

Research article

Different conformations of nascent polypeptides during translocation across the ER membrane

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Abstract

Background: In eukaryotic cells, proteins are translocated across the ER membrane through a continuous ribosome-translocon channel. It is unclear to what extent proteins can fold already within the ribosome-translocon channel, and previous studies suggest that only a limited degree of folding (such as the formation of isolated α -helices) may be possible within the ribosome.

Results: We have previously shown that the conformation of nascent polypeptide chains in transit through the ribosome-translocon complex can be probed by measuring the number of residues required to span the distance between the ribosomal P-site and the lumenally disposed active site of the oligosaccharyl transferase enzyme (J. Biol. Chem 271: 6241-6244). Using this approach, we now show that model segments composed of residues with strong helix-forming properties in water (Ala, Leu) have a more compact conformation in the ribosome-translocon channel than model segments composed of residues with weak helix-forming potential (Val, Pro).

Conclusions: The main conclusions from the work reported here are (i) that the propensity to form an extended or more compact (possibly α -helical) conformation in the ribosome-translocon channel does not depend on whether or not the model segment has stop-transfer function, but rather seems to reflect the helical propensities of the amino acids as measured in an aqueous environment, and (ii) that stop-transfer sequences may adopt a helical structure and integrate into the ER membrane at different times relative to the time of glycan addition to nearby upstream glycosylation acceptor sites.

Background

Most eukaryotic secretory proteins are translocated across the membrane of the endoplasmic reticulum (ER) through a continuous ribosome-translocon channel that is sealed to the cytoplasmic compartment and open towards the ER lumen [1]. Recent structural studies of ribosomes from *Haloarcula marismortui* suggest that the channel through the large ribosomal subunit is ~ 100 Å long and 10-20 Å wide [2], and the same appears to hold

true also for yeast and rabbit ribosomes [3]. The internal diameter of the translocon channel present in dog pancreas microsomes has been estimated to be 40-60 Å [4]. It is unclear to what extent proteins can fold already within the ribosome-translocon channel; studies on ribosome-nascent chain complexes suggest that only a limited degree of folding (such as the formation of isolated α -helices) may be possible within the ribosome [5,6,7].

