The general amino acid permease (Gap1p) of *Saccharomyces cerevisiae* is an integral membrane protein that contains 12 hydrophobic regions predicted to be membrane-spanning segments. A topological reporter construct, encoding an internal 53-amino acid peptide of invertase (Suc2p) containing three Asp-X-Ser/Thr glycosylation sites, was inserted in-frame into the hydrophilic N\(_2\)- and COOH-terminal domains and each of the 11 hydrophilic loops that separate the 12 hydrophobic segments of Gap1p. The resulting 13 gene sandwich fusion proteins were expressed in a gap1Δ null mutant strain; 9 of these retain amino acid transport activity.

A method for determining the in vivo topology of yeast polytopic membrane proteins demonstrates that Gap1p fully integrates into the membrane independently of Shr3p.*

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Amino acid uptake across the plasma membrane (PM)\(^1\) in *Saccharomyces cerevisiae* is catalyzed by a gene family of structurally related transport proteins known as amino acid permeases (AAPs). The AAP family contains 18 core members that share extensive homology, and 6 other members that exhibit less sequence conservation (1). Based upon available predictive algorithms, AAPs are thought to be integral membrane proteins comprising 12 membrane-spanning domains. The general AAP (GAPI) is a low affinity, high capacity permease with broad substrate specificity, which is capable of transporting most amino acids, even L-amino acids (2, 3). The majority of other AAP family members are low capacity, high affinity permeases, each exhibiting characteristic and rather narrowly defined substrate specificities (4). One of the core AAP members (SSY1) contains a unique NH\(_2\)-terminal extension not present in the other AAPs. Ssy1p has recently been shown to function as a sensor of extracellular amino acids that serves to transduce metabolic signals that differentially regulate the activity of the general and specific AAPs to control amino acid uptake under a variety of environmental conditions (5–8).

AAPs are co-translationally inserted into the membrane of the endoplasmic reticulum (ER). Subsequent to membrane insertion, these proteins must fold properly to attain native conformations in order to be transported to the PM. Protein folding is a process that often requires specific processing events (9). At an early stage in the secretory pathway, AAPs are co-transported (10) together with other secreted proteins from the ER to the Golgi apparatus via ER-derived COPII coated transport vesicles (reviewed in Refs. 9, 11, and 12). The AAP gene family members require Shr3p to be included in COPII transport vesicles (10, 13–15). Recently, several other complex polytopic PM proteins in *S. cerevisiae* have been found to require the assistance of accessory proteins to exit the ER (16–18). As is observed with shr3 mutations, when these accessory proteins are mutated or deleted, their cognate cargo do not enter COPII transport vesicles and accumulate within the ER. The secretory block in these mutants is specific; other plasma membrane, secretory, and vacuolar proteins are processed and targeted correctly.

Shr3p is an integral membrane protein with four membrane-spanning segments and a hydrophilic cytoplasmically oriented carboxyl-terminal domain (13). Shr3p has been shown to physically associate with Gap1p, but not with other polytopic membrane proteins such as Sec61p, Gal2p, or Pma1p, in a transient complex that can be purified from *N*-dodecylmaltoside solubilized membranes (15). The COPII coatomer components Sec13p, Sec23p, Sec24p, and Sec31p, but not Sar1p, bind Shr3p via interactions with its carboxyl-terminal domain. As Shr3p does itself not exit the ER (10), Shr3p must dissociate and diffuse away prior to completion of vesicle formation. By facilitating the membrane association and assembly of COPII coatomer components, Shr3p is thought to function as a packaging chaperone that directs the formation of vesicle buds around AAPs, thereby ensuring their inclusion into transport vesicles (15). An analysis of whether Shr3p influences the membrane structure of AAPs is essential to fully understand the molecular mechanisms governing the exit of permeases from the ER.

It is not trivial to determine the in vivo membrane association and orientation of hydrophobic domains within polytopic membrane proteins. Fusion proteins, with COOH-terminal reporter sequences, have been extensively used to study the topology of *Escherichia coli* membrane proteins (19, 20). Similar gene fusion approaches, often using the invertase gene (SUC2), have been used in yeast to study the topology of a

