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Effects of 'hydrophobic mismatch' on the location of transmembrane helices in the ER membrane

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Abstract We have studied the effects of 'hydrophobic mismatch' between a poly-Leu transmembrane helix (TMH) and the ER membrane using a glycosylation mapping approach. The simplest interpretation of our results is that the lumenal end of the TMH is located deeper in the membrane for both short (negative mismatch) and long (positive mismatch) TMHs than for poly-Leu segments of intermediate length. We further find that the position-specific effect of Lys residues on the location of short TMHs in the membrane varies with an apparent helical periodicity when the Lys residue is moved along the poly-Leu stretch. We discuss these findings in the context of models for peptide–lipid interactions during hydrophobic mismatch.

Key words: Membrane protein; Protein structure; Glycosylation; Transmembrane helix; Hydrophobic mismatch

1. Introduction

The large majority of all integral membrane proteins belong to the so-called helix bundle class [1], i.e. their membrane-embedded domains consist of tightly packed hydrophobic transmembrane α-helices (TMHs). Much interest is currently focused on trying to understand in molecular detail how protein–lipid interactions affect various aspects of the structure and membrane location of TMHs [2].

We have approached these kinds of questions using the so-called glycosylation mapping technique, by which it is possible to measure the location of a TMH relative to the ER membrane (or, more precisely, relative to the active site of the ER-bound enzyme oligosaccharyl transferase, OST). In previous work, we have studied the effects of proline residues [3], charged residues [4–6], and aromatic residues [7] on the location in the ER membrane of a model poly-Leu TMH using glycosylation mapping.

TMHs vary considerably in length, from less than 15 to nearly 40 residues [8], although their hydrophobic core is typically only around 20 residues long. Situations where a TMH is either shorter or longer than the membrane is thick have been referred to as negative and positive 'hydrophobic mismatch' between the TMH and the membrane bilayer [9]. Biophysical studies of synthetic peptides in artificial phospholipid bilayers of varying thickness have shown that negative mismatch tends to prevent a hydrophobic segment from partitioning into the bilayer whereas positive mismatch seems to be more easily compensated for, apparently by a combination of local membrane thickening, tilting of the TMH, and possibly formation of bent TMHs [10–16].

Here, we have studied hydrophobic mismatch of poly-Leu-based TMHs of variable lengths in the ER membrane by glycosylation mapping. We find that short (≤ 20 residues) and long (≥ 30 residues) poly-Leu TMHs have their ends located further from the OST active site (and hence deeper in the membrane) than poly-Leu segments of intermediate length. We have also studied how Lys and Asp residues affect the location in the membrane of a poly-Leu TMH, and extend previous findings suggesting that Lys side-chains can 'snorkel' towards the membrane surface. Finally, we have observed that the position-specific effect of Lys residues on the location of short TMHs in the membrane varies with an apparent helical periodicity when the Lys residue is moved along the poly-Leu stretch.

2. Materials and methods

2.1. Enzymes and chemicals

Unless otherwise stated, all enzymes were from Promega. Ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides, the cap analog m7G(5′)ppp(5′)G, T7 DNA polymerase, and [^{35}S]Met were from Amersham Pharmacia Biotech (Uppsala, Sweden). Plasmid pGEM1, DTT, BSA, RNasin and rabbit reticulocyte lysate were from Promega (Madison, WI, USA). Spermidine and PMSF were from Sigma (St. Louis, MO, USA). Oligonucleotides were from Kebo Lab (Stockholm, Sweden) and Cybergene (Stockholm, Sweden). PCR purification kit and RNeasy RNA clean up kit were from Qiagen (Hilden, Germany). QuickChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA, USA).

2.2. 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 [17]. 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 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. Residues 59–81 in H2 were thus replaced by the poly-Leu sequence IKKKKL$_n$VQQQP. Site-specific mutagenesis used to add *Bcl*I and *Nde*I restriction sites at the 3′ and 5′ ends of H2 in Lep, to insert lysine residues into poly-Leu stretches, and to introduce Asn-Thr-Ser acceptor sites for *N*-linked glycosylation was performed according to the method of Kunkel [18,19] or using the QuickChange site-directed mutagenesis kit. Glycosylation acceptor sites were designed as described previously [20], i.e. by replacing three

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appropriately positioned codons downstream of H2 with codons for the acceptor tri-peptide Asn-Ser-Thr. In all constructs, the naturally occurring glycosylation site at Asn^{214} was removed by a $Asn^{214} \rightarrow Gln$ mutation. In the glycosylation construct Asn^{84}-Ser^{85}-Thr^{86} (numbering corresponding to the Lep wild-type sequence), the two flanking Pro residues were changed to Gln since both proline residues were found to reduce the efficiency of glycosylation, cf. [21]. All mutants were confirmed by sequencing of plasmid DNA using T7 DNA polymerase.

