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Comparison of In Silico Signal Sequence-Phospholipid Results with Described In Vitro and In Vivo Protein Translocation Studies Seems to Underscore the Significance of Phospholipids

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Abstract: The precise role of protein–lipid interactions in protein translocation is, after almost four decades of research, still a matter of debate. The experimental evidence, as described in the literature, indicates that (anionic) phospholipids play a role in numerous events in protein translocation; however, its meaning and relevance are still a matter of debate. This study tries to fill some missing links in the experimental evidence by means of in silico experiments. The study presented here indicates not only that there is a direct signal sequence–phospholipid interaction but also that the corresponding signal peptides can translocate additional amino acids across a pure lipid membrane. Furthermore, results are presented when it comes to the extent of anionic phospholipids' dependence on this process. The correlations between the in silico results of pure signal peptide–phospholipid interactions and the observed experimental trends in the overall protein translocation effects are at least remarkable. The results emphasize that new models for protein translocation will have to be developed to take all these and previous experimental data into account.

Keywords: protein translocation; lipid-binding regions; protein–lipid interactions; Monte Carlo calculations; signal sequence

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1. Introduction

Protein translocation has been studied for almost four decades now, and for the so-called Sec-dependent translocation pathway, knowledge of this intriguing process has made impressive progress (see elsewhere [1–9] for some excellent reviews). In short, thanks to the pioneering work in this field by the groups of Wickner [10], Mizushima [11] and Silhavy [12], it has become clear which proteinaceous components are involved. The subsequent function of these (essential) Sec-proteins was elucidated by a combination of various approaches (in vivo and in vitro assays, model-system approaches and biophysical techniques). A major step was achieved by the successful attempts to reconstitute the minimal set of factors involved in the Sec-dependent protein translocation process (see, for example, [11,13]). Another breakthrough was the work that searched for the general characteristics of the signal sequence, which was conducted by Von Heijne and co-workers [14–17].

Last but not least, an important finding was the observation that anionic phospholipids are involved in the protein translocation process [18]. This observation was later confirmed in a reconstitution assay developed by Wickner and co-workers [10]. The proven involvement of phospholipids in protein translocation leads to roughly three different opinions about how to explain this. The first and most common view is that the anionic phospholipids are necessary to keep the various Sec-proteins in a so-called protein translocation competent state (see, for example, [19]). The second view can be summarized by an explanation that pictures protein–lipid interactions being involved and that the Sec machinery functions as a facilitator for more efficient protein translocation. For example, the various papers related to SecA–phospholipid interactions demonstrate that SecA–phospholipid

interactions without the SecYEG complex allow the protein translocation of a number of proteins/precursor proteins [20,21].

The most efficient protein translocation takes place upon the inclusion of the SecYEG complex [22,23], suggesting that the SecA–phospholipid binding is an intermediate step in the translocation process for the majority of the proteins. The third view can be summarized by an explanation of the active role of phospholipids. So, this is not merely a mediating role but a view where (part of) the protein translocation process takes place in the lipid phase of the membrane and that links, for example, the observed in vivo and in vitro anionic phospholipid dependence of the process of the anionic phospholipid dependence of the signal sequence–phospholipid interactions (see, for example, [24,25]) and possibly the SecA–phospholipid interactions (see, for example, [26–31]). Taking all these views into account, it is hard to envision how, when looking at all the data provided by numerous studies, the protein translocation process, and more particularly the passage of a protein across the membrane, could be an exclusive proteinaceous event.

Still, it is fair to say that the exact role of lipids in the protein translocation process remains a matter of debate. This study tries to link numerous studies with alterations in the signal sequence and the presence of anionic phospholipids and its overall consequences for the translocation efficiencies, as seen either in vivo or in vitro, of pure signal sequence–phospholipid results as obtained by in silico experiments. The in silico experiments are based on a combined use of lipid-binding prediction using Heliquest and a Heliquest-based Eisenberg plot approach [32], as well as the use of lipid–peptide interaction predictions using MCPep [33]. This approach has been successfully used and described in the literature [34–40]. The results as described here show remarkable correlations between, for example, the in vitro results of signal sequence alterations and its subsequent consequences on the overall anionic phospholipid dependence of the process and the in silico results of these alterations in the peptide–lipid interactions. This study seems to suggest that the overall consequences in protein translocation when one changes the signal sequence and/or where the anionic phospholipid dependence of the process is changed can be solely explained by signal sequence–phospholipid interactions. Consequently, the passage of a protein across the membrane cannot be an exclusive proteinaceous event and leaves questions about our current understanding of the putative protein conducting channel as part of our understanding of the protein translocation process.

2. Results

2.1. Signal Sequence Experimental Results of prePhoE Correlate with In Silico Data

The anionic phospholipid dependency of signal peptide–phospholipid interactions is well-studied. It has been demonstrated experimentally that the binding and insertion of signal peptides are stronger upon increasing the anionic phospholipid content [41], and this was also demonstrated in the context of the complete precursor [42,43]. The induction of α-helix upon interaction with negatively charged phospholipids was demonstrated with circular dichroism [25], and it was found to be a random coil in the absence of membranes [44]. According to the lipid-binding discrimination factor (D) [45] and the Heliquest-based Eisenberg plot approach [32], the signal peptide of prePhoE, indeed, corresponds to a potential helical lipid-binding region that can bind and be inserted into a lipid membrane with anionic phospholipids. The lipid discrimination factor D is 0.69 and therefore above the threshold value of 0.68 for a potential lipid-binding region (see Table 1 for details).

