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Hydrophobic Blocks Facilitate Lipid Compatibility and Translocon Recognition of Transmembrane Protein Sequences

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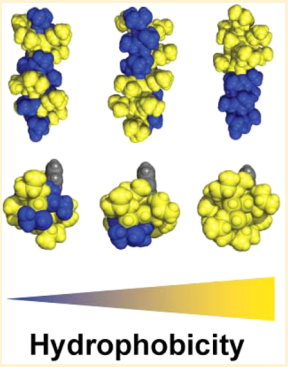
Supporting Information

ABSTRACT: Biophysical hydrophobicity scales suggest that partitioning of a protein segment from an aqueous phase into a membrane is governed by its perceived segmental hydrophobicity but do not establish specifically (i) how the segment is identified *in vivo* for translocon-mediated insertion or (ii) whether the destination lipid bilayer is biochemically receptive to the inserted sequence. To examine the congruence between these dual requirements, we designed and synthesized a library of Lys-tagged peptides of a core length sufficient to span a bilayer but with varying patterns of sequence, each composed of nine Leu residues, nine Ser residues, and one (central) Trp residue. We found that peptides containing contiguous Leu residues (Leu-block peptides, e.g., LLLLLLLLLWSSSSSSSSS), in comparison to those containing discontinuous stretches of Leu residues (non-Leu-block peptides, e.g., SLSLLSLSSWSLLSLSLLS), displayed greater helicity (circular dichroism spectroscopy), traveled slower during sodium dodecyl sulfate−polyacrylamide gel electrophoresis, had longer reverse phase high-performance liquid chromatography retention times on a C-18 column, and were helical when reconstituted into 1-palmitoyl-2-oleoylglycero-3-phosphocholine liposomes, each observation indicating superior lipid compatibility when a Leu-block is present. These parameters were largely paralleled in a biological membrane insertion assay using microsomal membranes from dog pancreas endoplasmic reticulum, where we found only the Leu-block sequences successfully inserted; intriguingly, an amphipathic peptide (SLLSSLLSSWLLSSLLSSL; Leu face, Ser face) with biophysical properties similar to those of Leu-block peptides failed to insert. Our overall results identify local sequence lipid compatibility rather than average hydrophobicity as a principal determinant of transmembrane segment potential, while demonstrating that further subtleties of hydrophobic and helical patterning, such as circumferential hydrophobicity in Leu-block segments, promote translocon-mediated insertion.



In both prokaryotic and eukaryotic cells, a majority of α-helical membrane proteins are inserted into the membrane with the aid of a Sec-type translocon complex. The central translocon subunit (called SecY in prokaryotes and Sec61α in eukaryotes) is composed of 10 transmembrane (TM) helices that form a channel through which translocating polypeptide chains may pass.[1] A lateral gate in the side wall of the channel may open to expose the channel interior and any passing polypeptide chain to the core of the lipid bilayer.[1−3] While significant progress has been made in identifying the sequence-specific "code" for the identification and insertion of TM segments into the lipid bilayer,[4−7] the process is not yet completely understood and is further complicated by the active involvement of the translocon itself in the selection and insertion process.[8−12]

Structural evidence indicating that opening of the translocon lateral gate is dependent on the sequence of the translocating polypeptide has emerged;[12] viz., sequences identified as membrane-targeted induce the lateral gate to open, exposing the passing segment simultaneously to the aqueous translocon channel and to the hydrophobic core of the bilayer.[13] The segment may then partition into the bilayer depending on its perceived hydrophobicity.[14] While the hydrophobicity of a potential TM segment can, in principle, be evaluated by simply averaging the hydrophobicity of its component (~20) amino acids, for which calculation numerous hydropathy scales exist,[15] there are TM segments that are abundant in polar residues,[16,17] termed "marginally hydrophobic", that may not be predicted *a priori* to reside within the membrane yet comprise ~25% of the TM segments in multispan membrane proteins.[17] Studies by Hessa et al., using an ER insertion assay, have led to a deeper understanding of such sequence- and position-specific influences on membrane insertion of hydrophobic and hydrophilic residues along the length of a TM helix and to the development of a "biological" hydrophobicity scale that can

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be used to predict insertion efficiency and membrane protein topology.[4,5,18] More recently, it has been demonstrated that insertion efficiency of a polyalanine segment was increased when hydrophobic (Leu) residues were clustered together.[11]

The hydrophobicity of a TM segment can thus be expected to be influenced concomitantly by residue patterning of local sequence, secondary structure propensity, and environment (water vs membrane). These overall considerations raise the questions of how a segment is identified as belonging in an integral membrane protein and, more broadly, given that hydrophobicity is a water-driven phenomenon, what the role of a TM segment's lipid compatibility with the target bilayer is in determining the candidacy of a protein segment for prospective membrane insertion. To address these issues systematically, here we have synthesized a library of peptides of identical composition but varied sequence, with average segmental hydrophobicity suitable for membrane insertion (≥0.4 by the Liu−Deber hydropathy scale),[19] and undertaken an *in vitro* biophysical analysis of their membrane compatibility, complemented by a translocon-dependent ER insertion assay for the corresponding sequences. The results reveal the importance of local residue patterning, particularly with respect to the presence and positioning of hydrophobic blocks, in terms of the suitability of a given peptide segment for transmembrane insertion.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Ten Ser-Leu peptides were synthesized on a PS3 peptide synthesizer (Protein Technologies, Inc.) using standard solid state Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] chemistry on a low-load PAL−PEG resin (Applied Biosystems) that produced an amidated C-terminus after cleavage. The SL_{scr2} peptide was purchased from GenScript. Peptides were purified using high-performance liquid chromatography (HPLC) with a C4 semipreparative column (Phenomenex). Typically, linear acetonitrile/water gradients were employed with initial conditions of 80% solvent A (95% water, 5% acetonitrile, and 0.1% TFA) and 20% solvent B (95% acetonitrile, 5% water, and 0.1% TFA). Peptides were quantified using the absorbance at 280 nm in 2,2,2-trifluoroethanol (TFE) and a molar extinction coefficient of 4806 M^{-1} cm^{-1}.