2.3. Expression in vitro

The constructs in pGEM1 were transcribed by SP6 RNA polymerase for one h 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 mg/ml), 5 μl of m7G(5′)ppp(5′)G (10 mM), 5 μl of DTT (50 mM), 5 μl of rNTP mix (10 mM ATP, 10 mM CTP, 10 mM UTP, 5 mM GTP), 18.5 μl of H_2O, 1.5 μl of RNase inhibitor (50 units), 0.5 μl of SP6 RNA polymerase (20 units). Translation was performed in reticulocyte lysate in the presence and absence of dog pancreas microsomes [22]. Proteins were analyzed by SDS–PAGE and gels were quantitated on a Fuji FLA-3000 phosphorimager using the Fuji Image Reader 8.1j 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. In general, the glycosylation efficiency varies by no more than ±5% between different experiments using the same batch of microsomes, and the precision in the MGD determinations is ±0.3 residues.

3. Results

3.1. The glycosylation mapping technique

The glycosylation mapping technique has been described in detail in previous work [3]. Briefly, the lumenally oriented active site of the ER enzyme OST is used as a fixed point of reference against which the position of a TMH in the ER membrane can be measured; in particular, mutations in a TMH 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 reference residue at the end of the TMH and the OST active site.

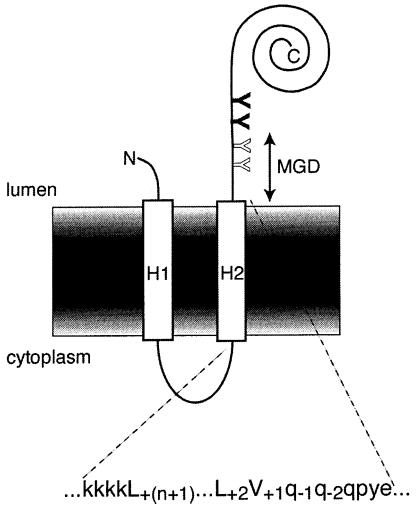

Fig. 1. The Lep-derived model protein used in this study. The H2 segment was replaced by a segment with four Lys at the N-terminus, three Gln and a Pro at the C-terminus and a hydrophobic stretch composed of *n* Leu residues and a Val. Potential glycosylation acceptor sites (Asn-Ser-Thr) were placed in various positions downstream of H2 (**Y** and 𝕐 denote glycosylated and non-glycosylated acceptor sites, respectively). MGD counting from Q_{-1}.

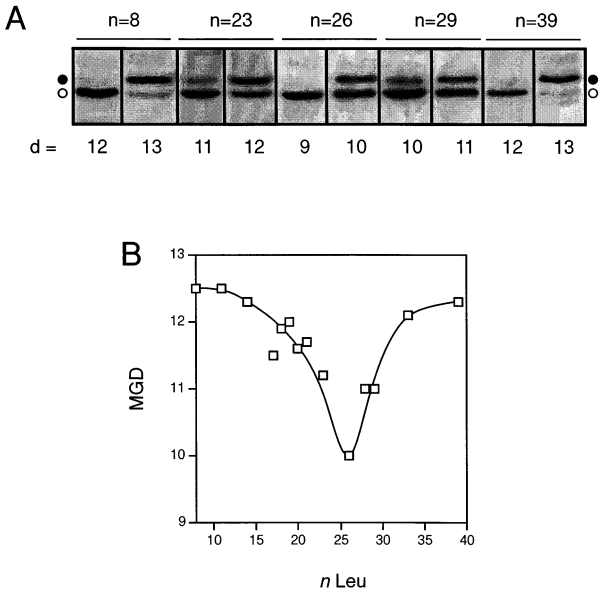

Fig. 2. Both negative and positive hydrophobic mismatch result in high MGD values. A: L_nV constructs with $8 \leq n \leq 39$ and with glycosylation acceptor Asn residues situated *d* residues downstream of Val_{+1} were analyzed by in vitro translation in the presence of dog pancreas microsomes (top). Glycosylated and non-glycosylated forms are indicated by black and white dots, respectively. Constructs with *d*-values bracketing the MGD are shown (MGD is defined as the value of *d* for which 40% of the molecules are glycosylated, and is determined by interpolation between the nearest integer *d*-values). B: MGD values determined for a large collection of L_nV constructs.

