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Formation of Helical Hairpins during Membrane Protein Integration into the Endoplasmic Reticulum Membrane. Role of the N and C-terminal Flanking Regions

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The helical hairpin, two closely spaced transmembrane helices separated by a short turn, is a common structural element in integral membrane proteins. Previous studies on the sequence determinants of helical hairpin formation have focussed on the role of polar and charged residues placed centrally in a long stretch of hydrophobic residues, and have yielded a "propensity scale" for the relative efficiency with which different residues promote the formation of helical hairpins. In this study, we shift our attention to the role of charged residues flanking the hydrophobic stretch. Clusters of charged residues are known to hinder membrane translocation, and thus flanking charged residues may conceivably force a long hydrophobic segment to form a helical hairpin even if there are no or only weakly turn-promoting residues in the hydrophobic stretch. We indeed find that Lys and, more surprisingly, Asp residues strongly affect helical hairpin formation when placed next to a poly-Leu-based transmembrane segment. We also find that a cluster of four consecutive Lys residues can affect the efficiency of helical hairpin formation even when placed ~30 residues downstream of the hydrophobic stretch. These observations have interesting implications for the way we picture membrane protein topogenesis within the context of the endoplasmic reticulum (ER) translocon.

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Introduction

The helical hairpin, i.e. two closely spaced hydrophobic transmembrane helices separated by a short turn,[1] is a common structural element in integral membrane proteins and is thought to serve as an important "topogenic element" during membrane protein assembly.[2] In previous studies, we have shown that the efficiency of formation of helical hairpins during protein insertion into the endoplasmic reticulum (ER) membrane depends both on the overall length of the hydrophobic segment and on the identity of the central, potentially turn-forming residues.[3–6] Thus, charged, polar, and "classical" helix-breaking residues are good turn-formers and effectively induce helical hairpin formation when placed near the middle of a sufficiently long hydrophobic stretch, whereas centrally placed apolar residues do not induce helical hairpin formation but rather cause the hydrophobic stretch to insert as a single, long transmembrane helix that spans the membrane only once.

While the basic sequence determinants behind the formation of helical hairpins are thus beginning to be mapped out, one aspect that has so far not been addressed is the possible role of the residues that flank the hydrophobic stretch. As charged residues are known to hinder membrane translocation when present in certain sequence contexts, and thus to act as powerful topological determinants in both prokaryotic and eukaryotic membrane proteins,[2] we considered the possibility that charged residues in the regions flanking a long hydrophobic stretch may specifically affect helical hairpin formation. We now report that both Lys and Asp residues strongly affect helical hairpin formation when present close to a poly-Leu hydro-

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phobic stretch, the formation of a helical hairpin with a lumenally oriented turn is promoted by C-terminally flanking charged residues, whereas the formation of a helical hairpin with a cytoplasmically oriented turn is prevented by such flanking residues. We also find that a cluster of four consecutive Lys residues can affect the efficiency of helical hairpin formation even when placed ~30 residues downstream of the hydrophobic stretch. These findings show that a helical hairpin consists of a long hydrophobic stretch with a central tight turn, and a rather extended region of flanking residues must be included in the definition of this basic "folding unit" in integral membrane proteins.

Results

Model proteins and topology assay

As in our previous studies of helical hairpin formation in transmembrane helices, we have used the well-characterized *Escherichia 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 followed by a large C-terminal periplasmic domain (P2). When expressed *in vitro* in the presence of dog pancreas microsomes, Lep adopts the same membrane topology as in its natural environment in the inner membrane of *E. coli*,[7] i.e. with the N and C termini on the lumenal side,[8] Figure 1(a) (left).

To study the formation of helical hairpins with the short turn oriented towards the lumenal side of the ER membrane, H2 was substituted by poly-Leu stretches of the general composition ...P^{58}-LI $K_4L_{29}VL_{10}Q_3P$-E^{82}... (superscript numbers refer to residues in Lep , subscripts indicate the number of consecutive residues of a given kind), cf. Monné *et al*.[4,5] We have shown that this stretch forms a single transmembrane segment when the model protein is integrated *in vitro* into dog pancreas