SDS Solubilization. Ser-Leu peptides were reconstituted in micellar sodium dodecyl sulfate (SDS) solutions using a protocol adapted from ref 20. Briefly, TFE-solubilized peptides were added to solubilized detergent and shaken for 15 min. Samples were lyophilized, and the resulting peptide−detergent powder was resuspended in water. It was determined empirically that a peptide to detergent ratio of 1:7000 was adequate to allow solubilization.

Liposome Preparation. The TFE-solubilized peptide was added to chloroform-solubilized 1-palmitoyl-2-oleoylglycero-3-phosphocholine (POPC) (10 μM peptide and 2.5 mM lipid) and dried under N_2. The lipid−peptide film was washed with water prior to resuspension in aqueous buffer [10 mM Tris-HCl and 10 mM NaCl (pH 7.4)] and underwent three freeze−thaw cycles. Samples were then passed through a 0.2 μm filter until the solution became clear. Samples were equilibrated overnight.

SDS−PAGE. Peptide−detergent samples were dissolved in 1× NuPAGE native sample buffer (without SDS) and equilibrated for 1 h at room temperature prior to loading. Samples were run on 12% Bis-Tris NuPAGE gels (Life Technologies) in MES running buffer at 200 V for approximately 30 min and stained with GelCode Blue Stain Reagent (Pierce). Migration rates were calculated as percent peptide gel shifts as previously described.[21]

Tryptophan Fluorescence. Fluorescence spectra were recorded on a Photon Technology International fluorimeter using a 1 cm path length quartz cuvette. Tryptophan was excited at 280 nm, and emission spectra were recorded between 300 and 400 nm. Lyophilized peptide−detergent powders were dissolved in water to final concentrations of 5 μM peptide and 35 mM SDS and equilibrated at room temperature for at least 1 h before spectra were recorded. POPC-solubilized samples (10 μM peptide and 2.5 mM lipid) were equilibrated overnight after extrusion. Samples were background subtracted, and the wavelength of maximal fluorescence emission intensity was recorded.

Circular Dichroism (CD) Spectroscopy. Lyophilized peptide−detergent powder was dissolved in ultrapure water to final concentrations of 25 μM peptide in 175 mM SDS, and the sample was allowed to equilibrate for 1 h at room temperature. POPC-solubilized samples (10 μM peptide and 2.5 mM lipid) were equilibrated overnight after extrusion. CD spectra were recorded on a Jasco J-810 CD spectropolarimeter at room temperature in a 0.1 cm path length cuvette. Spectra represent the average of at least three replicates [each replicate was an accumulation of three (SDS) or seven (POPC) scans]. Spectra were background subtracted and converted to mean residue molar ellipticity (MRE) using standard formulas.

HPLC Retention Times. Reverse phase HPLC on a Zorbax StableBond C-18 analytical column (Agilent Technologies) was performed using 20 μg of peptide dissolved in 1 mL of mobile phase solvent. The retention time of each peptide sample was normalized to the retention time of an internal standard (uracil, 5 μg in 50 μL of water) injected prior to the addition of the sample to the column. The mobile phase composition was 60% solvent A (95% water, 5% acetonitrile, and 0.1% TFA) and 40% solvent B (95% acetonitrile, 5% water, and 0.1% TFA).

Prediction of Translocon-Mediated Free Energies of Insertion. Ser-Leu bilayer insertion energies were predicted using the online ΔG_{app} predictor (allowing both length correction and identification of the subsequence with the lowest ΔG_{app}) available at http://dgpred.cbr.su.se.[4,S] The core of the Ser-Leu sequences with added GGPG···GPGG flanks was input (Lys tags omitted), and the corresponding ΔG_{app} values and predicted TM segments were recorded.

Experimental Determination of Translocon-Mediated Free Energies of Insertion. LepB constructs encoding Ser-Leu sequences were generated by modifying the *lepB* gene in the pGEM-1 vector containing *Spe*I and *Kpn*I restriction sites as previously described.[4] Constructs cloned in pGEM1 were transcribed and translated in the TNT Quick coupled transcription/translation system. An apparent equilibrium constant between the membrane-integrated and nonintegrated forms was calculated as $K_{app} = f_{1g}/f_{2g}$, where f_{1g} is the fraction of singly glycosylated LepB molecules and f_{2g} is the fraction of doubly glycosylated LepB molecules. The results were then converted to apparent free energies of membrane insertion via the equation $\Delta G_{app} = -RT \ln K_{app}$.

RESULTS

Design of Ser-Leu Peptide Sequences with Varied Patterns of Hydrophobic and Polar Residues. We hypothesized that detecting subtle differences in peptide

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partitioning among micellar or isotropic apolar media might best be achieved by initially imposing "extremes" of TM sequence polarity and/or amphipathicity. We therefore designed 19-residue TM sequences that contain an equal number of polar and hydrophobic residues, nine Ser and nine Leu residues, each with a centrally positioned Trp residue as a fluorescent probe. As the most commonly occurring amino acid in native TM helices, Leu was a natural choice,[22] while Ser is the most commonly occurring polar residue (similar to Thr) and can participate in both side chain−side chain and side chain−backbone H-bonds.[22] In addition, synthesis requirements of β-branch-rich peptides rendered Thr as a less feasible choice. When averaged over the full 19-residue core segment, this "9 × 9 × 1" residue composition exceeds the hydrophobicity threshold (0.4 by the Liu−Deber hydropathy scale) required for partitioning of the peptide into apolar phases with an averaged hydrophobicity of 1.16[19] yet remains similar to that of "marginally hydrophobic" TM helices ($\Delta G_{app} \sim 1.4$ kcal/mol) as measured by the "biological" hydrophobicity scale of Hessa et al.[4,5]