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‡ The abbreviations used are: PM, plasma membrane; AAP, amino acid permease; ER, endoplasmic reticulum; endo H, endoglycosidase H; PAGE, polyacrylamide gel electrophoresis.
variety of ER and PM proteins (21–24). Suc2p, comprising 530 amino acids, functions as a topological reporter as it becomes efficiently glycosylated at multiple sites when translocated across the ER membrane. Unfortunately, COOH-terminal fusion approaches have proven to be unreliable in determining the topology of polytopic membrane proteins. In several instances the NH2-terminal membrane segments of complex polytopic proteins have been shown to require downstream COOH-terminal membrane domains to attain their correct membrane orientation (23–25). Similarly, we have observed that hybrid proteins consisting of full-length Suc2p fused at various sites, into hydrophilic loops has also proven to be a useful alternative to COOH-terminal fusion approaches (24, 29). However, this approach requires isolation of homogeneous preparations of intact membranes, many tedious control experiments, and experimental difficulties associated with protease accessibility are well documented (29, 30). The insertion of N-glycosylation consensus sequences (NXS/T) into hydrophilic loops (30, 31) has been successfully used to study the topology of several mammalian polytopic membrane proteins. Primarily due to the fact that glycosylation sites are rather inefficiently used in yeast, similar glycosylation scanning approaches have not been used to examine the in vivo topology of proteins in yeast.

We have adapted the glycosylation scanning method for use in yeast and have determined the in vivo topology of Gap1p in SHR3 and shr3Δ6 cells. A novel gene sandwich reporter construct, encoding an internal 53-amino acid segment from Suc2p containing three NXS/T glycosylation sites, was inserted in-frame into the hydrophilic NH2- and COOH-terminal domains and each of the 11 hydrophilic loops that separate the 12 hydrophobic segments of Gap1p. Nine of these fusion proteins are correctly targeted to the PM and retain amino acid transport activity. By analyzing the glycosylation state of these fusion proteins, we have found that each of the 12 hydrophobic segments of Gap1p span the membrane, and both the NH2- and COOH-terminal domains are cytoplasmically oriented. This model was tested by isolating sealed right-side-out microsomes from sec12–1 strains expressing Gap1p constructs containing cleavable factor Xa protease sites. The proteolytic fragments derived from the Gap1p constructs corroborated the 12-membrane-spanning model. Additionally, our data clearly indicate that Gap1p fully integrates into the membrane independently of Shr3p. The Suc2p-based topological reporter cassette should be useful in studies aimed at determining the in vivo structure of other polytopic membrane proteins in yeast.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Microbiological Techniques—**Yeast strains are listed in Table I, and plasmids used are listed in Table II. The temperature-sensitive sec12–1 mutant CKY42 was kindly provided by C. A. Kaiser (Massachusetts Institute of Technology, Cambridge, MA). A diploid strain, constructed by crossing CKY42 with FGY58 (gap1Δ), was sporulated, and tetrad analysis indicated that the temperature sensitive and gap1Δ phenotypes segregated independently, and with the expected ratio of 2:2. Strains FGY11 (sec12–1 gap1Δ) and FGY15 (sec4Δ) were grown in YPD media or in the absence of leucine.

**TABLE I**

| Strain          | Genotype               | Source or reference |
|-----------------|------------------------|---------------------|
| AA288           | MATa ura3–52 leu2–3,112 lys2Δ201 ade2 | (53)               |
| FGY58           | MATa ura3–52 leu2–3,112 lys2Δ201 ade2 gap1Δ: LEU2 | (15)               |
| FGY60           | MATa ura3–52 leu2–3,112 lys2Δ201 ade2 gap1Δ: LEU2 shr3Δ6 | (15)               |
| sec– mutant strains |                       |                     |
| CKY42           | MATa ura3–52 sec12–1   | Kaiser Laboratory   |
| FGY11           | MATa ura3–52 leu2–3,112 lys2Δ201 sec12–1 gap1Δ: LEU2 | This work          |
| FGY14           | MATa ura3–52 leu2–3,112 lys2Δ201 sec12–1 gap1Δ: LEU2 shr3Δ6 | This work          |
| FGY15           | MATa ura3–52 leu2–3,112 lys2Δ201 gap1Δ: LEU2 | This work          |
| FGY135          | MATa ura3–52 leu2–3,112 lys2Δ201 gap1Δ: LEU2 shr3Δ6 | This work          |

