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Importance of Hydrophobic Matching for Spontaneous Insertion of a Single-Spanning Membrane Protein†

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ABSTRACT: In this study, we have investigated the effect of hydrophobic mismatch between the thickness of the membrane and a transmembrane segment of a protein that directly inserts into the membrane bilayer. For this purpose we used mutants of the single-spanning Pf3 coat protein that can spontaneously insert into *Escherichia coli* membrane vesicles and large unilamellar vesicles (LUVs). The thickness of the liposomal bilayer could be altered by using lipids with different acyl chain lengths or by incorporation of cholesterol. The insertion efficiency of the protein clearly depended on the bilayer thickness, with most efficient insertion under hydrophobic matching conditions. To discriminate between effects of length and hydrophobicity, mutants with different synthetic transmembrane segments were constructed. These mutants inserted into LUVs in a mismatch-dependent manner. However, in particular for longer and less hydrophobic mutants, most efficient insertion was generally observed in thinner bilayers than expected on the basis of hydrophobic matching.

Integral membrane proteins are mainly anchored in the membrane by hydrophobic interactions between their transmembrane segments and the apolar acyl chains of the lipids. It is energetically unfavorable if hydrophobic residues are exposed to the aqueous environment or hydrophilic amino acids are in contact with lipid acyl chains in the interior of the membrane. The hydrophobic lengths of transmembrane segments are therefore expected to approximately match the thickness of the lipid bilayer.

Many studies have been performed to investigate how membrane proteins and peptides respond to hydrophobic mismatch (reviewed in refs *1* and *2*). The activity of some proteins, such as Ca^{2+}- and Na^+,K^+-ATPase, melibiose permease, or diacylglycerol kinase, clearly depends on bilayer thickness, with maximum activity presumably under hydrophobic matching conditions (*3*−*9*). The different activities in membranes with different bilayer thickness could be caused by several factors, such as altered aggregation states of the protein or changed tilting of its transmembrane helices. Mismatch can even lead to exclusion of a peptide from the bilayer as was shown with synthetic transmembrane peptides (*10*−*13*).

This exclusion from the bilayer under mismatch conditions could play a role in membrane protein biogenesis. For instance, in the eukaryotic secretion pathway the membranes contain increasing amounts of cholesterol from the ER[1] (endoplasmic reticulum) to the Golgi to the plasma membrane, which presumably leads to a progressive increase in bilayer thickness. Concomitantly, the length of transmembrane segments of proteins residing in these membranes also increases (*14*). Sorting of membrane proteins between compartments seems to depend on hydrophobic matching, since proteins with shortened or lengthened transmembrane segments are missorted to the membrane with the appropriate thickness (*15*−*18*).

Not only the length of a transmembrane segment but also its hydrophobicity plays an important role in the membrane integration of proteins. For stop-transfer sequences it was observed that their hydrophobicity is even more important for efficient membrane integration than their length (*19*, *20*). Moreover, both the length and hydrophobicity of signal anchor sequences were found to affect the topology of ER proteins (*21*−*24*). However, these observations may not be related to interaction with the lipid bilayer itself but could also be caused by characteristics of protein translocation channels.

To study to what extent hydrophobic mismatch with the membrane and hydrophobicity can play a role in membrane protein insertion, we used Pf3 coat protein as a model protein. This small, single-spanning protein does not need the Sec machinery for its insertion (*25*, *26*). Mutants of Pf3 coat protein have been shown to insert efficiently into pure lipid

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[1] Abbreviations: DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; ER, endoplasmic reticulum; LUVs, large unilamellar vesicles; PA, phosphatidic acid; PC, phosphatidylcholine; PMSF, phenylmethylsulfonyl chloride; TCA, trichloroacetic acid.

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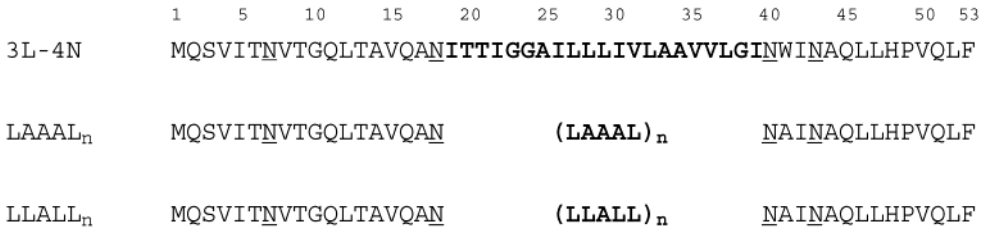

FIGURE 1: Amino acid sequences of the Pf3 mutants used in this study. The asparagines which were charged residues in the wild-type protein are underlined. The hydrophobic transmembrane segment is shown in boldface type. The *n* denotes the number of LAAAL or LLALL stretches in the protein ($n = 3-5$).

