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Analysis of Transmembrane Helix Integration in the Endoplasmic Reticulum in *S. cerevisiae*

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What sequence features in integral membrane proteins determine which parts of the polypeptide chain will form transmembrane α-helices and which parts will be located outside the lipid bilayer? Previous studies on the integration of model transmembrane segments into the mammalian endoplasmic reticulum (ER) have provided a rather detailed quantitative picture of the relation between amino acid sequence and membrane-integration propensity for proteins targeted to the Sec61 translocon. We have now carried out a comparative study of the integration of N_{out}–C_{in}-orientated 19-residue-long polypeptide segments into the ER of the yeast *Saccharomyces cerevisiae*. We find that the 'threshold hydrophobicity' required for insertion into the ER membrane is very similar in *S. cerevisiae* and in mammalian cells. Further, when comparing the contributions to the apparent free energy of membrane insertion of the 20 natural amino acids between the *S. cerevisiae* and the mammalian ER, we find that the two scales are strongly correlated but that the absolute difference between the most hydrophobic and most hydrophilic residues is ~2-fold smaller in *S. cerevisiae*.

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Keywords: membrane protein; membrane insertion; endoplasmic reticulum; hydrophobicity; *Saccharomyces cerevisiae*

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Introduction

In eukaryotic cells, integral membrane proteins destined for the plasma membrane and subcellular organelles in the exo- and endocytic pathways are cotranslationally targeted and integrated into the endoplasmic reticulum (ER) membrane.[1] The N-terminal signal peptide (SP; or the first transmembrane helix) in the protein is recognized by the signal recognition particle as it comes out of the ribosome, targeting the ribosome–nascent chain complex to the Sec61 translocon in the ER membrane. Finally, transmembrane helices in the nascent chain are inserted into the lipid bilayer through a 'lateral gate' in the wall of the Sec61 translocon channel.[2]

While the basic mechanism of membrane protein insertion into the ER is thus understood in outline, less is known about the quantitative relationships between membrane-insertion efficiency and the amino acid sequence of transmembrane segments. In recent years, we have measured the membrane-insertion efficiency of an extensive set of model hydrophobic segments (H-segments) into dog pancreas rough microsomes (RMs), using an *in vitro* transcription–translation system (or, in a few cases, *in vivo* expression in BHK cells) and a model protein based on the *Escherichia coli* inner-membrane protein leader peptidase (Lep). Using this system, we have studied the effects of overall hydrophobicity, length, and orientation of the H-segment on membrane insertion and have also derived a table of position-specific contributions to the insertion efficiency for all the 20 natural amino acids.[3–5] A subset of these H-segments has also been analyzed in *E. coli* using a different model protein.[6]

An important question in the field of membrane protein biogenesis is whether different kinds of translocons in different organisms 'read' the nascent polypeptide chain in similar ways. The aim of the

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Abbreviations used: ER, endoplasmic reticulum; SP, signal peptide; H-segment, hydrophobic segment; RM, rough microsome; OST, oligosaccharyl transferase; Endo H, endoglycosidase H; OD_{600}, optical density at 600 nm.

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current study is to provide quantitative data on Sec61-mediated insertion of H-segments into the ER membrane in the yeast *Saccharomyces cerevisiae* that can be directly compared to the results already obtained with the mammalian system. For technical reasons, we have used a modified model protein where the two N-terminal transmembrane helices in Lep have been replaced by a cleavable SP from Suc2p, a secreted *S. cerevisiae* protein. Our results show a strong correlation between the results obtained by *in vitro* transcription–translation in the presence of dog pancreas RMs and by *in vivo* expression in yeast. Interestingly, it appears that the absolute difference in the contributions to the apparent free energy of membrane insertion between the most hydrophobic and most hydrophilic amino acids is significantly smaller in *S. cerevisiae* than in the mammalian ER.