Broadly, the peptides may be categorized into two groups, i.e., Leu-block versus non-Leu-block (Table 1), in which a "Leu-block" peptide was defined as one containing a contiguous stretch of more than four Leu residues in the primary sequence. Sequences failing to meet these criteria were categorized as "non-Leu-block" peptides. The peptide LSL was categorized as a non-Leu-block peptide because of the presence of a Ser block that is larger than the present Leu-block and the lack of overlap of the present Leu-block with the center of the peptide. While numerous permutations may be envisaged, here sequences were varied to create patterns of hydrophobic and helical character in the form of (i) continuous stretches of hydrophobic residues in the primary sequence (Leu-blocks), (ii) an amphipathic sequence (Leu face and Ser face) when folded into a helix, and (iii) examples of equally or randomly distributed Leu and Ser residues (Table 1 and Figure 1A). Top-down views of the Ser-Leu peptides (Figure 1B) illustrate how variations in hydrophobic patterning lead to varying degrees of exposure of Leu residues on the surface of the peptides (i.e., hydrophobic character that extends around the circumference of the peptide vs hydrophobicity that is concentrated on a single face of the helix).

Table 1. Sequences of Designed Ser-Leu Peptides

Peptide	Sequence*
	Non-Leu-block
SL_n	KKSLSLSLSLSWLSLSLSLSLKK
LSL	KKLLLLSSSSWSSSSSLLLLKK
SL_{scr1}	KKSLSLLSLSSWSLLSLSLLSKK
SL_{scr2}	KKSSLSLLSLSWLSLSLLSLSKK
SL_{amp}	KKSLLSSLLSSWLLSSLLSSLKK
	Leu-block
S9L9	KKSSSSSSSSWLLLLLLLLLKK
S5L9	KKSSSSLLLLWLLLLLSSSSKK
S3L9	KKSSSLLLLLWLLLSSSSSSKK
S2L9	KKSSLLLLLLWLLSSSSSSSKK
S1L9	KKSLLLLLLLWLSSSSSSSSKK
S0L9	KKLLLLLLLLWSSSSSSSSSKK

*Ser, Leu, and Trp residues are colored blue, yellow, and gray, respectively. Peptides are tagged with three Lys residue tags[31] at each of the N- and C-termini. Sequences are categorized as either non-Leu-block or Leu-block as defined in Materials and Methods.

SDS−PAGE Migration Rates and Trp Blue Shifts of the Ser-Leu Peptide Library. Interactions between membrane proteins and detergents are complex and have been shown to occur in a sequence-specific manner, leading to variations in detergent coating, SDS−PAGE migration rates, and Trp fluorescence.[21,23−26] These effects are manifested in the wide variations of migration rates for the Ser-Leu peptide library observed via SDS−PAGE, with Leu-block peptides tending to migrate more slowly on the gel than the compositionally identical non-Leu-block peptides (Figure 2). Clustered Leu residues may increase a peptide's local hydrophobicity, resulting in an increased number of interaction with the hydrophobic detergent and thus an increase in the size of the observed peptide−detergent complex. The peptides SL_{scr1} and SL_{scr2} migrate the fastest, consistent with the absence of a Leu-rich locus in the scrambled peptides that would promote favorable burial into a hydrophobic micelle. The similarly fast migration of the amphipathic SL_{amp} peptide is likely a result of lengthwise exposure of both hydrophobic (Leu face) and polar (Ser face) residues. A combination of favorable interactions of the Leu face with the interior of the micelle and favorable interactions of the Ser face with water position the peptide on the micelle surface rather than buried in the interior, decreasing the size of the observed peptide−detergent complex. Interestingly, significant variations are found in the SDS−PAGE migration rates (percent peptide gel shift) among the Leu-block peptides themselves, with S3L9 traveling the slowest (Table S1 of the Supporting Information). The limited solubility of the non-

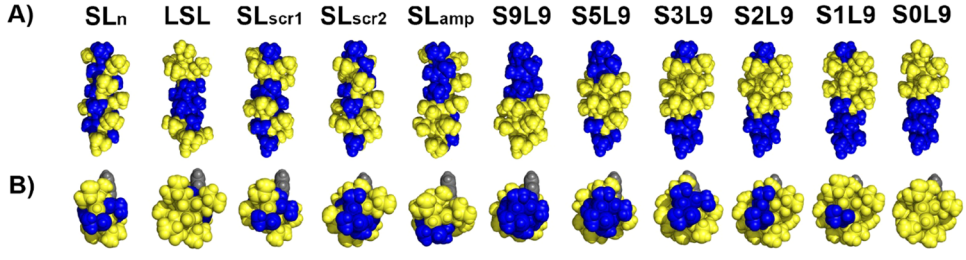

Figure 1. Helical models of Ser-Leu peptides. Peptides (excluding Lys tags) were modeled as α-helix monomers and are shown with the van der Waals radii of Ser (blue), Leu (yellow), and Trp (gray) side chains. (A) View perpendicular to the helix axis. The N-terminus is at the top and the Trp residue oriented into the plane of the page. (B) Top-down view parallel to the helix axis from the peptide N-terminus. Peptide sequences are listed in Table 1.

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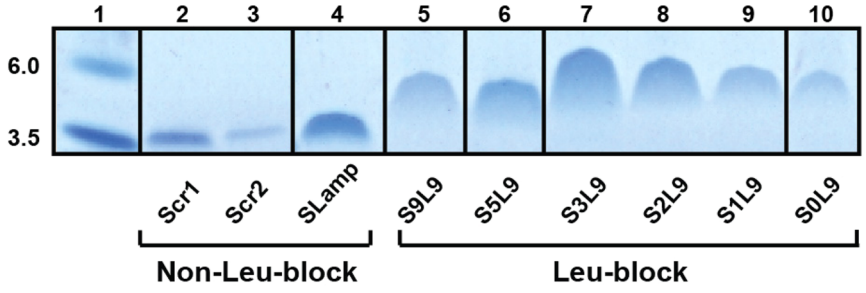

Figure 2. SDS–PAGE gel migration of the Ser-Leu peptide. SDS–PAGE gel of SDS-soluble Ser-Leu peptides. Ser-Leu peptides have identical molecular masses (2774 Da) and compositions but display significant differences in percent peptide gel shift [$p < 0.05$ (Table S1 of the Supporting Information)]. Peptide samples were run on a single gel and then arranged according to Leu-block properties. Lane 1 contained the molecular mass marker (Mark12). Lanes 2–4 contained the non-Leu-block Ser-Leu sequences. Lanes 5–10 contained the Leu-block sequences. Peptides SL_n and LSL were omitted from the gel because of their low solubility.