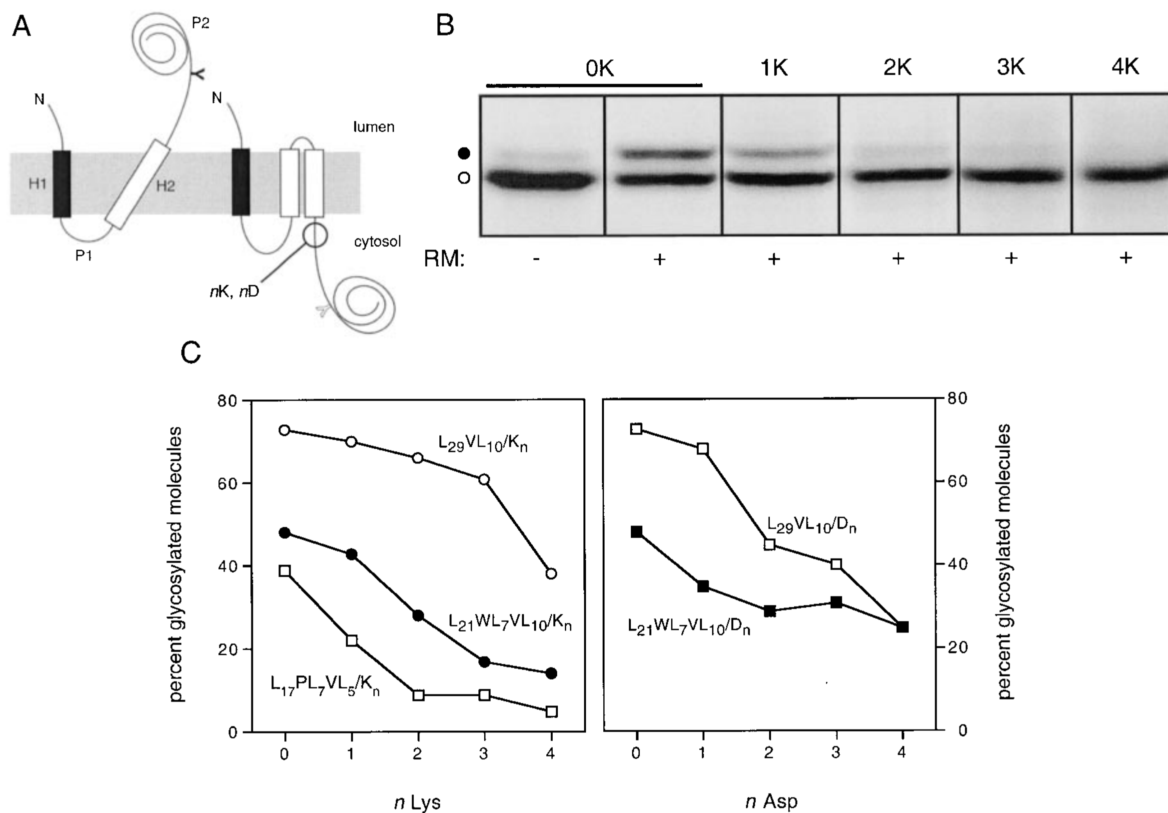


Figure 1. Lys and Asp residues at the C-terminal end of H2 promote the formation of helical hairpins with a lumenal turn. (a) The model protein used in this study. The H2 transmembrane segment in Lep (white) was replaced with three different poly-Leu-based segments of the composition $K_4L_{29}VL_{10}Q_3P$, $K_4L_{21}WL_7VL_{10}Q_3P$, and $K_4L_{17}PL_7VL_5Q_3P$, and one to four Lys or Asp residues were introduced at the C-terminal end of the poly-Leu stretch. A glycosylation acceptor site was placed 20 residues downstream of H2 (counting from the first Gln after the hydrophobic stretch). Depending on the lumenal or cytoplasmic localization of the P2 domain, the glycosylation acceptor site will either be modified (filled **Y**) or not (open **Y**). (b) $L_{17}PL_7VL_5$-derived constructs with different numbers of C-terminally flanking Lys residues were translated *in vitro* in the absence (−) and presence (+) of rough microsomes (RM) and analyzed by SDS-PAGE. Black and white dots indicate the glycosylated and non-glycosylated forms of the proteins, respectively. (c) Quantification of the full set of data. The percentage glycosylation was calculated as $100 \times I_+/(I_+ + I_-)$, where I_+ (I_-) is the intensity of the glycosylated (non-glycosylated) band.

microsomes, 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.[5] 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 H2; 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(a), right). We have shown, using alkaline extraction, that poly-Leu-based helical hairpins are properly assembled into the ER membrane, ruling out the possibility that low levels of glycosylation are caused by a failure in membrane integration rather than helical hairpin formation.[9] Turn formation can thus be assessed by *in vitro* transcription/translation of the relevant constructs in the presence of microsomes followed by quantification of the efficiency of glycosylation of the engineered *N*-glycosylation site.