**TABLE II**

| Plasmids        | Description                  | Source or reference |
|-----------------|------------------------------|---------------------|
| pPL247          | 3.5-kb SpeI-SalI fragment containing GAP1 in pRS316 | (13)               |
| pPL257          | GAP1::FLU1 in pRS316         | (13)               |
| pPL288          | shr3Δ5::hisG-URA3-neo-hisG in pBSII KSI (+) | (10)               |
| pFG6            | 1.8-kb SalI-XhoI fragment containing SUC2 in pBSII KS (+) | (15)               |
| pFG112          | 150-bp BamHI-flanked reporter cassette A within SUC2 in pFG6 | This work          |
| pFG113          | 162-bp BamHI-flanked reporter cassette B within SUC2 in pFG6 | This work          |
| pFG130–pFG142   | BamHI restriction sites introduced into GAP1 in pPL247 | This work          |
| pFG150–pFG162   | Reporter cassette A from pFG112 inserted into BamHI sites of pFG130–pFG142 | This work          |
| pFG170–pFG182   | Reporter cassette B from pFG113 inserted into BamHI sites of pFG130–pFG142 | This work          |
| pFG190          | GAP1::41/Xa in pRS316       | This work          |
| pFG195          | GAP1–271/Xa in pRS316       | This work          |
| pFG196          | gap1–312/Xa in pRS316       | This work          |
| pFG197          | GAP1–355/Xa in pRS316       | This work          |
| pFG200          | GAP1–481/Xa in pRS316       | This work          |
| pFG202          | GAP1–564/Xa in pRS316       | This work          |
22 °C, and transformation plates were incubated at 20 °C. Ura transformations were carried out with overnight cultures grown at DNA containing minus a squares creating plasmids pFG130 through pFG142, respectively (see Fig. 1 three stages. In the first stage, alleles with topological reporter cassettes A and B were constructed in molecular biological procedures. Plasmids containing lacking uracil. The successful deletion of SHR3 genes flanked by 13–14 nucleotides of complementary GAP1 and SUC2 sequences corresponding to amino acid 81 and following amino acid 133; pair B corresponding to sequences preceding amino acid 133; pair A corresponding to sequences preceding amino acid 81 and following amino acid 103; pair B corresponding to sequences preceding amino acid 357 and following amino acid 410), were annealed to single-stranded pPL257, non-functional alleles are indicated by black boxes (see “Experimental Procedures”). These cassettes, flanked by BamHI and SpeI-BamHI endonuclease sites, are cloned into plasmids pFG112 and pFG113, respectively. Asterisks indicate potential glycosylation sites within Sue2p. The plus symbols (+) indicate positions of positively charged amino acids (arginine or lysine), and the minus symbols (−) indicate negatively charged amino acids (glutamate or aspartate). B, native Gap1p (pPL247), cassette A gene fusion proteins Gap1p-5A (pFG150) and Gap1p-275A (pFG155), and cassette B gene fusion proteins Gap1p-5B (pFG170) and Gap1p-275B (pFG175) were expressed in FGY58. Extracts of total cell protein were prepared from transformants grown in SPD (plus lysine and adenine), and analyzed as described under “Experimental Procedures.” Briefly, protein preparations were solubilized in SDS-PAGE sample buffer, treated with endo H where indicated, resolved in 10% polyacrylamide gels, and immunoblotted with polyclonal anti-Gap1p antibody. The positions of molecular mass (kDa) markers are indicated.

gapΔ) are meiotic segregants from this cross. Strains FGY11 and FGY15 were transformed to Ura minus with a linear EcoRI-SalI fragment of DNA containing shr355::hisG-URA3-neo-hisG from pPL288 (10). The transformations were carried out with overnight cultures grown at 22 °C, and transformation plates were incubated at 20 °C. Ura− transformants were propagated on medium containing 5-fluoroorotic acid to obtain the unmarked shr3Δ6 deletion resulting in strains FGY14 and FGY135, respectively. The successful deletion of SHR3 was confirmed by Southern blot analysis. Standard yeast media were prepared, and yeast genetic manipulations were performed as described in Ref. 32. SPD and SCitD media, respectively containing proline and citrulline as sole nitrogen sources, were prepared as described (13). Yeast transformations were performed as described (33) using 50 μg of heat-denatured calf thymus DNA. Transformants were selected on solid SC media lacking uracil.