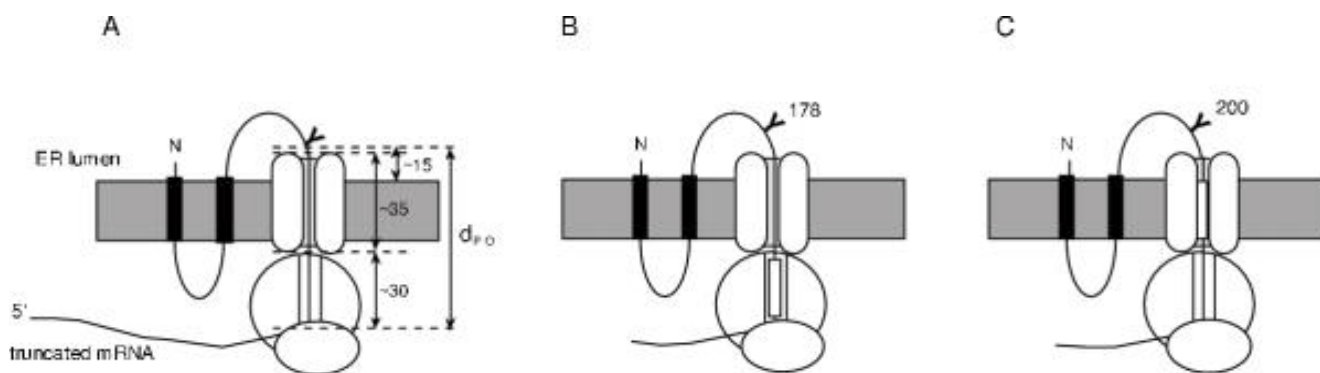


Figure 1

Determination of the minimum number of residues required to span the distance between the ribosomal P-site and the active site of oligosaccharide transferase (d_{p-O} values). Ribosome-bound truncated nascent chains of different lengths are generated by *in vitro* translation, in the presence of dog pancreas microsomes, of mRNAs lacking a stop-codon. The d_{p-O} value is the number of residues between the Asn residue in an Asn-Ser-Thr glycosylation acceptor site (Y) and the C-terminal end of the nascent chain required for half-maximal glycosylation of the acceptor site. (A) The model protein used in this study (*E. coli* Lep) has two transmembrane segments (black) and a C-terminal luminal domain. The approximate number of residues in an extended chain needed to span the indicated distances are given [10]. (B) Truncated Lep with a model segment (white) placed in the ribosome. The glycosylation site is placed in position 178 in Lep. (C) Truncated Lep with a model segment (white) placed in the translocon. The glycosylation site is placed in position 200 in Lep. In both panels B and C, the model segment is introduced between positions 214 and 220 in Lep.

In this paper, we report experiments where we have used truncated nascent chains trapped within the ribosome-translocon complex to measure the number of residues of nascent polypeptide (d_{p-O}) required to span the distance between the P-site on the ribosome (located at the entrance to the ribosomal channel) and the active site of the oligosaccharyl transferase (located near the luminal end of the translocon channel). By placing 18-residue long stretches of model sequences (e.g., $L_{17}V$, $A_{17}V$, V_{18} , P_{18}) in locations that are either within the ribosome or the translocon in the truncated nascent chain we find that d_{p-O} values tend to fall near either 65 or 70 residues, and we equate these two values with extended and helical conformations of the model stretches, respectively. We conclude that the $L_{17}V$ and $A_{17}V$ segments tend to form α -helices both in the ribosomal and translocon parts of the channel, while the V_{18} , P_{18} , and the transmembrane segment from glycoporphin A (GpA) have more extended conformations. Since the GpA transmembrane segment (and, presumably, the V_{18} segment) is helical in the lipid-embedded form of the protein [8, 9], this suggests that a hydrophobic segment with low helix potential in water may enter the translocon as an extended stretch of polypeptide and will only fold into a helical conformation upon lateral exit from the translocation channel into the lipid bilayer. On the other hand, stretches in the nascent polypeptide with strong helix propensity but insufficient hydrophobicity to insert into the lipid bilayer (e.g., $A_{17}V$) may form individually stable α -helices already in the ribosomal channel.

Results

The glycosylation mapping assay

The number of residues required to span the distance between the ribosomal P-site and the active site of the oligosaccharide transferase (OST) can be conveniently measured by a previously described "glycosylation mapping" assay [10]. Ribosome-nascent chain complexes attached to the ER translocon are generated by translation, in the presence of dog pancreas microsomes, of truncated mRNA molecules coding for a membrane protein (*E. coli* Lep) lacking a 3' stop codon, Fig. 1A. A series of neighboring truncation points on the mRNA is chosen such that a unique Asn-Ser-Thr acceptor site for N-linked glycosylation is moved from a position ~ 60 codons to a position ~ 75 codons away from the 5' end of the mRNA, and the degree of glycosylation is measured for each truncated chain. As we have shown previously [10], glycosylation of the ribosome-attached nascent chain is generally observed when the glycosylation acceptor site is placed at a distance of ~ 65 -75 residues away from the ribosomal P-site. As the maximum level of glycosylation typically observed for the truncated Lep constructs discussed below is $\sim 60\%$, we define the "critical" number of residues (d_{p-O}) required to span the distance between the P-site and the OST active site to be the chain length where 30% glycosylation is observed.

Ala- and Leu- but not Val- or Pro-based segments have a compact conformation both in the ribosome and the translocon

In the earlier study referred to above [10], we found that 65 residues from the C-terminal P2 domain of wild type Lep are required to span the distance between the P-site and the OST active site, i.e. $d_{p-o} = 65$. We further found that when a model hydrophobic stretch with the sequence ...QQQL₁₇VKKKK... was inserted 15 residues downstream of the glycosylation site (i.e., in a location that is largely inside the translocon when the nascent chain is long enough to allow glycosylation, Fig. 1C), d_{p-o} increased to 71 residues, suggesting a more compact, possibly α -helical, conformation of the L₁₇V segment compared to the corresponding stretch from the wildtype P2 domain. In a fully extended chain (~ 3.3 Å per residue), approximately 11 residues need to be converted to an α -helix (~ 1.5 Å per residue) to account for the observed change in d_{p-o} of ~ 6 residues.