A similar strategy was used to study the formation of helical hairpins with the turn oriented towards the cytoplasmic side of the ER membrane (see Figure 4(a)).[6] In this case, an extra transmembrane segment (H3) composed of a 40 residue poly-Leu stretch of the composition ...E^{225}-$TSL_{40}RS$-V^{233}... was introduced into the periplasmic P2 domain. This poly-Leu stretch is long enough to form either a single transmembrane segment or a helical hairpin, depending on the identity of residues placed near the middle of the hydrophobic stretch. The topology of the poly-Leu stretch can be determined easily by analyzing the glycosylation status of two strategically placed N-glycosylation acceptor sites, one upstream and one downstream of the hydrophobic stretch. Only the first site will be modified in constructs where the poly-Leu stretch spans the membrane once (Figure 1(a), left), whereas both will be modified when a helical hairpin is formed (Figure 4(a), right).

C-terminally flanking Lys and Asp residues promote the formation of helical hairpins with a lumenally oriented turn

As a first test of the effect of flanking charged residues on helical hairpin formation, we studied three constructs where the H2 transmembrane segment in Lep was replaced by poly-Leu-based segments of the composition $K_4L_{29}VL_{10}Q_3P$, $K_4L_{21}WL_7VL_{10}Q_3P$, and $K_4L_{17}PL_7VL_5Q_3P$. As shown previously,[5] the first, uniformly hydrophobic stretch forms a single transmembrane segment, while the two other constructs both have a mixed topology with a helical hairpin formed in approximately 50% of the molecules. The overall length of the hydrophobic segment (31 residues) in the $K_4L_{17}PL_7VL_5Q_3P$ construct is close to the minimum length required for helical hairpin formation.[5]

One to four positively charged Lys residues were inserted immediately downstream of the three hydrophobic stretches (replacing one to four residues of the Q_3P sequence: KQ_2P, K_2QP, K_3P, K_4) and helical hairpin formation was assayed by determining the fraction of glycosylated molecules, Figure 1(b). For all three constructs, the fraction of glycosylated molecules decreases with the number of C-terminally flanking Lys residues, Figure 1(c) (left panel). Two extra lysine residues are sufficient to ensure fully efficient helical hairpin formation in the $L_{17}PL_7VL_5$ construct, and a significant degree of helical hairpin formation is evident even in the uniformly hydrophobic $L_{29}VL_{10}$ construct when four lysine residues are added.

Since the difference in degree of helical hairpin formation between the two ''hairpin-prone'' and the uniformly hydrophobic segments was maximal for three added lysine residues, we compared helical hairpin formation in the absence and presence of three C-terminally flanking Lys residues for an additional series of constructs with the general composition $K_4L_{21}XL_7VL_{10}K_3P$ (where X is any of the 20 natural amino acids). The X residues were chosen on the basis of our earlier results with the $K_4L_{21}XL_7VL_{10}Q_3P$ constructs[4,5] to reflect the relative efficiency of helical hairpin formation induced by hydrophobic and mildly polar amino acids. As seen in Figure 2, the effect of adding three C-terminal lysine residues is minor for the hydrophobic residues, but is quite dramatic for Trp, Ser, Cys, and Thr (compare the white and black bars). As a comparison, Figure 2 also shows previously obtained results[5] for the same poly-Leu stretch lacking flanking charged residues, but with a pair of residues inserted in the middle of the stretch (gray bars). From this comparison, it is clear that the effect of three flanking lysine residues is similar to that seen when a second polar X residue is introduced near the middle of the hydrophobic stretch.

We also tested the effects on helical hairpin formation by negatively charged Asp residues. Again, the $K_4L_{29}VL_{10}Q_3P$ and $K_4L_{21}WL_7VL_{10}Q_3P$ constructs were used, and the Q_3P sequence was progressively replaced by one to four aspartic acid residues, Figure 1(c) (right panel). While overall the effects were similar to those seen for Lys (cf. Figure 1(c), left panel), Asp appears to promote helical hairpin formation somewhat more efficiently than Lys for the uniformly hydrophobic $K_4L_{29}VL_{10}Q_3P$ construct, while the differences seen for the $K_4L_{21}WL_7VL_{10}Q_3P$ construct are less conspicuous.

