doi:10.1016/j.jmb.2003.10.019 J. Mol. Biol. (2003) 334, 803–809

JMB

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Inter-helical Hydrogen Bond Formation During Membrane Protein Integration into the ER Membrane

Marika Hermansson and Gunnar von Heijne*

Department of Biochemistry and Biophysics, Arrhenius Laboratory, Stockholm University, SE-106 91 Stockholm, Sweden

*Corresponding author

Recent work has shown that efficient di- or trimerization of hydrophobic transmembrane helices in detergent micelles or lipid bilayers can be driven by inter-helix hydrogen bonding involving polar residues such as Asn or Asp. Using *in vitro* translation in the presence of rough microsomes of a model integral membrane protein, we now show that the formation of so-called helical hairpins, two tightly spaced transmembrane helices connected by a short loop, can likewise be promoted by the introduction of Asn-Asn or Asp-Asp pairs in a long transmembrane hydrophobic segment. These observations suggest that inter-helix hydrogen bonds can form within the context of the Sec61 translocon in the endoplasmic reticulum, implying that hydrophobic segments in a nascent polypeptide chain in transit through the Sec61 channel have immediate access to a non-aqueous subcompartment within the translocon.

Keywords: membrane protein; protein structure; hydrogen bond; transmembrane helix; helical hairpin

Introduction

In eukaryotic cells, most integral membrane proteins insert into and fold in the membrane of the endoplasmic reticulum (ER) from which they may then be transported further along the secretory pathway or to the nuclear envelope. The initial recognition of the hydrophobic segments that form the transmembrane α-helices takes place during polypeptide transfer through the Sec61 translocon complex in the ER membrane.[1,2] It appears that the transmembrane helices can either be recognized and transferred laterally into the surrounding lipid bilayer one by one, or in pairs,[3–6] or even as higher-order multimers,[7] though these early steps on the folding pathway are only poorly understood at present.

Recent biochemical studies of model peptides in detergent micelles or liposomes have shed some light on the basic physical chemistry of helix–helix interactions in a non-polar environment. Tight close-packing between helices mediated by the so-called GxxxG-motif, or inter-helical hydrogen bond formation between pairs of polar residues such as Asn and Asp, or more complex motifs involving Thr or Ser[8–16] have been shown to drive efficient dimer formation in these systems. These interactions have been confirmed by two-hybrid assays in *Escherichia coli* where the formation of a dimeric protein complex in the inner membrane ultimately results in the activation of a reporter gene.[17,18] It is thus quite clear that hydrogen bonding can drive helix–helix interactions in biological membranes; what is not clear, however, is if inter-helix hydrogen bonds can form already within the context of the translocon.

Inspired by previous observations that suggest that transmembrane helices may encounter a bilayer-like environment almost immediately upon entering the translocon channel,[2,19] we have carried out a detailed analysis of the possible influence of Asn-Asn and Asp-Asp pairs on the formation of so-called helical hairpins (i.e. two closely spaced transmembrane helices connected by a short loop) during co-translational membrane protein insertion into the ER. We have already reported that helical hairpin formation is facilitated by the presence of charged, polar, and weakly hydrophobic residues in the short connecting loop,[20–22] and by charged residues placed immediately downstream of a long, uniformly hydrophobic segment.[23] Here, we show that Asn-Asn or Asp-Asp pairs can promote the formation of helical hairpins in a strictly position-specific manner. The observed pattern of hairpin-promoting pairs

Abbreviations used: ER, endoplasmic reticulum; Lep, leader peptidase; RM, rough microsomes.

E-mail address of the corresponding author: gunnar@dbb.su.se

is consistent with the formation of inter-helical hydrogen bonds. If this interpretation is correct, it provides yet another argument for the contention that transmembrane helices are formed within a non-aqueous subcompartment in the Sec61 translocon.

Results

Model protein and topology assay

As in our previous studies of helical hairpin formation in membrane proteins, we have used the well-characterized *E. coli* inner membrane protein leader peptidase (Lep) as a model protein. Lep consists of two transmembrane segments (H1 and H2) connected by a short cytoplasmic loop (P1) and a large C-terminal periplasmic domain (P2). When expressed *in vitro* in the presence of dog pancreas rough microsomes (RM), Lep adopts the same membrane topology as in its natural environment in the inner membrane of *E. coli*,[24] i.e. with the N terminus and the large C-terminal P2 domain on the lumenal side,[25] Figure 1(a) (left).