Here, we have extended these studies, both by analyzing a wider selection of model segments (V₁₈, A₁₇V, P₁₈, and the transmembrane α -helix from GpA) and by changing the position of the glycosylation site relative to the model segment such that the model segment is located within the ribosome rather than the translocon at chain lengths around the d_{p-o} value, Fig. 1B. Results for the two Val-based constructs with the hydrophobic stretch in the ribosome (upper panel) and in the translocon (lower panel) are shown in Fig. 2A. In both cases, $d_{p-o} \cong 64$ residues, Fig. 2B, i.e., close to the value found for wild type Lep. The results for the GpA and P₁₈ constructs are similar, Fig. 2B, with $d_{p-o} \cong 65$ residues. In contrast, the d_{p-o} values for the L₁₇V and A₁₇V segments are 70-71 residues when they are present in the ribosome, and somewhat larger (~ 73 residues) for L₁₇V in the translocon, Fig. 2B. Assuming that an increase in d_{p-o} corresponds to a more compact conformation of the model segment, we conclude that the L₁₇V and A₁₇V segments have a compact, possibly helical, conformation when located in the ribosome-translocon channel, while the V₁₈, P₁₈, and GpA segments are more extended.

The differences in d_{p-o} values do not correlate with the ability of the model segments to insert into the ER membrane

To assess the ability of the model segments to insert into the ER membrane (i.e., their stop-transfer function), a second glycosylation acceptor site was added at the C-terminal end of the protein (see Methods). As shown in Fig. 3A, model segments with efficient stop-transfer function will only be glycosylated on the upstream acceptor site, while those lacking stop-transfer function will be glycosylated on both sites. Since acceptor sites located close to the C-terminus of a protein are only about 30%

glycosylated even if efficiently translocated into the ER lumen [11], we expect a mixture of singly and doubly glycosylated molecules in the latter case.

As seen in Fig. 3B, the L₁₇V and V₁₈ constructs were glycosylated on only one site, while 19% and 27% of the A₁₇V and P₁₈ constructs were doubly glycosylated, respectively. Protease treatment of microsomes also demonstrated that only a small fragment (corresponding to the H2-V₁₈ region) was protected in the V₁₈ construct while a major part (corresponding to the H2-P2 region) of wild type Lep was protected inside the microsome, Fig. 3C. Thus, although the V₁₈ and GpA (8) segments do not appear to fold into a compact conformation inside the ribosome-translocon channel, they are efficient stop-transfer sequences. In contrast, the A₁₇V sequence adopts a compact (possibly α -helical) conformation in the channel, but has little or no stop-transfer function. The L₁₇V segment is both compact and an efficient stop-transfer sequence, while the P₁₈ segment has an extended conformation (possibly forming a poly-proline II helix with a rise of 3.2 Å per residue [12], very close to the rise of ~ 3.3 Å per residue for a fully extended chain) and no stop-transfer function.

Poly-Val and poly-Leu TMH segments behave differently during integration into the ER membrane

Given that all stop-transfer sequences, once integrated into the membrane, are expected to form transmembrane α -helices [13], the extended conformation of the V₁₈ and GpA model segments in the translocon channel was somewhat surprising. To study this further, we determined the "minimal glycosylation distance" (MGD) for the full-length V₁₈ and A₁₇V constructs, and also for a construct where the third Val residue from the N-terminal end of the V₁₈ stretch had been changed to Pro.

