The Aromatic Residues Trp and Phe Have Different Effects on the Positioning of a Transmembrane Helix in the Microsomal Membrane

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ABSTRACT: We have examined the effect of Trp and Phe residues on the positioning of a poly-Leu transmembrane helix relative to the microsomal membrane by employing a previously described "glycosylation mapping" technique [Nilsson, I. M., Sääf, A., Whitley, P., Gafvelin, G., Waller, C., and von Heijne, G. (1998) *J. Mol. Biol. 284*, 1165−1175]. Both Trp and Phe tend to push the transmembrane helix into the membrane when inserted in positions flanking the poly-Leu stretch, and Trp (but not Phe) pulls the transmembrane helix toward the lipid−water interface when inserted inside the poly-Leu segment. Thus, the preference of Trp for the lipid−water interface previously suggested on the basis of biophysical studies of model peptides can also be observed for a bona fide transmembrane helix in a biological membrane. We further show that a sufficiently long poly-Trp segment functions as an efficient stop-transfer sequence during protein translocation across the microsomal membrane, despite the preference of Trp residues for the lipid−water interface region.

Statistical analysis of the few existing high-resolution structures of α-helical integral membrane proteins shows that the occurrence of aromatic residues in transmembrane α-helices is remarkably position-dependent (*1*). Trp and Tyr are enriched near the ends of the helices, suggesting that they interact favorably with the lipid headgroups. Phe, in contrast, is more abundant in the central core region of the transmembrane helices.

A number of recent biophysical studies have investigated the apparent affinity of aromatic residues for the lipid−water interface or central hydrophobic regions of phospholipid membranes. Data for small model peptides show that the partitioning of Trp into the interface of lipid bilayers is highly favorable (*2, 3*), and NMR measurements with Trp analogues lacking polar groups in the indol ring demonstrate that partitioning is not much affected by these groups (*4*). Hence, both aromaticity, which is thought to favor partitioning into the electrostatically complex interface environment, and unfavorable interactions of the flat, rigid Trp side chain with the hydrocarbon core seem to be responsible for the residue's preference for the interface (*4*). Other in vitro studies have addressed the effect of Trp on the membrane insertion and orientation of synthetic model peptides (*5*) and its role in hydrophobic matching between transmembrane peptides and lipid bilayers (*6*).

While in vitro studies with various model systems thus support a role for Trp and Tyr residues in positioning transmembrane helices relative to the lipid bilayer, it has so far not been possible to study this phenomenon in a biological membrane. Here, we have compared the effect of Trp and Phe residues on the positioning of a poly-Leu transmembrane helix in microsomal membranes derived from dog pancreas

endoplasmic reticulum (ER),[1] using the recently introduced "glycosylation mapping" technique. This technique allows any transmembrane segment to be positioned relative to a known reference transmembrane helix in the direction perpendicular to the membrane plane (*7, 8*), and is particularly useful for detecting changes in the position of a transmembrane helix caused by systematically engineered point mutations.

We find that Trp and Phe have strikingly different effects on the position of the poly-Leu transmembrane helix relative to the membrane plane. While both residues tend to push the transmembrane helix into the membrane when inserted in positions flanking the poly-Leu stretch, Trp (but not Phe) pulls the helix toward the lipid−water interface when inserted inside the poly-Leu segment. Thus, the affinity of Trp for the lipid headgroup region and of Phe for the core of the membrane can be demonstrated for a bona fide transmembrane helix inserted into a biological membrane. Interestingly, the effects seen for Trp are very similar to what we have previously found for Arg and Lys, whereas Asp and Glu affect the positioning of the poly-Leu transmembrane helix in a distinctly different way (*8*).

Considering that Trp thus behaves as a residue considerably less hydrophobic than Phe, we have also studied the ability of poly-Trp stretches to act as stop-transfer sequences during polypeptide translocation across the ER membrane. We find that a stretch of seven consecutive Trp residues does not halt translocation, whereas one with 27 Trp residues is an efficient stop-transfer sequence.