Results

Model protein and membrane-insertion assays

In preliminary experiments, we found that the Lep model protein used in our studies of membrane protein insertion into the mammalian ER does not attain a unique topology when expressed in *S. cerevisiae*. In particular, for strongly hydrophobic H-segments, a fraction of the molecules insert into the membrane with a partially inverted topology (data not shown). To circumvent this problem, we replaced the two N-terminal transmembrane helices in Lep with the cleavable SP of the yeast secretory protein Suc2p (Fig. 1a). Membrane insertion of H-segments introduced into the SP-Lep construct was probed by assessing the glycosylation status of two engineered acceptor sites for N-linked glycosylation (G1 and G2) (Fig. 1b), as has been done in previous studies.[3–5,8] If the H-segment is inserted into the membrane, only the G1 site is translocated into the lumen of the ER and becomes glycosylated by the lumen-facing oligosaccharyl transferase (OST) enzyme, whereas if the H-segment is translocated across the membrane, both the G1 and G2 sites become glycosylated. For detection of radiolabeled SP-Lep molecules by immunoprecipitation, a triple hemagglutinin tag was fused at the C-terminus of Lep.

After radiolabeling *in vivo*, singly and doubly glycosylated SP-Lep molecules were separated by SDS-PAGE, together with a sample treated with endoglycosidase H (Endo H) to remove the glycans. A typical gel is shown in Fig. 1c. No unglycosylated protein was seen with the SP-Lep constructs, indicating that they were efficiently targeted to the ER membrane. The lack of unglycosylated molecules also rules out the possibility that an H-segment may be integrated with an 'inverted' N_{in}–C_{out} orientation since the G2 site is too close to the membrane (only five residues away from the end of the H-segment) for modification by the OST.[9] Had a population of SP-Lep molecules been integrated into the membrane with N_{in}–C_{out} orientation, unglycosylated Lep molecules would have accumulated. Thus, singly glycosylated SP-Lep molecules have an H-segment integrated in the ER membrane with N_{out}–C_{in} topology, and doubly glycosylated SP-Lep molecules have a non-membrane-integrated H-segment that has been translocated into the ER lumen.

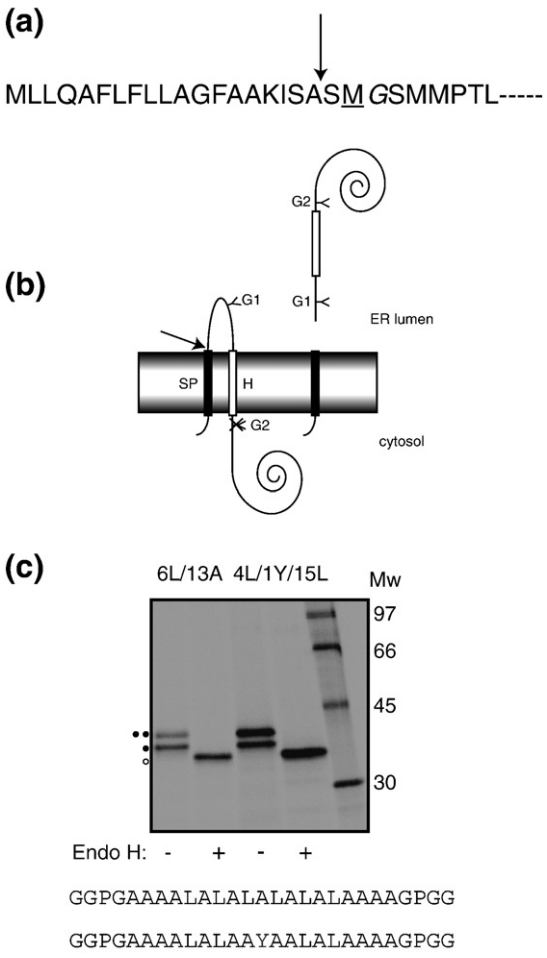

Fig. 1. The SP-Lep model protein. (a) Residues 1–88 of Lep were replaced by the SP of Suc2p (residues 1 to 21). The signal peptidase cleavage site[7] is indicated by the arrow, Gly89, the first Lep residue is in italics, and the alternative initiator Met is underlined. (b) Membrane-insertion assay. If the H-segment (white) is inserted into the membrane, only the first N-glycan acceptor site (G1) is glycosylated by the lumenal OST enzyme (left); if the H-segment is translocated across the ER membrane, both N-glycan sites (G1 and G2) become glycosylated (right). The Suc2p SP (black) is indicated. (c) SP-Lep molecules with the indicated H-segments were labeled with [^{35}S]Met *in vivo*, immunoprecipitated, subjected to Endo H digestion or mock treatment, and analyzed by SDS-PAGE. Two filled circles indicate doubly glycosylated, one filled circle indicates singly glycosylated, and one open circle indicates unglycosylated SP-Lep molecules.