Leu-block sequences SL_n and LSL led to their exclusion from further experiments in detergent and liposomes.

In conjunction with SDS–PAGE experiments, the Ser-Leu peptides were examined for the occurrence and extent of blue shifts in Trp fluorescence spectra, ostensibly a measure of the "degree of burial" of the Trp moiety in the hydrophobic region of the SDS micelles. We found that all Ser-Leu sequences exhibit Trp blue shifts in the presence of SDS micelles, varying between 320 and 335 nm versus the typical aqueous position near 350 nm, indicating their overall micelle compatibility and detergent coating of the peptides (Table S1 of the Supporting Information); however, no pattern could be discerned between the extent of the Trp blue shift and either the peptide migration position on SDS–PAGE or the presence or absence of Leu-blocks.

Structural Assessment of Ser-Leu Peptides in Detergent Micelles by Circular Dichroism (CD) Spectroscopy. CD spectra of the Ser-Leu peptide series in SDS micelles established that peptide secondary structure is highly sensitive to sequence and patterning (Figure 3A,B). Peptides exhibited

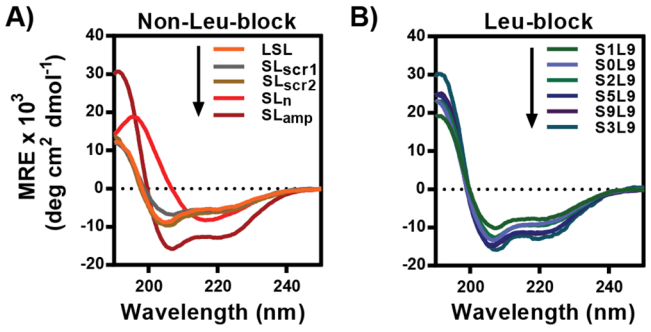

Figure 3. Ser-Leu peptide helicities in SDS micelles. CD spectra of Ser-Leu peptides in SDS micelles (1:7000 peptide:SDS ratio). (A) Non-Leu-block peptides. SL_n notably adopts a β-sheet structure. (B) Leu-block peptides. Significant differences in helicities exist between the different categories of Ser-Leu peptides [$p < 0.05$ (Table S1 of the Supporting Information)]. Spectra shown are an average of at least three independent experiments.

helical CD patterns, with ellipticities ranging from approximately −5000° (non-Leu-block peptides) to −13000° (SL_{amp} and Leu-block peptides) at 222 nm (Table S1 of the Supporting Information). The only peptide not to display at least a partial helical conformation was SL_n, a classic "silk-like" sequence for β-sheet structures, that perhaps unsurprisingly exhibited a CD spectrum consistent with β-sheet features in SDS micelles (Figure 3A) and could not be observed via SDS–PAGE, suggesting poor solubility and/or aggregation.

Assessment of Peptide Apparent Hydrophobicity by Reverse Phase HPLC. Consistent with the trends observed above in SDS–PAGE migration positions and helicities in CD spectra, measurements of apparent peptide hydrophobicity through HPLC retention times on a C-18 column indicated an increased hydrophobicity for peptides with continuous stretches or "blocks" of hydrophobic character. Thus, as shown in Table 2, the overall range in normalized retention

Table 2. Ser-Leu Peptide Retention Times Determined via HPLC

Peptide	Retention Time*
Non-Leu-Block	
SL_n	0.53
LSL	0.31
SL_{scr1}	0.63
SL_{scr2}	1.05
SL_{amp}	2.10
Leu-Block	
S9L9	1.73
S5L9	1.90
S3L9	3.48
S2L9	4.14
S1L9	2.57
S0L9	1.72

*Retention time in a C-18 column with an isocratic mobile phase (60% solvent A and 40% solvent B). Retention time is normalized to elution of uracil. Non-Leu-block peptides (except SL_{amp}) elute significantly faster than Leu-block peptides ($p < 0.01$). Values are an average of at least three independent experiments. Error values were no larger than ±0.2 standard deviation for each point.

times for this series of peptides with identical compositions varied from 0.31 to 4.14, with values that could qualitatively be subdivided into earlier-eluting non-Leu-block sequences (0.31–1.05) and later-eluting Leu-block sequences (1.72–4.14). An important exception was the SL_{amp} peptide, which eluted at 2.10, essentially within the center of the Leu-block range (Table 2). This result indicates that in the relatively isotropic environment of the solvent–column interface, the hydrophobic "Leu face" of this amphipathic peptide, similar to a Leu-block, exhibits a strong interaction with the alkyl chains of the C-18 column. In this regard, an amphipathic peptide with a Leu face is found to display hydrophobic character similar to that of a

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Leu-block peptide. Assessment of peptide helical character in the HPLC solvents (60% solvent A and 40% solvent B), revealed the majority of the Ser-Leu peptides (SL_n, SL_{scr2}, SL_{amp}, and the Leu-block peptides) to have similar helicities (Figure S1 of the Supporting Information). LSL notably adopted a mixed random coil−α-helix conformation, while SL_{scr1} failed to adopt any helical character. No correlation was observed between Ser-Leu peptide helicity in HPLC solvents and retention time within a C-18 column (data not shown).