vesicles using an in vitro translation/translocation protocol (*27*). Using LUVs with different bilayer thicknesses and Pf3 mutants with synthetic transmembrane segments, we found that both the extent of hydrophobic mismatch and the hydrophobicity of the protein play a role in determining the efficiency of direct membrane insertion.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol was obtained from Merck. The phosphatidylcholines (PCs, 1,2-diacyl-*sn*-glycero-3-phosphocholines), di-14:1Δ9c-PC (dimyristoleoyl-PC), di-16:1Δ9c-PC (dipalmitoleoyl-PC), di-18:1Δ9c-PC (dioleoyl-PC, DOPC), di-20:1Δ11c-PC (dieicosenoyl-PC), and di-22:1Δ13-PC (dierucoyl-PC) were purchased from Avanti Polar Lipids, as well as the di-18:1Δ9-PA (1,2-dioleoyl-*sn*-glycero-3-phosphate, DOPA). The other phosphatidic acids (PAs) were prepared from the corresponding PCs by treatment with phospholipase D (Boehringer), which removes the choline headgroup. This was done by incubating 20−50 mg/mL PC in diethyl ether and an equal volume of buffer (0.1 M $CaCl_2$, 0.1 M sodium acetate, pH 5.8) with 1 mg of phospholipase D at around 40 °C with vigorous stirring for 30 min. The reaction was stopped by the addition of 1 volume of 0.1 M EDTA, pH 8.0. Complete conversion was checked by thin-layer chromatography, and subsequently the lipids were extracted (*28*).

Construction of Mutants. The 3L-4N mutant (Figure 1) has been described in ref *26*. Pf3 mutants with artificial transmembrane segments (Figure 1) were prepared as follows. We used the DD mutant (*29*) as a starting point. This mutant differs from the 3L-4N mutant in that it has four aspartate residues instead of the asparagine residues and lacks the three leucines in the middle of the transmembrane segment. In a single QuikChange reaction (Stratagene) a mutant was constructed in which residues 18−40 (the transmembrane segment and its flanking residues) were deleted and *Sfi*I and *Blp*I restriction sites were introduced around the deletion. The aspartate at position 7 was subsequently changed to asparagine by QuikChange. The resulting mutant was the starting point for cassette mutagenesis. After *Sfi*I and *Blp*I digestion, synthetic oligonucleotides were ligated into this vector, coding for a 10-residue hydrophobic segment (LLALLLLALL or LAAALLAAAL) with the appropriate flanking sequences. These mutants could be lengthened in 5-residue steps by subsequent rounds of cassette mutagenesis. For this purpose, the vector was digested with *Nhe*I and *Eco*47III (for the LLALL mutant) or *Not*I and *Nhe*I (for the LAAAL mutants), removing five codons. Consequently, oligonucleotides coding for 10 amino acids were ligated into the vector. In this manner, mutants with LLALL or LAAAL sequences of different lengths were prepared (see Figure 1), and all mutants were checked by DNA sequencing.

Preparation of Large Unilamellar Vesicles (LUVs). Dry lipid films were prepared by mixing the appropriate amounts of lipids dissolved in chloroform and evaporating the solvent under a stream of nitrogen. These films were dried overnight under vacuum to remove all solvent. They were then rehydrated at room temperature in LUV buffer (104 mM Na_2SO_4, 40 mM HEPES, pH 8.0) to a final concentration of 10 mM phospholipid and vortexed. After 10 freeze−thawing cycles, the vesicle suspensions were extruded 10 times through 0.4 μm polycarbonate filters. The LUVs were stored under nitrogen at 4 °C for maximally 1 day before use.

In Vitro Translocation. S-135 cell extracts were prepared from *Escherichia coli* MRE600 as described (*30*). In vitro transcriptions, translations, and translocations were carried out as described (*31*) and included 5 μCi of [^{35}S]methionine and 10 μL of LUV suspension. The final concentration of phospholipid was thus 2 mM, which is a large excess compared to the amount of Pf3 that is produced in a translation reaction, which is in the order of a picomole. The total volume of each translation reaction was adjusted to 50 μL with LUV buffer. After 30 min translation and translocation at 37 °C an aliquot of 10 μL was withdrawn to serve as 20% standard. The LUVs were reisolated by centrifugation to ensure that only membrane-bound protein was analyzed. To pellet the LUVs, the salt concentration was lowered by the addition of 120 μL of H_2O, and the mixture was centrifuged in a TLA100 rotor at 100000 rpm for 60 min at 4 °C. After centrifugation, the supernatant was removed, and the pellets were resuspended in 40 μL of LUV buffer. The pellet fraction was divided into two equal aliquots, one of which was treated with 40 μL of proteinase K (1 mg/mL) for 30 min at room temperature. The amount of protease-protected fragments relative to the amount of protein bound to the LUVs is termed the N-terminal translocation efficiency (see Results section). All samples were trichloroacetic acid (TCA) precipitated and analyzed on 16.5% SDS−tricine gels containing 6 M urea (*32*). Protein bands were quantified on a PhosphoImager (Molecular Dynamics).