For each H-segment tested, the relative amounts of singly *versus* doubly glycosylated molecules were quantified and expressed as an apparent equilibrium constant, $K_{app}=f_{1g}/f_{2g}$, where f_{1g} is the fraction of

singly glycosylated Lep molecules and f_{2g} is the fraction of doubly glycosylated Lep molecules. K_{app} was then used to calculate an apparent free energy of membrane insertion, $\Delta G_{app} = -RT\ln K_{app}$ (where R is the gas constant and T is the absolute temperature, $T = 298$ K).

Membrane-insertion efficiency of Leu/Ala-based H-segments

First, we measured the membrane-insertion efficiency of 19-residue-long Leu/Ala-based H-segments of the composition GGPG-nL/(19−n)A-GPGG (Gly-Pro flanks are included in order to 'insulate' the H-segment from the surrounding sequence; Fig. 2a). As shown in Fig. 2b, ΔG_{app} is roughly linear with the number of Leu residues (n), and $\Delta G_{app} = 0$ kcal/mol (50% membrane insertion) for $n \approx 4$. The results obtained in mammalian RMs and in BHK cells for the same H-segments[3] are shown for comparison. Qualitatively, the mammalian and yeast systems yield similar results (in particular, the number of Leu residue required for $\Delta G_{app} = 0$ kcal/mol is 3–5 for both systems). The slope of the $\Delta G_{app} = f(n)$ curve indicates that $\Delta\Delta G_{app}$ for an Ala → Leu replacement in the H-segment is smaller in yeast than in the mammalian RM system (−0.27 *versus* −0.67 kcal/mol). In BHK cells, the slope is even steeper (−1.16 kcal/mol). Assuming a simple additive model for the contributions of Leu and Ala residues to the apparent free energy of insertion, $\Delta G_{app} = n \cdot G_{app}^{Leu} + (19-n) \cdot \Delta G_{app}^{Ala} = -0.27n + 1.2$, and ignoring any contributions to ΔG_{app} from the Gly-Pro flanks,[4] we obtain $\Delta G_{app}^{Leu} = -0.21$ kcal/mol and $\Delta G_{app}^{Ala} = 0.06$ kcal/mol. The corresponding values found using the mammalian RM system are −0.43 and 0.13 kcal/mol.[4]

As a control, two SP-Lep constructs were also expressed *in vitro* in the presence of dog pancreas RMs (Fig. 2c). In agreement with a previous study[10] where Suc2p was translated *in vitro*, two translated products were observed in the absence of RM. The longer form includes the SP, while the shorter form is produced by ribosomes initiating translation at Met21 (underlined in Fig. 1a). The construct with an H-segment derived from the polar C-terminal P2