Ser-Leu Peptides Partition into POPC Liposomes. To assess whether the Ser-Leu peptides could satisfactorily interact not only with micellar detergent but also with phospholipid bilayer phases, we reconstituted each of the peptides into POPC liposomes and measured their propensity to adopt helical structures, accompanied by observation of the anticipated blue shifts of their central Trp residue fluorescence. CD spectra confirmed helical conformations of the Leu-block peptides in the presence of POPC liposomes (Figure 4), with

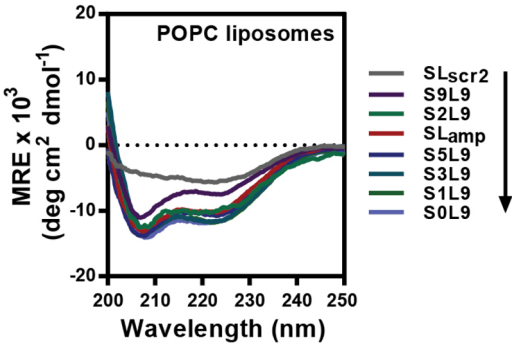

Figure 4. Ser-Leu peptide helicity in POPC liposomes. CD spectra of Ser-Leu peptides in POPC liposomes (1:250 peptide:lipid ratio). SL_{scr1} was insoluble in POPC, while SL_{scr2} adopts a β-sheet-like conformation. SL_{amp} and the Leu-block sequences all adopt helical conformations. Spectra shown represent an average of three independent experiments.

ellipticity values comparable to those observed in SDS (Table S2 of the Supporting Information). Of the non-Leu-block peptides, only SL_{amp} was soluble, similarly adopting a helical conformation (Figure 4). SL_{scr1} visibly aggregated, and SL_{scr2} adopted a β-sheet-like conformation. All soluble, helical peptides exhibited strong blue shifts in a manner expected for a Trp residue buried in the interior of the bilayer, with values clustered around 320−328 nm (Table S2 of the Supporting Information). SL_{amp} exhibited the weakest blue shift (to 332 nm).

Translocon-Mediated Insertion of Ser-Leu Sequences into the ER Membrane. The data from liposome partitioning confirm that the Ser-Leu sequences containing a Leu-block are lipid compatible and, therefore, good candidates for membrane insertion. Thus, in these instances, the high hydrophobicity imparted by nine Leu residues is apparently capable of masking the polarity of some of the Ser residues. To examine this extreme scenario in the context of translocon-mediated membrane insertion, the corresponding Ser-Leu sequences were incorporated into a Lep construct[4] and translated *in vitro* in the presence of ER-derived dog pancreas rough microsomes (Figure 5A). In this assay, the sequence of interest (red) is placed downstream of two native TM segments (black) and flanked by glycosylation sites (G1 and G2). If the translocon inserts the Ser-Leu sequence (red) into the membrane, only

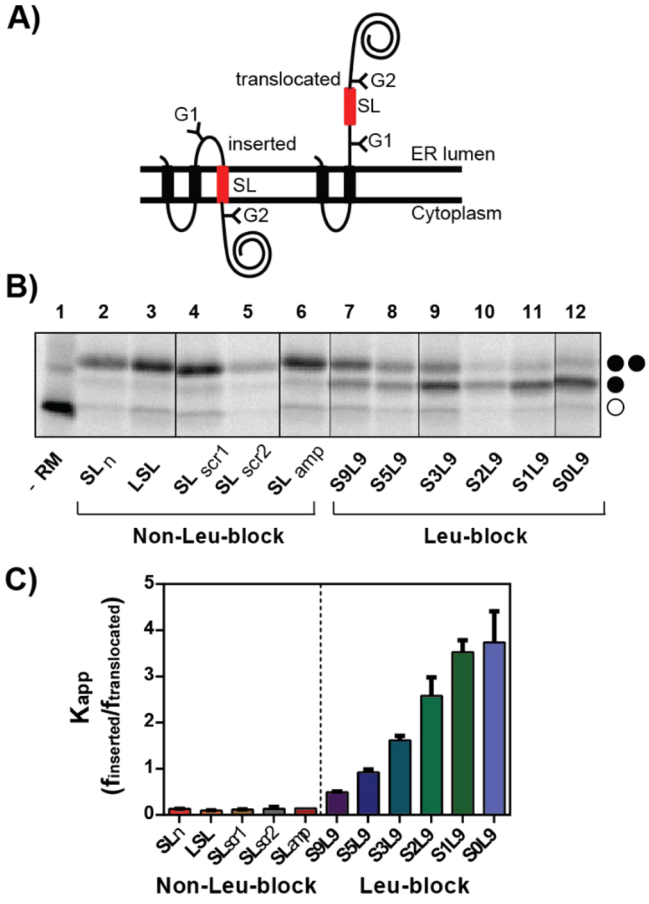

Figure 5. SDS−PAGE gel of Lep constructs containing Ser-Leu sequences. (A) Ser-Leu sequences were inserted into the Lep construct as depicted in the cartoon (the Ser-Leu sequence is colored red, and the two glycosylation sites are indicated as G1 and G2) and expressed in the presence of rough microsomes. Ser-Leu sequences that insert into the ER become monoglycosylated at position G1, and sequences that are translocated across the ER become diglycosylated at positions G1 and G2. (B) Protein samples were run on two separate gels and then arranged according to Leu-block properties. Lane 1 (−RM) contained the Lep construct run in the absence of rough microsomes. Lanes 2−6 contained the non-Leu-block Ser-Leu sequences. Lanes 7−12 contained the Leu-block sequences. Translocated, doubly glycosylated sequences are indicated by the upper band (●●), inserted, singly glycosylated sequences by the middle band (●), and unglycosylated sequences by the lower band (○). Sequences are listed in Table 1. (C) Fraction of inserted segment over translocated (K_{app}). K_{app} values represent an average of three independent experiments. Error bars are reported as the standard deviation.

one glycosylation site (G1) will be exposed to the oligosaccharide transferase enzyme within the ER lumen and become glycosylated (monoglycosylation). If the Ser-Leu sequence fails to insert and is translocated across the membrane into the ER lumen, both glycosylation sites (G1 and G2) will be exposed to oligosaccharide transferase and glycosylated (diglycosylation). The degree of glycosylation may be differentiated by size on SDS−PAGE with diglycosylated proteins running slower than monoglycosylated proteins. In this manner, the relative extents of mono- and/or diglycosylation provide a direct measurement of the extent of translocon-mediated insertion of a given Ser-Leu segment [K_{app} (see Materials and Methods)]. We found that Ser-Leu sequences

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containing a Leu-block generally inserted well (K_{app} > 1), while non-Leu-block sequences failed to insert (K_{app} < 1) (Figure 5B,C). Perhaps most notably, the amphipathic SL_{amp} sequence failed to insert into the microsomal membrane, despite its strong interaction with the membrane mimetics reported above.