MATERIALS AND METHODS

Enzymes and Chemicals. Unless otherwise stated, all enzymes were from Promega (Madison, WI). Ribonucle-

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[1] Abbreviations: ER, endoplasmic reticulum; Lep, leader peptidase; MGD, minimal glycosylation distance; OST, oligosaccharyl transferase.

otides, deoxyribonucleotides, dideoxyribonucleotides, the cap analogue m7G(5′)ppp(5′)G, T7 DNA polymerase, and [^{35}S]-Met were from Amersham-Pharmacia (Uppsala, Sweden). Plasmid pGEM1, DTT, BSA, RNasine, and rabbit reticulocyte lysate were from Promega. Spermidine and PMSF were from Sigma. The Expand High Fidelity PCR System and EndoH were from Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were from Kebo Lab (Stockholm, Sweden), Cybergene (Stockholm, Sweden), DNA Technology (Copenhagen, Denmark), and Interactiva (Ulm, Germany).

DNA Techniques. For cloning into and expression from the pGEM1 plasmid, the 5′ end of the *lepB* gene was modified, first by the introduction of an *Xba*I site and second by changing the context 5′ to the initiator ATG codon to a "Kozak consensus" sequence (*9*) as described previously (*8*). Thus, the 5′ region of the gene was modified to ATAAC-CCTCTAGAGCCACCATGGCGAAT (the *Xba*I site and initiator codon being underlined). Replacement of the H2 region in Lep was performed by first introducing *Bcl*I and *Nde*I restriction sites in codons 59 and 80 flanking the H2 region and then replacing the *Bcl*I−*Nde*I fragment with the poly-Leu sequence PGLIKKKKL$_{23}$VQQQP. Site-specific mutagenesis was used to add *Bcl*I and *Nde*I restriction sites at the 3′ and 5′ ends of H2 in Lep, to insert aromatic residues into the poly-Leu stretch, and to introduce Asn-Thr-Ser acceptor sites for N-linked glycosylation which was performed either according to the method of Kunkel (*10*, *11*) or according to a method adapted from Hemsley (*12*, *13*). Glycosylation acceptor sites were designed as described previously (*14*), i.e., by replacing three appropriately positioned codons downstream of H2 with codons for the acceptor tripeptide Asn-Ser-Thr. In the glycosylation constructs Asn$_{84}$-Ser$_{85}$-Thr$_{86}$ and Asn$_{88}$-Ser$_{89}$-Thr$_{90}$ (numbering corresponding to the Lep wild-type sequence), the flanking Pro residues were changed to Gln since they were found to reduce the efficiency of glycosylation (*15*).

Poly-Trp stretches were inserted into a modified *lepB* gene (*16*) between a *Spe*I site (codons 226 and 227) and a *Bgl*II site (codons 231 and 232) by the method of Hemsley (*12*, *13*). The gene that was used also carried an Asn$_{214}$ → Gln mutation (removing the potential glycosylation site in wild-type Lep) and mutations converting residues 96−98 and residues 258−260 to Asn-Ser-Thr and Asn-Ala-Thr, respectively (introducing two new potential glycosylation sites). All mutants were confirmed by DNA sequencing of plasmid and single-stranded M13 DNA using T7 DNA polymerase.

Expression in Vitro and Analysis of Trp and Phe Mutants. Synthesis of mRNA from pGEM1 by SP6 RNA polymerase and translation in reticulocyte lysate in the presence and absence of dog pancreas microsomes were performed as described previously (*17*). Proteins were analyzed by SDS−PAGE, and gels were quantitated on a Fuji BAS1000 phosphoimager using the MacBAS 2.31 software. The extent of glycosylation of a given mutant was calculated as the quotient between the intensity of the glycosylated band divided by the summed intensities of the glycosylated and nonglycosylated bands. In general, the glycosylation efficiency varied by no more than ±5% between different experiments, and the precision in the MGD determinations was ±0.2 residue. For EndoH treatment, the translation mix was diluted (1:4) with 50 mM sodium citrate (pH 6) and
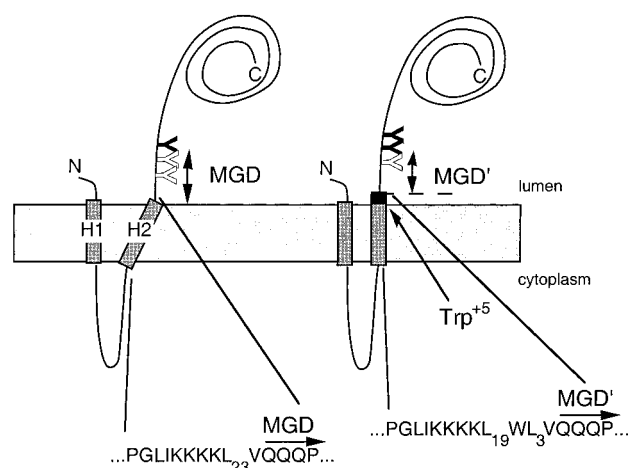