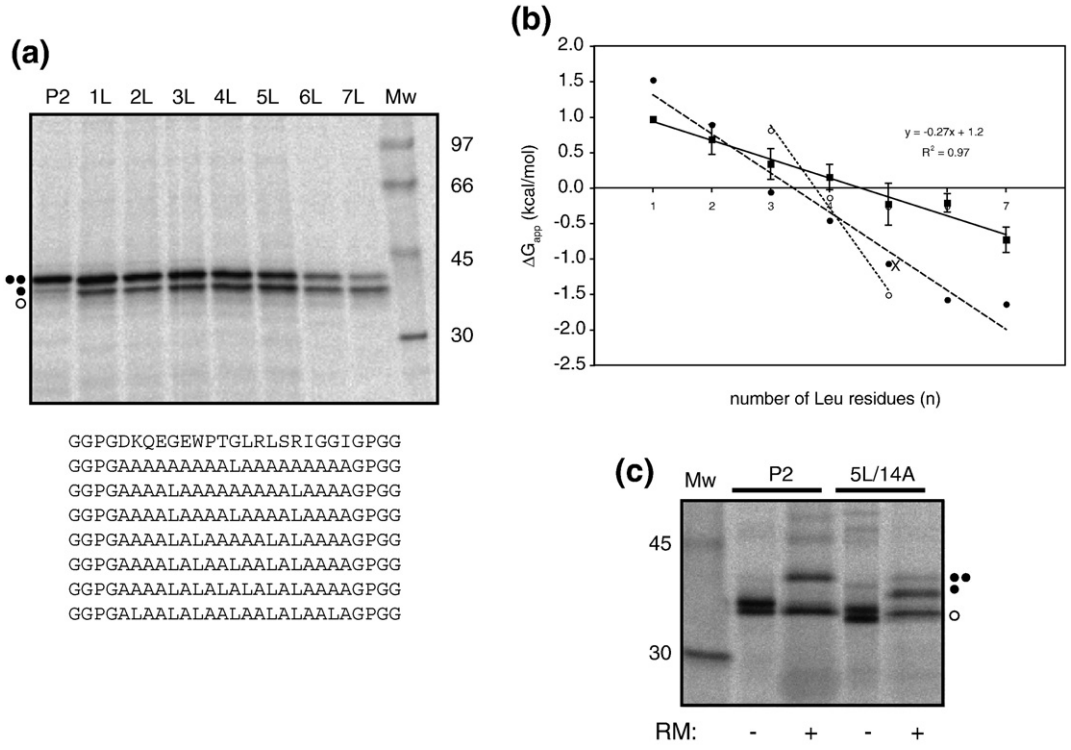

Fig. 2. Membrane-insertion efficiency into the yeast ER of Leu/Ala-based H-segments of the composition GGPG-nL/(19−n)A-GPGG. (a) SP-Lep molecules with the indicated H-segments were labeled with [^{35}S]Met, immunoprecipitated, and subjected to SDS-PAGE. Two filled circles indicate doubly glycosylated, one filled circle indicates singly glycosylated, and one open circle indicates unglycosylated SP-Lep molecules. The P2 segment is a polar segment from periplasmic C-terminal domain of *E. coli* Lep. (b) Insertion free energy (ΔG_{app}) plotted against n, the number of Leu residues in the H-segment (black squares). Averages and standard deviations from at least three independent experiments are shown. A linear least-squares fit to the data is shown as a continuous line. For comparison, data for the same H-segments obtained with the dog pancreas RM system and in BHK cells[3] are shown as black and white circles, respectively, and the linear least-squares fit is shown as broken lines. The value measured by *in vitro* translation in the presence of dog pancreas RMs for the SP-Lep(5L/14A) construct is shown by 'X'. (c) SP-Lep molecules with the indicated H-segments were transcribed and translated *in vitro* in the absence (−) and presence (+) of dog pancreas RMs and analyzed by SDS-PAGE. Two filled circles indicate doubly glycosylated, one filled circle indicates singly glycosylated, and one open circle indicates unglycosylated SP-Lep molecules.

Table 1. ΔG_{app} values and standard deviations (SDs) for the H-segments analyzed in this study

Construct	H-segment	ΔG_{app} (kcal/mol)	SD (kcal/mol)
SP-P2	GGPGDKQEGEWPTGLRLSRIGGIGPGG	0.86	0.13
SP-19A	GGPGAAAAAAAAAAAAAAAAAAGPGG	0.43	0.07
SP-2L/17A	GGPGAAAALAAAAAAAAALAAAAGPGG	0.56	0.13
SP-3L/16A	GGPGAAAALAAAALAAAALAAAAGPGG	0.20	0.07
SP-4L/15A	GGPGAAAALALAAAAALALAAAAGPGG	0.06	0.17
SP-5L/14A	GGPGAAAALALAALAALALAAAAGPGG	−0.23	0.29
SP-6L/13A	GGPGAAAALALALALALALAAAAGPGG	−0.21	0.13
SP-7L/12A	GGPGALAALALAALAALALAALAGPGG	−0.83	0.09
SP-19L	GGPGLLLLLLLLLLLLLLLLLLGPGG	>3	—
SP-4L/1C/16A	GGPGAAAALALAACAALALAAAAGPGG	−0.27	0.07
SP-9L/1D/9A	GGPGALAALALLLDLALALAALAGPGG	−0.35	0.11
SP-6L/1E/12A	GGPGAAAALALALELALALAAAAGPGG	−0.02	0.07
SP-6L/1G/12A	GGPGAAAALALALGLALALAAAAGPGG	−0.05	0.04
SP-6L/1H/12A	GGPGAAAALALALHLALALAAAAGPGG	0.18	0.05
SP-2L/1I/16A	GGPGAAAALAAAAIAAAALAAAAGPGG	0.37	0.01
SP-8L/1K/10A	GGPGALAALALALKLALALAALAGPGG	−0.28	0.10
SP-6L/1N/12A	GGPGAAAALALALNLALALAAAAGPGG	−0.02	0.06
SP-6L/1P/12A	GGPGAAAALALALPLALALAAAAGPGG	0.10	0.07
SP-6L/1R/12A	GGPGAAAALALALRLALALAAAAGPGG	0.13	0.03
SP-4L/1S/14A	GGPGAAAALALAASAALALAAAAGPGG	0.36	0.09
SP-4L/1T/14A	GGPGAAAALALAATAALALAAAAGPGG	0.12	0.03
SP-4L/1W/14A	GGPGAAAALALAAWAALALAAAAGPGG	0.03	0.03
SP-4L/1Y/14A	GGPGAAAALALAAYAALALAAAAGPGG	0.18	0.08
SP-6L/1Q/12A	GGPGAAAALALALQLALALAAAAGPGG	0.04	0.07
SP-4L/1F/14A	GGPGAAAALALAAFAALALAAAAGPGG	−0.09	0.03
SP-4L/1M/14A	GGPGAAAALALAAMAALALAAAAGPGG	−0.05	0.04
SP-4L/1V/14A	GGPGAAAALALAAVAALALAAAAGPGG	−0.07	0.10