**Plasmid Constructions—**Plasmids were constructed using standard molecular biological procedures. Plasmids containing GAP1 gene fusion alleles with topological reporter cassettes A and B were constructed in three stages. In the first stage, BamHI restriction sites were inserted at 13 positions along the GAP1 gene at sequences corresponding to amino acids 5, 121, 154, 198, 226, 275, 307, 360, 412, 444, 491, 525, and 567, creating plasmids pFG130 through pFG142, respectively (see Fig. 1B). Synthetic oligomers comprising 32–34 nucleotides containing BamHI sites flanked by 13–14 nucleotides of complementary GAP1 sequence were annealed to single-stranded pPL247 prepared with helper phage M13K07 (34) in the dut ung E. coli host RZ1032 (35). After elongation, ligation, and transformation into a dut ung host, plasmids were screened for the presence of the BamHI restriction sites diagnostic for successful mutagenesis. In stage 2, in two separate reactions, pairs of synthetic oligomers containing a BamHI and SpeI-BamHI sites flanked on each side by 16 bases complementary to the SUC2 sequence (pair A corresponding to sequences preceding amino acid 51 and following amino acid 139; pair B corresponding to sequences preceding amino acid 357 and following amino acid 410), were annealed to single-stranded pFG6. This procedure created plasmids pFG112 and pFG113, respectively (see Fig. 2A). In stage 3, plasmids pFG130-pFG142 were restricted with BamHI, and topological reporter cassettes A (168 base pair BamHI fragment from pFG112) and B (171 base pair BamHI fragment from pFG113) were separately ligated into each plasmid, creating plasmids pFG150–pFG162 and pFG170–pFG182, respectively.

**FIG. 1.** Hydrodynamic profile of Gap1p and positions of fusion insertions. A, the hydrodynamic profile of Gap1p, the mean hydrodynamic index calculated using a window size of 11 amino acids (18), was used. B, the boxes depict the 12 hydrophobic domains (I–XII). The positions of the suc2 topological reporter cassette insertions are indicated by open circles, the positions of the factor Xa (fXa) protease cleavage site insertions are shown by open squares, and the position of the hemagglutinin epitope tag is shown as a solid triangle. The numbers refer to the amino acid to which the insertions were fused. Fusion alleles that maintained functional Gap1p activity are indicated with a plus (+), non-functional alleles are indicated with a minus (−).

**FIG. 2.** Topological reporter cassettes A and B, derived from internal regions of SUC2, exhibit differential effects on Gap1p fusion protein stability. A, schematic presentation of the 1.8-kilobase pair SalI-XmnI DNA fragment in pFG6 containing the coding sequence of mature Sue2p. Single-stranded pFG6 was used as template for the construction of topological reporter cassettes A and B indicated as black boxes (see “Experimental Procedures”). These cassettes, flanked by BamHI and SpeI-BamHI endonuclease sites, are cloned into plasmids pFG112 and pFG113, respectively. Asterisks indicate potential glycosylation sites within Sue2p. The plus symbols (+) indicate positions of positively charged amino acids (arginine or lysine), and the minus symbols (−) indicate negatively charged amino acids (glutamate or aspartate). B, native Gap1p (pPL247), cassette A gene fusion proteins Gap1p-5A (pFG150) and Gap1p-275A (pFG155), and cassette B gene fusion proteins Gap1p-5B (pFG170) and Gap1p-275B (pFG175) were expressed in FGY58. Extracts of total cell protein were prepared from transformants grown in SPD (plus lysine and adenine), and analyzed as described under “Experimental Procedures.” Briefly, protein preparations were solubilized in SDS-PAGE sample buffer, treated with endo H where indicated, resolved in 10% polyacrylamide gels, and immunoblotted with polyclonal anti-Gap1p antibody. The positions of molecular mass (kDa) markers are indicated.
μl, equivalent to ODnano of 0.2 cell suspension) were diluted with an equal volume of 100 mM sodium citrate, pH 5.5, and heated for 10 min at 37 °C. Three milliliters of endoglycosidase H (endo H; Roche Molecular Biochemicals) was added to half of the samples, and all samples were incubated overnight at 37 °C. Proteins were resolved by 10% SDS-PAGE and immunoblotted with polyclonal anti-Gap1p antibody. The topological orientation, as indicated (cyt, cytosolic; lum, luminal), of each reporter cassette was identical in both strains.

Yeast Microsome Preparation—Strains FGY135 (shr3Δ), FGY11 (sec12–1), and FGY14 (shr3Δ sec12–1) transformed with plasmids encoding gene fusion proteins containing factor Xa cleavage sites were grown in SC (minus uracil) to an OD600 of 2. Cells from 50 ml of culture were harvested, washed once in water, resuspended in 5 ml of water and incubated at 34 °C for 10 min. Temperature-shifted cells were used to inoculate 200 ml of SPD (plus lysine and adenine), and analyzed as described under “Experimental Procedures.” Protein preparations were treated with endo H as indicated, and resolved by SDS-PAGE in 10% polyacrylamide gels, immunoblotted with polyclonal anti-Gap1p antibody. The topological orientation, as indicated (cyt, cytosolic; lum, luminal), of each reporter cassette was identical in both strains.