Proteinase K Incubation. In order to follow the proteinase K incubation in time, translocation reactions were performed in 100 μL volumes. An aliquot of 10 μL was withdrawn to serve as a 10% standard, while the rest was mixed with 120 μL of H_2O and centrifuged as described above. The pellets were resuspended in 100 μL of LUV buffer. Of this suspension, 10 μL was TCA-precipitated, while the remaining 90 μL was incubated with 180 μL of 1 mg/mL proteinase K solution at room temperature. At various time points, aliquots of 30 μL were withdrawn and immediately TCA-

precipitated. The samples were then analyzed by gel electrophoresis and autoradiography as described above.

Urea/Carbonate Extraction. Translocation reactions were performed as described above in 200 μL volumes. An aliquot of 10 μL was used as a 5% standard, and the rest was mixed with an equal volume of water and centrifuged as described. The pellets were resuspended in 100 μL of LUV buffer. Of this suspension, 10 μL was TCA-precipitated, while the rest was incubated with 180 μL of 1 mg/mL proteinase K solution at room temperature for 30 min. After the addition of 3 μL of 100 mM phenylmethylsulfonyl chloride (PMSF) to stop the protease digestion, the LUV suspension was divided into 60 μL aliquots. Urea (6.6 or 20 μL of 10 M urea) or carbonate (60 μL of 0.2 M Na_2CO_3) was added, and the suspensions were incubated on ice for 5 min. Subsequently, the volume was adjusted to 400 μL with H_2O, and the samples were centrifuged in a TLA 100.1 rotor at 100000 rpm for 30 min at 4 °C. The supernatant and pellet fractions were TCA-precipitated and then analyzed by gel electrophoresis and autoradiography.

RESULTS

We have shown in previous studies that the Pf3 mutant 3L-4N (Figure 1) is able to insert efficiently in a transmembrane fashion into *E. coli* inner membrane vesicles as well as in LUVs with different lipid headgroup compositions (*27*, *31*). In this study we have used the same mutant and variants of it to investigate for the first time the influence of bilayer thickness on direct membrane protein insertion. Because translocation of the N-terminus of this protein across the membrane is stimulated by the presence of negatively charged lipids (*31*), we used LUVs composed of 75% PC and 25% PA as the receiving membrane. The thickness of this bilayer can readily be altered by varying the length of the acyl chains from 14 to 22 carbon atoms, all containing one unsaturated bond to ensure that the bilayer is in the fluid phase. The 3L-4N protein is produced in an in vitro translation reaction, to which LUVs are added cotranslationally. The N-terminus of the protein contains a unique methionine which becomes ^{35}S-labeled. Subsequently, N-terminal translocation efficiencies are determined by reisolation of the LUVs and treatment with proteinase K. In previous studies, it was observed that this removes part of the small C-terminal tail, resulting in a radioactive protein with a slightly lower molecular weight. This protein was considered to be oriented in a transmembrane fashion, with the N-terminus translocated and the C-terminus cleaved off (*29*). It should be noted that the 3L-4N mutant is a very small protein of only 54 amino acids. Since during synthesis of a protein about 30−40 amino acids are inside the ribosome (*33*−*35*), its hydrophobic segment will not emerge before translation is terminated. The actual insertion of this protein must therefore be posttranslational.

Protease Accessibility of 3L-4N Depends on Bilayer Thickness. To investigate whether proteinase K cleavage of the protein is influenced by bilayer thickness, translocation reactions were performed with the 3L-4N mutant and LUVs of 14:1, 18:1, or 22:1 PC/PA. After reisolation of the LUVs, proteinase K was added, and at various time points aliquots were withdrawn and TCA-precipitated. The results of this experiment are shown in Figure 2. In all three bilayer thicknesses, protease-protected protein fragments can be observed (Figure 2A). The total amount of protected protein