The P2 segment is a polar segment from the normally periplasmic C-terminal domain of Lep.

domain of Lep is doubly glycosylated, as expected, while for the construct with a 5L/14A H-segment, $\Delta G_{app} = -1.15$ kcal/mol, to be compared with $\Delta G_{app} = -0.23$ kcal/mol in yeast (Table 1) and $\Delta G_{app} = -1.07$ kcal/mol for the original Lep construct with the 5L/14A H-segment expressed *in vitro*.[3] Therefore, replacing the TM1–TM2 part of Lep with the Suc2p SP makes little difference to the results obtained using *in vitro* translation in the presence of RMs, as was found previously with another SP.[8] The differences between the mammalian and yeast systems seen in Fig. 2b are therefore not due to the use of the Suc2p SP in the latter.

'Biological' ΔG_{app}^{X} scale for insertion into the yeast ER membrane

To determine how much each of the remaining 18 natural amino acids contributes to ΔG_{app}, we measured a second set of H-segments (see Supplement S1), each with one test amino acid in the middle of the segment. Thus, the test residue is embedded in the hydrophobic core of the lipid bilayer when the H-segment is integrated as a transmembrane helix. Since the maximal sensitivity of the glycosylation assay is when $\Delta G_{app} \approx 0$ kcal/mol,[3] the number of Leu and Ala residues was adjusted depending on the test amino acid in the middle position to keep the ΔG_{app} of the H-segment close to zero; that is, the number of leucines was increased when the middle amino acid was charged or polar and was decreased when it was hydrophobic.

For each amino acid (X), we calculated its contribution to the apparent free energy of insertion, ΔG_{app}^{X}, from the ΔG_{app} value measured for the relevant H-segment and the values given above for ΔG_{app}^{Leu} and ΔG_{app}^{Ala}: $\Delta G_{app}^{X} = \Delta G_{app} - (n \cdot \Delta G_{app}^{Leu} + (18-n) \cdot \Delta G_{app}^{Ala})$. The results are shown in Fig. 3a. As expected, the hydrophobic amino acids have $\Delta G_{app}^{X} < 0$ and the charged amino acids are found at the other end of the scale. A comparison with the scale obtained using dog pancreas RMs[3] is shown in Fig. 3b. The correlation is good ($R^2 = 0.9$) and the zero points on the two scales coincide, but the overall range in the ΔG_{app}^{X} values is smaller in the case of yeast (−0.3 to +1.0 kcal/mol *versus* −0.5 to +1.9 kcal/mol). The yeast scale is thus a 'compressed' version of the mammalian scale.

Discussion

Are the 'rules' for membrane protein assembly different between different organisms? In this report, we have measured how well 19-residue-long model H-segments characterized earlier in mammalian systems integrate into the ER membrane in *S. cerevisiae*.

While the overall relation between the Leu/Ala balance in H-segments of the composition GGPG-*n*L/(19−*n*)A-GPGG and the apparent free energy of membrane insertion (ΔG_{app}) in yeast is similar to what we found previously for the mammalian RM system and BHK cells,[3] with 50% membrane integration ($\Delta G_{app} = 0$ kcal/mol) observed for $n = 3$–5, the measured $\Delta\Delta G^{Ala \rightarrow Leu}$ is smaller for yeast by a factor of ~2.