Table 1. Overview of the results of the studied signal peptides with the use of the combined Heliquest discrimination factor and the Heliquest-based Eisenberg plot methodology in the identification of potential lipid-binding regions (LBR).

Name	Sequence	z	<μH>	<H>	LBR [a]
PhoE SP	MKKSTLALVVMGIVASASVQA	2	0.027	0.578	D
PhoE SP G10L	MKKSTLALVVMLIVASASVQA	2	0.105	0.673	D
LamB SP	MMITLRKLPLAVAVAAGVMSAQAMA	2	0.222	0.582	D
LamB SP A13D	MMITLRKLPLAVDVAAGVMSAQAMA	1	0.262	0.522	No
LamB Δ78	MMITLRKLP----VAAGVMSAQAMA	2	0.178	0.476	D
LamB Δ78r1	MMITLRKLP----VAACVMSAQAMA	2	0.176	0.562	D
LamB Δ78r2	MMITLRKLL----VAAGVMSAQAMA	2	0.206	0.531	D
2K7L SP	MMKKNNLLLLLLLGTANAAS	2	0.165	0.637	D
2K8L SP	MMKKNNLLLLLLLLGTANAAS	2	0.086	0.714	D
2K9L SP	MMKKNNLLLLLLLLLGTANAAS	2	0.095	0.842	TM/D
2K8V SP	MMKKNNVVVVVVVVGTANAAS	2	0.107	0.501	D
2K9V SP	MMKKNNVVVVVVVVVGTANAAS	2	0.080	0.602	D
2K10V SP	MMKKNNVVVVVVVVVVGTANAAS	2	0.136	0.652	D
CLY SP	MRSLLILVLCFLPLAALG	1	0.081	1.098	TM
CLY R3	MRRRSLLILVLCFLPLAALG	3	0.100	0.918	TM/D
CLY R17D	MDSLLILVLCFLPLAALG	−1	0.067	1.112	TM
M13 coat SP	MKKSLVLKASVAVATLVPMLSFA [b]	3	0.072	0.559	D
M13 coat SP	MKKSLVLKASVAVATLVPMLSFA [b]	1	0.109	0.863	TM
M13 coat SP ΔN	M--------NSVAVATLVPMLSFA	0	0.164	0.784	TM
M13 coat SP ΔN + 3	M--------NSLRRRNSVAVATLVPMLSFA	3	0.250	0.440	D
OmpA SP	MKKTAIAIAVALAGFATVAQA	2	0.251	0.620	D
OmpA SP I8N	MKKTAIANAVALAGFATVAQA	2	0.118	0.487	D
OmpA SP L12N	MKKTAIAIAVANAGFATVAQA	2	0.174	0.492	D

[a] The lipid binding region (LBR) is identified either by the lipid discrimination factor D or by the identification of the transmembrane (TM), see Methods section for further details. [b] Depending on the selected 18AA window (underlined) an LBR based on the discrimination factor (D), or a transmembrane sequence (TM) can be predicted. Where applicable, the (point) mutations are indicated in red.

In Figure S1, the anionic phospholipid dependency of the signal peptide interaction is depicted as found by the Monte Carlo simulations using MCPep [46]. Both the effects of the total free energy difference between the prePhoE peptide in the aqueous phase and in the membrane (ΔG_{Total}) as well as the average distance from the membrane midplane (Z_{center}) of the peptide in the so-called surface configuration are depicted. The increase in these parameters, as measures of the interaction with a lipid membrane, are dependent on the percentage of anionic phospholipids and in line with what was observed in both Trp-fluorescence [41] and circular dichroism experiments [25]. For a discussion on how the detailed shape of the observed curve can be due to a conformational change from the so-called surface configuration towards a transmembrane configuration, see elsewhere [47]. This in silico experiment with the use of the prePhoE signal peptide not only reinforces the validity of the used approach, as already briefly discussed in the introduction, but also demonstrates a strong correlation between the predictions as obtained with this bioinformatics approach and earlier observed experimental data.

2.2. In Silico Results Suggest a Possible In Silico Protein Translocation Assay

An interesting paper demonstrated the possibility of translocation of a part of the mature protein of prePhoE across a lipid membrane without any proteinaceous component of the Sec machinery [48]. Additionally, the study demonstrated that when the Gly at position −10 was replaced by a Leu the translocation of an additional seven amino acids (AA) can occur even without a proton motor force (pmf). Indeed, according to the Heliquest data, both signal peptides can be considered potential helical lipid-binding regions (see

Table 1), where the PhoE G10L peptide sequence leads to an even higher discrimination factor D (0.76) than the wild-type signal peptide (0.69). Both the wild-type as well as the mutant signal peptide with an additional 7 AA attached lead in MCPep to so-called significant clusters for both surface and transmembrane configurations (see Table S1 for details). In other words, the spontaneous translocation of seven additional amino acids could be mimicked in MCPep. It is tempting to speculate that the lower energy levels as seen for the mutant signal peptide compared to the wild-type signal peptide is the reason why the mutant signal peptide no longer requires a proton motor force (pmf) in the described in vitro assay [48].

These in silico results, strongly inspired by this experimental assay as described for the prePhoE signal peptide [48], led subsequently to the question as to whether an in silico-based protein translocation assay could be developed accordingly.