We further observed that the sequence with the most centrally located Leu-block (S5L9) does not exhibit the most favorable insertion energy (Table 3). Instead, the sequence with

Table 3. Predicted and Experimental Free Energy of Insertion Values (ΔG_{app}) for Ser-Leu Sequences

Peptide	ΔG_{app} pred. (kcal/mol)*	ΔG_{app} exp. (kcal/mol)†	Predicted TM segment‡
		Non-Leu-block	
SL_n	1.8	1.3	GGPGSLSLSLSLSWLSLSLSLSLGPGG
LSL	2.9	1.4	GGPGLLLLLSSSSWSSSSSLLLLGPGG
SL_{scr1}	1.8	1.4	GGPGSLSLLSLSSWSLLSLSLLLSGPGG
SL_{scr2}	1.4	1.3	GGPGSSLSLLSLSWLSLSLLSLSGPGG
SL_{amp}	1.8	1.2	GGPGSLLSSLLSSWLLSSLLSSLGPGG
		Leu-block	
S9L9	- 0.4	0.4	GGPGSSSSSSSSSSWLLLLLLLLLLGPGG
S5L9	0.3	0.0	GGPGSSSSSLLLLLWLLLLLSSSSGPGG
S3L9	0.0	-0.3	GGPGSSSLLLLLLLWLLLSSSSSSGPGG
S2L9	-0.3	-0.6	GGPGSSLLLLLLLLWLLSSSSSSSGPGG
S1L9	-0.3	-0.8	GGPGSLLLLLLLLLWLSSSSSSSSGPGG
S0L9	-0.4	-0.8	GGPGLLLLLLLLLLWSSSSSSSSSGPGG

*Predicted free energies of insertion (ΔG_{app}) of Ser-Leu peptides according to the ΔG predictor (http://dgpred.cbr.su.se) with "Allow subsequence" turned on.[4,5] †Experimentally determined free energies of insertion (ΔG_{app}). Values calculated using the fraction of mono- and diglycosylated bands in Figure 5 (see Materials and Methods). ΔG_{app} values represent an average of three independent experiments. Error values were no larger than ±0.3 standard deviation for each point. Significant differences are found between the ΔG_{app} of all Leu-block peptides (p < 0.001), except between S0L9 and S1L9. ‡Segments analyzed in the ER insertion assay. TM segments predicted by the ΔG predictor[4,5] are underlined.

the most N-terminally located Leu-block (S0L9) displayed the most favorable experimental ΔG_{app} value (−0.8 kcal/mol) (Table 3). Indeed, insertion efficiency overall increased as the Leu-block was moved closer to the N-terminus (i.e., the luminal side); this trend is emphasized in the comparison of S0L9 (ΔG_{app} = −0.8 kcal/mol) to S9L9 (ΔG_{app} = 0.4 kcal/mol) (Table 3 and Figure 5C).

The experimental ΔG_{app} values are in good correspondence with their predicted values (Table 3), except that the trend toward lower ΔG_{app} values when the Leu-block is moved toward the luminal, N-terminal end of the segment is not captured by the ΔG predictor, as expected, because the underlying model assumes symmetric effects of N- and C-terminally located residues.[5] It is further interesting to note that the predicted TM stretches are considerably shorter than 19 residues for the Leu-block segments, leaving terminal Ser residues outside the membrane (Table 3), a behavior that has been seen in molecular dynamics simulations of short hydrophobic peptides.[27] The good correlation between the measured and predicted ΔG_{app} values largely disappears if the entire 19-residue Ser-Leu stretch is confined to the membrane in the calculation of ΔG_{app} for the Leu-block segments (data not shown).

DISCUSSION

In vitro hydrophobicity measurements of a Ser-Leu library of peptides with identical composition but varying sequence patterning, using helicity in SDS micelles, percent peptide gel shift during SDS−PAGE, retention times on a C-18 column, and partitioning in helical form into POPC bilayers, are shown to be relatively accurate predictors of candidacy for membrane insertion. In combination with these observations, insertion studies with the mammalian Sec61 translocon demonstrate that the relatively high apparent hydrophobicity of the peptides containing Leu-block sequences is biologically relevant, as this feature clearly promotes membrane insertion (Table 3). Significant correlations are found between the experimentally determined free energy of insertion of the Ser-Leu sequences and both peptide HPLC retention times (Figure 6A; R^2 = 0.65) and percent peptide gel shifts during SDS−PAGE (Figure 6B; R^2 = 0.80).