We have also derived a biological hydrophobicity scale for yeast by analyzing H-segments with a

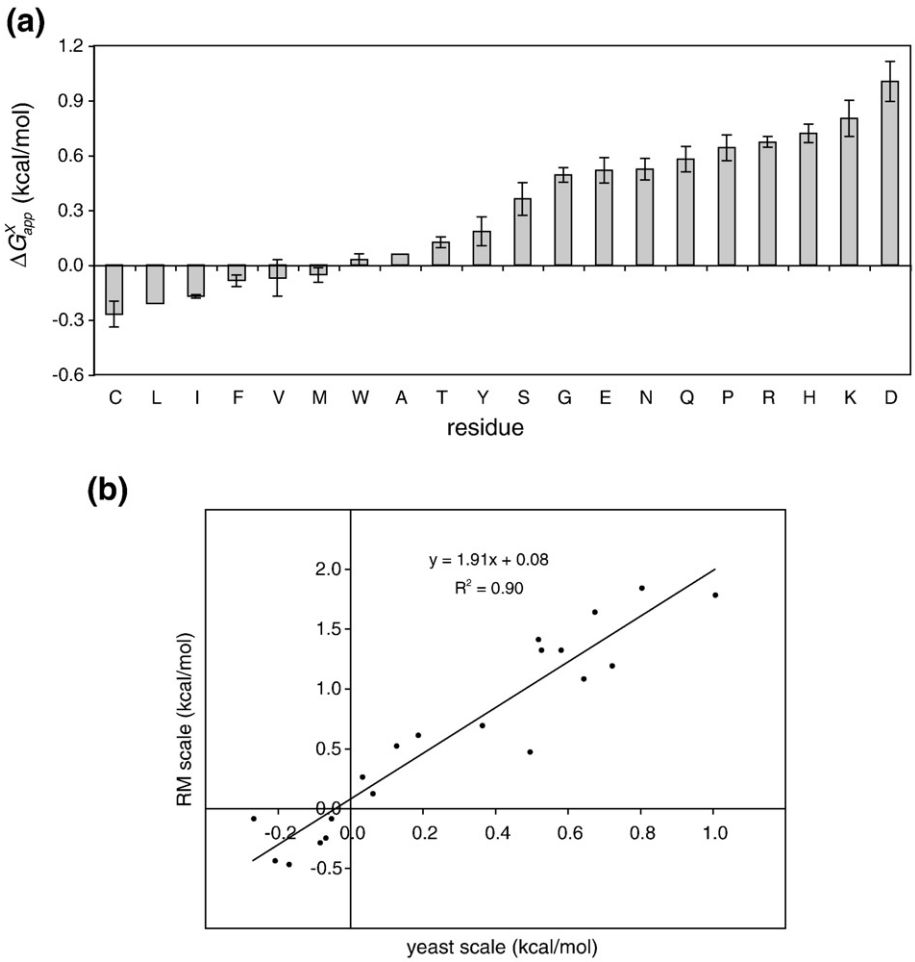

Fig. 3. Biological ΔG_{app} scales. (a) The yeast ΔG_{app} scale derived from Leu/Ala-based H-segments with the indicated amino acid placed in the middle of the H-segment (see Supplement S1). Averages and standard deviations from at least three independent experiments are shown. (b) Comparison between the ΔG_{app} scales obtained for yeast (present study) and for the mammalian RM system.[4] The continuous line shows the linear least-squares fit to the data.

single test residue located in the middle of a 19-residue-long Leu/Ala segment (Fig. 3). The yeast scale correlates well with the scale previously measured using the mammalian RM system,[4] but again, the apparent free-energy values for the different residues measured in the yeast system are scaled down by a factor of ~2.

Taken together with previous studies, we now have quantitative data on the membrane integration of H-segments into mammalian RMs, the ER of mammalian and yeast cells, and the inner membrane of *E. coli*.[3,4,6] We have tested H-segments with both N_{out}–C_{in} and N_{in}–C_{out} membrane orientations[5] and have started to analyze the effects of residues flanking the H-segment and of interactions between neighboring transmembrane segments on the insertion behavior.[8,11] In spite of the diversity in experimental systems, the emerging picture of transmembrane helix recognition by the Sec61/SecY family of translocons is simple: unless flanked by multiple charged residues, the 'hydrophobicity threshold' for 50% membrane integration of a 19-residue-long H-segment corresponds to an nL/(19−n)A sequence with n=3–5 (with longer H-segments requiring fewer leucines[4]). The relative contributions of other amino acids to the overall insertion efficiency are also similar between systems, although the absolute range of the free-energy values differs. This may reflect subtle differences in pore architecture between the various Sec translocons, resulting in slight differences in the way borderline H-segments are handled. Differences in lipid composition or the possible use of the posttranslational translocation pathway in *S. cerevisiae* may also play a role.