Table 2 indicates the results of all signal sequences studied. For all wild-type signal sequences, the maximum number of additional mature part amino acids that can be translocated across a pure lipid membrane are indicated. The results indicate that the different signal peptides have different potential to translocate a number of additional amino acids in a spontaneous way across a membrane containing anionic phospholipids and no proteinaceous components. This might reflect a possible step in the translocation process where after initial binding of the signal sequence to anionic phospholipids and subsequent insertion of the signal sequence into the membrane a number of amino acids of the mature part of a precursor protein are translocated across the membrane before any involvement of the Sec machinery.

Table 2. Overview of the studied signal peptides with the detailed sequence info and maximum added amino acids for which significant clusters were found in MCPep [46].

Signal Peptide	Peptide Sequence	Add. AA [a]
PhoE	**MKKSTLALVVMGIVASASVQA**AEIYNKDGNKLDVYGKV	+17
PhoE G10L	**MKKSTLALVVML IVASASVQA**AEIYNKDGNKLDVYGKV	+17
LamB	**MMITLRKLPLAVAVAAGVMSAQAMA**VDFHGYARSG	+10
LamB A13D	**MMITLRKLPLAVDVAAGVMSAQAMA**	0
LamBΔ78	**MMITLRKLP----VAAGVMSAQAMA**	0
LamBΔ78r1	**MMITLRKLP----VAACVMSAQAMA**VDFH	+4
LamBΔ78r2	**MMITLRKLL----VAAGVMSAQAMA**VDFHGYARSG	+10
OmpA	**MKKTAIAIAVALAGFATVAQA**ATSTKKLHKEPATLIKAIDGT	+21
OmpA I8N	**MKKTAIANAVALAGFATVAQA**ATSTKKLHKEPATLIKAI	+18
OmpA I8D	**MKKTAIADAVALAGFATVAQA**ATSTKKLHKEP	+11
OmpA L12N	**MKKTAIAIAVANAGFATVAQA**ATSTKKLHKE	+11
OmpA L12D	**MKKTAIAIAVADAGFATVAQA**	0
2K7L	**MMKKNNLLLLLLLGTANAAS**EIYNKDGNKVDLYGKAV	+17
2K8L	**MMKKNNLLLLLLLLGTANAAS**EIYNKDGNKVDLYGKAVGL	+19
2K9L	**MMKKNNLLLLLLLLLGTANAAS**EIYNKDGNKVDLYGKAVGL	+19
2K8V	**MMKKNNVVVVVVVVVGTANAAS**EIYNKDGNKVDLYGK	+15
2K9V	**MMKKNNVVVVVVVVVVGTANAAS**EIYNKDGNKVDLYGK	+15
2K10V	**MMKKNNVVVVVVVVVVVGTANAAS**EIYNKDGNKVDLYGKAV	+17
CLY	**MRSLLILVLCFLPLAALG**KVFERCELARTLKRLGMDGYRGIS	+24
CLY R3	**MRRRSLLILVLCFLPLAALG**KVFERCELARTLKRLGMDGYRGIS	+24
CLY R17D	**MDSLLILVLCFLPLAALG**KVFERCELARTLKRLGMDGYRGIS	+24
M13 coat SP	**MKKSLVLKASVAVATLVPMLSFA**AEGDDPAKAAFNSLQASATEYI	+22
M13 coat SP ΔN	**M--------NSVAVATLVPMLSFA**	0
M13 coat SP ΔN + 3	**M--------NSLRRRNSVAVATLVPMLSFA**AEGDDPAKAAFNSL	+14

[a] Maximum number of amino acids added to signal peptide with significant clusters found. The signal sequence is indicated in bold. Where applicable, the (point) mutations are indicated in red.

2.3. In Silico Analysis Correlations with In Vitro and In Vivo Findings of Protein Translocation Effects of Signal Sequence Mutations

The LamB signal sequence has been, like the PhoE signal sequence, extensively studied (see, for example, [49]). In the present study, the in vivo results [49] are compared with the in silico results. As mentioned above, the WT LamB signal sequence can translocate an additional 10 amino acids spontaneously across the membrane without any proteinaceous component (see Table 1). The results of three studied deletion mutants are summarized in Tables 2 and 3. It was demonstrated experimentally that the LamB Δ78 signal sequence mutation leads to a non-functional precursor unable to demonstrate protein translocation in vivo [49]. Looking at the lipid binding potential, it is clear that the LamB Δ78 peptide by itself can be considered as a lipid-binding region (see Table 1). However, no significant clusters were found using MCPep when additional amino acids were attached, implicating that, indeed, this signal sequence mutation yields a non-functional signal sequence. A SignalP 4.1 analysis [50] was used to see whether all studied LamB signal sequences were identified as signal sequence and, indeed, no signal peptide identification was found for the LamB Δ78 while all other LamB varieties were identified positively. The in silico results indicate that this mutation renders a non-functional signal sequence as was found experimentally in vivo. It can be argued that one of the reasons why this signal sequence is non-functional in silico is because the middle part of the signal sequence is too short. For this reason, the standard 30 Å membrane width was reduced to 22 Å; under these circumstances, no relevant clusters were found with MC Pep as well, in other words still no functional signal sequence functioning can be predicted and thus expected. The two other deletion mutants LamB 78r1 and LamB 78r2 were found to restore in vivo translocation with 50 and 90%, respectively [49]. Initially no significant clusters were found using MCPep although the Heliquest analysis predicted lipid-binding region features (Table 1). However, when a reduced membrane width, 22 Å instead of the standard 30 Å, was used, indeed, significant clusters were found with MCPep. The in silico results indicated a 40 and 100% translocation of additional amino acids, respectively (see Table 3). So, under the assumption that it is allowed and relevant to adjust the membrane width, it is clear that again a remarkable correlation between in silico results and in vivo results was found. In order to exclude the possibility that only by adjusting membrane width can a correlation be found, another described LamB mutation A13D was investigated as well (see Tables 2 and 3).