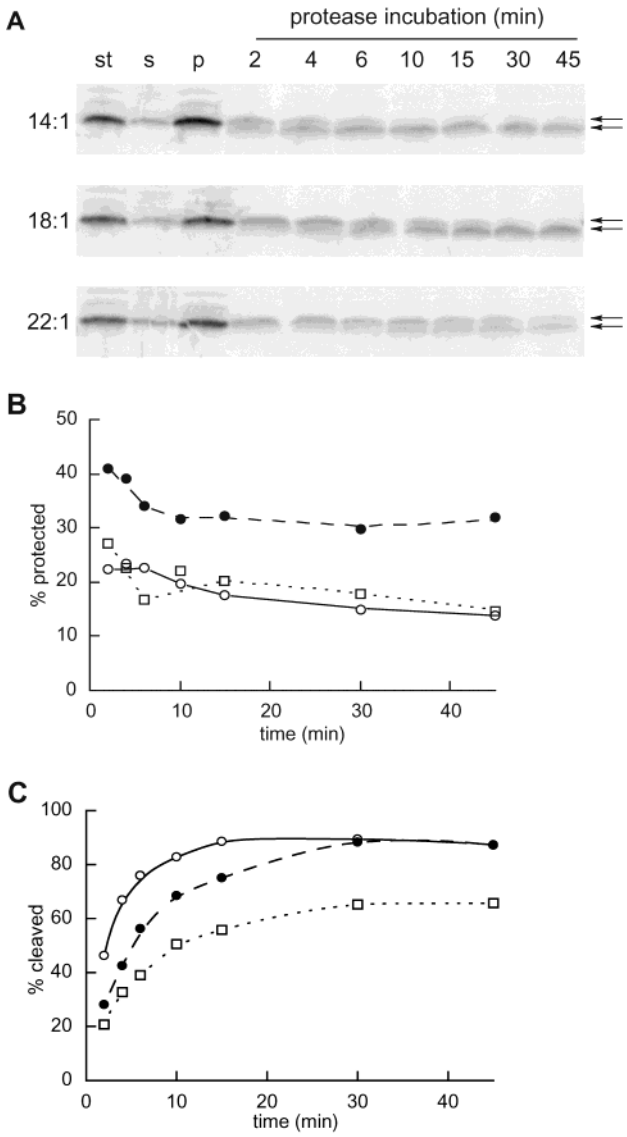

FIGURE 2: Time dependency of proteinase K digestion of 3L-4N in different bilayer thicknesses. (A) Translocation of 3L-4N into LUVs with different thicknesses was performed as described in Experimental Procedures and divided into standard (st), supernatant (s), and pellet (p) fractions. The pellet was treated with proteinase K at room temperature for the indicated time intervals. The arrows indicate the uncleaved (upper band) and cleaved (lower band) form of the protein. (B) Quantification of the data in panel A. The total amount of protease-protected protein (cleaved and uncleaved) is expressed as the percentage of the amount of protein originally present in the pellet fraction for 14:1 (○), 18:1 (●), and 22:1 (□) bilayers. (C) The amount of the cleaved form of the protein is expressed as the percentage of the total amount of protease-protected protein present at each time point. The symbols correspond to those in panel B.

remains practically constant during the protease incubation and depends on the bilayer thickness, since it is lower in 14:1 and 22:1 LUVs than in 18:1 LUVs (Figure 2B). This lipid dependency is discussed in more detail below.

The 3L-4N molecules are in time converted from full-length to a protease-cleaved form, most likely due to removal of the C-terminus. This occurs at different rates and to different extents, depending on the bilayer thickness (Figure 2A). In the thin 14:1 bilayers, already after 2 min a fraction of the protein is cleaved, as indicated by the shift in molecular weight, and cleavage is complete after 15 min. In 18:1 LUVs

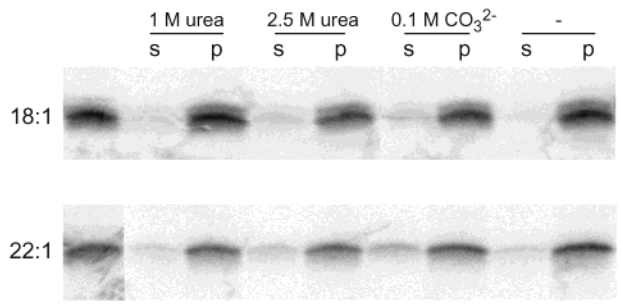

FIGURE 3: Membrane association of 3L-4N with 18:1 and 22:1 LUVs. The 3L-4N mutant was translocated into the LUVs and treated with proteinase K as described (left lane). Subsequently, the protein-containing LUVs were subjected to treatment with 1 or 2.5 M urea or 0.1 M carbonate or not treated (−). The samples were centrifuged and divided into supernatant (s) and pellet (p) fractions.

cleavage is somewhat slower, with the appearance of the cleaved fragment after about 6 min and complete cleavage at 30 min. In the thickest bilayers of the 22:1 LUVs, protease cleavage can be observed only after 10 min, and a portion of the protein still remains uncleaved after 45 min. Quantification of these results (Figure 2C) shows that the fraction of cleaved fragment indeed increases most rapidly in 14:1, somewhat slower in 18:1 and slowest in 22:1 LUVs. Protease cleavage thus clearly becomes less efficient in thicker bilayers. These results indicate that the upper, uncleaved fragment most likely originates from proteins that are correctly inserted with their labeled N-terminus inside the LUVs, but whose proteinase K cleavage sites are less accessible, since they are located close to the membrane surface (*29*). Both forms of the protein can be degraded by proteinase K if the detergent Triton X-100 is used to dissolve the membrane (data not shown). More direct evidence for the fact that the N-terminus of both forms of the protein has actually crossed the membrane was obtained by inserting the protein into microsomes. It was observed that proteins that could not be cleaved by proteinase K in the C-terminus were nevertheless glycosylated in the N-terminal tail (data not shown).

Since for all bilayer thicknesses the fraction of cleaved protein, as well as the total amount of labeled protein, remains constant after 30 min of incubation with proteinase K, we have used this incubation time in the other experiments in this paper.