The MGD value is defined as the minimum number of residues required to bridge the distance between the luminal end of a hydrophobic transmembrane segment in a membrane protein and the OST active site [14], Fig. 4A. MGD measurements can be used to roughly position the luminal end of a transmembrane segment relative to the ER membrane by comparison to MGD values for transmembrane helices where the position relative to the lipid bilayer has been derived from various biophysical experiments [15, 16]. In our previous studies, we have mainly measured MGD values for poly-Leu based sequences. As an example, the glycosylation profile for the L₁₇V construct is shown in Fig. 4B, yielding an MGD value of 15.7 residues [14].

Since, as shown above, the A₁₇V construct is efficiently translocated into the lumen of the microsomes, it is not expected to have a "minimal glycosylation distance". In-

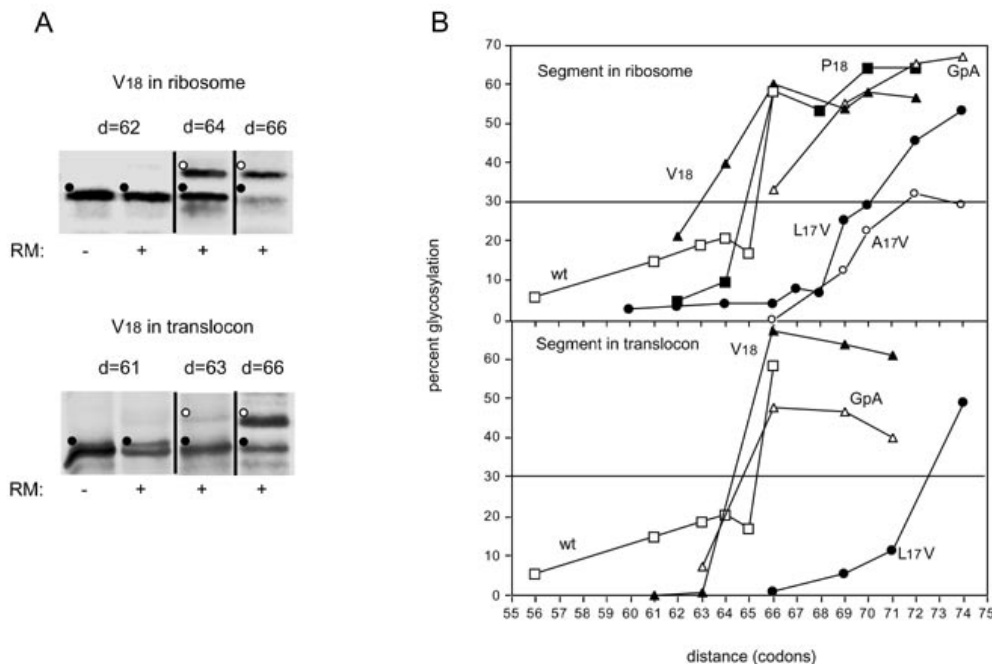


Figure 2
 Determination of $d_{p,O}$ values for constructs where a model polypeptide segment has been inserted into the middle of the C-terminal P2 domain of Lep. (A) Two constructs with a ...QQQV¹⁸KKKK... model segment in the C-terminal domain were translated *in vitro* in the absence (-) and presence (+) of rough dog pancreas microsomes (RM). The 3' codon in the truncated mRNA was placed *d* codons downstream of the Asn residue in the Asn-Ser-Thr glycosylation acceptor site. The Asn residue in the acceptor site was placed in *lep* codon 178 (upper panel) or 200 (lower panel), and the model segment was inserted between *lep* codons 214 and 220 in both constructs. For the indicated range of *d* values, the model segment is located in the ribosome (upper panel) or in the translocon (lower panel). Non-glycosylated and glycosylated molecules are indicated by black and white dots, respectively. (B) Glycosylation profiles for constructs with model segments composed of the indicated residues flanked by QQQ...KKKK. The Asn residue in the acceptor site was placed in *lep* codon 178 (upper panel) or 200 (lower panel), and the model segment was inserted between *lep* codons 214 and 220 in all constructs. Half-maximal glycosylation (30%) is indicated by the horizontal line.