Finally, we compared the effects of N and C-terminally flanking Asp and Lys residues on helical hairpin formation in a $X_4L_{29}VL_{10}Z_4$ construct (X, Z = K or D), where four consecutive Lys or Asp residues were inserted in all four combinations immediately upstream and downstream of the hydrophobic stretch, Figure 3. Quantification of the degree of glycosylation of the various con-

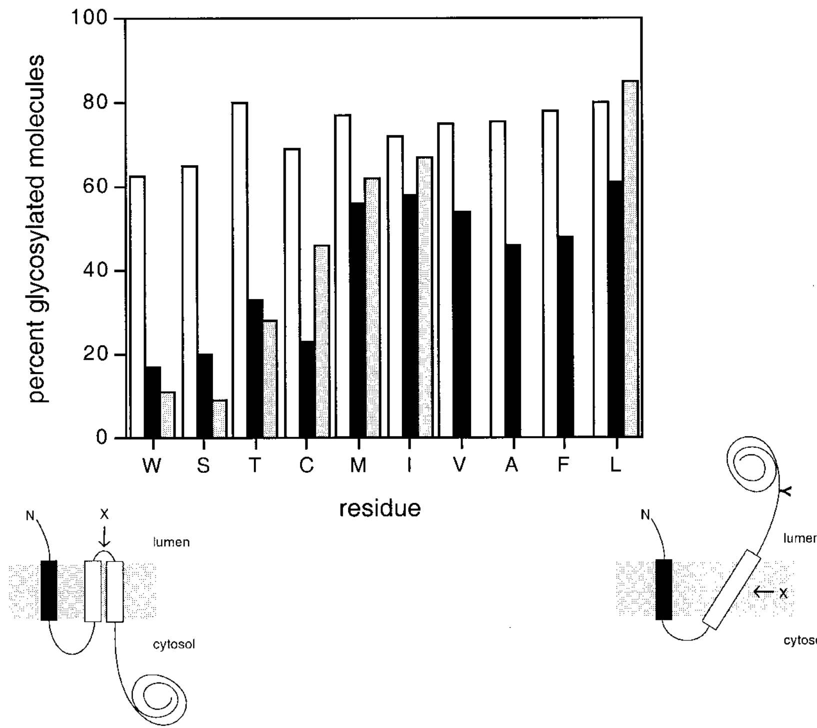
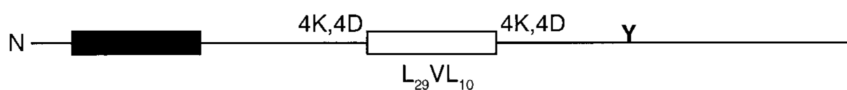


Figure 2. Helical-hairpin formation in H2-segments of the general composition $K_4L_{21}XL_7VL_{10}K_3P$ (black bars), where X is any of the 20 natural amino acids. Results from our earlier turn propensity study,[5] where H2 was substituted by a stretch of the design $K_4L_{21}XL_7VL_{10}Q_3P$ (white bars) or $K_4L_{20}X_2L_7VL_{10}Q_3P$ (gray bars) are included for comparison.

structs revealed that the identity of the N-terminally flanking charged residues made no difference, ruling out an effect dependent on, e.g. charge-pairing between the N and C-terminal flanking regions.

To make sure that the low levels of glycosylation seen for the these constructs were not caused by a failure of the helical hairpin to insert into the membrane, we expressed a truncated form of the $K_4L_{29}VL_{10}D_4$ construct where residues 5-46 (including the H1 transmembrane segment) were deleted, thus leaving the $L_{29}VL_{10}$ stretch as the only potential membrane-spanning segment. As expected, the molecules were not glycosylated and were associated with the membrane pellet after alkaline extraction of the



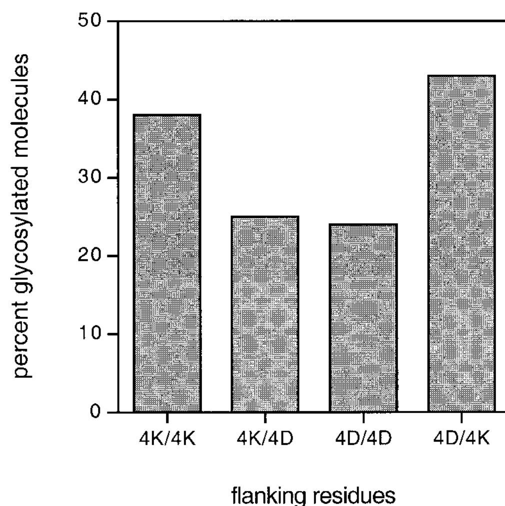


Figure 3. N-terminally flanking residues do not affect helical hairpin formation in H2 (white). Four Lys or four Asp residues were introduced simultaneously at both ends of an H2-stretch of the composition $L_{29}VL_{10}$, and the degree of glycosylation was determined for each combination of K_4 and D_4 flanking segments.

microsomes,[10] thus demonstrating efficient membrane integration (data not shown).