Uncleaved and Cleaved Forms of the Protein Are Both Stably Integrated into the Bilayer. If the uncleaved fragment observed in thicker bilayers originates from protease-inaccessible but otherwise correctly inserted protein as we proposed above, then it should be stably integrated in the membrane, just like the cleaved form of the protein. This was investigated using urea and carbonate extraction, treatments that solubilize peripherally bound membrane proteins, while integral membrane proteins remain in the membrane fraction. Translocation reactions were performed with the mutant 3L-4N in 18:1 or 22:1 LUVs that were subsequently reisolated and treated with proteinase K for 30 min as described above. For 18:1 LUVs, this resulted predominantly in the cleaved form of the protein, whereas in 22:1 LUVs the protein remained mostly uncleaved. These protease-treated LUVs were subsequently incubated with urea or carbonate. As seen in Figure 3, in both LUV preparations the labeled protein predominantly remained in the pelleted

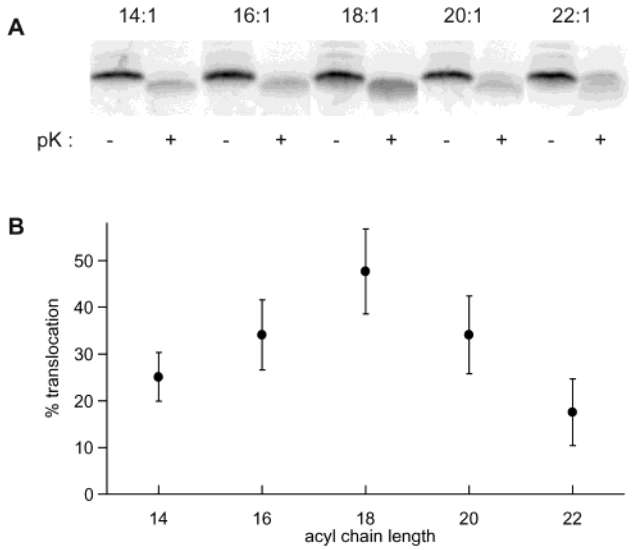

FIGURE 4: Translocation of 3L-4N into LUVs with different bilayer thicknesses. (A) The 3L-4N mutant was translocated into LUVs with the indicated acyl chain lengths. Only the pellet fractions are shown, which were treated with proteinase K (pK) where indicated. (B) Quantification of the data in panel A. The amount of translocation is expressed as the percentage of the amount of protein present in the pellet fraction. Average values (●) and standard deviations ($n = 2-6$) are shown.

membrane fraction. Moreover, the ratio of uncleaved to cleaved 3L-4N protein did not change upon extraction (data not shown), indicating that both forms remain in the membrane to the same extent. This demonstrates clearly that also the uncleaved form of the protein is stably integrated into the membrane and thus provides yet another piece of evidence for our interpretation that this is protein which is inserted correctly, with its N-terminus translocated across the membrane. Therefore, in the following experiments, all protease-protected molecules, uncleaved and cleaved, are taken to represent N-terminally translocated membrane-inserted proteins. We will refer to these proteins as either inserted or N-terminally translocated protein.

N-Terminal Translocation Is Dependent on Bilayer Thickness. To investigate the influence of membrane thickness on membrane protein insertion, we have performed translocation reactions with the 3L-4N mutant in LUVs with lipid acyl chains ranging from 14 to 22 carbon atoms. As can be seen from Figure 4A and the quantification in Figure 4B, N-terminal translocation of 3L-4N in 18:1 PC/PA LUVs is very efficient, in agreement with our previous results (*31*). Interestingly, the translocation efficiency is less in the thinner 14:1 and 16:1 bilayers as well as in the thicker 20:1 and 22:1 bilayers, where the uncleaved form of the protein appears (see above). The insertion of this protein therefore clearly depends on the thickness of the bilayer, which appears optimal at 18 carbon atoms.

Cholesterol Influences Insertion by Increasing Bilayer Thickness. An additional method to alter membrane thickness is the addition of cholesterol, which increases lipid acyl chain order and, thereby, the thickness of the bilayer (*36*). We investigated the effect of cholesterol on insertion of the 3L-4N mutant. Translocation reactions were performed with 18:1 LUVs composed of 75% DOPC and 25% DOPA, in which the zwitterionic lipid PC was gradually replaced by the neutral cholesterol. As seen in Figure 5, increasing amounts of cholesterol in 18:1 LUVs lead to a decrease in the

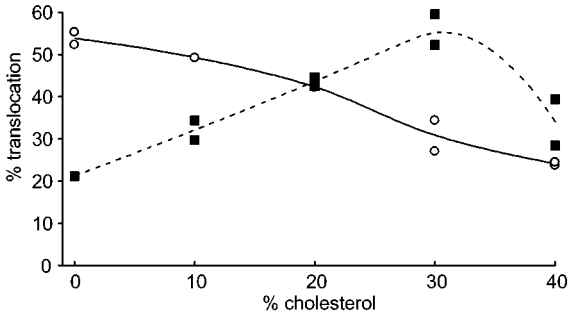

FIGURE 5: Cholesterol dependency of translocation of 3L-4N into bilayers with different thicknesses. The 3L-4N mutant was translocated into 18:1 (○) or 14:1 (●) LUVs with increasing amounts of cholesterol. The translocation efficiencies were determined as described. The experiment was performed twice, and both values are indicated.