For the studies presented here, we used the well-characterized *Escherichia coli* inner membrane protein leader peptidase (Lep) which has two TMHs (H1 and H2) and N_{lum}/C_{lum} orientation when assembled into microsomal membranes [23]. As shown in Fig. 1, H2 was replaced with hydrophobic stretches composed of $n = 8$ to $n = 39$ leucines and one valine (L_nV) flanked by four N-terminal lysines and a C-terminal Gln-Gln-Gln-Pro segment. Various point mutants where individual residues in the L_nV segments were replaced by Lys or Asp residues were also studied.

Consensus *N*-glycosylation sites were engineered at different positions downstream of the L_nV stretch. All constructs were expressed in vitro in the presence of ER-derived dog pancreas microsomes, and MGD values were obtained by determining the position of the glycosylation acceptor Asn (counting from the Q_{-1} reference residue at the lumenal end of the L_nV segment, see Fig. 1) for which half-maximal glycosylation (i.e. 40%; ∼80% glycosylation is the maximum level seen in our in vitro system) was observed. The transition from no glycosylation to full glycosylation generally requires that the glycosylation acceptor site is moved only two or three residues, and is thus quite sharp. Partial glycosylation of constructs in the transition region may be caused either by small fluctuations in the location of the TMH relative to the OST active site or by small conformational fluctuations near the helix end or in the flexible segment between the TMH and the glycosylation site.

3.2. MGD values are minimal for poly-Leu segments of intermediate length

While earlier glycosylation mapping data have shown that short TMHs (negative mismatch) have their lumenal end lo-

cated deeper in the ER membrane than well-matched TMHs [3], positive mismatch has not been studied before using this technique. Thus, we used glycosylation mapping to study how positive hydrophobic mismatch affects the location of long L_nV segments in the ER membrane. As seen in Fig. 2, when the length of the L_nV stretch was varied from 9 to 40 residues, the MGD values first decreased from 12.5 ($n=8$; negative mismatch) to 10.0 ($n=26$), and then increased back to 12.3 ($n=39$; positive mismatch). It thus appears that the lumenal end also of very long TMHs is located deeper in the membrane than the lumenal end of intermediate length TMHs, suggesting that the lumenal end of the L_nV TMH is closest to the lumenal surface of the membrane when the hydrophobic mismatch is minimal.

The part of the curve between $n=8$ and $n=29$ is essentially identical to previous measurements [3], except for the $n=23$ and $n=29$ points, for which the previously reported MGD values were 9.7 and 9.6 rather than 11.2 and 11.0 as we now find. Prompted by this discrepancy, we have re-measured MGD values for a large number of previously analyzed constructs [3,4,7] using different batches of microsomes, and have only found minor differences (± 0.3 residues) except for a couple of constructs that will be specifically mentioned below. At present, we have no explanation for these discrepancies, but note that for very precise comparisons between different constructs, the same batch of microsomes should be used throughout. All data reported here were obtained with a single batch of microsomes.

3.3. Effects of Lys and Asp residues on the membrane location of L_nV TMHs

We have previously found that Lys and Asp residues placed at different positions in the lumenal, C-terminal half of the $L_{23}V$ stretch have distinct effects on the MGD values [4], suggesting that the side-chain of Lys residues may 'snorkel' towards the membrane–water interface. These observations have now been extended to include Lys and Asp residues placed further away from the lumenal end of the $L_{23}V$ TMH. As seen in Fig. 3, Lys has little effect on MGD when placed in positions -1 and +1 (i.e. at the lumenal end of the TMH; note that Lys positions are counted in a C- to N-terminal direction starting from V_{+1}, Fig. 1). When placed in positions +2 to +10, Lys causes a progressive decrease in MGD, with the most dramatic drop seen between positions +1 and +2. Finally, the effect on MGD rapidly disappears when Lys is placed in positions +13 and +16, i.e. in the cytoplasmic, N-terminal half of the TMH. As compared to our earlier results [4], the MGD values measured for the $L_{23}V(K_{-1})$ and $L_{23}V(K_{+1})$ constructs are now smaller by about one residue, while all other points agree within ± 0.3 residues.