We conclude that the efficiency of formation of helical hairpins with a lumenally oriented turn is strongly affected by the presence of C-terminally (but not N-terminally) flanking charged residues in our model protein, that a helical hairpin can be induced even in a uniformly hydrophobic segment provided that the number of C-terminally flanking charged residues is sufficiently high, and that the efficiency of helical hairpin formation can be increased significantly by flanking charged residues in constructs such as $K_4L_{17}PL_7VL_5Q_3P$, where the overall length of the hydrophobic segment is close to the minimum length required for the formation of a helical hairpin. Furthermore, both positively and negatively charged C-terminally flanking residues promote helical hairpin formation in this context, i.e. the sign of the charge has little effect. It should be noted, though, that the lumenal or cytoplasmic location of the N-terminal end of the poly-Leu segment may be fixed by the topology of the N-terminal part of the protein (i.e. the H1-P1 part), and thus that the effects of N-terminally flanking residues may well be different in constructs where the poly-Leu stretch is at the N terminus.

C-terminally flanking Lys and Asp residues inhibit the formation of helical hairpins with a cytoplasmically oriented turn

We have shown that helical hairpins with a cytoplasmically oriented turn can be induced in a long poly-Leu stretch by the introduction of pairs or triplets of polar or charged residues near the middle of the hydrophobic stretch.[6] To study the effect of C-terminally flanking charged residues in this context, we used a construct with a poly-Leu-based stretch of the composition $TSL_{19}P_2L_{19}RS$ placed in the middle of the P2 domain, and the topology of this stretch (a single transmembrane span or a helical hairpin) was determined by assessing the glycosylation status of two *N*-glycosylation acceptor sites flanking the hydrophobic stretch, Figure 4(a). The $L_{19}P_2L_{19}$ segment was chosen because it is poised near the threshold for helical hairpin formation and has an intermediate level of doubly glycosylated molecules (56 %).

Again, from one to four Lys or Asp residues were introduced by replacing one to four residues immediately C-terminally to the L_{19} P_2L_{19} stretch, and the fraction of doubly *versus* singly glycosylated molecules was determined, Figure 4(b). As seen in Figure 4(c), the degree of helical hairpin formation decreased with increasing numbers of charged residues in this case, and the effect of Lys was somewhat stronger than that of Asp. We conclude that C-terminally flanking charged residues inhibit the formation of helical hairpins with a cytoplasmically oriented turn.

The effect on helical hairpin formation of C-terminally flanking Lys and Asp residues decreases with the distance from the hydrophobic stretch

Finally, we studied the effect of increasing the separation between the charged residues (four Lys or four Asp) and the hydrophobic stretch for both "lumenal turn" ($K_4L_{29}VL_{10}Q_3P$ construct) and "cytoplasmic turn" ($TSL_{19}P_2L_{19}RS$ construct) helical hairpins. As shown in Figure 5, the K_4 segment promoted the formation of a helical hairpin with a lumenally oriented turn even when placed 32 residues downstream of the hydrophobic stretch (left panel), while the effect of a D_4 segment decreased much more rapidly with the separation distance. Similarly, the K_4 segment inhibited the formation of a helical hairpin with a cytoplasmically oriented turn up to larger separation distances than did the D_4 segment, although the effect drops off more rapidly in this case (right panel). Thus, the efficiency of helical hairpin formation can depend on sequence determinants located quite far away from the hydrophobic transmembrane segment.

Discussion

The helical hairpin appears to be a basic folding unit in multi-spanning integral membrane proteins, and is thus central to our understanding of membrane protein topology. Based on analyses of model membrane proteins specifically designed to facilitate the study of helical hairpin formation during membrane protein insertion into the ER membrane, we previously quantified the relative propensities of the 20 natural amino acids to induce the formation of helical hairpins when placed in the middle of a long poly-Leu stretch, both for helical hairpins with lumenally and cytoplasmically oriented turns.[4–6,9] We have also determined the minimal length of the hydrophobic segment required for efficient helical hairpin formation.[5]

Membrane protein topology is, however, not determined by only the hydrophobic transmembrane helices themselves. Charged residues in short loops connecting the hydrophobic segments are potent topogenic determinants; the best-known effect in this regard is codified in the so-called positive inside rule, which states that positively charged loops tend to remain on the cytoplasmic side of the membrane.[11,12] Based on this premise, we hypothesized that the tendency for helical hairpin formation in a long hydrophobic stretch would depend on the number of charged residues in the immediate flanking regions, in addition to the characteristics of the hydrophobic stretch itself.