deed, all A₁₇V-based glycosylation mutants tested are 60%-80% glycosylated, Fig. 4B. Interestingly, the V₁₈ construct, which, like the L₁₇V construct, forms a transmembrane segment, nevertheless has a glycosylation profile that is clearly distinct from that of the L₁₇V construct: an initial drop from ~ 80% to ~ 40% glycosylation at roughly the same glycosylation distance as L₁₇V (~ 15.5 residues) is followed by a plateau, and background levels of glycosylation are approached only at a glycosylation distance of ~ 10.5 residues. One possible interpretation is that there are two populations of V₁₈ molecules at the time when the glycan moiety is added to the growing nascent chain: one that has a similar disposition relative to the OST active site as the L₁₇V molecules (i.e., presumably α -helical and membrane-integrated with an MGD value of ~ 15.5 residues) and one with a significantly smaller MGD value (~ 10.5 residues).

To test this idea further, we also analyzed a construct where the third Val residue in the V₁₈ segment was

changed to Pro. We have previously shown that the introduction of a Pro residue in corresponding positions in a L₂₃V transmembrane segment leads to a reduction in the MGD value of about 2.5 residues, presumably as a result of a break in the poly-Leu α -helix caused by the Pro residue [14]. Indeed, the initial drop in the glycosylation profile for the V₁₈(P³) construct was ~ 2 residues, Fig. 4B, while the shift in the location of the second drop was only ~ 1 residue. This is consistent with the possibility that V₁₈ molecules with MGD ~ 15.5 residues indeed have already formed a transmembrane α -helix at the time of glycosylation, whereas the remaining ones have not. More detailed kinetic studies will be needed to further substantiate this idea.

Discussion

In this paper, we have analyzed the conformation of model polypeptide segments as they traverse the ribosome-translocon channel during translocation across the ER membrane. The study extends previous data, from