Both the in vivo and in silico results indicate a non-functional signal sequence. This seems to suggest that in vivo differences found with different signal sequence mutations can be attributed primarily to signal sequence–phospholipid interactions during the protein translocation process most likely at the initial stages.

M13 coat protein is well-studied and one study in particular looked at the effects of different signal sequence mutations [51]. The wild-type M13 coat protein is able to translocate 22 additional amino acids across the membrane spontaneously as was found when using the MCPep simulation (see Tables 1 and 2). The deletion of parts of the N-terminus did not result in significant clusters in MCPep indicating that this mutation of signal sequence renders a non-functional sequence (see Table 2) and this also correlates with what was found in vivo [51]. Reintroduction of three positive charges partially restored protein translocation more than 60% according to the in silico data (see Table 3) and around 90% according to the in vivo results [51]. In conclusion, results as described and obtained for both the LamB and M13 coat protein showed remarkably good correlations between overall in vivo effects of protein translocation as a result of signal sequence mutations and the signal peptide–phospholipid interactions as studied using in silico results.

Due to the intrinsic limitations of both in vivo and in silico approaches, it is not always possible to compare these types of experiment on a one-to-one base. This was clear when looking in somewhat more detail to the finding of the CLY peptides. In vivo studies showed three distinct outcomes for the WT and two mutations of the precursor [52], ranging from a rather low efficiency up to a two-fold better protein translocation efficiency as summarized in Table 3. Apparently, the transmembrane tendency as indicated by the

Heliquest prediction (Table 1) and the ΔG predictor program (Table 3) is too strong to enable the MCPep to distinguish between these three sequences; indeed, all three are predicted to be able to translocate 24 AA across a lipid membrane (Tables 2 and 3). For the OmpA mutations, it was a somewhat mixed picture. The two peptides OmpA I8N and L12N corresponding to the signal sequences of the precursors with a point mutation as studied in vivo [53] were predicted by MCPep to able to translocate a significant portion of additional AA compared to WT (86 and 52%, respectively) while the in vivo results indicated a fully non-functional precursor. Interestingly enough, this same paper [53] briefly described that the OmpA I6N mutation was functional. Since MCPep cannot distinguish between I6N and I8N (for being a too subtle difference), then from this perspective the MCPep prediction was rather good. The OmpA SP L12D (closely related to L12N) was predicted by MCPep to be non-functional and this would suggest that this prediction was rather good as well. Most likely the observed discrepancies find their origin in the same limitations of the MCPep program as discussed for the CLY peptides.

Table 3. Comparison of the in silico results obtained by ΔG predictor (ΔG_{app}) and MCPep and the in vivo protein translocation efficiency as described in previous studies (see main text for sources). The + and − assignments relate to the value of ΔG_{app}, low is positive and high is negative.

Name	Translocated Mature	ΔG_{app}	$MCPep_{pred}$/ ΔG_{app} [c]	In Vivo/In Vitro Activity
PhoE SP WT	+7 AA (TM)	1.972	100/+	100 (+pmf)
PhoE SP WT	+17 AA (TM)	1.972	100/+	ND [a]
PhoE SP G10L	+7 AA (TM)	0.958	100/++	100 (−pmf)
PhoE SP G10L	+17 AA (TM)	0.958	100/++	ND
LamB WT	+10 AA (TM)	1.633	100/+	100
LamB A13D	+1 AA (Surf.)	3.818	0/−	10
LamB Δ78	+5 AA (Surf.) [b]	No pred.	0/−	0
LamB Δ78r1	+4 AA (TM) [b]	3.798	40/−	50
LamB Δ78r2	+10 AA (TM) [b]	2.533	100/+	90
OmpA SP WT	+21 AA (TM)	1.382	100/+	100
OmpA SP I8N	+18 AA (TM)	2.581	86/−	0
OmpA SP I8D	+11 AA (TM)	2.933	52/−	ND
OmpA SP L12N	+11 AA (TM)	2.961	52/−	0
OmpA SP L12D	+2 AA (Surf.)	3.468	0/−	ND
CLY WT	+24 AA	−1.184	100/++	100
CLY R3	+24 AA	−1.569	100/++	216
CLY R17D	+24 AA	−0.536	100/++	17
M13 SP WT	+22 AA (TM)	2.806	100/+	100
M13 coat SP ΔN	+3 AA (Surf.)	3.610	0/−	0
M13 coat SP ΔN + 3	+14 AA (TM)	4.562	64/−	90

[a] ND, not determined. [b] Membrane width 22 Å. [c] The intuitive assignment of + and − is defined as follows: ΔG value below 1.0 (++), between 1.0 and 2.8 (+) and above 2.8 (−).