Table 1: Hydrophobic Lengths and Bilayer Thicknesses

mutant	hydrophobic length (Å)[a]	acyl chains	bilayer thickness (Å)[b]
3L-4N	31.5	14:1	22.5
$LAAAL_3$	22.5	16:1	26.0
$LAAAL_4$	30.0	18:1	29.5
$LAAAL_5$	37.5	20:1	33.0
		22:1	36.5

[a] Calculated lengths of the hydrophobic segments presuming an α-helical conformation using 1.5 Å/residue. [b] Estimated values of the carbonyl to carbonyl distance for PC bilayers adapted from ref *13*.

N-terminal translocation efficiency of 3L-4N. Similar to the situation in longer lipids, the addition of cholesterol leads to the appearance of the uncleaved fragment (data not shown). This suggests that the decrease in translocation efficiency upon addition of cholesterol is caused by its tendency to thicken the membrane. If this is true, the addition of cholesterol should improve the translocation efficiency in thin membranes. Indeed, in 14:1 PC/PA bilayers the addition of cholesterol increases the percentage of N-terminal translocation up to a maximum of about 50%, which is similar to the 18:1 bilayers without cholesterol (Figure 5). This suggests that insertion is low in the 14:1 bilayer because the membrane is too thin, and cholesterol thickens the membrane until maximal insertion can take place.

Insertion of a Mutant with a Synthetic Transmembrane Segment Depends on Bilayer Thickness. The previous experiments clearly showed that translocation efficiency depends on the membrane thickness, suggesting that the extent of hydrophobic matching between the bilayer thickness and the protein transmembrane segment is important (see Table 1). However, since so far we have only investigated one protein, it is not possible from these results to distinguish between the influence of the bilayer thickness alone or of its hydrophobic matching with the protein, nor is it possible to establish the potential role of the hydrophobicity of the transmembrane segment. To be able to study these aspects separately, we constructed mutants in which the transmembrane segment of 3L-4N was replaced by multiple five-residue stretches of leucines and alanines. In this manner, mutants with different hydrophobic lengths could be created, and the hydrophobicity could be altered by varying the leucine content of the stretches (see Figure 1).

First, we investigated whether such mutants with artificial transmembrane stretches are still able to insert into bilayers.

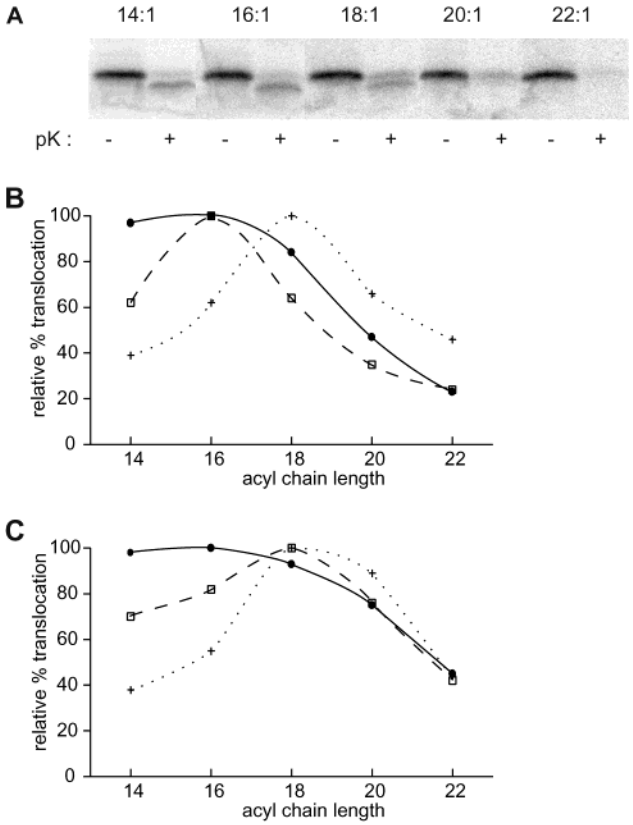

FIGURE 6: Translocation of mutants with synthetic transmembrane segments into LUVs with different bilayer thicknesses. (A) The $LAAAL_3$ mutant was translocated into LUVs with the indicated acyl chain lengths. Only pellet fractions are shown, which were treated with proteinase K where indicated. (B) Relative translocation efficiencies of the LAAAL mutants. Translocation efficiencies were determined two times as described. The values were averaged, and the highest value for each mutant was set at 100%. The highest absolute values are $LAAAL_3$ (●) 36%, $LAAAL_4$ (□) 17%, and $LAAAL_5$ (+) 33%. (C) Relative translocation efficiencies of the LLALL mutants, calculated as described in panel B. The highest absolute values are $LLALL_3$ (●) 43%, $LLALL_4$ (□) 21%, and $LLALL_5$ (+) 26%.