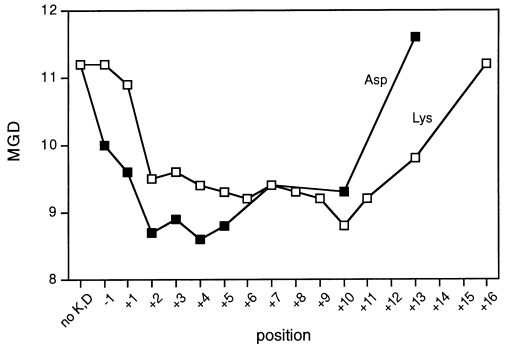

Fig. 3. Lys and Asp residues only affect MGD when located in the lumenal half of the $L_{23}V$ TMH. Single Lys (white squares) and Asp (black squares) residues were inserted in different positions in the $L_{23}V$ TMH and MGD values were determined. Lys and Asp positions are counted in a C- to N-terminal direction from Val_{+1} (cf. Fig. 1).

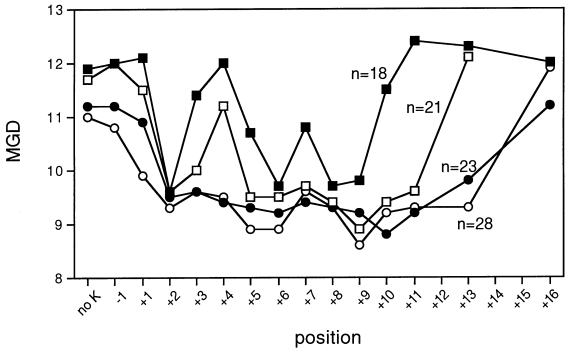

Fig. 4. MGD values vary strongly with the position of single Lys residues inserted into short but not long L_nV TMHs. A single Lys residue was inserted in different positions in L_nV TMHs and MGD values were determined ($n=18$: black squares; $n=21$: white squares; $n=23$ black dots; $n=28$: white dots). Lys positions are counted in a C- to N-terminal direction from Val_{+1}.

For Asp, the situation is somewhat different, with an initial drop in MGD seen for Asp in position -1 and a minimum MGD value reached already for position +2. The effect becomes weaker for positions +5 to +10, and disappears altogether for Asp in position +13.

We have also made a detailed analysis of the position-specific effects on MGD of Lys residues introduced into L_nV TMHs of different lengths, Fig. 4. While the results for long TMHs ($n=23$, $n=28$) are similar to each other and vary smoothly with the position of the Lys residue, there is a dramatic periodicity in the MGD values for the shorter $n=18$ and $n=21$ constructs. High MGD values are seen for Lys in position +4 and, to a lesser degree, position +7, while low values are seen for Lys in positions +2, +6, and +9. Further, the effect on MGD disappears when Lys is placed in position +10 for the $L_{18}V$ construct and in position +13 for the $L_{21}V$ construct. Interestingly, the MGD values for Lys in position +2 are very similar for all L_nV constructs, irrespective of their lengths.

To rule out that the periodicity is caused by charge-paring between the Lys residue and a Glu residue present in position -6 (see Fig. 1), Glu_{-6} was changed to Gln in the $L_{18}V(K_{+4})$ construct. The MGD value did not change as a result of this mutation (data not shown).

We conclude that Lys and Asp residues have distinct effects on the MGD values when introduced into the $L_{23}V$ TMH, and that the effect of Lys residues on the MGD values is strongly position-dependent for short L_nV TMHs, i.e. under conditions of negative mismatch.

4. Discussion

Hydrophobic mismatch between TMHs and lipid bilayers has so far been studied mostly by analyzing synthetic peptides incorporated into model membranes [10,14,15,24,25]. These

studies have suggested that very short peptides (negative mismatch) do not form TMHs but rather bind peripherally to the membrane surface, while very long peptides (positive mismatch) at least in part relieve the mismatch by tilting relative to the membrane normal.