Since the threshold hydrophobicity required to reach ΔG_{app}=0 kcal/mol is approximately the same, the scaling between the different systems has little effect on topology prediction schemes based on ΔG_{app} calculations.[12] This observation may also explain why functional expression of membrane proteins in heterologous hosts is often—though not always—possible. Whether failures in heterologous expression in some cases can be explained by the finer differences in the handling of transmembrane helices observed between different host organisms is an interesting question for future study.

Materials and Methods

Plasmid constructions

All plasmids were constructed from p424GPDHA[5] by overlap PCR[13] and homologous recombination.[14] To construct a plasmid encoding a protein with the SP of Suc2p replacing the two N-terminal transmembrane helices of Lep, we amplified a DNA segment encoding the SP of Suc2p (MLLQAFLFLLAGFAAKISASM) by PCR using genomic DNA isolated from W303-1a (*MAT a*, *ade2*, *can1*, *his3*, *leu2*, *trp1*, *ura3*) as a template and two primers, 5′GTTTCGACGGATTCTAGAACTAGTGGATC-CATGCTTTTGCAAGCTTTC3′ (Primer A) and 5′AGTA-GAGTTCAGAGTCGGCATCATCGAACCCATTGATG-CAGATATTTTG3′ (Primer B; underlined sequences are the sequences complementing the start and the end of Suc2p SP, and the rest are the overhangs for homologous recombination in Primer A and overlap PCR in Primer B). In parallel, a segment from the *E. coli lepB* gene (codons 89–324) lacking the region encoding the two N-terminal transmembrane helices was amplified by PCR using the primers 5′CAGGTTCGATGATGCCCG3′ (Primer C) and 5′ AGGAACATCGTATGGGTAAGATGGCTGCAGATG-GATGCCGCCAAT 3′ (Primer D) and various pGEM1-derived *E. coli* Lep-H-segment constructs[3,4] as templates. The PCR fragments encoding the Suc2p SP and the truncated Lep-H-segment constructs were mixed and used as template in a second round of PCR using Primers A and D. Full-length PCR products encoding the SP-Lep-H-segment constructs were confirmed by sizing on an agarose gel. The PCR products were transformed into strain W303-1a (*MAT a*, *ade2*, *can1*, *his3*, *leu2*, *trp1*, *ura3*) or STY50 (*MATa*, *his4-401*, *leu2-3* and *leu112*, *trp1-1*, *ura3-52*, *HOL1-1*, *SUC2*::*LEU2*) together with SmaI-linearized p424GPDHA. Yeast transformants were selected on -Trp plates, plasmid was isolated, and the correct sequence was confirmed by DNA sequencing. Basic yeast transformation techniques were followed as described in Ref. [15]. All confirmed constructs were retransformed into W303-1a or STY50 strains, and transformants were selected on -Trp plate and subjected to further analysis.