For the studies reported here, H2 was substituted by a poly-Leu stretch of the general composition …P^{58}-$LIK_4L_{29}VL_{10}Q_3P$-E^{82}… (superscript numerals refer to residues in Lep, subscript numerals indicate the number of consecutive residues of a given kind).[20,22] The segment has four N-terminal lysine residues intended to anchor this end to the cytoplasmic side of the membrane, a 40 residue stretch composed of 39 leucine and one valine (the latter resulting from an engineered restriction site) that is long enough to span the membrane either as a single or as two closely spaced transmembrane helices, and four uncharged polar residues (Q_3P) demarcating the C-terminal end of the hydrophobic stretch. We have shown previously that this stretch forms a single transmembrane segment when the model protein is integrated *in vitro* into RMs,[26] but that the introduction of "turn-promoting" residues such as Pro, Asn, Arg, or Asp near the middle of the poly-Leu stretch leads to the formation of a helical hairpin.[20]

As an easily scored marker for the lumenal or cytoplasmic localization of the P2 domain, an N-glycosylation site (Asn-Ser-Thr) was introduced 20 amino acid residues downstream of the poly-Leu stretch. In constructs where the poly-Leu stretch spans the membrane only once, this site will be glycosylated by the lumenally disposed oligosaccharyl transferase enzyme (Figure 1(a), left), while it will not be modified in constructs where the poly-Leu stretch has been mutated to form a helical hairpin (Figure 1(b), right).[27] Turn formation can thus be assayed by *in vitro* transcription/translation of the relevant constructs in the presence of RM followed by quantification of the efficiency of glycosylation of the engineered N-glycosylation site.

Typical gels for three constructs described below (D8, D33, and D8-D33) are shown in Figure 1(b); in these constructs, the poly-Leu segment in D8-D33 inserts almost exclusively as a helical hairpin (very little glycosylation), while essentially no helical hairpin formation is seen for D8 (almost complete glycosylation). The D33 construct has a