Here, we have used the glycosylation mapping technique [3] to study hydrophobic mismatch involving poly-Leu-based TMHs present within a bona fide membrane protein, and using an in vitro system (dog pancreas microsomes) generally considered to closely mimic the situation in a living cell. In contrast to studies using synthetic peptides and artificial membranes, the hydrophobic stretch in our model protein is fixed in a transmembrane disposition by the flanking parts of the protein (unless it is too short to form a TMH, in which case the large C-terminal domain will remain on the cytoplasmic side of the membrane, cf. Fig. 1), and thus cannot equilibrate between transmembrane and non-integrated forms. The experimental set-up is in this respect distinct from and complementary to that used in the biophysical studies.

It should be noted that there is no formal proof that MGD values reflect the location of a fully lipid-embedded TMH: it is conceivable that glycan addition to the engineered glycosylation acceptor sites happens while the L_nV segment is held within the translocon channel. However, it has recently been shown that lipid exposure of the H1 TMH in Lep precedes glycan addition to an acceptor site engineered into the lumenal N-terminal tail [26], suggesting that also the L_nV segment used here is exposed to lipids and thus most likely folded into a bona fide TMH before glycan is added. Previous glycosylation mapping studies using TMHs whose location in the membrane has been determined using independent means are also consistent with a lipidic environment [3,4]. Even so, we cannot exclude that the poly-Leu TMHs studied here are still at least partly in contact with proteins in the Sec61 translocon complex at the time of glycan addition, and that this may to some extent affect their conformation and their location relative to the bilayer.

In a first set of experiments, the overall length of the hydrophobic stretch in a poly-Leu-based TMH of the composition K_4 L_nV Q_3P was varied from 9 to 40 residues, Fig. 2. A clear minimum in MGD was observed for $n = 26$. In the interval $14 \leq n \leq 26$, MGD drops by about 2.5 residues, i.e. by about 0.2 residues per Leu residues added to the TMH. This is in accord with a previously proposed model, Fig. 5 (left), in which it is assumed that short TMHs (negative hydrophobic mismatch) are oriented perpendicular to the membrane and that the hydrophobic segment is positioned symmetrically relative to the bilayer [3]. What is new in the present work is the extension of the curve to large n values (positive hydrophobic mismatch), and the surprising observation that the MGD values increase when the TMH becomes very long ($n > 26$). While tilting of the TMH in the membrane may contribute to this increase, it is not obvious why a highly tilted TMH would 'pull back' into the membrane to the extent observed here. Instead, we suggest a model where very long TMH segments would adapt to positive hydrophobic mismatch by forming both a transmembrane helix (perpendicular to the membrane surface) and a short terminal helix that is buried in the acyl chain region and oriented parallel to the membrane surface, Fig. 5 (right). Considering that highly titled TMHs are likely to induce unfavorable distortions in the lipid bilayer, it is not unreasonable to assume that the proposed

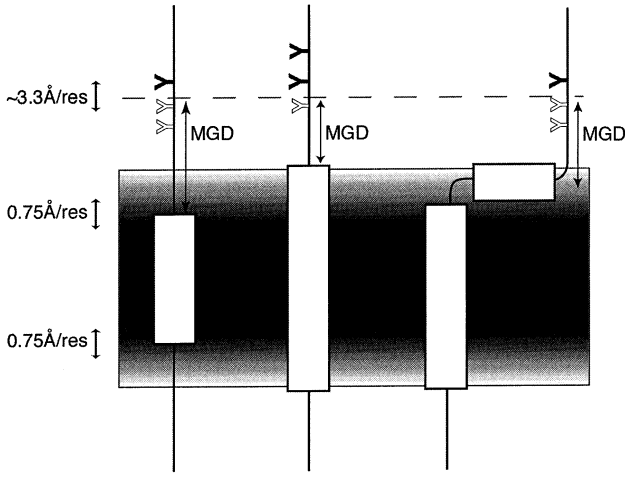

Fig. 5. Model for the membrane location of L_nV TMHs under conditions of negative hydrophobic mismatch (left), no mismatch (middle), and positive mismatch (right). For short L_nV TMHs (left), it is assumed that a perpendicular orientation has the lowest free energy. An increase in length by one hydrophobic residues extends the helix by 1.5 Å; if the locations of the two helix ends are symmetrically affected, each end moves 0.75 Å closer to the membrane surface. To reduce the MGD-value by one residue, the linker segment (assumed to be in a flexible, extended conformation) between the transmembrane helix and the OST active site needs to move 3–3.5 Å, i.e. MGD $\sim 0.75\ n/3.3 \approx 0.2\ n$. For very long L_nV TMHs (right), it is proposed that the transmembrane part of the L_nV segment remains perpendicular to the membrane and that the remaining part of the segment forms a helix that is oriented parallel to the membrane and is buried in the lumenal leaflet of the membrane. **Y** = glycosylated acceptor site, **Y** = non-glycosylated acceptor site.