2.4. In Silico Analysis of Signal Peptide–Lipid Interactions Correlate with In Vitro and In Vivo Findings That Increasing Hydrophobicity in Signal Sequences Changes the Degree of Anionic Phospholipid Dependency in Protein Translocation

Phoenix et al. have published intriguing results that demonstrated that increasing the hydrophobicity of the signal sequence led to a decreased anionic phospholipid-dependency of the process [24]. This is interesting in multiple ways. First, this seems to suggest that the anionic phospholipid dependence of protein translocation cannot merely be a matter of keeping Sec proteins in a protein translocation competent way, since at low levels of anionic phospholipids, the protein translocation efficiencies are at wild-type levels with no other change than alterations in the signal sequence. Second, this seems to suggest that the signal sequence–phospholipid interactions are the predominant factor for the overall anionic phospholipid dependency of the protein translocation process. However, it has never been

demonstrated that the corresponding signal sequences bind to membranes with low levels of anionic phospholipids when the hydrophobicity of the signal sequences was increased. This was studied in the present study and results are summarized in Tables 1 and 2.

The results with leucines (2K8L and 2K9L) already correspond reasonably well to the observed experimental tendencies (see Figure 1 and for more details Table S2) [24]. In order to look into this somewhat deeper, in silico experiments were performed using valine instead of leucine. Valine is less hydrophobic than leucine, so it was checked whether the MCPep program was more sensitive to changes in hydrophobicity using valine mutants instead of leucine mutants. Indeed, a very similar tendency as found experimentally was observed. Where Phoenix et al. observed an anionic independent protein translocation with two lysines present and increased the number of leucines from eight to nine, the in silico experiments showed a similar tendency when two lysines were present and when the number of valines was increased from nine to ten (see Figure 1 and Table S2). The fact that the leucine containing peptides could not be 'perfectly' compared with the in vivo results is most likely due to the fact that leucine is too hydrophobic and therefore MCPep cannot distinguish between a peptide with eight or nine leucines. This is most likely due to the inevitable limitation of the hydrophobicity scale used in MCPep (or any other comparable program) which has been elaborated further elsewhere [33].

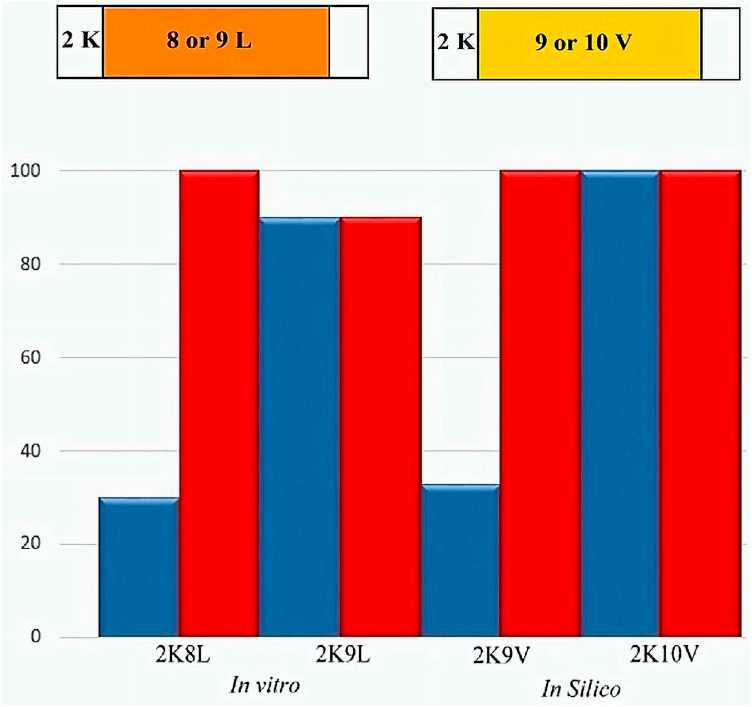

Figure 1. Comparison of protein translocation efficiency of in vitro [24] and in silico experiments (this study). Two sets of peptides where compared, one with 8 or 9 leucines (L) and one with 9 or 10 valines (V). The anionic phospholipid content used is 5% (blue) and 20% (red), respectively.

Having said this, the in silico results of the leucine and/or valine containing peptides not only suggest, as was found previously [54], that the effect of removing one or more positive charges can be compensated by an increased hydrophobic region in the signal sequence, but also that an increased hydrophobic region can make the signal sequence–phospholipid interaction anionic phospholipid-independent and consequently makes the overall protein translocation process anionic phospholipid-independent as was already suggested experimentally in vitro [24].

2.5. In Vivo Proven Signal Sequence Lipid Contacts in ER Translocation

An important paper in relation to experimentally demonstrated signal sequence–phospholipid interactions in vivo was found in the protein translocation process in the