FIGURE 1: Determination of MGD values. A model transmembrane helix with the sequence PGLIKKKKL$_{23}$VQQQP (*7*) or mutants thereof were inserted in place of the H2 transmembrane helix in Lep, and the minimal glycosylation distance (MGD) was determined by placing potential glycosylation acceptor sites (Asn-Ser-Thr) in various positions downstream of H2 and analyzing the constructs by in vitro translation in the presence of microsomes (left). A mutation that changes the position of the transmembrane helix relative to the membrane will result in a corresponding change in the MGD (right; the sequence of the W^{5+} mutation is shown). Glycosylated (**Y**) and nonglycosylated (**Y**) glycosylation acceptor sites on either side of the MGD are depicted.

1% SDS and boiled for 3 min. Aliquots were then incubated with 0−1 milliunits of EndoH for 1 h at 37 °C.

RESULTS

Glycosylation Mapping Technique. The glycosylation mapping technique has been described in detail in previous papers (*7*, *8*). Briefly, the lumenally oriented active site of the ER enzyme oligosaccharyl transferase (OST) is used as a fixed point of reference against which the position of a transmembrane helix in the ER membrane can be measured; in particular, point mutations in a transmembrane helix that affect its position in the membrane will change the "minimal glycosylation distance" (MGD), i.e., the number of residues in the nascent chain needed to bridge the distance between a given residue at the end of the transmembrane helix and the OST active site (Figure 1). The analysis can be conveniently performed by in vitro transcription and/or translation in the presence of dog pancreas microsomes.

For the studies reported here, we used the well-characterized *Escherichia coli* inner membrane protein leader peptidase (Lep) which has two transmembrane helices (H1 and H2) and a large C-terminal domain (P2) (Figure 1). Lep assembles into microsomal membranes with a N$_{lum}$−C$_{lum}$ topology (*18*). To provide a simplified sequence context and to reduce the likelihood of specific interactions between H1 and H2, H2 was replaced with a hydrophobic stretch composed of either 17 or 23 leucines and one valine flanked by four N-terminal lysines and a C-terminal Gln-Gln-Gln-Pro segment (constructs L$_{17}$V and L$_{23}$V) (*7*), and various point mutants with individual residues in the poly-Leu segment replaced with aromatic amino acids were analyzed.

Asn-Ser-Thr N-glycosylation sites were introduced at different positions downstream of the poly-Leu transmembrane helix. All constructs were expressed in vitro in the presence of dog pancreas microsomes, and MGD values were

obtained by determining the number of residues between a given reference residue at the end of the transmembrane helix (the first Gln after the poly-Leu stretch) and the glycosylation acceptor Asn needed to achieve half-maximal glycosylation (see Figure 2B). We have shown previously that the P2 domain in mutants where the acceptor Asn is too close to the membrane for efficient glycosylation is still translocated into the lumen of the microsomes (*18*), and thus that the degree of glycosylation is an accurate measure of the location of the Asn relative to the OST active site.

Effects of Trp and Phe Residues on the Position of Poly-Leu Transmembrane Segments in the Microsomal Membrane. To study the effects of aromatic amino acids on the position of a transmembrane helix in the microsomal membrane, we introduced single Trp or Phe residues in various positions in the model $L_{23}V$ transmembrane helix and determined the MGD for the resulting constructs (Figure 2A,B).