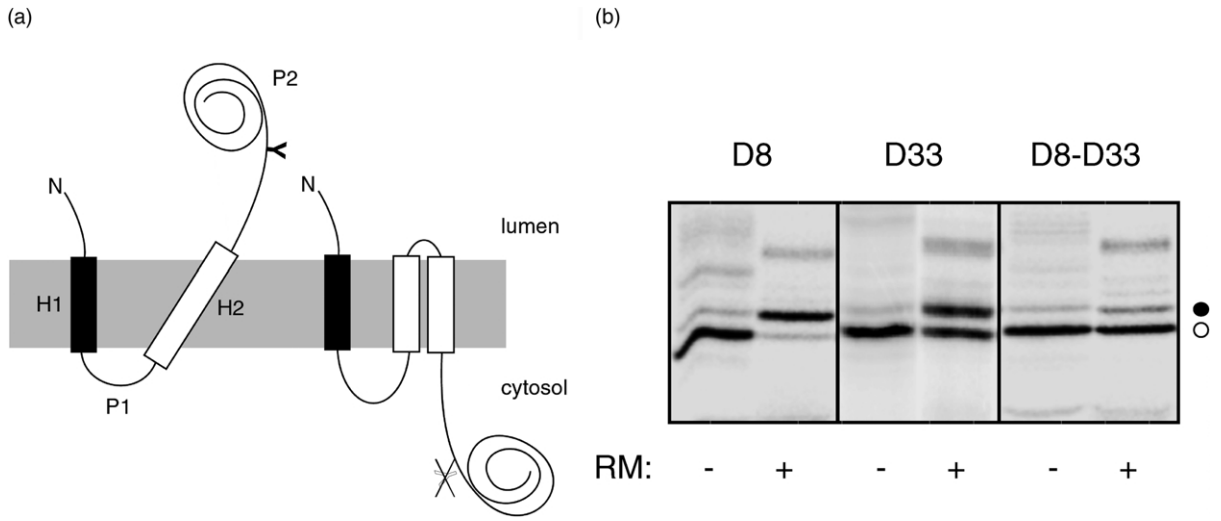

Figure 1. (a) The Lep model protein. Wild-type Lep has two transmembrane segments (H1, H2), and inserts into dog pancreas rough microsomes *in vitro* with the N_{lum}–C_{lum} orientation shown on the left. In the studies reported here, the H2 segment has been replaced by a poly-Leu-based sequence ($LIK_4L_{29}VL_{10}Q_3P$) into which one or two Asn or Asp residues have been inserted. Constructs where H2 spans the membrane once are glycosylated by the lumenally disposed oligosaccharyl transferase enzyme on a unique Asn-Ser-Thr glycosylation acceptor site (**Y**) in the P2 domain (left); those where the H2 segment forms a helical hairpin are not (right). In this study, one or a pair of Asn or Asp residues has been inserted in the areas in the H2 segment encircled on the right-hand model. (b) The D8, D33, and D8-D33 constructs were translated *in vitro* in the absence (−) and in the presence (+) of dog pancreas rough microsomes (RM). Unglycosylated and glycosylated molecules are indicated by the white dot and the black dot, respectively.

mixed topology with about half the molecules being glycosylated.

Effects on helical hairpin formation of single Asn and Asp residues in a (39L + V) transmembrane segment

To test the effect on helical hairpin formation when an Asn or Asp residue is placed in different positions in a poly-Leu stretch, we introduced Asn or Asp in positions 8, 10, 12 and 31–39 in the (39L + V) segment, and helical hairpin formation was assayed by determining the fraction of non-glycosylated molecules (f^{ng}) for each construct.

None of the single Asn-mutations induce efficient formation of a helical hairpin (Figure 2(a)). We always observe a background level of $f^{ng} \sim 0.2$, most likely a result of somewhat inefficient targeting and glycosylation in the *in vitro* system. The only positions in which an Asn leads to a slight increase in f^{ng} are 12 and 37. We have already shown that an Asn-residue placed in position 22 near the middle of the (39L + V) segment induces almost complete formation of a helical hairpin ($f^{ng} = 0.85$),[22] and it is therefore not very surprising that we see a slight effect with the N12 mutation. It is less obvious why the N37 mutation should have a comparable effect (but see Discussion).

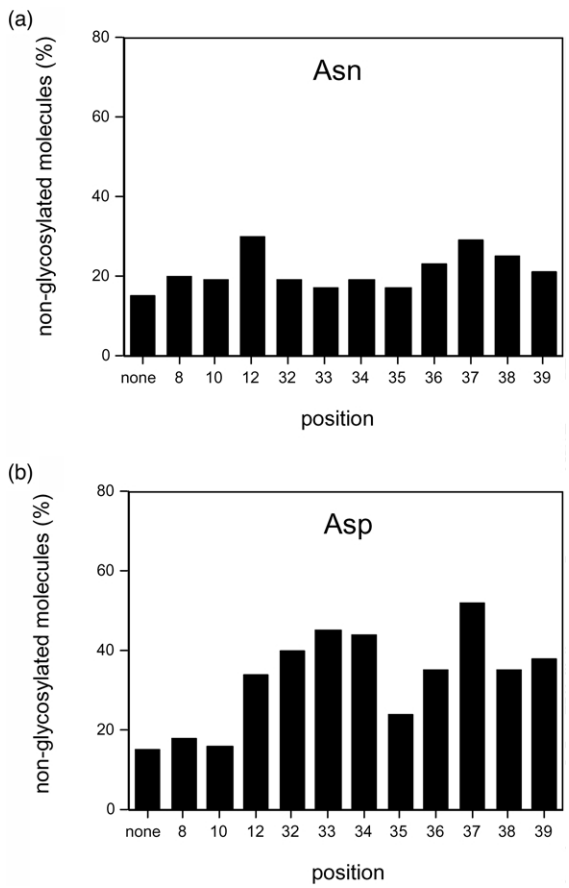

Figure 2. Degree of helical hairpin formation measured as the fraction of non-glycosylated molecules (f^{ng}) observed after *in vitro* translation in the presence of RM for single Asn (a) and single Asp (b) mutations in the (39L + V) H2 segment. The position of the Asn or Asp residue is counted from the N-terminal end of the (39L + V) segment. All measurements are mean values of two or three independent experiments; in general, repeat f^{ng}-value measurements differ by no more than ±0.05 from the mean value.