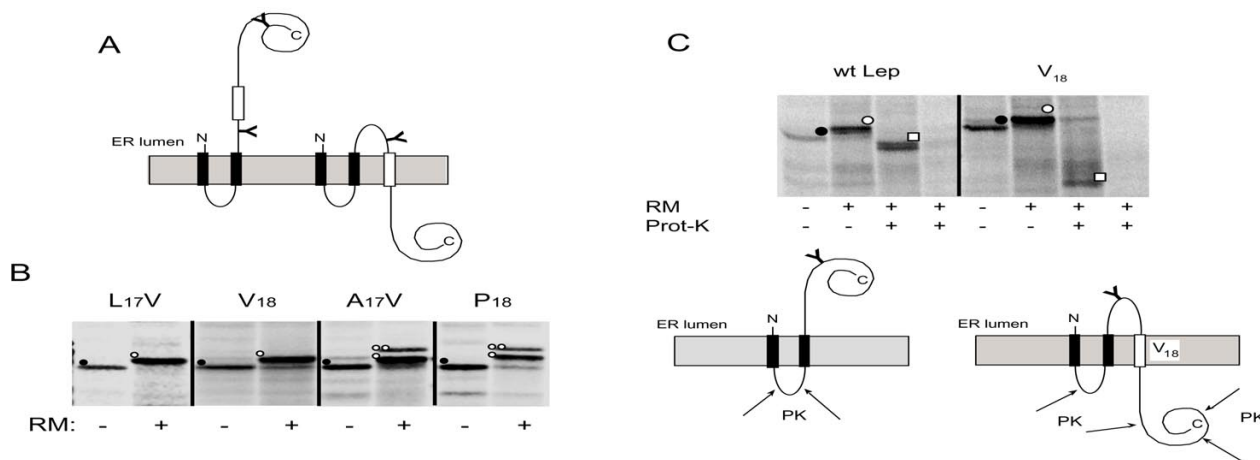


Figure 3

Determination of stop-transfer activity of different full-length constructs. (A) Model segments (white) lacking stop-transfer activity are translocated into the ER lumen and both glycosylation acceptor sites become modified (left). For model segments with stop-transfer function, only the upstream site will become modified (right; Ψ denotes a glycosylated site, Φ a non-glycosylated site). The C-terminal glycosylation site is 6 residues away from the C-terminus of the protein (see Materials and Methods). (B) *In vitro* translation in the absence (-) and presence (+) of rough dog pancreas microsomes (RM) of constructs with the indicated model segments. Non-glycosylated and mono- and di-glycosylated molecules are indicated by a black dot, a white dot, and two white dots, respectively. (C) Proteinase K (PK) treatment of microsomes carrying *in vitro* translated wild type Lep (left panel) or the full-length V_{18} construct (right panel). The detergent Triton X-100 was included to dissolve the microsomal membrane in lanes 4 and 8. Non-glycosylated and glycosylated molecules are indicated by a black dot and a white dot, respectively. Protease-protected fragments are indicated by a white square.

which we concluded that a segment from the globular P2 domain of the *E. coli* leader peptidase (Lep) protein most likely traverses the ribosome-translocon channel as an extended chain, whereas a model $L_{17}V$ segment placed in the P2 domain adopts a more compact, possibly α -helical, conformation when placed in the translocon part of the channel [10].

The main conclusions from the work reported here are (i) that the propensity to form an extended or more compact (possibly α -helical) conformation in the ribosome-translocon channel does not depend on whether or not the model segment has stop-transfer function, but rather seems to reflect the helical propensities of the amino acids as measured in an aqueous environment, and (ii) that stop-transfer sequences may adopt a helical structure and integrate into the ER membrane at different times relative to the time of glycan addition to nearby upstream glycosylation acceptor sites.

Conclusion (i) follows from the observation that the $A_{17}V$ and $L_{17}V$ model stretches - which are composed of residues that are strong helix-formers in an aqueous environment [17] - both adopt a compact conformation in the ribosome-translocon channel (Fig. 2), whereas the V_{18} and GpA model segments do not. Val has a low helix po-

tential in water but readily forms helices in micellar and vesicular media [9, 17], and the GpA segment also includes a number of residues with low helix potential in water (Gly, Val, Ile, Thr). The $A_{17}V$ segment does not have stop-transfer function, as previously found for similar constructs composed of up to 19 Ala residues [18], while $L_{17}V$ and V_{18} do (Fig. 3).

The known dimensions of the ribosomal channel (minimum diameter 10-20 Å [2, 3]) are consistent with the formation of an α -helix, but would not allow bigger structures to form. The walls of the ribosomal channel are mostly composed of RNA and have a hydrophilic character, suggesting that they do not interact strongly with hydrophobic segments in the nascent polypeptide chain. Our results are also consistent with a recent study where it was found that the N-termini of ribosome-attached nascent proteins become accessible to antibodies at different chain-lengths depending on the protein [5], also suggesting different degrees of structure formation within the ribosome channel. The reported size of the channel in the mammalian ER translocon is considerably larger (40-60 Å diameter [4]), and would seem to allow the formation of larger folded structures; we see no evidence for this, however.

