7.5%) followed by autoradiography.

Membrane Topology of the N-terminal Half of Ste6 Expressed in S. cerevisiae, as Assessed by Glycosylation of Inv—To study the membrane topology of the N-terminal half of Ste6 in yeast, we examined a series of Ste6-Inv hybrids in which Inv is joined to amino acids 7 and 8 or at its C terminus. Cells bearing these HA-tagged Ste6 constructs were pulse-labeled with [35S]Met and Cys, radioactivity was chased for varying lengths of time, labeled proteins were immunoprecipitated with anti-HA antibodies, and separated by SDS-PAGE. Signal sequence cleavage is a rapid process that occurs either during or immediately after the completion of protein synthesis. Thus, if a cleavable N-terminal signal peptide were present in Ste6, we would expect significantly more rapid disappearance of the radioactive label from the N-terminally tagged molecule due to its removal by signal peptidase than from the C-terminally tagged molecule. However, as shown in Fig. 3, there is no difference in the stability of the tagged molecules. Instead, both tagged versions of Ste6 exhibit the same half-life (~20 min); this moderately unstable pattern is typical of Ste6, and reflects its degradation in the vacuole (27). Overall, in contrast to a previous report (23), our analysis shows that Ste6 does not appear to contain a cleaved signal sequence.

ste6 does not contain a cleavable signal sequence—in the experiment described above we noted an anomalous migration pattern for the Endo H-treated deglycosylated forms of hybrids Ala60 and Val72. These hybrids should contain an additional 60 and 72 amino acid residues, respectively, as compared to the deglycosylated form of mature Inv, yet the mobility of all three species appears to be identical (Fig. 2A, compare lanes 1, 5, and 7). One possible explanation for this result is that Ste6 contains a cleaved N-terminal signal sequence and that after cleavage, the final molecular weight of the hybrids is actually similar to mature wild-type Inv. A second explanation is that anomalous migration arises due to a technical artifact, namely that certain glycosyl groups in mature Inv (which has 9–10 asparagine-linked oligosaccharide chains per subunit) are not accessible to Endo H (37). According to this explanation, Endo H-treated Inv would still contain some glycosyl moieties and thus would be expected to migrate more slowly than true, non-glycosylated mature Inv. To distinguish these possibilities, cells expressing wild-type Inv or various hybrids were treated in vivo with tunicamycin, a treatment which completely blocks glycosylation. Following tunicamycin treatment, cells were labeled with [35S]methionine, proteins were immunoprecipitated, and separated by SDS-PAGE. As shown in Fig. 2B, after tunicamycin treatment wild-type Inv now exhibits its expected mobility, which is notably faster than that of hybrid Val72 (Fig. 2B, lanes 3 and 1, respectively), and similar to hybrid Arg21 (Fig. 2B, lanes 3 and 5) thus obviating the need to propose a cleaved Ste6 signal sequence. Based on the equivalent migration pattern of mature Inv and the Val72 hybrid, after Endo H treatment, Kolling and Hollenberg (23) concluded that Ste6 contains a cleavable leader peptide; they also noted the presence of a potential signal sequence cleavage site between Gly62 and Ser63, according to an algorithm developed by von Heijne (38). However, our results clearly do not support this interpretation. Instead, our studies indicate that the results of Kolling and Hollenberg (23) can be explained as an artifact of incomplete deglycosylation of Inv by Endo H.