For constructs with a single Asp in the (39L + V) stretch, the picture is the same as for Asn for positions 8, 10, and 12 (Figure 2(b)). The Asp mutations near the C-terminal end of the segment have significantly higher f^{ng} values than the corresponding Asn mutations, with the D37 mutation having the strongest effect. It thus appears that an Asp residue near the C-terminal end of the (39L + V) stretch induces a significant level of helical hairpin formation, in agreement with earlier studies where Asp residues immediately flanking the C-terminal end of the (39L + V) stretch were shown to induce a helical hairpin conformation.[23] We have shown that a single Pro residue placed 10–12 residues from either end of the (39L + V) stretch induces a significant amount of the helical hairpin conformation,[20] suggesting that the higher f^{ng} values seen for the more centrally placed D12, D32, D33, and (possibly) D34 mutations might be caused by the strong hairpin-inducing effect demonstrated earlier for a centrally placed Asp residue.[20]

Position-specific effects on helical hairpin formation by Asn-Asn and Asp-Asp pairs

We next examined the tendencies of Asn-Asn and Asp-Asp pairs to mediate helical hairpin formation by introducing such pairs in the (39L + V) stretch using the single-residue constructs reported above for comparison.

After an initial screening of a small number of Asn-Asn pairs, we focused mainly on the N8 position, and made a complete set of pairs with the second Asn in positions 32–39. For the N10 and N12 pairs, we made constructs with the second Asn in positions 33, 35, 37, 38 (for N12), and 39. Strikingly, there is an almost twofold increase in f^{ng} for the N8–N33, N8–N37, and N12–37 constructs over the corresponding single-Asn constructs, while all the other Asn-Asn constructs have f^{ng} values similar to those of the corresponding single-Asn constructs.

The same set of constructs but with Asp instead of Asn were tested (Figure 3(b)). The D8-D33 and D8-D37 constructs have ~1.5-fold higher f^{ng} values than seen for the corresponding individual mutations. In addition, the D10–D35, D10–D37, and D10–D39 constructs, and all constructs that include D12, have higher f^{ng} values than those of the corresponding single-Asp constructs.

As a control to ensure that the poly-Leu segments in even the poorly glycosylated constructs were integrated properly into the microsomal membrane, we made a final series of constructs where the H1 transmembrane segment and a part

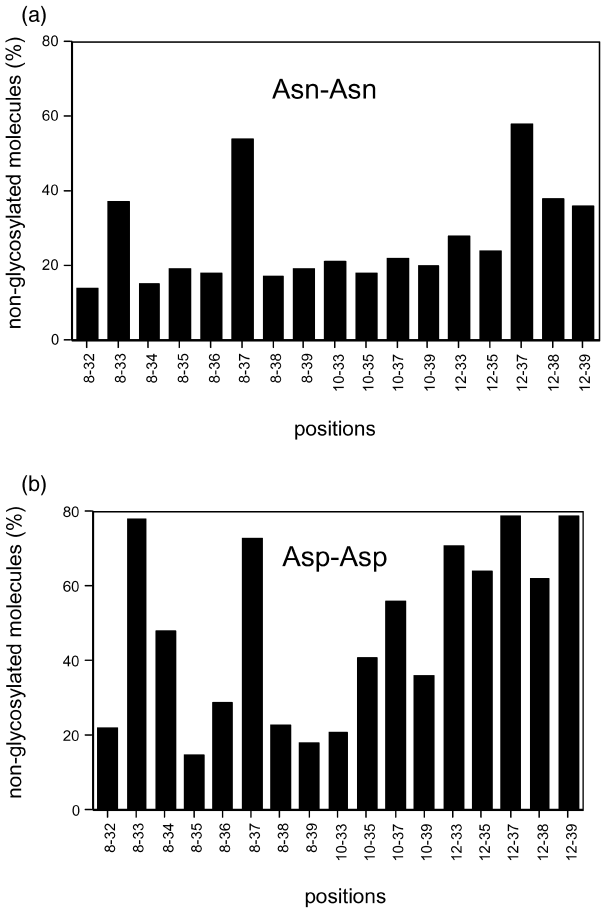

Figure 3. Degree of helical hairpin formation measured as the fraction of non-glycosylated molecules (f^{ng}) observed after *in vitro* translation in the presence of RM for Asn-Asn (a) and Asp-Asp (b) pairs in the (39L + V) H2 segment. The positions of the Asn or Asp residues are counted from the N-terminal end of the (39L + V) segment.

of P1 (residues 5–46) were deleted, leaving the poly-Leu stretch as the only hydrophobic segment in the protein. These constructs were expressed in the presence of RM, and their membrane integration was tested by alkaline extraction of the membranes.[28,29] Two typical examples are shown in Figure 4. The D33(ΔH1) construct is partially glycosylated when expressed in the presence of RM, and both the glycosylated and the non-glycosylated molecules remain in the membrane pellet (lanes 3 and 4). Likewise, even though the D8-D33(ΔH1) construct is glycosylated only weakly, the bulk of the molecules remain in the membrane pellet when the protein is expressed in the presence of RM. Similar results were obtained for the other ΔH1 constructs tested (D33(ΔH1), D37(ΔH1), D8-D35(ΔH1), and D8-D37(ΔH1); data not shown).