In Vivo Membrane Topology of Gap1p

RESULTS

The Gap1p Sequence Contains 12 Potential Membrane-spanning Domains—Gap1p comprises 602 amino acids. Hydrophathic profile analysis indicates that Gap1p has 12 hydrophobic regions (Fig. 1A), numbered I–XII, each of which are of sufficient length and hydrophobicity to function as membranespanning domains (39). Using single-stranded mutagenesis, BamHI restriction sites were individually introduced into GAP1 sequences encoding the amino- and the carboxyl-terminal domains, and into each of the hydrophilic loops (L1–L11) that separate the hydrophobic domains (see “Experimental Procedures”). The BamHI sites were inserted in such a manner as not to disrupt the reading frame of the Gap1p coding sequence, and enabled the subsequent construction of in-frame gene sandwich fusions with Suc2p based topological reporter cassettes A or B (Fig. 1B, open circles). Additionally, tandemly repeated in-frame factor Xa protease sites were inserted into the Gap1p sequence at positions within the NH2- and COOH-terminal domains, and into hydrophilic loops L5, L6, L7, and L10 (Fig. 1B, open squares). The factor Xa protease recognizes the tetrapeptide motif IEGR and specifically cleaves the protein sequence COOH-terminal of the arginine residue (40). The recognition motif was tandemly (IEGRIEGR) inserted to increase the probability of cleavage (29). The ability of factor Xa protease to cleave the Xa fusion constructs was initially tested in Nonidet P-40-solubilized membrane preparations; in each case, the fusion constructs were efficiently cleaved. Factor Xa sites inserted into the other hydrophilic loops of Gap1p were refractory to added protease; consequently, these fusion constructs were not further analyzed.

We examined whether the modified Gap1p fusion proteins retained the capacity to transport amino acids. Gap1p is the only AAP capable of transporting citrulline at rates sufficient to support growth on media containing citrulline as sole nitrogen source (3). Strain FGY58 (gap1Δ::LEU2), lacking its chromosomal copy of GAP1, was individually transformed with plasmids pFG150–pFG162 (encoding cassette A gene fusions), pFG170–pFG182 (encoding cassette B gene fusions), and pFG190, pFG195, pFG200, and pFG202 (encoding factor Xa insertions). Plasmids capable of supporting growth on media containing citrulline as sole nitrogen source were judged to carry functional Gap1p alleles (Fig. 1B, plus signs) within mutant symbols). The fusion constructs are designated by the amino acid to which they are fused, functional constructs begin with a capital letter, e.g., Gap1p-198A (cassette A fused at position 198), and non-functional constructs begin with a lowercase letter, e.g., gap1p-312Xa (factor Xa cleavage sites fused at position 312).

Construction of Topological suc2 Reporter Cassettes A and B—Two regions within Suc2p, amino acids 81–133 and 357–410 (the amino acid coordinates are derived from the sequence of the mature secreted form of Suc2p), were selected to construct topological reporter cassettes for the in vivo analysis of the membrane structure of Gap1p. These regions, each comprising approximately 50 amino acids, contain three NXS/T sequences for asparagine-linked glycosylation that are known to be glycosylated in the secreted form of invertase (41). It has
been shown that the distance between the luminal end of a transmembrane segment and a potential glycosylation acceptor site influences whether the site is efficiently glycosylated (42); the reporter cassettes were therefore constructed to place the first and last NXST acceptor sites at least 12 amino acids away from the ends. Both cassettes contain equal numbers of negatively and positively charged amino acids, and thus are not expected to influence the orientation of adjacent membrane-spanning segments. Plasmids pFG112 (cassette A) and pFG113 (cassette B) contain the sequences encoding these regions flanked by BamHI sites (Fig. 2A).