In this study, we have thus asked whether helical hairpin formation can be influenced by flanking positively and negatively charged residues (Lys and Asp). We find that N-terminally flanking residues have no effect on helical hairpin formation in our model protein (possibly because the lumenal

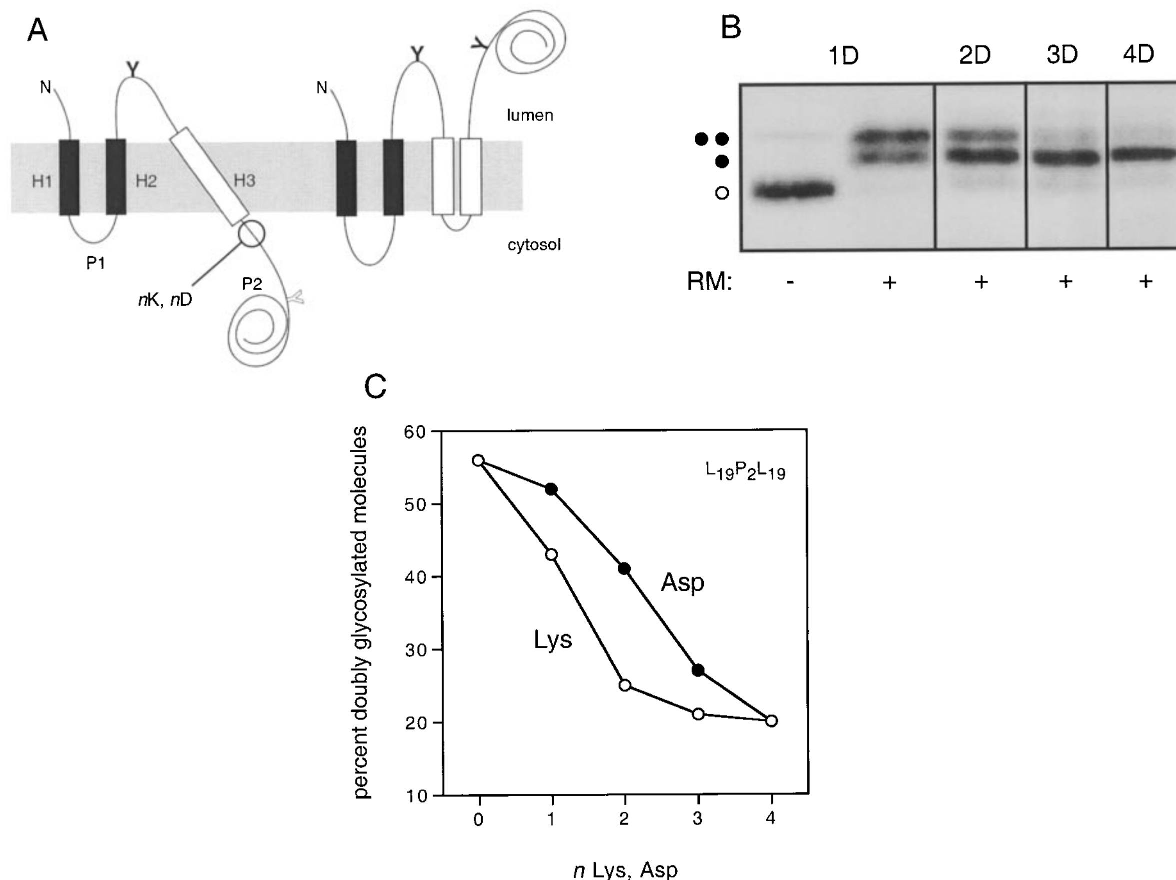


Figure 4. Lys and Asp residues at the C-terminal end of H2 inhibit the formation of helical hairpins with a cytoplasmic turn. (a) A 40 residue poly-Leu stretch (H3, white) was inserted into the middle of the P2 domain, and the glycosylation status of two Asn-X-Thr glycosylation acceptor sites flanking H3 was determined after *in vitro* translation in the presence of dog pancreas microsomes (glycosylated and non-glycosylated glycosylation acceptor sites are indicated, respectively, by filled **Y** and open **Y**). Constructs where H3 forms a single transmembrane segment (left) will be glycosylated on only one site, whereas those where H3 forms a helical hairpin (right) will be glycosylated on both sites. (b) $TSL_{19}P_2L_{19}RS$-derived constructs with different numbers of C-terminally flanking Asp residues were translated *in vitro* in the absence (−) and presence (+) of rough microsomes (RM) and analyzed by SDS-PAGE. Molecules with zero, one, and two modified glycosylation sites are indicated by one white dot, one black dot, and two black dots, respectively. (c) Quantification of the full set of data for C-terminally flanking Lys (white dots) and Asp (black dots) residues. The percentage of doubly glycosylated molecules was calculated as the quotient between the intensity of the doubly glycosylated band divided by the summed intensities of the doubly and singly glycosylated bands.