Discussion

In earlier work on helical hairpin formation, we have shown that single polar or charged residues introduced in the middle of a sufficiently long poly-Leu transmembrane segment can induce an almost complete conformational transition from a long single-spanning transmembrane segment to a helical hairpin.[20–22,26,30] We have further shown that charged residues placed immediately downstream of the poly-Leu transmembrane segment can induce the formation of helical hairpins even when no polar or charged residue is present within the hydrophobic stretch itself.[23]

Inspired by the observation that hydrogen bonding between Asn or Asp residues in transmembrane helices in detergent micelles, model membranes, or the *E. coli* inner membrane can drive oligomerization,[12,13,31] we have now tested whether pairs of Asn or Asp residues, i.e. residues shown to mediate strong dimerization in these studies, can promote the formation of intramolecular helical hairpins during membrane protein assembly into the ER membrane, i.e. within the context of the Sec61 translocon.

As shown in Figures 2 and 3, Asn-Asn and Asp-Asp pairs placed in a model (39L + V) transmembrane segment can increase the efficiency of helical hairpin formation by 1.5–2-fold over that seen for the corresponding single Asn or Asp residues. Even more strikingly, the effect is highly position-specific. For both Asn and Asp, pairs in positions 8–33 and 8–37 in the 40 residue long hydrophobic segment induce the formation of a helical hairpin, as does the N12–N37 pair and all pairs with Asp in positions 10 or 12 combined with a second Asp in positions 33, 35, 37 or 39.

The Asn and Asp 8–33 and 8–37 pairs are especially interesting, as they are clearly position-specific (a shift of either of the two residues by one or two positions abolishes the effect) and further involve residues that are both one to two turns from the ends of the (39L + V) stretch. Moreover, if one assumes a helical hairpin conformation for the (39L + V) stretch, residues 33 and 37 will be located one turn apart on the same face of the C-terminal helix of the hairpin. These pairs of residues would thus be in a perfect position to stabilize the helical hairpin conformation by inter-residue hydrogen bonding.

For Asn, the only other pair that has an increased level of helical hairpin formation compared to the two corresponding single-Asn constructs is N12–N37. Presumably, N12 is near the middle of the first helix in the hairpin, while N37 is close to the C-terminal end of the second helix, making a direct interaction a less likely explanation in this case. Since both N12 and N37 have a slightly higher level of helical hairpin conformation than the other single-Asn constructs, there may be an additive effect of the two Asn residues that reflects, e.g. interactions with the translocon rather than inter-residue hydrogen bonding. Further studies will be necessary to resolve this point.

The Asp-Asp pairs are less informative, since the single-Asp mutations, in general, have a stronger effect than the single-Asn mutations (Figure 2(b)).