As described in the preceding section, the reporter cassettes were inserted into the GAP1 sequence at the BamHI sites of plasmids pFG130-pFG142. Each of the resulting 26 gene fusion constructs were tested for Gap1p activity in a growth-based assay. Fusion proteins with either cassette A or B inserted into 9 of the 13 positions enabled strain FGY58 to grow on citrulline-based media (Fig. 1B); however, the strains expressing the functional cassette A gene fusions formed larger colonies. We compared the steady-state levels and glycosylation state of wild-type Gap1p, Gap1p-5A, Gap1p-275A, Gap1p-5B, and Gap1p-275B proteins expressed in strain FGY58 (Fig. 2B). Compared with extracts prepared from the wild-type Gap1p-expressing strain (Fig. 2B, lanes 1 and 2), extracts obtained from strains expressing Gap1p-5A and Gap1p-275A (Fig. 2B, lanes 3–6) contained similar amounts of fusion proteins. In contrast, the extracts isolated from Gap1p-5B- and Gap1p-275B-expressing strains contained significantly lower levels of fusion proteins (Fig. 2B, lanes 7–10). Strains expressing the remaining 11 cassette B reporter constructs invariably contained less fusion proteins than strains expressing the corresponding cassette A hybrids (data not shown). Despite differences in protein levels, Gap1p-275A and Gap1p-275B were similarly glycosylated as evidenced by their increased electrophoretic mobility after incubation with endo H (Fig. 2B, compare lanes 5 with 6, and lanes 9 with 10). These results indicate that the loop containing the reporter cassettes are oriented in the ER lumen. The observed 4–5-kDa decrease in molecular mass suggests that all three NXST sites were glycosylated. Treatment with endo H did not affect the mobility of wild-type Gap1p, Gap1p-5A, and Gap1p-5B proteins, indicating that these proteins are not glycosylated.

**Analysis of Cassette A Fusion Protein Topology**—Due to the consistently low levels of cassette B gene fusions in protein extracts, we present our analysis of Gap1p topology obtained using the reporter cassette A containing gene fusions. It should be noted that cassette B constructs were analyzed in parallel, and identical patterns of glycosylation were obtained. Preparations of total cell protein were isolated from strains FGY58 (SHR3) and FGY60 (shr3Δ) expressing the 13 cassette A reporter constructs. Proteins were fractionated by SDS-PAGE before and after treatment with endo H (Fig. 3). The extracts contained different amounts of fusion proteins; the observed differences are likely a consequence of differential stability, a phenomenon that has been described for other gene sandwich systems. Each of the 13 fusion proteins exhibited an identical pattern of endo H sensitivity, regardless of the SHR3 genotype of the strain in which it was produced (Fig. 3, compare the upper and lower panels), indicating that the membrane structure of Gap1p is not dependent upon the presence of Shr3p.

Fusion proteins Gap1p-5A, Gap1p-154A, Gap1p-228A, Gap1p-307A, Gap1p-412A, Gap1p-491A, and Gap1p-567A were insensitive to endo H treatment, indicating that the NH2 and COOH termini, and the even-numbered loops are oriented toward the cytoplasm. The remaining fusion proteins gap1p-121A, Gap1p-198A, Gap1p-275A, Gap1p-360A, Gap1p-444A, and Gap1p-525A were sensitive to endo H treatment, indicating that the odd-numbered loops are accessible to glycosyl transferase and thus localized to the ER lumen. These in vivo findings suggest that Gap1p contains 12 membrane-spanning domains.

Prior to endo H treatment, fusion proteins gap1p-121A, Gap1p-198A, Gap1p-275A, Gap1p-360A, Gap1p-444A, and Gap1p-525A expressed in strain FGY58 (SHR3) migrated as diffuse heterogeneous high molecular weight smears. After
endo H treatment, the resulting unglycosylated proteins migrated as single faster migrating bands. This pattern of migration, particularly notable in preparations containing Gap1p-444A and Gap1p-525A, suggests that these proteins exit the ER and receive extensive outer chain glycosylation in the Golgi apparatus (43). Fusion proteins gap1p-121A and Gap1p-525A expressed in strain FGY60 (shr3Δ6) were core-glycosylated, but were not further glycosylated; a clear 4–5-kDa decrease in mass was observed following treatment with endo H, and the bands before and after treatment were of similar intensity. These results are consistent with the requirement of Shr3p for AAPs to exit the ER. Surprisingly, fusion proteins Gap1p-198A, Gap1p-275A, Gap1p-360A, and Gap1p-444A migrated similarly in either FGY58 and FGY60.