structure may have a lower free energy than a uniformly tilted helix, although this hypothesis obviously needs further experimental and theoretical verification. As this extreme degree of positive hydrophobic mismatch is outside the range normally seen for TMHs in natural membrane proteins [8], this observation may not be of immediate biological relevance but rather addresses the more general biophysical problem of lipid–protein interactions.

We have also extended our previous studies [4] of the effects of Lys and Asp residues on the location of poly-Leu-based TMHs in the ER membrane. By moving the charged residue further away from the lumenal end of the TMH, we now clearly demonstrate that the location of the lumenal end relative to the OST active site is only affected by charged residues in the lumenal half of the TMH, and not when the charged residue is placed in the cytoplasmic half of the poly-Leu stretch, Figs. 3 and 4. Presumably, the effect exerted by a charged residue in the cytoplasmic half affects the location of the cytoplasmic end of the TMH, although this cannot be detected using the glycosylation mapping approach.

The differences between the effects on MGD of Lys and Asp residues placed close to the lumenal end of the $L_{23}V$ TMH seen in Fig. 3 are in accord with the so-called 'snorkel model' [2,4,27,28] which postulates that the long, aliphatic part of the Lys side-chain reaches up along the surface of the helix, placing the basic amino group in the vicinity of the acidic phospholipid head groups.

Interestingly, the effects on MGD exerted by a Lys residue are dramatically different depending on the length of the TMH, Fig. 4. For L_nV TMHs of a length close to the 'MGD minimum' discussed above (i.e. $n = 23$, 28), there is a

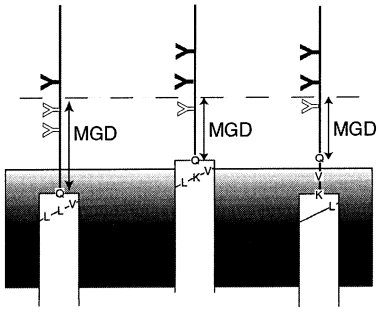

Fig. 6. MGD values can change either as a consequence of an en bloc movement of the TMH (middle), or as a consequence of local unwinding of the lumenal end of the TMH (right). The K_{+2} mutation is indicated. **Y** = glycosylated acceptor site, 𝕐 = non-glycosylated acceptor site.

large drop in MGD between Lys positions +1 and +2, followed by a more gradual decrease as the Lys residue is moved further towards the middle of the hydrophobic segment.

For the two shorter TMHs studied ($n = 18$, 21), the situation is different, however. In this case, there is a dramatic apparent periodicity in the MGD curve, with maxima for Lys in position +4 and, to a lesser degree, position +7, and minima for Lys in positions +2, +6, and +9. A much weaker periodicity of the same kind seems to be present also for the $n = 23$, 28 TMHs, as noted before [4]. The periodicity is suggestive of a helical pitch, where constructs with the Lys residues placed on the same side of the helix as the first flanking Gln residue (Q_{-1}) have high MGD values and those with the Lys residue on the opposite face have low MGD values.

Further work will be required to establish the precise reason for the strong periodicity seen for the short constructs; for now, we note that changes in MGD can be brought about either by a shift in membrane location of the whole TMH or by a local winding/unwinding of the lumenal end of the TMH, Fig. 6. It may be argued that the dramatic changes in MGD between, e.g. the $L_{18}V(K_{+1})$, $L_{18}V(K_{+2})$, and $L_{18}V(K_{+3})$ constructs are too large to be easily explained by a simple shift of the TMH relative to the membrane but rather suggest a local unwinding of the TMH when Lys is placed in position +2. It is also possible that the periodicity reflects an interaction between short TMHs and components of the Sec61 translocon, as has been observed in crosslinking experiments for signal peptides (which also have short hydrophobic regions) during early stages of the translocation of a nascent chain [29,30].