Labeling with [^{35}S]Met and immunoprecipitation

Yeast transformants carrying the SP-Lep-H-segment constructs were grown in 5 ml -Trp medium at 30 °C overnight. When cells reached an OD_{600} (optical density at 600 nm) between 0.2 and 0.8, 1 to 1.5 OD_{600} units of cells was harvested by centrifugation at 3000*g*, washed twice with -Met medium without ammonium sulfate, and incubated at 30 °C for 10 min. Cells were centrifuged and resuspended in 150 μl of -Met medium without ammonium sulfate and labeled with [^{35}S]Met (50 to 100 μCi/1 OD_{600} unit of cells) for 5 min at room temperature. Labeling was stopped by addition of 750 μl ice-cold stop solution buffer (20 mM Tris–HCl, pH7.5, and 20 mM sodium azide). Cell pellets were harvested by centrifugation at 20,000*g* and left at −80 °C until further use. Cell pellets were resuspended in 100 μl lysis buffer (20 mM Tris–HCl, pH7.5, 1% SDS, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail composed of 3 μg/ml antipain, 0.1 mg/ml pefablock, 2 μg/ml leupeptin, 8.8 μg/ml aprotinin, 100 μg/ml benzamidine, 150 μg/ml chymostatin, and 2 μg/ml pepstatin) and mixed with an equal volume of ice-cold acid-washed glass beads (Sigma). Cell suspensions were vortexed at maximum speed for 1 min three times with 1 min on ice between each vortexing. Then, samples were incubated at 60 °C for 15 min and centrifuged at 20,000*g* for 5 min, and supernatant fractions were transferred to new tubes. The solubilized fractions were mixed with 500 μl IP buffer (15 mM Tris–HCl, pH7.5, 0.1% SDS, 1% Triton X-100, and 150 mM NaCl), 1.5 μg anti-hemagglutinin (mouse) antibody (Covance, California, USA), and 50 μl of prewashed protein G–agarose beads (GE Healthcare, Uppsala, Sweden; 33% slurry in IP buffer) and rotated at 4 °C overnight. The agarose beads were then washed twice with IP buffer, once with urea buffer (2 M urea, 200 mM NaCl, 100 mM Tris–HCl, pH7.5, and 1% Triton X-100), once with ConA buffer (500 mM NaCl, 20 mM Tris–HCl, pH7.5, and 1% Triton X-100), and finally with 1 ml of buffer containing 50 mM NaCl and 10 mM Tris–HCl, pH7.5. The beads were resuspended in 50 μl SDS-PAGE sample buffer, boiled for 5 min, and centrifuged down, and the supernatant fractions were loaded onto 10% SDS gels. For Endo H digestion, 9 μl of the sample was mixed with 9 μl dH_2O, 2 μl Endo H buffer (800 mM sodium acetate, pH5.8), and 1.25 μl of Endo H (5 U/ml; Roche) or dH_2O for the mock treatment and incubated at 37 °C for 1.5 h. Radiolabeled bands on SDS gels were quantified on a Fuji FLA-3000 phosphoimager using the Image Reader V1.8J/Image Gauge V 3.45 software.

Expression in vitro

The SP-Lep gene was amplified by PCR using Primers A and D above and subcloned into the pCRII-TOPO vector (Invitrogen). Correct insertion of the PCR fragment was assessed by restriction enzyme digestion as instructed by the manufacturer's protocol (Invitrogen) and by DNA sequencing. Constructs were transcribed and translated with [^{35}S]Met in the TNT® Quick Coupled Transcription/Translation System (Promega) in the absence and presence of dog pancreas RMs (a kind gift from Dr. B. Dobberstein, Heidelberg) for 90 min at 30 °C. Translation products were analyzed by SDS-PAGE. Since different microsome preparations give highly correlated ($R^2 \approx 0.95$) but slightly different ΔG_{app} values when tested with a panel of standard H-segment containing Lep constructs (data not shown), the measured ΔG_{app} values were corrected such that they can be directly compared with the values reported in the original papers by Hessa *et al.*[3,4] For the microsomes used here, the corrected values are given by the linear regression $\Delta G_{app}(\text{Hessa}) = 0.85 \times \Delta G_{app}$ (new microsomes) − 0.43 [kcal/mol].

Quantification and calculation of membrane-insertion efficiency

The degree of membrane integration of each H-segment was quantified from SDS-PAGE gels by measuring the relative amounts of singly and doubly glycosylated SP-Lep molecules. The relative amounts of the two products were used to calculate an apparent equilibrium constant between the membrane-integrated and nonintegrated forms: $K_{app} = f_{1g}/f_{2g}$, where f_{1g} is the fraction of singly glycosylated SP-Lep molecules and f_{2g} is the fraction of doubly glycosylated SP-Lep molecules. Finally, membrane-insertion efficiency was expressed as an apparent free energy, $\Delta G_{app} = -RT\ln K_{app}$ between the integrated and nonintegrated forms. To derive a biological hydrophobicity scale for each amino acid (X), we calculated ΔG_{app}^{X} from the average ΔG_{app} measured for the H-segment in question (Table 1) and the values measured for ΔG_{app}^{Leu} (−0.21 kcal/mol) and ΔG_{app}^{Ala} (0.06 kcal/mol):

$\Delta G_{app} = n \cdot \Delta G_{app}^{Leu} + (18-n) \cdot \Delta G_{app}^{Ala} + \Delta G_{app}^{X}$. For each H-segment, at least three independent measurements were taken by analyzing three different yeast colonies.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.01.027

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