**Integrity and Sidedness of Microsomal Membrane Preparations**—The 12 membrane-spanning model of Gap1p was tested by introducing factor Xa protease sites into six diagnostic positions of Gap1p (Fig. 1B). Gap1p in SHR3 cells is predominantly localized to the plasma membrane, whereas Gap1p in shr3Δ6 cells is almost exclusively localized to the ER (13). In order to be able to compare the membrane topology of factor Xa fusion proteins in SHR3 and shr3Δ6 cells, we took advantage of the well characterized temperature-sensitive sec12Δ1 mutation (44, 45). At restrictive temperatures (≥34 °C), the transport of AAPs from the ER is blocked in strains carrying the sec12Δ1 mutation. Gap1p expression can be controlled by the nitrogen source supplied in the medium; in comparison to cells grown in synthetic complete (SC) media containing ammonia, cells grown in minimal synthetic proline medium (SPD) contain 15-fold higher levels of Gap1p (15).

Strains FGY11 (sec12Δ1 SHR3), FGY14 (sec12Δ1 shr3Δ6), and FGY135 (SEC12 shr3Δ6) were transformed with plasmids encoding the six factor Xa fusion proteins. The resulting 18 strains were pregrown at 25 °C (permissive temperature) in SC, harvested, and incubated in water for 10 min at 34 °C to initiate the secretory block imposed by the sec12Δ1 mutation. The temperature-shifted cells were used to inoculate SPD, and the cultures were incubated an additional 2 h at 34 °C. Microsomal membranes were isolated as described under “Experimental Procedures.”

The integrity and sidedness of microsomal preparations was determined by examining whether the ER luminal protein Kar2p, and the ER membrane proteins Wbp1p and Sec61p were accessible to degradation by protease K in the absence or presence of 0.2% Nonidet P-40. At the times indicated, samples were prepared for SDS-PAGE and analyzed by immunoblotting with anti-Kar2p, anti-Wbp1p, and anti-Sec61p antiserum (Fig. 4). We found that the luminal protein Kar2p (46) was protected from proteinase K digestion, even during extended incubations (8 h). Kar2p was rapidly degraded when membranes were permeabilized with 0.2% Nonidet P-40. Similarly, Wbp1p, a type I membrane protein with a large luminal domain (47), was only degraded in the presence of detergent. In contrast, the polytopic membrane protein Sec61p was rapidly degraded even in the absence of detergent, indicating that cytoplasmically localized COOH-terminal domain recognized by the anti-Sec61p antibodies was accessible to the protease (24). These results demonstrate that the microsomal membranes predominantly comprised sealed membrane preparations oriented with the cytoplasmic side facing out.

**Analysis of Factor Xa Fusion Protein Topology—Microsomes prepared from strains FGY11 (sec12Δ1 SHR3), FGY14 (sec12Δ1 shr3Δ6), and FGY135 (SEC12 shr3Δ6) expressing the Xa fusion proteins were incubated with factor Xa for 2 h in the absence or presence of 0.2% Nonidet P-40. The reaction products were resolved by SDS-PAGE and analyzed by immunoblotting with antiserum directed against the NH2-terminal domain of Gap1p (Fig. 5). The patterns of factor Xa cleavage were identical in all three strains, confirming that the membrane structure of Gap1p is not dependent upon the presence of Shr3p. In the absence of detergent, factor Xa cleavage products were generated from microsomes containing Gap1p-414Xa, gap1p-312Xa, Gap1p-481Xa, and Gap1p-564Xa proteins, consistent with the cytoplasmic localization of the NH2 and COOH termini, and hydrophilic loops 6 and 10. The Gap1p-271Xa and Gap1p-355Xa proteins were not cleaved without permeabilizing the membranes with Nonidet P-40. Native Gap1p does not contain factor Xa recognition sites and was not cleaved under the conditions described. The data indicate that these Xa sites, and hence hydrophilic loops 5 and 7, are oriented in the ER lumen.

**DISCUSSION**

The topological reporter cassettes described here form the basis of a general method to determine the membrane association and orientation of hydrophobic domains within polytopic yeast membrane proteins. In designing the reporter constructs, we attempted to satisfy the following criteria. We sought to establish a method for the in vivo analysis of yeast proteins, and to avoid using heterologous expression or in vitro translation systems. To enable the topological analysis to be carried out in the context of the complete protein sequence, i.e. in the