In conclusion, both negative and positive hydrophobic mismatch have strong effects on the location in the ER membrane of poly-Leu TMHs, and TMHs of different lengths also react differently to the introduction of a Lys residue in the hydrophobic stretch. Finally, Lys and Asp residues introduced into the cytoplasmic half of poly-Leu TMHs do not affect the membrane location of the lumenal end of the TMH.

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References

[1] von Heijne, G. (2000) Q. Rev. Biophys. 32, 285–307.
[2] Killian, J.A. and von Heijne, G. (2000) Trends Biochem. Sci. 25, 429–434.
[3] Nilsson, I., Sääf, A., Whitley, P., Gafvelin, G., Waller, C. and von Heijne, G. (1998) J. Mol. Biol. 284, 1165–1175.
[4] Monné, M., Nilsson, I., Johansson, M., Elmhed, N. and von Heijne, G. (1998) J. Mol. Biol. 284, 1177–1183.
[5] Chin, C.N. and von Heijne, G. (2000) J. Mol. Biol. 303, 1–5.
[6] Armulik, A., Nilsson, I., von Heijne, G. and Johansson, S. (1999) J. Biol. Chem. 274, 37030–37034.
[7] Braun, P. and von Heijne, G. (1999) Biochemistry 38, 9778–9782.
[8] Bowie, J.U. (1997) J. Mol. Biol. 272, 780–789.
[9] Killian, J.A. (1998) Biochim. Biophys. Acta Rev. Biomembr. 1376, 401–416.
[10] Harzer, U. and Bechinger, B. (2000) Biochemistry 39, 13106–13114.
[11] Opella, S., Marassi, F., Gesell, J., Valente, A., Kim, Y., Oblatt-Montal, M. and Montal, M. (1999) Nat. Struct. Biol. 6, 374–379.
[12] Marassi, F.M., Ma, C., Gratkowski, H., Straus, S.K., Strebel, K., Oblatt-Montal, M., Montal, M. and Opella, S.J. (1999) Proc. Natl. Acad. Sci. USA 96, 14336–14341.
[13] Kovacs, F.A., Denny, J.K., Song, Z., Quine, J.R. and Cross, T.A. (2000) J. Mol. Biol. 295, 117–125.
[14] Ren, J., Lew, S., Wang, Z. and London, E. (1997) Biochemistry 36, 10213–10220.
[15] Ren, J.H., Lew, S., Wang, J.Y. and London, E. (1999) Biochemistry 38, 5905–5912.
[16] de Planque, M., Goormaghtigh, E., Greathouse, D., Koeppe, R., Kruijtzer, J., Liskamp, R., de Kruijff, B. and Killian, J. (2001) Biochemistry, in press.
[17] Kozak, M. (1989) Mol. Cell. Biol. 9, 5073–5080.
[18] Geisselsoder, J., Witney, F. and Yuckenberg, P. (1987) BioTechniques 5, 786–791.
[19] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[20] Nilsson, I., Whitley, P. and von Heijne, G. (1994) J. Cell. Biol. 126, 1127–1132.
[21] Gavel, Y. and von Heijne, G. (1990) Protein Eng. 3, 433–442.
[22] Liljeström, P. and Garoff, H. (1991) J. Virol. 65, 147–154.
[23] Nilsson, I. and von Heijne, G. (1993) J. Biol. Chem. 268, 5798–5801.
[24] Webb, R., East, J., Sharma, R. and Lee, A. (1998) Biochemistry 37, 673–679.
[25] de Planque, M. (2000) Ph.D. Thesis, Department of Biochemistry of Membranes, Utrecht University, Utrecht.
[26] Heinrich, S., Mothes, W., Brunner, J. and Rapoport, T. (2000) Cell 102, 233–244.
[27] Segrest, J.P., De Loof, H., Dohlman, J.G., Brouilette, C.G. and Anantharamaiah, G.M. (1990) Proteins 8, 103–117.
[28] Shrivastava, I.H., Capener, C.E., Forrest, L.R. and Sansom, M.S. (2000) Biophys. J. 78, 79–92.
[29] Mothes, W., Jungnickel, B., Brunner, J. and Rapoport, T.A. (1998) J. Cell. Biol. 142, 355–364.
[30] Plath, K., Mothes, W., Wilkinson, B.M., Stirling, C.J. and Rapoport, T.A. (1998) Cell 94, 795–807.