or cytoplasmic location of the N terminus of the particular hydrophobic stretch studied here is determined by the topology of the preceding part of the model protein), whereas charged residues of either sign placed C-terminally to the hydrophobic stretch generally promote the cytoplasmic location of the C-terminal end of the hydrophobic stretch. Thus, charged C-terminally flanking residues promote helical hairpin formation in constructs where the turn is oriented towards the lumenal side of the ER membrane, and weaken the tendency to form helical hairpins in constructs where the turn is oriented towards the cytoplasmic side.

Interestingly, flanking positively and negatively charged residues have similar effects on helical hairpin formation when located close to the hydrophobic stretch (Figures 1 and 4), suggesting that they somehow "sense" the sidedness of the ER membrane during the co-translational membrane assembly of the model protein. Given the high hydrophobicity of the poly-Leu stretch used in these studies, it is possible that the hydrophobic stretch partitions into the lipid environment almost immediately upon entering the protein-conducting translocon channel in the ER membrane,[13,14] thus facilitating interactions between the charged flanking residues and the lipid head groups. The surprising observation that an effect on the formation of a helical hairpin with a lumenal turn of a stretch of four Lys residues can be detected even at quite large separation distances (~30 residues; Figure 5) between the hydrophobic stretch and the charge cluster further shows that a considerable length of nascent chain can impact the process of hairpin for-

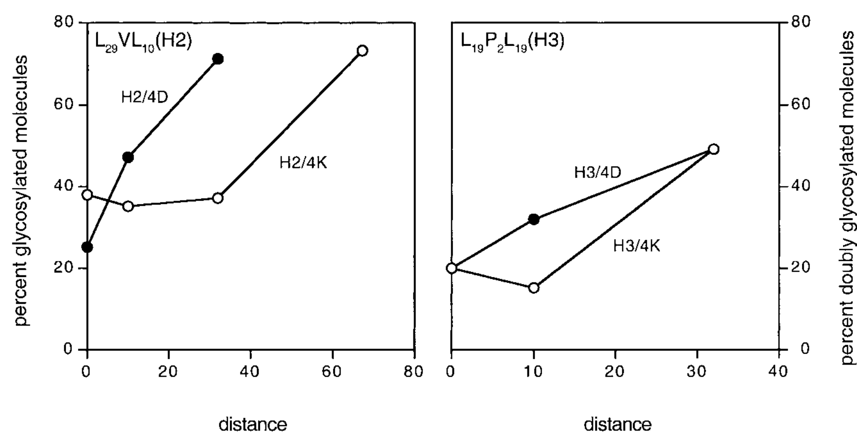


Figure 5. The effect on helical hairpin formation of C-terminally flanking Lys and Asp residues decreases with the distance from the hydrophobic stretch. Four Lys (white dots) or Asp (black dots) residues were placed at different distances C-terminally to the H2 $L_{29}VL_{10}$ stretch (left panel) or the H3 L_{19} P_2L_{19} stretch (right panel). The percentage singly (left panel) or doubly (right panel) glycosylated molecules were calculated as for Figures 1 and 4, respectively.

mation. This is somewhat reminiscent of the so-called charge block on protein translocation observed when a string of positively charged residues is placed downstream of a signal-anchor sequence[15,16] and indicates that a fairly sizeable portion of the nascent chain (the 40 residue hydrophobic stretch and at least 30 additional residues) may be present within the translocon when the helical hairpin forms. An alternative, though perhaps less likely, possibility is that a block of charged residues located in the ribosomal tunnel can trigger a change in the ribosome-translocon interaction that facilitates the helical hairpin formation, cf. Liao *et al*.[17]

The stronger effect on helical hairpin formation seen for positively charged Lys residues compared to the negatively charged Asp residues, both for helical hairpins with a cytoplasmic turn (Figure 4), and for helical hairpins with a lumenal turn at large separation distances (Figure 5), is in accord with the positive inside rule. From this point of view, it is surprising that the effects on helical hairpin formation exerted by negatively charged residues located close to the hydrophobic segment are very similar or even stronger than those seen for positively charged residues (Figure 1). In previous studies, negatively charged residues have been found to have only weak effects on the translocation of N-terminal tails across the membrane,[18,19] and on the translocation of C-terminal domains when placed downstream of a hydrophobic signal-anchor sequence.[20] We have no good explanation for their much stronger effects on helical hairpin formation.