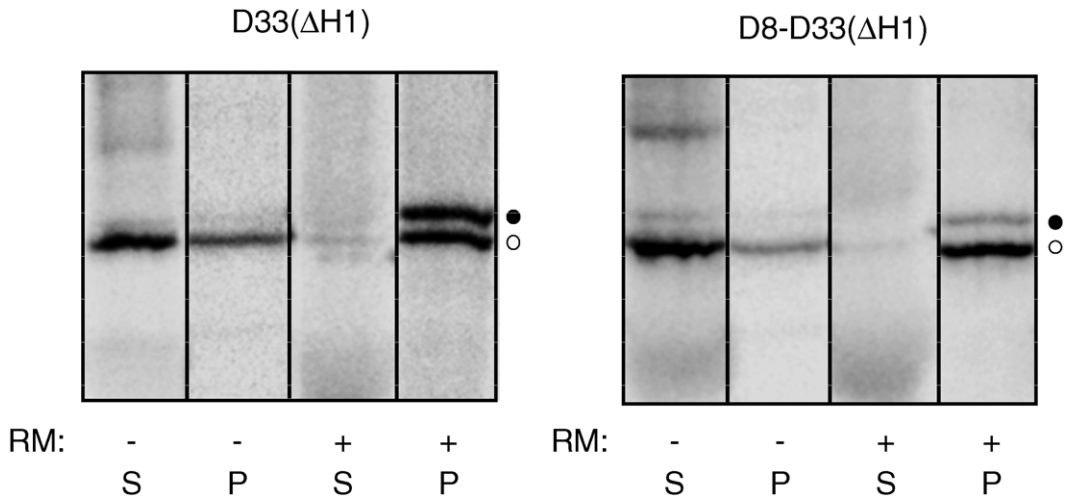

Figure 4. Alkaline extraction of the D33(ΔH1) and D8-D33(ΔH1) constructs expressed *in vitro* in the absence (−) and presence (+) of RM. Unglycosylated and glycosylated molecules are indicated by the white dot and the black dot, respectively. S, supernatant; P, membrane pellet.

It is thus difficult to discern an obvious pattern in the Asp-Asp pair constructs (Figure 3(b)). Nevertheless, it is interesting to note that helical hairpin formation is clearly increased in the 8–33 and 8–37 pairs also for Asp.

If our interpretation that the 8–33 and 8–37 Asn and Asp pairs indeed stabilize the helical hairpin conformation by hydrogen bonding is correct, it implies, first, that the formation of the helical hairpin must take place in a non-aqueous environment within the translocon, since stable inter-residue hydrogen bonding would not be expected in an aqueous environment; previous results from our laboratory also point in this direction.[21] Second, it further implies that the two halves of the helical hairpin formed in the (39L + V) segment cannot rotate freely relative to one another, as only positions on one face of the two helices (8–33 and 8–37 but not 8–35 or 10–33) are implicated. It is not immediately obvious why these faces are special; perhaps the polar residues flanking the hydrophobic stretch help orient the helices relative to the translocon. In any case, our results suggest that very specific helix–helix interactions can be formed within the context of the ER translocon and that such interactions can have a dramatic effect on membrane protein topology.

Materials and Methods

Enzymes and chemicals

Unless stated otherwise, all enzymes were from Promega (Madison, WI, USA). [^{35}S]Met, ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides, and the cap analog m7G(5′)ppp(5′)G were from Amersham-Pharmacia (Uppsala, Sweden). Plasmid pGEM1, DTT, transcription buffer and rabbit reticulocyte lysate were from Promega. Spermidine was from Sigma. Oligonucleotides were from Cybergene (Stockholm, Sweden).

DNA manipulations

For cloning into and expression from the pGEM1 plasmid, the 5′ end of the *lep* 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.[32] Thus, the 5′ region of the gene was modified to:

…ATAACCCTCTAGAGCCACCATGGCGAAT…

(*Xba* I site and initiator codon underlined).

Replacement of the H2 region in Lep was performed as described,[27] i.e. 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 by the appropriate double-stranded oligonucleotides. Site-specific mutagenesis used to add *Bcl* I and *Nde* I restriction sites at the 3′ and 5′ ends of H2 in Lep and to introduce an Asn-Ser-Thr acceptor site for N-linked glycosylation was performed according to the method of Kunkel.[33,34] The glycosylation acceptor site was designed as described,[35] i.e. by replacing three codons positioned 20 codons downstream of H2 with codons for the acceptor tripeptide Asn-Ser-Thr. In all constructs, the naturally occurring glycosylation site at Asn^{214} in Lep was removed by an Asn^{214} → Gln mutation. Residues 59–81 in H2 were replaced by a (39L + V) sequence of the design $LIK_4L_{29}VL_{10}Q_3P$ (subscripts indicate the number of consecutive residues).

The QuickChange site-directed mutagenesis kit (Stratagene) was used for the introduction of Asn or Asp residues in position 8, 10, and 12 and in positions 33–39 of the (39L + V) stretch. All mutants were confirmed by DNA sequencing of plasmids using ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Expression *in vitro*

The constructs in pGEM1 were transcribed by SP6 RNA polymerase for one hour at 37 °C. The transcription mixture was as follows: 1–5 μg of DNA template, 5 μl of 10 × SP6 H-buffer (400 mM Hepes–KOH (pH 7.4), 60 mM magnesium acetate, 20 mM spermidine–HCl), 5 μl of BSA (1 μg/μl), 5 μl of 10 mM m7G(5′)ppp(5′)G,