The MGD values obtained for an extensive set of Trp and Phe mutations in the C-terminal half of the $L_{23}V$ transmembrane helix are summarized in Figure 2C and compared to the MGD value for the unmutated $L_{23}V$ stretch (dashed line). An S-shaped dependence of the MGD on Trp substitutions with a maximum at position -2, an inflection point around position 5, and a minimum around position 11 is observed (note that the mutated positions are counted in a C- to N-terminal direction starting from the Val residue at the end of the hydrophobic stretch; cf. Figure 1). The dependence of the MGD on Phe substitutions is similar for positions -2 to 6 with a maximum at -2, but significantly different for positions $6-11$ where essentially no effect on the MGD is observed. Hence, the MGD increases in response to both Leu → Trp and Leu → Phe substitutions in the polar flanking segment and in the most C-terminal positions of the hydrophobic segment, indicating that the transmembrane helix is pushed into the membrane, away from the OST active site. In contrast, Leu → Trp (but not Leu → Phe) substitutions placed further into the hydrophobic segment (positions 7 and 11) tend to pull the helix out of the membrane, toward the OST active site. We also note that Trp has a similar effect on the MGD as has Arg (and Lys, not shown) insofar as it increases the MGD when placed in the flanking region and reduces it even when placed deep inside the core of the poly-Leu segment, but that the effect is clearly distinct from that of Glu (and Asp, not shown) (*8*).

A more detailed interpretation of these results suggests that both Trp and Phe, when placed in position -2 just outside the poly-Leu stretch, efficiently partition their aromatic rings into the interface, essentially extending the hydrophobic segment by up to one residue as reflected in the increase in MGD values relative to that of the nonmutated $L_{23}V$ stretch. Phe is noticeably more effective than Trp in pushing the helix into the membrane (larger increase in MGD values), possibly due to a deeper penetration toward the hydrophobic core of the membrane.

In more central locations (positions $7-11$), substitutions by Trp but not by Phe residues result in a drop in MGD values. The most straightforward explanation is that the indol ring is attracted toward the interface region, thus pulling the transmembrane helix out of the membrane. An alternative interpretation is that Trp induces the lipid molecules to adopt nonbilayer structures, thereby shortening the effective hydrophobic length of the bilayer and causing an opening of

the helix due to its exposure to water (*6*). Interestingly, we observed a more pronounced drop in the MGD of 1.7 residues for a Leu₉ → Trp substitution in the shorter $L_{17}V$ transmembrane helix (data not shown), possibly indicating that a short transmembrane helix is less tilted in the membrane and thus has to be pulled farther out of the membrane for a centrally located Trp to reach the interface region.

In any case, our results show that Trp, contrary to Phe, has a noticeable affinity for the lipid−water interface even when placed well within the hydrophobic core region of a transmembrane helix. It further appears that the localization of the phenyl ring, as opposed to Trp's indol ring, within the lipid core region is favored over an interfacial localization.

Poly-Trp Can Arrest Polypeptide Translocation across the ER Membrane. Given that Trp does not behave as a typical hydrophobic residue, either in model peptides or in the context of the poly-Leu transmembrane segments studied above, we tested whether poly-Trp segments can arrest polypeptide translocation across the ER membrane, i.e., whether poly-Trp can function as a stop-transfer sequence (*19*). To this end, stretches of 7 and 27 Trp residues were introduced into the middle of the P2 domain (between codons 226 and 232), and Asn-Ser-Thr glycosylation acceptor sites were placed in positions 96 and 258, upstream and downstream of the poly-Trp stretch, respectively. With this construct, stop-transfer activity can be easily measured since segments lacking stop-transfer activity will be glycosylated on both sites, whereas those that function as efficient stop-transfer sequences will only be glycosylated on Asn₉₆ (*16*) (Figure 3A).