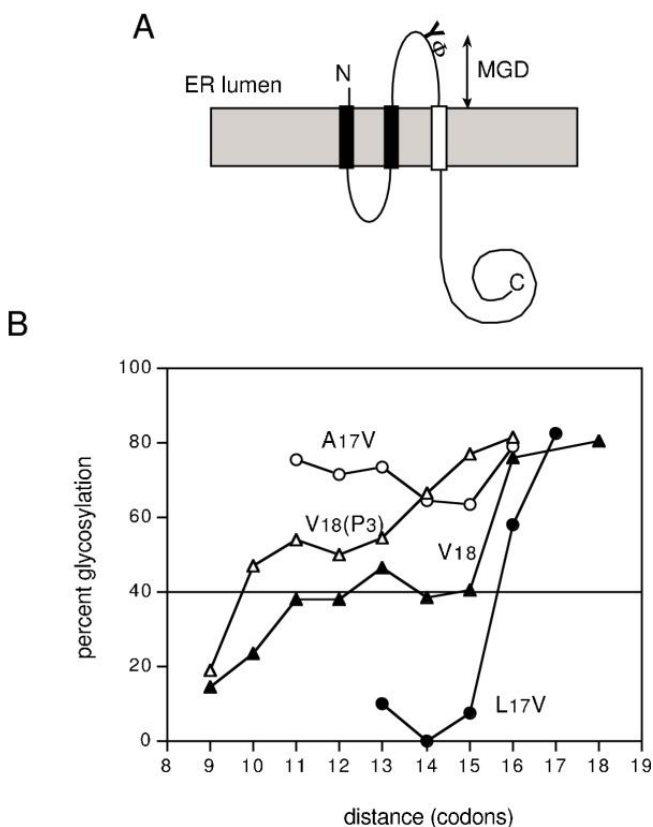


Figure 4
 Determination of the "minimal glycosylation distance" (MGD). (A) The MGD is defined as the minimum number of residues required to bridge the distance between the luminal end of a transmembrane segment (white) and the active site of oligosaccharyl transferase (Y denotes a glycosylated site, Φ a non-glycosylated site). (B) MGD measurements for constructs with the indicated model segments. Half-maximal glycosylation (40%) is indicated by the horizontal line. The MGD value is counted from the first polar residue before the transmembrane segment (Gln) up to the acceptor Asn residue as in [14].

The second conclusion, that stop-transfer sequences may adopt a helical structure and integrate into the ER membrane at different times relative to the time of glycan addition to nearby acceptor sites, is tentative and based on the finding that the L₁₇V stop-transfer segment (which has a compact structure in the translocon channel) has a well-defined MGD value of ~ 15 residues, while molecules containing the V₁₈ stop-transfer segment (which has an extended conformation in the translocon channel) appear to be characterized by two distinct MGD values (~ 15 and ~ 10 residues), Fig. 4. By comparison to the L₁₇V result and based on the observation that a V → P mutation near the N-terminal end of the V₁₈ segment causes a rather large drop in the larger of the two MGD values (as would be expected if a transmembrane α-helix

was broken by the Pro residue), we suggest that the V₁₈ segment in the population with MGD ~ 15.5 residues is helical and membrane-integrated whereas the population with MGD ~ 10.5 residues is not. It is thus possible that we have been able to catch the V₁₈ molecules in the process of converting from an extended conformation in the translocon channel to a helical, membrane-embedded state. This interpretation is broadly consistent with the results of a recent study [19], where it was shown that more hydrophobic transmembrane segments move from the translocon channel into the lipid bilayer more easily (and thus presumably more rapidly) than less hydrophobic segments.

In summary, it appears that our approach using engineered glycosylation sites, truncated nascent chains, and different model polypeptide sequences makes it possible to characterize the conformational propensities of different polypeptide segments during translocation across and integration into the ER membrane. Our results also point to the necessity to carry out a careful analysis of the glycosylation profile when MGD values are used to infer the position of a transmembrane segment relative to the ER membrane.

Materials and Methods

Enzymes and chemicals

Unless otherwise stated, all enzymes as well as plasmid pGEM1, The RiboMAX SP6 RNA polymerase system, and rabbit reticulocyte lysate were from Promega (Madison, WI) or New England Biolabs (Boston, MA). T7 DNA polymerase, Taq polymerase, [³⁵S]-Met, [¹⁴C]-methylated marker proteins, ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides, and the cap analog m7G(5')ppp(5')G were from Amersham-Pharmacia (Uppsala, Sweden). The PCR purification and RNeasy RNA clean up kits were from Qiagen (Hilden, FRG). The PCR mutagenesis kit was from Stratagene (La Jolla, CA). Proteinase K was from Boehringer Mannheim GmbH (Mannheim, Germany). PMSF (phenylmethylsulfonyl fluoride) was from ICN Biochemicals Inc (Aurora, Ohio). Puromycin was from Sigma (St. Louis, Missouri). Oligonucleotides were from Kebo Lab and Cybergene (Stockholm, Sweden).

DNA manipulations

Site-specific mutagenesis was performed according to the method of Kunkel [20, 21] or by PCR. All mutants were confirmed by sequencing of plasmid DNA. All cloning steps were done according to standard procedures.