Translocation reactions were performed with the mutant $LAAAL_3$, which has 15 hydrophobic residues, and PC/PA LUVs with different bilayer thicknesses. When the LUVs are pelleted, the $LAAAL_3$ mutant associates with them with an efficiency similar to that of 3L-4N (data not shown). As can be seen in Figure 6A, when this fraction is treated with proteinase K, a portion of the protein is protected. This indicates that this mutant is indeed able to insert into a membrane. Apparently, no specific amino acid sequence is required for direct insertion into membranes, but an artificial hydrophobic segment also suffices. In addition, it can be observed that for this short mutant the uncleaved fragment already appears in the thinnest bilayers and that protease cleavage hardly occurs in the thickest bilayers. This is consistent with our interpretation that the uncleaved fragment is due to protease inaccessibility of the C-terminus, as discussed above. Quantification of the data (Figure 6B) shows that N-terminal translocation of the $LAAAL_3$ mutant is most efficient in the thinnest bilayers of the 14:1 and 16:1 LUVs, while translocation becomes less efficient in thicker membranes.

Insertion of Mutants with Synthetic Transmembrane Segments Depends on Length as Well as Hydrophobicity. To investigate the influence of the length of the hydrophobic

stretch, we compared the insertion of the $LAAAL_3$ mutant with that of $LAAAL_4$ and $LAAAL_5$, mutants which have hydrophobic segments of 20 or 25 residues, respectively. To allow a better comparison between the different mutants, the values were plotted relative to the highest translocation efficiency for each mutant (Figure 6B,C).

While the $LAAAL_3$ mutant inserts efficiently in both the 14:1 and 16:1 bilayers, the insertion of the $LAAAL_4$ mutant is clearly most efficient in 16:1 LUVs, while the $LAAAL_5$ mutant inserts most efficiently in 18:1 LUVs (Figure 6B). Apparently, mutants which have longer hydrophobic stretches prefer to insert into thicker membranes. We also compared the translocation efficiencies of the LAAAL mutants with those of the LLALL mutants (Figure 6C). The $LLALL_3$ mutant, which is equally long but more hydrophobic than the $LAAAL_3$ mutant, behaves very similarly and inserts most efficiently in 14:1 and 16:1 LUVs. However, whereas the $LAAAL_4$ mutant prefers 16:1 LUVs, the more hydrophobic $LLALL_4$ mutant inserts most efficiently into the 18:1 bilayer. The 3L-4N mutant, which has a similar length and a hydrophobicity intermediate between these two mutants, also inserts most efficiently in the 18:1 LUVs (Figure 4). The translocation efficiencies of $LLALL_5$ are somewhat shifted to thicker bilayers compared to the $LAAAL_5$ mutant. Apparently, more hydrophobic mutants preferably insert into thicker bilayers. This demonstrates that not only the length of the transmembrane segment but also its hydrophobicity plays a role in determining in which bilayer thickness most efficient insertion can take place.

DISCUSSION

In this study, we have investigated the influence of hydrophobic matching between the membrane thickness and a protein transmembrane segment on its direct bilayer insertion. We have found that insertion depends on the bilayer thickness but that the length and hydrophobicity of the transmembrane segment are additional important factors that determine into which bilayers efficient insertion can take place.

We used an in vitro approach, with mutants of the single-spanning Pf3 coat protein and LUVs with different bilayer thicknesses. The mutants we have used are able to efficiently insert into bilayers without the translocase, allowing us to investigate the effect of hydrophobic mismatch with the bilayer itself on membrane protein insertion.

Insertion of the 3L-4N mutant clearly depended on the bilayer thickness and was most efficient in 18:1 lipids. This mutant has a hydrophobic stretch of 21 amino acids that most likely spans the membrane in a helical conformation. Since the length of an α-helix increases 1.5 Å per amino acid, the length of the hydrophobic segment of 3L-4N is about 31.5 Å (see Table 1). Since the hydrophobic thickness of a di-18:1-PC bilayer is approximately 29.5 Å (Table 1), insertion of this mutant is most efficient under hydrophobic matching conditions.

More evidence for the importance of hydrophobic matching was obtained by using LUVs with increasing amounts of cholesterol. This decreased the insertion of 3L-4N in 18:1 bilayers, but addition of 30% cholesterol to 14:1 LUVs caused a remarkable increase in translocation efficiency up to the level observed in 18:1 bilayers without cholesterol. These results suggest that a 14:1 bilayer is too thin for efficient insertion but that the increased membrane thickness caused by cholesterol produces a hydrophobic matching situation such that insertion becomes more favorable. Apparently, 30% cholesterol thickens the 14:1 membrane toward a 18:1 bilayer, which is in agreement with previous studies on the effect of cholesterol on bilayer thickness (*9*, *36*, *37*).