As shown in Figure 3B, the construct with an insert of seven Trp residues was efficiently glycosylated. Incubation with increasing concentrations of EndoH, a glycan-removing enzyme, revealed doubly and singly glycosylated and non-glycosylated molecules, thus demonstrating that a stretch of seven Trp residues is efficiently translocated across the microsomal membrane. In contrast, the construct with an insert of 27 Trp residues was glycosylated only on one site, as no intermediate forms became visible upon EndoH treatment. Hence, a stretch of 27 Trp residues can act as an efficient stop-transfer sequence, whereas a stretch of seven Trp cannot. It has been shown previously that a stretch of seven Leu residues already acts as an efficient stop-transfer sequence (*20*), consistent with a greater affinity for the hydrophobic core of the membrane.

DISCUSSION

The glycosylation mapping technique provides a sensitive way of detecting structural and positional changes in a transmembrane helix (*7, 8*). An obvious advantage with this technique is that the experiments are performed in the context of a biological membrane; the prize one pays is that the complexity of the experimental system only allows indirect structural interpretations of the data. However, previous determinations of the MGD for two natural transmembrane helices where the position relative to the lipid bilayer has been derived from X-ray crystallography, NMR, spin-labeling, and fluorescence quenching experiments have shown a good correspondence between the glycosylation mapping and the biophysical results (*7, 8*).
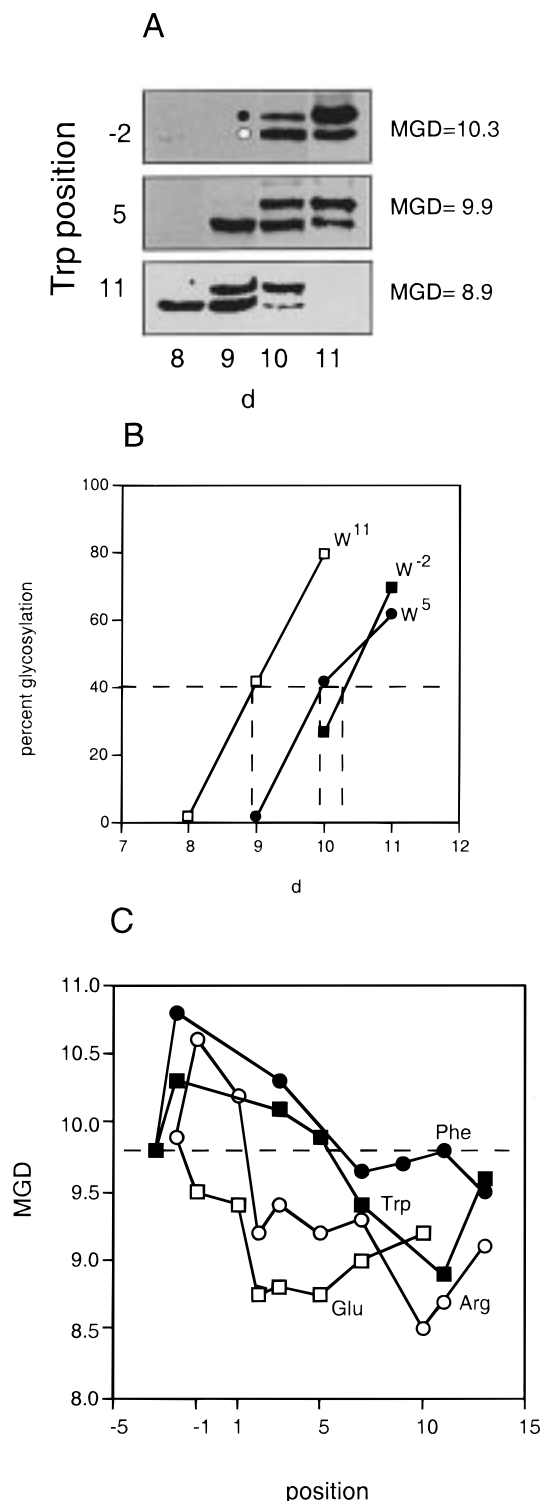
A