To verify that Ste6 does not contain a cleavable signal sequence, we compared the metabolic stability of full-length Ste6 tagged with an HA epitope, either at its N terminus (between amino acids 7 and 8) or at its C terminus. Cells bearing these HA-tagged Ste6 constructs were pulse-labeled with [35S]Met and Cys, radioactivity was chased for varying lengths of time, labeled proteins were immunoprecipitated with anti-HA antibodies, and separated by SDS-PAGE. Signal sequence cleavage is a rapid process that occurs either during or immediately after the completion of protein synthesis. Thus, if a cleavable N-terminal signal peptide were present in Ste6, we would expect significantly more rapid disappearance of the radioactive label from the N-terminally tagged molecule due to its removal by signal peptidase than from the C-terminally tagged molecule. However, as shown in Fig. 3, there is no difference in the stability of the tagged molecules. Instead, both tagged versions of Ste6 exhibit the same half-life (~20 min); this moderately unstable pattern is typical of Ste6, and reflects its degradation in the vacuole (27). Overall, in contrast to a previous report (23), our analysis shows that Ste6 does not appear to contain a cleaved signal sequence.
We next analyzed hybrids predicted to be cytosolic. Hybrids in L4 (Ala213 and Arg236) and L6 (Ile314 and Leu350) exhibit an identical pattern of migration on SDS-PAGE with or without tunicamycin treatment, indicating that these hybrid proteins do not undergo glycosylation (Fig. 4A). Likewise, as shown above using EndoH (Fig. 2A), the migration of hybrid Ile20 and Arg21 in the N-terminal region of Ste6 is also indifferent to prior treatment of cells with tunicamycin (data not shown). Thus, as predicted from hydropathy analysis, the N terminus of Ste6, L4, and L6 appear to lie on the cytosolic face of the membrane. A unique pattern of migration was observed for the L2 hybrid, Ser152, in which a glycosylated species as well as a distinct nonglycosylated species was detected. This “mixed phenotype” result is discussed in detail below.

Membrane Topology of the N-terminal Half of Ste6 Expressed in S. cerevisiae, As Assessed by Growth on Sucrose and Localization of Inv Activity—Hybrids that are glycosylated should promote growth on sucrose as the sole carbon source if they are targeted to the plasma membrane and their Inv moiety is exposed to the periplasm. This was tested by spotting cells expressed to the periplasm. This was tested by spotting cells expressing native Inv, or the Inv moiety of the hybrids, on plates containing 0.5% sucrose. Growth was monitored at 30°C for 24 h (Fig. 5). Cells expressing native Inv, or the glycosylated hybrid proteins with junctions in L1 (Ala60, Val72), L3 (Thr168), and L5 (Ile290) exhibit robust growth on sucrose as expected. Not surprisingly, cells harboring the vector alone or expressing the non-glycosylated hybrid proteins with junctions in cytosolic domains, i.e., the N terminus, Ile20, Arg21, L4, Ala213, and Arg236, and L6, Ile314 or Leu350 do not grow under these conditions. On the other hand, expression of the L2 hybrid Ser152, which exhibits the mixed glycosylation phenotype described above, enables yeast to grow somewhat on sucrose, but to a much lesser extent than is the case for the L1 (Ala60), L3 (Thr168), and L5 (Ile290) hybrids.

The localization of the Inv moiety of the hybrids was further analyzed by assaying enzyme activity, under conditions that allowed us to distinguish cytosolic invertase from translocated (periplasmic plus intracellular lumenal) invertase. The percentage of cytosolic invertase activity for each Ste6-Inv hybrid protein is shown in Fig. 6. For the control suc2Δsp, which contains no Ste6, about 90% of the Inv activity is cytosolic (Fig. 6, fusion location 0), while for wild-type SUC2, <5% of the invertase activity is cytosolic (data not shown). A high level of cytosolic invertase activity (50–90%, except for hybrid Ile20 with 42%) is detected for hybrids in the N terminus of Ste6 (Arg21), in L4 (Ala213, Arg236) and L6 (Leu350, Cys642), reconfirming the conclusions from the glycosylation and growth assays that the N terminus, L4 and L6 are cytosolic. Interestingly, the hybrids in L2 (Ala114, Ser152) which show a mixed phenotype in other assays also exhibit unexpectedly low cytoplasmic invertase activity, and are discussed in more detail below. As anticipated, for the presumed extracellular hybrids in loops L1 (Ala60, Val72), L3 (Thr168, Ser169), and L5 (Ile290), the cytosolic activity is low (20–30%), consistent with the prediction that L1, L3, and L5 are luminal. It should be noted, however, that the cytoplasmic activity in these fusions is not as low as for WT Inv (~5%). This may be due to incomplete translocation of Inv in these hybrids or, alternatively, to some inherent leakiness in the assay; for instance, a low level of organelle rupture during processing of our samples could contribute to apparent cytoplasmic activity. Nevertheless, the relatively low activity of the L1, L3, and L5 hybrids confirms the overall view that these loops of Ste6 are luminal.