Our results with mutants with synthetic transmembrane sequences show that not only mismatch but also the thickness of the bilayer itself and the hydrophobicity of the transmembrane segment are important factors determining efficient membrane insertion. For comparison, the lengths of the mutants and the approximate bilayer thicknesses are shown in Table 1. The $LAAAL_3$ mutant, which has a hydrophobic segment of 22.5 Å, inserts most efficiently in the thinnest 14:1 and 16:1 bilayers (22.5 and 26.0 Å, respectively), hence under conditions close to hydrophobic matching. The $LAAAL_4$ mutant, with a 30 Å hydrophobic segment, inserts most efficiently in the 16:1 bilayer, although one would expect it to behave similarly to the 3L-4N mutant, which prefers the more closely matching 18:1 bilayer (29.5 Å). The $LAAAL_5$ mutant, which theoretically matches the 22:1 bilayer, inserts most efficiently into 18:1 LUVs. The more hydrophobic LLALL mutants behave similarly to the LAAAL mutants but, in general, are able to insert more efficiently into thicker bilayers. These mutants with synthetic transmembrane segments therefore insert into bilayers in a mismatch-dependent manner. However, especially the longer and less hydrophobic mutants generally insert into thinner bilayers than could be expected solely on the basis of hydrophobic matching.

The thickness dependency of these mutants can be understood if one realizes that the N-terminal translocation efficiencies we determine are probably a combination of two factors, namely, translocation and hydrophobic matching. First, the N-terminus of the protein has to translocate across the bilayer, which is probably easier if the bilayer is thinner, because the distance that the hydrophilic tail has to travel is less and the energetic cost of disrupting the packing of a thin bilayer will be less than for a thick one. Hydrophobicity is also important in this process, since more hydrophobic mutants will have a stronger tendency to partition into the membrane and thus can more easily overcome the energy barrier of transiently burying the hydrophilic N-terminal tail within the hydrophobic membrane interior. Only after the N-terminus has reached the other side of the membrane will the extent of matching become important for stable integration of the protein into the bilayer. Since hydrophobic mismatch between the transmembrane segment and the bilayer is energetically unfavorable, under mismatch conditions the protein will be more likely to flip out of the membrane. Using reconstitution protocols, it has indeed been shown that the main response to hydrophobic mismatch is that incorporation of synthetic transmembrane peptides into lipid bilayers becomes less favorable, especially when the membrane becomes too thick (*10*−*13*). Furthermore, it can be expected that stable integration is promoted by increased hydrophobicity of the transmembrane segment. If the translocation efficiencies are indeed caused by such a combination of translocation and matching processes, this explains why the longer synthetic mutants which require thicker bilayers for hydrophobic matching conditions nevertheless have optima in slightly thinner bilayers and also why this is more pronounced for the less hydrophobic ones.

A remaining puzzle is the dynamic nature of the insertion pathway. If there were a thermodynamic equilibrium between

inserted and noninserted molecules, one would expect that the protease could eventually degrade all of the protein. However, Figure 2 shows that this is not the case. On the other hand, matching of the proteins' transmembrane segment and the bilayer thickness can only occur if a protein is actually already inserted into the membrane. Therefore, it is unclear how kinetic partitioning of the protein between two irreversible states, inserted or noninserted, can cause the insertion efficiency to be matching dependent.

We speculate that the measured insertion efficiencies are caused by a combination of these two mechanisms. One such possibility is as follows. Initially, the protein associates with the membrane in an undefined manner, and from this state different routes can be followed. One is acquiring an N-terminal translocated transmembrane orientation, which is stabilized under matching conditions. Proteins that do not achieve a stably integrated conformation irreversibly aggregate in a protease-accessible location.

Previous studies on the role of the transmembrane segment on membrane protein insertion were always performed in the context of translocation channels. It was observed that the orientation of signal-anchor proteins in the ER membrane depends on the hydrophobicity and to a lesser extent on the length of the apolar stretch. It was observed that N-terminal translocation is favored by long, hydrophobic sequences (*21*−*24*). On the basis of the results presented in this study, a possible explanation could be that such stretches are able to insert directly into the lipids, while shorter or less hydrophobic segments may have to insert via the translocon.

The concept of hydrophobic mismatch has been implicated in protein sorting in the eukaryotic secretion pathway, where the membranes are believed to thicken from the ER to the Golgi to the plasma membrane because of their increasing amounts of cholesterol. It was proposed that proteins are sorted to the membrane in which matching with their transmembrane segment occurs (*14*). It was shown in vivo that proteins indeed can be redirected to other compartments by changing the length of their transmembrane segment (*15*−*18*). Consistent with their observations, our results with cholesterol show that its tendency to thicken the membrane and thereby influence the extent of hydrophobic matching can substantially influence the insertion efficiency of proteins.

Hydrophobic matching might also play a role in direct membrane integration of other proteins. For example, in the thylakoid membrane of chloroplasts some proteins are known to insert without the aid of any known proteins and may insert spontaneously (*38*−*41*). In addition, in genome-sequencing projects small proteins are being identified that are predicted to be membrane-spanning and whose insertion might in some cases also be spontaneous.

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