ER [55]. This study was unfortunately unable to distinguish between the signal peptide–lipid interaction as the result of an initial interaction of the signal peptide with the lipids and the subsequent movement towards the channel or because of a binding of the signal peptide to lipids after the outward movement of the channel. A few years later, a paper strongly suggested an inward movement [56] and indicated important (initial) events at the interface of the channel and the lipid phase. Using Heliquest, both the signal anchor peptide of the type II membrane protein invariant chain as well as the preprolactin signal peptide used in the Martoglio paper [55] can be classified as potential lipid-binding regions (see Supplementary Materials for details). It was experimentally observed that the signal anchor region crosslinked roughly three times more lipids than the preprolactin signal peptide. Based on the Heliquest results, it was found that the signal anchor peptide gave rise to a higher mean hydrophobicity than the used signal peptide (0.798 and 0.418, respectively). The fact that the signal anchor peptide corresponds to a longer stretch of potential lipid-binding amino acids than the signal sequence (25 AA and 18 AA, respectively) indicates that the theoretical crosslinking ability of the signal anchor peptide is roughly three times higher than that of the signal peptide as was found experimentally [55]. This interpretation of the Heliquest results is substantiated with the MCPep results. In both cases, so-called significant clusters for both peptides were found, indicating spontaneous interactions with lipids. Furthermore, the preprolactin is predicted to interact with lipids in a surface configuration, while the SA peptide interacts with lipids in a transmembrane configuration (see Figure 2 for examples of these configurations). Again, not only the predicted lipid binding but also the subsequent extent of expected crosslinking correspond remarkably well with the experimentally observed results [55] and strongly indicate that the signal sequence–lipid interaction takes place before a possible inward movement towards the translocon.

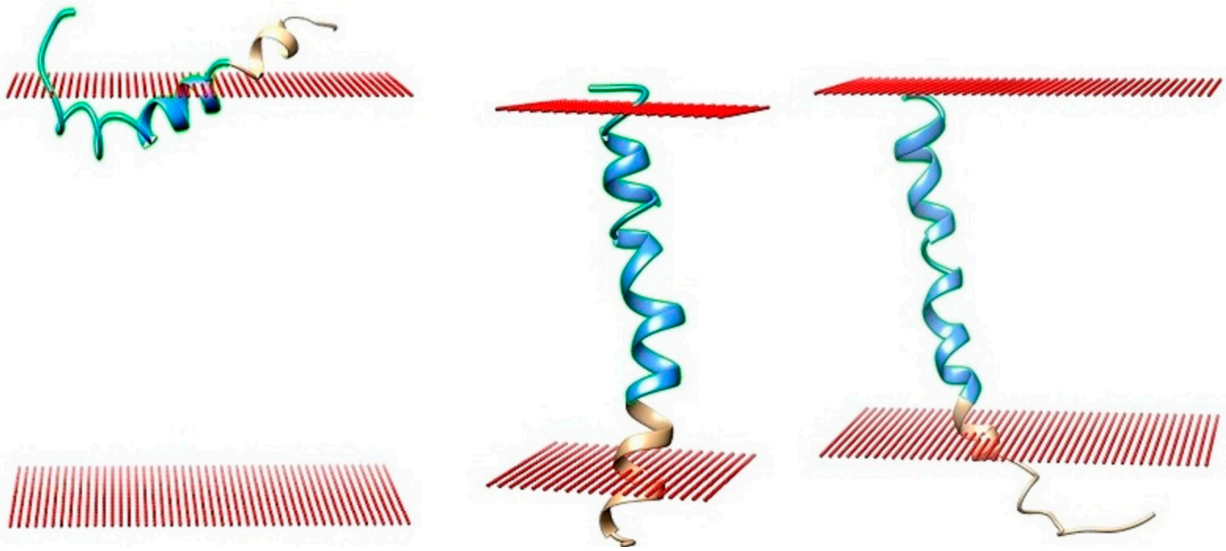

Figure 2. The model representations of the Monte Carlo results as obtained by MCPep [46] depict from left to right the PhoE SP with 10 extra amino acids (AA) interaction with the membrane in the surface and TM orientation, respectively. On the right, a transmembrane (TM) orientation of the PhoE SP with 16 additional AA is depicted. The signal sequence is indicated in blue/green, and the white/yellow color resembles the additional AA attached.

2.6. An Example of a Closely Related Transmembrane Insertion Experiment

Membrane protein insertion and integration in the membrane is closely related to protein translocation using the same Sec system. It has been demonstrated that certain transmembrane domains (TMDs) can spontaneously translocate a significant number of additional amino acids across a pure lipid membrane [57]. One particular experiment is repeated by the in silico approach presented herein. For the so-called b5-ops-29 protein, the results correspond nicely with what is found experimentally. First, the Heliquest-based

Eisenberg plot approach (Ref. [32] confirms the presence of a transmembrane region in this protein since the mean hydrophobicity is 1.138, which is above the threshold value of 0.75 characteristic for a potential transmembrane region. Furthermore, the MCPep result depicts a transmembrane helix with additional amino acids attached in a transmembrane configuration indicative of a spontaneous translocation of the additional amino acids across the membrane (for further details, see Supplementary Figure S2). It was shown experimentally that even up to 85 amino acids can be translocated unassisted [58]. The MCPep does not allow proteins or peptides longer than 50 AA so this experiment could not be mimicked completely. Overall, the in silico data indicate a strong correlation to what is found experimentally and again emphasize the validation of the approach as extensively used and described here in this study.

3. Discussion

It is discussed and provocatively concluded here that it is in the process of transmembrane helix insertion that the insertion takes place according to a proposed 'sliding' model where the insertion is merely mediated by the Sec translocon [59]; thus, it is implied that the insertion of a transmembrane helix does not take place within an exclusive proteinaceous environment. It is tempting to speculate on a similar 'sliding' model for signal sequences, i.e., the signal sequence inserts close to the lateral gate of the translocon while in constant contact with the lipid phase of the membrane.