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**FIG. 6. The proposed membrane topology of Gap1p.** Twelve membrane-spanning domains have been identified. Transmembrane domains I–XII are represented by shaded rectangles. The NH2 and COOH termini, and the even-numbered hydrophilic loops, were found to be cytoplasmically oriented. The odd-numbered loops were found to be accessible to luminal ER-processing components, and thus will be oriented toward the extracellular milieu when Gap1p is localized at the plasma membrane.
presence of all membrane-spanning domains, the cassettes were used to create gene sandwich fusion proteins. Due to the ease of the analysis, and the demonstrated success of glycosylation scanning approaches in mammalian systems (30, 31), glycosylation was chosen as the topological read-out. However, it is known that glycosylation sites are rather inefficiently used in yeast, and it is not understood why some potential glycosylation sites are not used. Recent work demonstrates an inverse relationship between protein folding reactions and glycosylation, i.e. regions of proteins containing glycosylation sequons that fold quickly are less likely to be glycosylated (48). To minimize complications we chose two regions of Suc2p that are known to be effectively used (41). The cassettes have a net neutral charge, each contains equal numbers of negatively and positively charged amino acids, to minimize influencing the orientation of adjacent membrane-spanning segments (49, 50).

Finally, the desired reporter constructs were designed to be as small as possible to minimize potential folding artifacts, protein instability, and hopefully to enable the enzymatic activity of target proteins to be maintained. The desire for compactness was balanced against the demand for a robust signal strength; three glycosylation sequons were included in each cassette with sufficient flanking sequence to facilitate access by glycosyl transferase.

We have used the Suc2p-based topological reporter cassettes to determine the membrane structure of Gap1p. Although the results presented here have focused on cassette A-containing fusions, due to the higher steady-state levels of fusion proteins (Fig. 2), identical topological results were obtained with both cassettes at all positions. We have not investigated the underlying reasons for the lower levels of cassette B-containing fusions. The insertion of factor Xa protease cleavage sites was used to test the validity of the topological model obtained using the reporter cassette. The data obtained from these two independent approaches show that the 12 hydrophobic domains of Gap1p traverse the membrane in a zigzag fashion connected by hydrophilic loops, with the hydrophilic NH$_2$ and COOH termini oriented in the cytoplasm (Fig. 6). This model is based on our finding that gene fusions in the NH$_2$- and COOH-terminal regions, and within the even-numbered hydrophilic loops were not glycosylated (Fig. 3) and were accessible to factor Xa protease even in the absence of detergent (Fig. 5). Conversely, gene fusions in the odd-numbered loops were extensively glycosylated (Fig. 3) and inaccessible to protease in the absence of detergent (Fig. 5), indicating that these regions of Gap1p are localized to the lumen of the ER. No structural ambiguities were detected, and it should be noted that the three data sets (cassette A, cassette B, and Xa) are internally consistent, we never observed conflicting data regarding the orientation of membrane-spanning segments.

Given the high level of sequence homology and similarity of hydrophobic profiles, the other members of the AAP gene family are likely to have similar membrane topologies. Gene sandwich approaches using alkaline phosphatase have been used to study the membrane topology of three E. coli permeases that share significant homology with the yeast AAPs. According to these studies, each of the 12 hydrophobic regions function as membrane-spanning domains (27, 28, 51). In contrast to these results and those presented here, earlier investigations analyzing the topology of the yeast arginine permease (CANT) did not result in a clear consensus regarding the number of hydrophobic segments that span the membrane, both 10 and 11 membrane-spanning domains were postulated (22, 52).

Finally, the data clearly demonstrate that the fusion proteins exhibited an identical pattern of endo H and protease sensitivity, and migrated the same regardless of the SHR3 genotype of the strain in which they were produced (Figs. 3 and 5). Since no differences were observed, our data suggest that Shr3p does not participate in the co-translational insertion of AAPs into the ER membrane. These results are consistent with our previous findings that suggested that AAPs are correctly integrated into the ER membrane and fold properly independent of Shr3p function. Thus Shr3p must function at a subsequent step in the secretory pathway to ensure AAPs are efficiently packaged in ER-derived COPII transport vesicles. We were surprised by the observation that fusion proteins Gap1p-198A, Gap1p-275A, Gap1p-360A, and Gap1p-444A expressed in FG60 (shr3Δ) appeared to receive outer chain glycolyctic linkages, suggesting that these proteins exit the ER independently of Shr3p (Fig. 3, lower panel). The fact that Gap1p-525A did not exit the ER raises the interesting possibility that core glycosylation, or the Suc2p moiety, within internal hydrophilic loops may enable Gap1p to be packaged for transport without the assistance of Shr3p. The model of Gap1p topology (Fig. 6) forms the basis for further dissection of the molecular mechanisms governing Gap1p function and its intracellular transport through the secretory and endocytic pathways.

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