In summary, our results show that flanking residues need to be included in the definition of the helical hairpin when viewed as a fundamental folding unit in integral membrane proteins. As a first approximation, membrane proteins may thus be pictured as being composed of single transmembrane helices (and their immediate flanking segments) spaced far apart in the sequence and of helical hairpins (again including their immediate flanking segments). This view rests on the assumption that the topology of a (long or short) hydrophobic segment can be influenced directly only by the part of the nascent chain that is present within the ribosome-translocon channel at the time when the segment integrates into the lipid bilayer, and thus that topology is, to an important extent, determined locally. The topological determinants for single transmembrane helices are quite well understood,[21] and our recent work on helical hairpin formation has now shed some light on this second kind of topogenic element. Finally, we note that the observations reported here may be relevant for improving current methods of membrane protein topology prediction,[22] since these methods do not distinguish between single transmembrane helices and helical hairpins.

Materials and Methods

Enzymes and chemicals

Unless stated otherwise, all enzymes were from Promega (Madison, WI, USA). Phage T7 DNA polymerase, *Bcl*I, [^{35}S]Met, ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides, and the cap analog m7G(5′)ppp(5′)G were from Amersham-Pharmacia (Uppsala, Sweden). Plasmid pGEM1, transcription buffer and rabbit reticulocyte lysate were from Promega. 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.[23] 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,[9] 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.[24,25] The glycosylation acceptor site was designed as described,[26] 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 Asn214 in Lep was removed by an Asn214 → Gln mutation. Residues 59-81 in H2 were replaced by a poly-Leu sequence of the design $LIK_4L_{29}VL_{10}Q_3P$ (subscripts indicate the number of consecutive residues) for the 40 residue poly-Leu construct. In the 31 residue poly-Leu constructs, four leucine residues were deleted from the N terminus and five leucine residues from the C terminus of the 40 residue poly-Leu stretch by PCR mutagenesis.

Introduction of the H3 region into the periplasmic P2 domain of Lep was performed as described,[6] i.e. by using a pING1 vector containing a *lep* gene with *Spe*I and *Bgl*II restriction sites introduced into codons 226-227 and 231-232, and then replacing the *Spe*I-*Bgl*II fragment by a double-stranded oligonucleotide encoding the sequence ...E^{225}-$TSL_{19}P_2L_{19}RS$-V^{233}... (superscript numbers refer to the wild-type Lep sequence) flanked by *Spe*I and *Bam*HI (compatible with *Bgl*II) restriction sites. The *lep* gene was further modified by an Asn214 → Gln mutation (to remove a potential glycosylation site present in the wild-type Lep sequence) and by mutations converting residues 96-98 and 258-260 to, respectively, Asn-Ser-Thr and Asn-Ala-Thr (thus introducing two new potential glycosylation sites flanking the H3 segment). The modified *lep* gene was re-cloned into the pGEM1-derived vector described above for the *in vitro* expression experiments.

The QuickChange site-directed mutagenesis kit (Stratagene) was used for the introduction of flanking charged Lys and Asp residues and all L → X substitutions. Asp residues flanking the H2 poly-Leu stretches on the N-terminal side were introduced by replacing the K_4 segment by a D_4 segment; Lys and Asp residues flanking the H2 poly-Leu stretches on the C-terminal side were introduced by replacing the Q3P segment (KQ_2P, K_2QP, K_3P, K_4, and the same for D); and Lys and Asp residues flanking the H3 poly-Leu stretch on the C-terminal side were introduced by replacing one to four residues immediately following the poly-Leu stretch. All mutants were confirmed by DNA sequencing using T7 DNA polymerase.

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 1 μg/μl BSA, 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 33 units/μl RNase inhibitor, 0.5 μl of 40 units/μl SP6 RNA polymerase. Translation of 1 μl of mRNA was performed as described[27] at 30 °C for one hour in 9 μl of nucelase-treated reticulocyte lysate, 1 μl of 40 units/μl RNase inhibitor, 1 μl of 15 μCi/μl [^{35}S]Met, 1 μl of amino acids mix (1 mM of each amino acid except Met), 1 μl of mRNA, and 1 μl of 2 units/μl dog pancreas microsomes (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 gels 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 quotient between the intensity of the glycosylated band divided by the summed intensities of the glycosylated and non-glycosylated bands for the H2-based constructs, and as the quotient between the intensity of the doubly glycosylated band divided by the summed intensities of the doubly and singly glycosylated bands for the H3-based constructs.

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