5 μl of 50 mM DTT, 5 μl of rNTP mix (10 mM ATP, 10 mM CTP, 10 mM UTP, 5 mM GTP), 18.5 μl of water, 1.5 μl of RNase inhibitor (33 units/μl), 0.5 μl of SP6 RNA polymerase (40 units/μl). Translation of 1 μl of mRNA was performed as described[36] at 30 °C for one hour in 9 μl of nuclease-treated reticulocyte lysate, 1 μl of RNase inhibitor (40 units/μl), 1 μl of [^{35}S]Met (15 μCi/μl), 1 μl of amino acids mix (1 mM each amino acid except Met), 1 μl of mRNA, and 1 μl of dog pancreas microsomes (2 units/μl; one unit is defined as the amount of microsomes required for 50% translocation of *in vitro* synthesized preprolactin). Translation products were analyzed by SDS-PAGE and gel bands were quantified on a Fuji FLA-3000 phosphoimager using the Fuji Image Reader 8.1j software. The glycosylation efficiency of a given mutant was calculated as the intensity of the glycosylated band divided by the summed intensities of the glycosylated and non-glycosylated bands. Sodium carbonate extraction of microsomes was carried out as described.[29]

Acknowledgements

This work was supported by grants from the Swedish Cancer Foundation, the Swedish Research Council, and the Margareta and Marcus Wallenberg Foundation to G.v.H. Dog pancreas microsomes were a kind gift from Dr M. Sakaguchi, Fukuoka.

References

1. Goder, V. & Spiess, M. (2001). Topogenesis of membrane proteins: determinants and dynamics. *FEBS Letters*, **504**, 87–93.
2. von Heijne, G. (2003). Membrane protein assembly *in vivo*. *Advan. Protein Chem.* **63**, 1–18.
3. Oliver, J., Jungnickel, B., Görlich, D., Rapoport, T. & High, S. (1995). The Sec61 complex is essential for the insertion of proteins into the membrane of the endoplasmic reticulum. *FEBS Letters*, **362**, 126–130.
4. Do, H., Falcone, D., Lin, J., Andrews, D. W. & Johnson, A. E. (1996). The cotransational integration of membrane proteins into the phospholipid bilayer is a multistep process. *Cell*, **85**, 369–378.
5. Heinrich, S., Mothes, W., Brunner, J. & Rapoport, T. (2000). The Sec61p complex mediates the integration of a membrane protein by allowing lipid partitioning of the transmembrane domain. *Cell*, **102**, 233–244.
6. Kanki, T., Young, M. T., Sakaguchi, M., Hamasaki, N. & Tanner, M. J. A. (2003). The N-terminal region of the transmembrane domain of human erythrocyte band 3—residues critical for membrane insertion and transport activity. *J. Biol. Chem.* **278**, 5564–5573.
7. Borel, A. C. & Simon, S. M. (1996). Biogenesis of polytopic membrane proteins: membrane segments assemble with translocation channels prior to membrane integration. *Cell*, **85**, 379–389.
8. Russ, W. P. & Engelman, D. M. (2000). The GxxxG motif: a framework for transmembrane helix–helix association. *J. Mol. Biol.* **296**, 911–919.
9. Zhou, F. X., Cocco, M. J., Russ, W. P., Brunger, A. T. & Engelman, D. M. (2000). Interhelical hydrogen bonding drives strong interactions in membrane proteins. *Nature Struct. Biol.* **7**, 154–160.
10. Senes, A., Gerstein, M. & Engelman, D. M. (2000). Statistical analysis of amino acid patterns in transmembrane helices: the GxxxG motif occurs frequently and in association with beta-branched residues at neighboring positions. *J. Mol. Biol.* **296**, 921–936.
11. Zhou, F. X., Merianos, H. J., Brunger, A. T. & Engelman, D. M. (2001). Polar residues drive association of polyleucine transmembrane helices. *Proc. Natl Acad. Sci. USA*, **98**, 2250–2255.
12. Choma, C., Gratkowski, H., Lear, J. D. & DeGrado, W. F. (2000). Asparagine-mediated self-association of a model transmembrane helix. *Nature Struct. Biol.* **7**, 161–166.
13. Gratkowski, H., Lear, J. D. & DeGrado, W. F. (2001). Polar side chains drive the association of model transmembrane peptides. *Proc. Natl Acad. Sci. USA*, **98**, 880–885.
14. Li, R., Mitra, N., Gratkowski, H., Vilaire, G., Litvinov, R., Nagasami, C. *et al.* (2003). Activation of integrin αIIbβ3 by modulation of transmembrane helix associations. *Science*, **300**, 795–798.
15. Dawson, J. P., Melnyk, R. A., Deber, C. M. & Engelman, D. M. (2003). Sequence context strongly modulates association of polar residues in transmembrane helices. *J. Mol. Biol.* **331**, 255–262.
16. Dawson, J., Weinger, J. & Engelman, D. (2002). Motifs of serine and threonine can drive association of transmembrane helices. *J. Mol. Biol.* **316**, 799–805.
17. Langosch, D., Brosig, B., Kolmar, H. & Fritz, H. J. (1996). Dimerisation of the glycophorin A transmembrane segment in membranes probed with the ToxR transcription activator. *J. Mol. Biol.* **263**, 525–530.
18. Russ, W. P. & Engelman, D. M. (1999). TOXCAT: a measure of transmembrane helix association in a biological membrane. *Proc. Natl Acad. Sci. USA*, **96**, 863–868.
19. Mothes, W., Heinrich, S., Graf, R., Nilsson, I., von Heijne, G., Brunner, J. & Rapoport, T. (1997). Molecular mechanisms of membrane protein integration into the endoplasmic reticulum. *Cell*, **89**, 523–533.
20. Monné, M., Nilsson, I., Elofsson, A. & von Heijne, G. (1999). Turns in transmembrane helices: determination of the minimal length of a "helical hairpin" and derivation of a fine-grained turn propensity scale. *J. Mol. Biol.* **293**, 807–814.
21. Nilsson, I., Johnson, A. E. & von Heijne, G. (2003). How hydrophobic is alanine? *J. Biol. Chem.* **278**, 29389–29393.
22. Monné, M., Hermansson, M. & von Heijne, G. (1999). A turn propensity scale for transmembrane helices. *J. Mol. Biol.* **288**, 141–145.
23. Hermansson, M., Monné, M. & von Heijne, G. (2001). Formation of helical hairpins during membrane protein integration into the endoplasmic reticulum membrane. Role of the N and C-terminal flanking regions. *J. Mol. Biol.* **313**, 1171–1179.
24. Wolfe, P. B., Wickner, W. & Goodman, J. M. (1983). Sequence of the leader peptidase gene of *Escherichia coli* and the orientation of leader peptidase in the bacterial envelope. *J. Biol. Chem.* **258**, 12073–12080.
25. Nilsson, I. & von Heijne, G. (1993). Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J. Biol. Chem.* **268**, 5798–5801.
26. Nilsson, I. & von Heijne, G. (1998). Breaking the