Based on the in silico protein translocation assay as used here, it is tempting to speculate that not only the signal sequence but also the first part of the mature part of the precursor protein is translocated according to this sliding model, i.e., a pure lipidic environment event (see Figure 3 for a schematic representation; a more realistic depiction of the conformation and orientation of the SP is depicted in Figure 2).

As argued in an excellent review by the group of Randall [60], it is vital and I quote: "Sometimes the early literature is overlooked because powerful, new techniques have been developed and structures have become available. However, the early data are robust and hold an enormous amount of valuable information. . . .One may discover important insight into interpretation of early data when seen in retrospect and armed with knowledge gained from subsequent work. . ." Indeed, the study presented herein emphasizes the importance of having a closer look at some of the key papers published a significant time ago. For example, the paper by Phoenix et al. [24] strongly suggested that increasing the hydrophobicity of the signal sequence results in an anionic independent protein translocation process. However, it did not demonstrate a direct correlation with signal peptide–phospholipid interactions. The results presented here provide this 'missing link' and make it more likely that, indeed, the only reasonable explanation for the results as observed previously [24] is a direct signal sequence–phospholipid interaction during the earlier stages of the protein translocation process and might explain how alterations in signal sequences can influence the anionic phospholipid dependency of the overall protein translocation process. Even stronger, the current study seems to suggest that not only the signal sequence but also the first number of amino acids of the mature part of precursor proteins translocate across the membrane in a spontaneous fashion presumably according to the 'sliding model' as was proposed for the insertion of transmembrane helices [59]. The most recent paper that focused on the phospholipid dependence of membrane protein insertion [61] revealed an interesting inventory of which factors are most likely attributed to this, and which ones are not. A report that described the role of signal peptides in a SecA transport [62] strongly demonstrated a signal peptide–lipid interaction and indicated a model where this interaction was part of a clustering of both substrate and accessory proteins closely related to the model that was proposed earlier for the Sec system in *E. coli* [31] and used the original idea that described the benefits of the reduction in dimensionality in biological processes [63]. The idea of the sliding model is supported by the earlier findings by P. C. Tai and co-workers. They demonstrated intriguing results that some proteins can translocate across the membrane in the presence of SecA but without the presence of the SecYEG

complex [21,22]. It was furthermore demonstrated that the SecYEG complex increased the efficiency and signal peptide specificity of the process [23]. These findings are in line with the proposed sliding model and with the finding of spontaneous translocation of the first part of the mature part, along with a direct signal peptide–lipid interaction being an intrinsic part of the protein translocation process where the SecYEG complex is not a 'real' pore but much more a facilitator for more efficient and specific protein translocation. The often-used name for the SecYEG complex putative 'protein conducting channel' (PCC) might be better called 'protein translocation mediating complex' (PTMC). A paper that was published recently made a first attempt to incorporate an explanation for the earlier observed signal peptide–phospholipid interaction [25] into their model based on the demonstrated, enriched anionic phospholipid content around the translocon [64]. The demonstrated intermediate state of the translocon seemed to suggest partial membrane partitioning and folding of a nascent chain that suggested more events at the lipid–PTMC interface than anticipated before [65]. So, one might speculate that the membrane interface in the vicinity of the translocon plays an important role in translocon-guided protein translocation in a similar way as suggested for TM helix insertion [66].