Hybrids in L2 of Ste6 Exhibit Anomalous Behavior—In general, the enzymatic activities of nearly all of the hybrids are in agreement with the glycosylation patterns and with the sucrose growth experiments, and confirm the predictions based on hydropathy analysis (Fig. 1B). An exceptional entity is hybrid Ser152 (in predicted L2) which displays a less clearcut result. By hydropathy, Ser152 is predicted to lie in a cytosolic loop, L2. However, by the Inv activity assay, glycosylation analysis, and sucrose growth experiments Ser152 exhibits an ambiguous phenotype. One possible explanation for the mixed topology of hybrid Ser152 is that TM2, which precedes it, is not stably anchored in the membrane in the context of a C-terminally truncated Ste6, causing downstream sequences, including Inv, to exist on both sides of the membrane. To determine whether sequences within L2 might be responsible for desta-
bilizing TM2, we constructed another hybrid, Ala114, in which the fusion junction is located on the N-terminal side of L2. An additional hybrid was also constructed, which serves as a control, and contains Inv joined to Val200 located at the N-terminal side of the adjacent cytoplasmic loop (L4). Cells expressing these new hybrids were either treated or not treated with tunicamycin, labeled, and analyzed after immunoprecipitation and SDS-PAGE. The control, hybrid Val200 (Fig. 4B, lanes 3 and 4), exhibits the same mobility with or without tunicamycin treatment, confirming its cytosolic orientation as predicted. However, just as with hybrid Ser152, only a fraction of the molecules of hybrid Ala114 undergo glycosylation (Fig. 4B, lanes 1 and 2), indicating that a significant portion of the invertase molecules (the non-glycosylated species) are retained in the cytoplasm. These results further suggest that the mixed properties of hybrids Ser152 and Ala114 are most likely caused by the instability of TM2 and not by signals located in L2. Therefore, we suggest that L2 is cytoplasmic, and that this region of the protein is conformationally unstable. Notably, a similar observation suggesting two alternative membrane topologies has been described for Mdr (17).

Membrane Topology of the N-terminal Half of Ste6 Expressed in E. coli—Recently, the membrane topology of the mouse Mdr1 protein has been studied using Mdr-AP hybrids in E. coli (1, 2). Although the information obtained from that study is in general agreement with conclusions reached regarding the topology of Mdr1 in eukaryotic expression systems (17, 18), a direct comparison between the outcome of topology studies of a single eukaryotic membrane protein in the prokaryotic and eukaryotic systems, using similar molecular tools, has not been undertaken. For this reason, we felt it would be extremely valuable to directly compare the topogenic behavior of the eukaryotic polytopic membrane protein Ste6 in both the eukaryotic and the prokaryotic systems in vivo.

To study the topology of Ste6 in E. coli, we generated a series of STE6-phoA fusions corresponding to a subset of the STE6-SUC2 fusions analyzed above (Fig. 1A). To enable direct comparative analysis, the AP is joined to Ste6 in these fusions at exactly the same Ste6 junctions as in the Ste6-Inv fusions. In E. coli, AP becomes active only when translocated to the periplasmic space; non-translocated cytosolic AP is inactive. Therefore, the location of the reporter with respect to the cytoplasmic membrane can be determined by assaying the specific activity of AP in the hybrids. To calculate the specific activity of various Ste6-AP hybrids, we first examined their level of expression. One problem that we encountered with respect to the expression of Ste6 in E. coli is proteolysis, which is significant even in E. coli UT5600, a strain devoid of the outer membrane protease OmpT. This problem is apparent in the immunoprecipitation experiments with cells expressing various Ste6-AP hybrids; in addition to the full-length hybrid protein, we also observed rapidly migrating species corresponding to breakdown products (Fig. 7). Nevertheless, all the rapidly migrating bands shown in the autoradiogram must contain AP epitopes, since we used anti-AP antibodies for immunoprecipitation. Previous studies on the properties of Mdr-AP proteolytic fragments suggest that the AP is still connected to its immediate N-terminal topogenic determinant (22) and thus may be inactivated after the cleavage event. This is consistent with the finding that the Ste6-AP hybrid has the same migration pattern as the full-length Ste6-Inv hybrid in SDS-PAGE (Fig. 7). The specific activity of the Ste6-AP hybrids was determined as described under "Experimental Procedures." The number adjacent to each data point is the STE6 codon to which SUC2 is fused. The chimera designated "0" contains only mature Inv and no STE6 codons and corresponds to suc2-sp. A topology model based on the hydropathy plot shown in Fig. 1B is drawn to scale under the data.