camel's back: proline-induced turns in a model transmembrane helix. *J. Mol. Biol.* **284**, 1185–1189.
27. Nilsson, I., Sääf, A., Whitley, P., Gafvelin, G., Waller, C. & von Heijne, G. (1998). Proline-induced disruption of a transmembrane α-helix in its natural environment. *J. Mol. Biol.* **284**, 1165–1175.
28. Fujiki, Y., Hubbard, A. L., Fowler, S. & Lazarow, P. B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment. *J. Cell Biol.* **93**, 97–102.
29. Sakaguchi, M., Mihara, K. & Sato, R. (1987). A short amino-terminal segment of microsomal cytochrome P-450 functions both as an insertion signal and as a stop-transfer sequence. *EMBO J.* **6**, 2425–2431.
30. Sääf, A., Hermansson, M. & von Heijne, G. (2000). Formation of cytoplasmic turns between two closely spaced transmembrane helices during membrane protein integration into the ER membrane. *J. Mol. Biol.* **301**, 191–197.
31. Howard, K. P., Lear, J. D. & DeGrado, W. F. (2002). Sequence determinants of the energetics of folding of a transmembrane four-helix-bundle protein. *Proc. Natl Acad. Sci. USA*, **99**, 8568–8572.
32. Kozak, M. (1992). Regulation of translation in eukaryotic systems. *Annu. Rev. Cell Biol.* **8**, 197–225.
33. Kunkel, T. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.
34. Geisselsoder, J., Witney, F. & Yuckenberg, P. (1987). Efficient site-directed *in vitro* mutagenesis. *BioTechniques*, **5**, 786–791.
35. Nilsson, I., Whitley, P. & von Heijne, G. (1994). The C-terminal ends of internal signal and signal-anchor sequences are positioned differently in the ER translocase. *J. Cell Biol.* **126**, 1127–1132.
36. Liljeström, P. & Garoff, H. (1991). Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. *J. Virol.* **65**, 147–154.

Edited by F. E. Cohen

(Received 18 June 2003; accepted 8 October 2003)