SDS−PAGE Migration of Ser-Leu Peptides. When subjected to SDS−PAGE, the Ser-Leu peptides migrate at a range of positions despite having identical molecular masses (Figure 2), with the Leu-block peptides traveling the slowest at

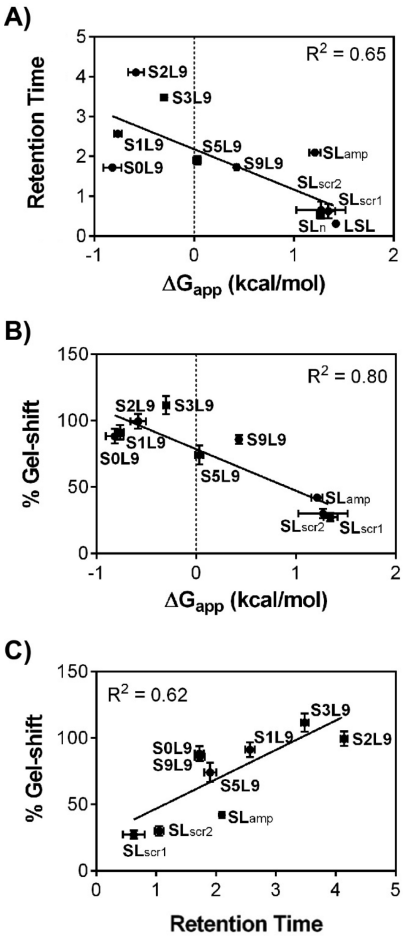

Figure 6. Comparison of biophysical measurements of hydropathy and translocon-mediated membrane insertion. (A) Comparison of percent peptide gel shift and normalized retention time within a C-18 column (R^2 = 0.62). (B) Comparison of percent peptide gel shift during SDS−PAGE to experimentally determine ΔG_{app} for insertion into rough microsomes (R^2 = 0.80). (C) Comparison of normalized retention time within a C-18 column to experimentally determined ΔG_{app} for insertion into rough microsomes (R^2 = 0.65).

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molecular masses corresponding to approximately double their actual monomeric molecular mass (2.7 kDa). In concert with previous analyses of membrane protein migration rates during SDS−PAGE, we suggest that the relatively slow migration of the Leu-block peptides is a consequence of a greater local binding of SDS to these hydrophobic blocks, resulting in a relatively larger peptide-detergent complex size.[21] Interestingly, the amphipathic sequence (SL_{amp}) traveled like the scrambled sequences (SL_{scr1} and SL_{scr2}), implying that despite similar hydrophobicity readings, a Leu face does not bind as much detergent as a Leu-block. Förster resonance energy transfer (FRET) experiments to assess oligomeric state(s) (viz., monomeric vs dimeric) were inconclusive, as the covalent addition of dansyl/dabsyl probes tended to render the peptides insoluble in the required medium. While the possibility of higher-order oligomers in detergent cannot be excluded, we believe it to be unlikely, given that the Leu-block peptides lack a specific interface that can contribute to oligomer formation, and the previously shown inability of poly-Leu sequences to self-associate.[28,29] Further, comparison of peptide retention times in the isotropic environment of the C-18 column, a medium that is not conducive to oligomer formation, to percent peptide gel shift during SDS−PAGE, reveals a moderate correlation (Figure 6C; $R^2 = 0.62$), supporting the idea that the observed slow migration rates during SDS−PAGE arise predominantly from the relatively high local hydrophobic character of Leu-block peptides. In addition, because TM segments enter the translocon as monomers, the strong correlation between translocon-mediated insertion (ΔG_{app}) and percent peptide gel shift (Figure 6B; $R^2 = 0.80$) implies the observed increased migration rates of Leu-block peptides during SDS−PAGE are due to the increased hydrophobic character of these peptides rather than self-association. Nevertheless, the possibility of peptide self-association in detergent remains and could be responsible for the observed lower helicity values in detergent of the Ser-Leu series, as in such circumstances, the peptide molecular mass may be confounded by the existence of larger peptide complexes.

Translocon-Mediated Insertion of Ser-Leu Sequences into Native Bilayers. As predicted by *in vitro* measurements of membrane insertion candidacy, the Leu-block peptides all produced favorable insertion values, establishing that they are capable of translocon-mediated membrane insertion (Table 3). The significant difference in insertion energy between S0L9 and S9L9, which vary only in the extreme positioning of the Leu-block on the peptide N-terminus versus the C-terminus, has implications for the role of the translocon in insertion. As these two peptides display essentially parallel *in vitro* properties in detergent, on the C-18 column, and in POPC liposomes, it is likely that the difference observed in translocon-mediated insertion is imparted by the translocon itself rather than the membrane. In addition, the inner and outer leaflets of the ER membrane are believed to be symmetrical, supporting the idea that the position-dependent differences arising in insertion efficiency of TM segments are not dictated by the lipid bilayer.[30]

Similar biases that favor the positioning of hydrophobic residues at one helix terminus over the other have been previously observed.[5,6,11] Thus, in yeast, a cluster of three Leu residues within a poly-Ala segment similarly experienced increased insertion efficiency when positioned closer to the luminally disposed N-terminus of the segment, away from the hydrophobic constriction ring within the translocon channel.[11] Further, on the basis of cryo-EM structures of the mammalian translocon, the lateral gate is presumed to open when highly hydrophobic membrane sequences enter the channel,[12] implying an early recognition of incipient TM sequences. Thus, if the translocon "senses" an area of high hydrophobic character, such as a Leu-block, perhaps the gate is opened, allowing exposure of the threading segment to the lipid bilayer, allowing favorable partitioning to occur. In this scenario, the Leu-block is recognized as membrane-competent, leading to opening of the lateral gate, with insertion efficiency decreasing as the position of the Leu-block is shifted toward the cytosolically disposed C-terminus of the TM segment.