**FIG. 6. Invertase activity in the cytoplasm of Ste6-Inv chimeras.** The amount of Inv activity in the cytoplasm of cells harboring different STE6-SUC2 fusions was determined as described under "Experimental Procedures." The number adjacent to each data point is the STE6 codon to which SUC2 is fused. The chimera designated "0" contains only mature Inv and no STE6 codons and corresponds to suc2-sp. A topology model based on the hydropathy plot shown in Fig. 1B is drawn to scale under the data.

**TABLE I**

| Fusion | Number of methionines | Alkaline phosphatase activity (unit) | Expression level (pixel/1000) | Normalized activity |
|--------|------------------------|-------------------------------------|-------------------------------|---------------------|
| Arg<sub>21</sub> | 8                      | 5                                   | 5                            | 0.8                 |
| Ala<sub>60</sub> | 10                     | 415                                 | 15.4                          | 27                  |
| Ala<sub>114</sub> | 14                     | 5.9                                 | 5.5                           | 1.5                 |
| Ser<sub>152</sub> | 15                     | 38                                  | 10.5                          | 5.4                 |
| Thr<sub>168</sub> | 15                     | 305                                 | 12.8                          | 35.7                |
| Val<sub>200</sub> | 19                     | 5.4                                 | 30.8                          | 0.3                 |
| Ala<sub>113</sub> | 16                     | 13                                  | 10                            | 2.1                 |
| Arg<sub>236</sub> | 17                     | 14                                  | 5.2                           | 4.6                 |
| Ile<sub>290</sub> | 19                     | 216                                 | 6.8                           | 60.4                |
| Ile<sub>280</sub> | 20                     | 4                                   | 8.9                           | 0.1                 |

2 R. Edgar and E. Bibi, unpublished data.
high specific activity (27–60.4 units) while hybrids in predicted cytosolic regions, the N terminus of Ste6 (Arg21), L2 (Ala114, Ser152), L4 (Val200, Ala213, Arg239), and L6 (Ile314), all exhibit low specific activity (0.1–5.6 units). Overall, the analysis of AP hybrids in E. coli strongly supports the predicted secondary structure model of Ste6 containing 6 TMs. Notably, unlike Inv fusions in S. cerevisiae, the AP hybrids Ala114 and Ser152 do not exhibit any controversial behavior.

**DISCUSSION**

Membrane Topology of Ste6—Although proteins in the ABC superfamily exhibit significant similarity in their nucleotide binding domains, their MSDs are more variable and in some cases have been shown to exhibit different numbers of TMs. For example, systematic studies on certain prokaryotic ABC proteins have suggested that HlyB, a hemolysin transporter, may contain 8 TMs (39); MalF and MalG, the membrane subunits involved in maltose transport, appear to contain 8 and 6 TMs, respectively (reviewed in Ref. 40); and the histidine periplasmic permease contains two separately expressed membrane domains, each composed of 5 TMs (41). Nevertheless, several eukaryotic members of the ABC superfamily that have been examined in detail, including Mrd (1, 20, 19) and CFTR (42), appear to exhibit similar membrane topologies, with their N-terminal halves containing 6 transmembrane segments. In addition, extensive hydropathy comparison predicts that the MSDs of Ste6 share features in common with MSDs of Mrd and CFTR, namely, 6 TMs and intervening loops of conserved length (43). Indeed, the gene fusion analysis presented in this paper in both the eukaryotic and the prokaryotic expression systems strongly supports the proposed topology in the N-terminal half of Ste6.