B

C

FIGURE 2: Determination of MGD values for Trp and Phe mutations in the poly-Leu transmembrane helix. (A) In vitro translation in the presence of dog pancreas microsomes for three constructs with either Gln_{-2}, Leu_5, or Leu_{11} replaced with Trp. The acceptor Asn is located at 8, 9, 10, and 11 residues from the first Gln residue after the $L_{23}V$ segment as indicated in Figure 1. The glycosylated and nonglycosylated forms of the proteins are represented by a black and a white dot, respectively. (B) Quantitation of the data in panel A. The MGD value corresponds to half-maximal (i.e., 40%) glycosylation and was determined by interpolation. (C) MGD values for Trp (■) and Phe (●) mutations in the $L_{23}V$ transmembrane helix. The dashed line represents the MGD value for the nonmutated $L_{23}V$ transmembrane helix. Results for Glu (□) and Arg (○) are shown for comparison (8). Mutated positions are counted in a C- to N-terminal direction from the C-terminal end of the $L_{23}V$ segment; position 1 is the C-terminal Val residue (cf. Figure 1).
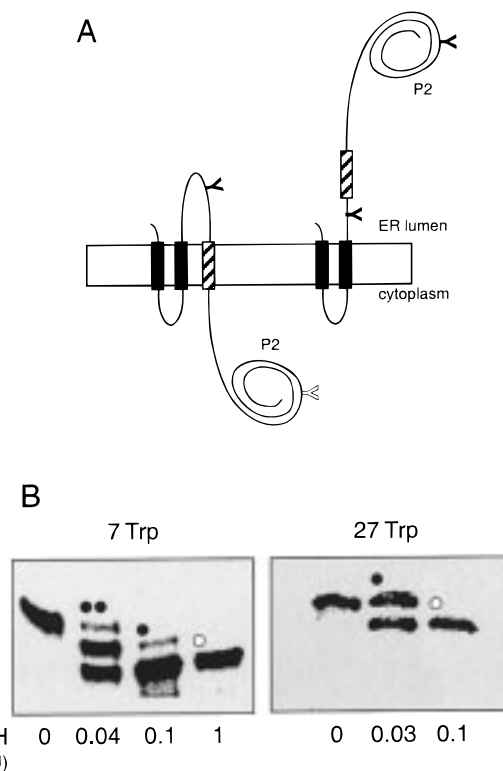
A

B

FIGURE 3: Poly-Trp has stop-transfer activity. (A) A strecth of 7 or 27 contiguous Trp residues (hatched) was cloned into the Lep P2 domain, and glycosylation acceptor sites were placed both up- and downstream of the poly-Trp stretch. A fully translocated P2 domain will be glycosylated on both sites (right), whereas one where the poly-Trp segment functions as a stop-transfer sequence will only be modified on the upstream site (left). (B) The two Lep constructs were translated in vitro in the presence of microsomes and digested with increasing concentrations of EndoH. Doubly and singly glycosylated and nonglycosylated forms of the constructs are represented by two black dots, one black dot, and one white dot, respectively.

Here, we have used glycosylation mapping to analyze the effect of Trp and Phe residues on the position of a model poly-Leu transmembrane helix relative to the microsomal membrane. Both Phe and Trp appear to push the transmembrane helix into the membrane when placed adjacent to the poly-Leu stretch, but only Trp pulls the helix out of the membrane when present in the central core of the poly-Leu segment (Figure 2C).

These results are consistent with earlier biophysical and statistical studies where a strong preference for an interfacial location has been found for Trp (1, 21), and further show that this preference is strong enough to cause significant repositioning of a poly-Leu transmembrane helix relative to a biological membrane. Phe, in contrast, appears to have no preference for the interface over the core of the membrane, as is also evident from statistical studies of transmembrane helices (1, 22, 23). Trp has a MGD profile similar to that of Arg, suggesting that its relatively long side chain may "snorkel" toward the lipid−water interface (24−28).

Essentially no Trp but many Phe residues are found in fully lipid-exposed helix core regions of helix bundle membrane proteins (1), and poly-Trp (in contrast to poly-Phe) segments have been shown not to be functional in the context of an idealized signal peptide (29). Nevertheless, as is clear from Figure 3, a stretch of 27 Trp residues functions

as an efficient stop-transfer sequence, demonstrating its ability to become anchored in the lipid bilayer rather than translocated. Despite its clear preference for an interfacial location, Trp can thus be considered a moderately hydrophobic residue in the context of predicting transmembrane helices from the amino acid sequence.

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