Construction of full-length and truncated Lep glycosylation mutants

Insertion of the model sequences QQQL₁₇VK₄KKK, QQQA₁₇VK₄KKK, QQQV₁₈K₄KKK, and QQQP₁₈K₄KKK and

the transmembrane helix from glycoporphin A MITLIIF-GVMAGVIGTILLISYGIKKKKH (GpA-derived residues underlined) into the P2 domain of Lep was performed by introducing BclI and NdeI restriction sites in lep codons 214 and 220, respectively. Double-stranded oligonucleotides coding for the model sequences were then cloned between the BclI and NdeI restriction sites. Site-specific mutagenesis was used to introduce an Asn-Ser-Thr glycosylation acceptor site in lep codons 200 and 178 and to introduce the restriction enzyme cleavage sites. For cloning into and expression from the pGEM1 plasmid, the 5' end of the lep gene was modified by introducing a Kozak consensus sequence [22] for enhanced translation and a XbaI site for cloning: ...ATAACCCTCTAGAGCCACCATGGCGAATATG...(XbaI site and initiator codon underlined). Mutants of Lep were cloned into pGEM1 behind the SP6 promoter as an XbaI-SmaI fragment.

Templates for *in vitro* transcription of truncated mRNA with or without a 3' stop codon were prepared using PCR to amplify fragments from pGEM1 plasmids containing the relevant Lep constructs. The 5' primer was the same for all PCR reactions and had the sequence 5'-TTCGTC-CAACCAAACCGACTC-3'. This primer is situated 210 bases upstream of the translational start, and all amplified fragments thus contained the SP6 transcriptional promoter from pGEM1. The 3' primers were designed according to the desired C-terminal end of the truncated protein and either contained no stop codon or a TAG stop codon. All primers were designed to have approximately the same annealing temperature. PCR amplification was performed with a total of 30 cycles using an annealing temperature of 52 °C. The amplified DNA products were purified using the Qiagen PCR purification kit as described in the manufacturers protocol and verified on a 1.2 % agarose gel.

To introduce a C-terminal glycosylation site in the V₁₈ and P₁₈ constructs (Fig. 3B), the templates were amplified as above but with a 3' primer encoding the C-terminal sequence ...PTGLRLSNSTGIH(stop) corresponding to the C-terminal end of Lep but with the underlined residues changed to encode a glycosylation acceptor site.

MGD values (Fig. 4) were measured as described in [14] for the L₁₇V and V₁₈ constructs, and also for a V₁₈-derived construct where the third Val from the N-terminal end was replaced by Pro. Briefly, full-length constructs with the Asn residue in a Asn-Ser-Thr glycosylation acceptor site placed 9-18 residues upstream of the respective hydrophobic segments were translated *in vitro* in the presence of dog pancreas microsomes, and the MGD value was determined from a plot of the efficiency of glycosylation versus the position of the glycosylation site as shown in Fig. 4.

Expression *in vitro*

Templates for *in vitro* transcription were prepared as described in [23] or by PCR amplification with the pGEM1 construct as template as described above. Amplified PCR fragments were transcribed from the SP6 promoter using the Large Scale RNA Synthesis kit with the RiboMAX SP6 RNA polymerase system. Transcriptions were carried out at 30°C for 12 hours. The mRNAs were purified using Qiagen RNeasy clean up kit and verified on a 1% agarose gel.

Translation in reticulocyte lysate in the presence of dog pancreas microsomes was performed as described in [23] at 30°C for 1 h (when generating full-length, properly terminated chains) or at 22°C for 30 min. (when generating truncated ribosome-nascent chain complexes). 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 non-glycosylated bands, or as the quotient between the intensities of the doubly glycosylated band divided by the summed intensities of the singly and doubly glycosylated bands (for the experiment in Fig. 3B)

Translocation of polypeptides to the luminal side of the microsomes was assayed by resistance to exogenously added proteinase K and by analysis of constructs with two glycosylation acceptor sites flanking the model polypeptide segments as in [24].

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Ismael Mingarro and IngMarie Nilsson contributed equally to this work.

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