Leu Faces versus Leu-Blocks within the Translocon Channel. A striking difference is seen between the *in vitro* biophysical techniques and the biological ER insertion assay in their relative ability to identify an amphipathic segment as a candidate for insertion. In the majority of the *in vitro* studies using detergent and a C-18 column, SL_{amp} exhibits behavior very similar to that of Leu-block peptides, displaying comparable helicity, Trp burial, and HPLC retention time. It is not until SL_{amp} is confronted by the translocon that the Leu face becomes clearly distinguishable from the Leu-block sequences (Table 3 and Figure 5B,C). While the Leu-block sequences insert efficiently, SL_{amp} fails to insert into the ER membrane. Previously, an increase in amphipathicity has been seen to decrease insertion efficiency.[4] Also, the amphipathic SL_n and the scrambled Ser-Leu sequences fail to insert (Table 3 and Figure 5B,C). These results are consistent with the notion that the translocon requires a hydrophobic surface that extends around the entire circumference of a nascent TM segment to open its lateral gate (Figure 7A); in the Leu-block peptides used in this study, nine consecutive Leu residues would comprise two or three turns of a helical peptide. As such, the translocon constriction ring, itself replete with a circle of hydrophobic residues, may be acting as a surrogate for recognition of the destination environment, wherein phospholipids would similarly surround the inserted segment. Thus, lacking both circumferential hydrophobicity and compatibility with the bilayer, the SL_{amp} peptide is directed by the translocon into the lumen along with the remaining non-Leu-block sequences (Figure 7B). From this perspective, the poly-Ala/Leu segments (e.g., ALLLLAAAAAAAAAAAAAA) studied by Demirci et al.[11] would satisfy this "hydrophobic ring" requirement at all points along their sequence, rendering the gate open regardless of Leu positioning. The insertion efficiency of these poly-Ala/Leu sequences would then be dependent solely on interaction of the Leu-block with the constriction ring.

Whether the observed positional bias for the proximity of the Leu-block to the luminally disposed N-terminus is due to an earlier opening of the lateral gate and therefore immediate exposure to lipid, the unfavorable positioning of numerous bulky Leu residues near the narrow hydrophobic constriction ring, or a combination of both remains uncertain. Nevertheless, we find that the clustering of nine Leu residues increases the perceived hydrophobicity of these marginal TM segments to allow the insertion of some or all of the nine Ser residues as "cargo".

CONCLUSION

In vitro biophysical analysis by several biochemical and biophysical techniques in membrane-mimetic environments established the sequence-dependent lipid compatibility of a

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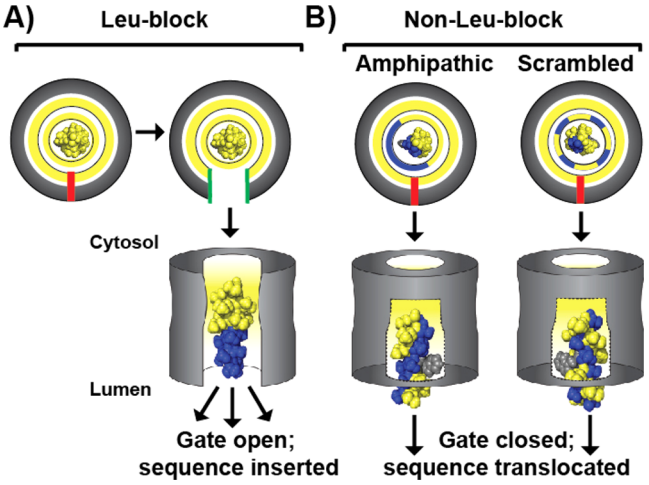

Figure 7. Schematic translocon model for insertion of Leu-block sequences vs translocation of amphipathic and scrambled sequences of Ser-Leu peptides. (A) Top-down view (from the C-terminus) of the Leu-block sequence S9L9 (SSSSSSSSSWLLLLLLLLL) when within the Lep construct, in contact with the translocon channel (colored gray). The hydrophobic constriction ring is colored yellow and the translocon lateral gate red (closed) and then green (open). The circumferential hydrophobic surface area of the passing peptide is signified by the continuous yellow circle encompassing the peptide. Below is a lateral view of the translocon with S9L9 within the channel with the lateral gate open toward the viewer. (B) Top-down view of non-Leu-block sequences, shown for the amphipathic sequence SL_{amp} (SLLSSLLSSWLLSSLLSSL) and the scrambled sequence SL_{scr1} (SLSLLSLSSWSLLSLSLLS), passing through the channel. The discontinuous hydrophobic surface area common to both peptides is represented by the yellow (hydrophobic) and blue (polar) circle encompassing each peptide. The translocon remains closed during passage of the non-Leu-block sequences. Below are lateral views of the translocon translocating the amphipathic and non-Leu-block sequences.

series of synthetic peptides of identical Leu/Ser/Trp composition. Most prominently, an overall increase in a TM segment's apparent hydrophobicity is observed when the peptides present an extensive continuous hydrophobic face (Leu-block peptides; SL_{amp}, Leu face). This work further demonstrates that simply averaging the hydrophobicity of a segment is not an adequate measure of the segment's "actual hydrophobicity" or likelihood for membrane insertion.

Intriguingly, only two of the six biophysical techniques (SDS–PAGE migration and tryptophan fluorescence in liposomes) could distinguish between an amphipathic helix and the Leu-block sequences. The more complex ΔG_{app} predictor that takes both positional variation in residue hydrophobicity and amphiphilicity and overall length of the membrane-embedded segment into account works better in this regard.[5] The overall results thus suggest that while current *in vitro* partitioning techniques are generally excellent predictors of potential TM segments, they may not completely capture the subtleties of patterning of polar and apolar residues along a protein segment that the translocon can discern.

■ ASSOCIATED CONTENT

Supporting Information

Exact values of percent peptide gel shifts, tryptophan blue shifts, and helicities (MRE) in SDS detergent micelles (Table S1), exact values of helicities (MRE) and tryptophan blue shifts in POPC liposomes (Table S2), and CD spectra of non-Leu-block and Leu-block peptides in HPLC solvents (60% solvent A and 40% solvent B) (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RP-HPLC, reverse phase high-performance liquid chromatography; POPC, 1-palmitoyl-2-oleoylglycero-3-phosphocholine; ER, endoplasmic reticulum; TM, transmembrane; Fmoc, fluorenylmethyloxycarbonyl; PAL–PEG, peptide amide linker polyethylene glycol; TFA, trifluoroacetic acid; TFE, trifluoroethanol; MES, 2-(*N*-morpholino)ethanesulfonic acid; CD, circular dichroism; MRE, mean residue ellipticity.

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