Use of Inv as a Reporter Protein—The methodology of gene fusions provides an attractive approach to deduce topology of a membrane protein in vivo. In this report we compare the use of AP and Inv fusions to study Ste6 topology in vivo, in both a prokaryotic and eukaryotic organism, E. coli and S. cerevisiae, respectively. Previous work has established the use of another reporter, the histidinol dehydrogenase (HD) protein encoded by HIS4C, as a topologically sensitive monitor for gene-fusion analysis of the topology of ER membrane proteins in S. cerevisiae (44) and of a plasma membrane protein, arginine permease (45). Here we have chosen to examine the use of Inv as a topology detector for the plasma membrane protein Ste6. Invertase has been utilized in a previous topology study for examining the ER membrane resident protein Sed52p (46). The advantages of using Inv to study topological elements in plasma membrane proteins are 3-fold: the protein becomes heavily glycosylated upon translocation into the lumen of the ER which can be detected as a mobility shift by SDS-PAGE, the secreted form of Inv enables cells to utilize sucrose as a sole carbon source for growth of cells, and finally, its enzymatic activity can be assayed in vivo by growth of cells on indicator agar plates or by an enzyme activity assay. Importantly, while the non-secreted form of Inv is also fully active, it is unable to promote growth on sucrose. On the other hand, the use of Inv in this study has proved to be informative, lending support for the proposal that the N-terminal half of Ste6 contains 6 TMs. On the other hand, our study suggests that caution should be taken when utilizing Inv in gene-fusion technology, as exemplified by the mixed phenotypes observed with hybrids in which Inv is joined to residues in the rather large cytoplasmic loop (L2) between TM2 and TM3. It is not yet clear whether the reason for the complex phenotype of these hybrids is due to a particular structure within the Ste6 protein or to the specific properties of Inv. It is possible that for proper assembly of L2 between TM2 and TM3, C-terminal domains of Ste6 are essential. Alternatively, the mixed topology we observe with the fusions could represent a mixed or alternating topology actually adopted by Ste6 in S. cerevisiae. However, we cannot exclude the possibility that the mature portion of Inv harbors signals that under certain conditions become sufficient to promote translocation across the ER membrane.

Use of the Heterologous E. coli System for Topological Analysis—When AP was used as a reporter for Ste6 in the heterologous E. coli expression system, no discrepancy was observed. The results clearly support a secondary structure model in which Ste6 contains 6 TMs in its N-terminal half. Therefore, we conclude that in this case, the E. coli-phoA-fusion system, unlike the S. cerevisiae-SUC2 fusion system, provides information that is less controversial. Interestingly, and in line with this conclusion, previous gene-fusion studies with Mdr show that results obtained in the E. coli system could clarify uncertainties observed in analogous gene-fusion studies in eukaryotic expression systems (2). What, then, is the possible explanation for the clear phenotypes obtained with gene fusions in E. coli, in contrast to the mixed phenotypes obtained in eukaryotic systems. Schematically, polytopic membrane proteins are composed of two types of transmembrane segments, signal anchor and stop transfer sequences (reviewed in Ref. 47). The topology of these proteins is determined by the orientation of the signal anchor and the stop transfer stretches. Flanking charged residues are probably major determinants of the transmembrane orientation of these sequences. More specifically, orientation preferences are imparted by positively charged amino acids, termed by von Heijne (48) as the positive-inside rule. However, predictive methods which are based on positive charge distribution in addition to hydrophobicity profile predictions seem to perform most reliably on bacterial membrane proteins (49). Electrochemical potential across the bacterial membrane may in part provide a mechanistic basis for the positive-inside rule in bacteria (50). Therefore, the substantial difference between the ER membrane (of which the membrane potential is substantially lower than that in the eukaryotic plasma membrane which has a Δψ(M) of approximately ~60 mV) and the inner membrane of E. coli (Δψ(M) of about ~150 mV) may explain the clear-cut results that we obtain with Ste6-AP hybrids in E. coli. The hydrophilic stretch of amino acid residues N-terminal to the fusion joint in hybrid Ala114, which exhibits a mixed phenotype in yeast, contains only one net positive charge (the sum of 3 Arg residues and 2 Glu residues). Therefore, it is possible that this weak positive charge performs as a better stop transfer signal in E. coli than in S. cerevisiae.

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