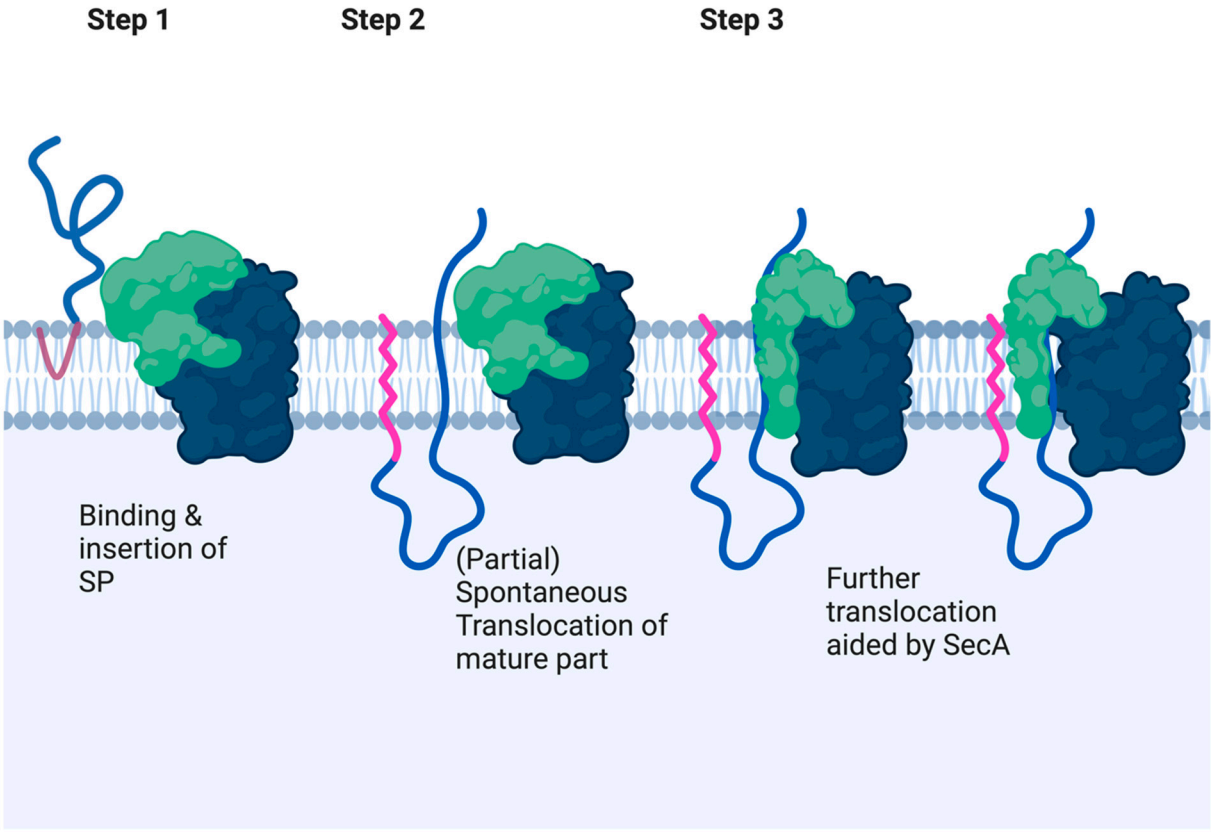

Figure 3. A schematic representation of the various (initial) steps in protein translocation. Step 1 is the binding and partial insertion of the SP (in purple). In Step 2, the SP adopts a close to transmembrane orientation and subsequently translocates additional amino acids (colored in blue) across the membrane. In Step 3, additional parts of the mature protein translocate either purely through the lipid phase of the membrane or 'slide' along the surface of the Sec machinery (dark blue) with the possible aid of SecA (green) that provides a 'pushing' movement. Created using https://www.biorender.com (accessed 10 January 2023).

It cannot be ruled out that in the presence of the translocon, the process proceeds preferentially through the translocon but the ability of the signal sequence to interact with anionic phospholipids and the subsequent ability to translocate a substantial number of amino acids across the membrane (without a proteinaceous component) makes it more

than feasible that the protein translocation process utilizes these intrinsic abilities when it comes to the signal sequence–phospholipid interactions.

It is good to realize that, for example, our idea of cell membranes as once proposed by Singer and Nicolson [67] is nowadays more complex than anticipated ever before (for excellent reviews, see elsewhere [68,69]). As ideas change on how cell membranes appear and how they function, it might very well be that our ideas about the role of phospholipids in a process like protein translocation and their importance and relevance need to be re-evaluated as well.

4. Methods

4.1. Primary and Secondary Structure

The primary structure that is used in the analysis was obtained from information in the indicated references and/or from the Uniprot database [70]. The extent of helicity was routinely checked by secondary structure prediction like the program SOPMA available at http://npsa-pbil.ibcp.fr/ (accessed on 20 August 2020), see for more details [33] and references herein.

4.2. Determination Lipid-Binding Potential and Eisenberg Plot Approach

The identification of potential lipid-binding regions was determined using the Heliquest software available at website http://heliquest.ipmc.cnrs.fr/ (accessed on 25 January 2024). See elsewhere for detailed information [45]. In short, the Heliquest server provides information about the mean hydrophobicity (<*H*>), the hydrophobic moment (<μH>) and the net charge (z) for a given region. The so-called lipid-binding discrimination factor (D) depends on the hydrophobic moment (μH) and the net charge (z) and is defined according to: $D = 0.944$ (<μH>) + 0.33 (z). D-values above 0.68 are considered to be a (potential) lipid-binding α-helix [45]. Only regions are considered that are predicted to be predominantly helical.

4.3. Heliquest-Based Eisenberg Plot Approach

For the Eisenberg plot approach, both the mean hydrophobicity (<*H*>) and the hydrophobic moment (μH) were plotted using Heliquest generated data (see elsewhere [32] for a detailed description). In short, this approach allows the identification of lipid-binding regions missed by the Heliquest lipid-binding discrimination factor and additionally it can characterize the identified regions as either possibly surface seeking (S) or as a transmembrane (TM) helix. Since a <*H*> value above 0.75 indicates a possible transmembrane regions and positions in the Eisenberg plot above the following line, <μH> = 0.645 − 0.324 (<*H*>) indicates the presence of a surface-seeking region [32].

4.4. Monte Carlo Simulations Using MCPep

The MCPep server allows the performance of Monte Carlo (MC) simulations of the interaction of helical peptides with lipid bilayers. If a likely interaction is predicted, then the program provides information on whether the peptide prefers the transmembrane (TM) or the surface orientation configuration. A typical analysis (see elsewhere [46] for more detailed information) includes the input of the corresponding sequence in FASTA format, a membrane width of 30 Å and an RMSD cut-off of 3 Å. The anionic lipid content used is 25% unless stated otherwise in the Results and Discussion sections. The number of independent MC runs (three) and the number of MC cycles in each independent run (500,000) were fixed for each analysis. The MCPep server [46] used to be available at http://bental.tau.ac.il/MCPep/ (accessed on 28 August 2020) and was transferred to a newly designed website: https://www.bentalab.com/program-and-databases (accessed on 2 August 2023). See Supplementary Materials for further details on how to handle this program properly and some remarks about the possible alternatives for MCPep.

4.5. Structural Modelling

I-TASSER was used to generate the 3D structures and corresponding PDB files of a number of proteins studied. This program is available at website http://zhanglab.ccmb.med.umich.edu/I-TASSER/ (accessed on 1 December 2023) and although the use of this program requires a one-time subscription (with the use of a valid e-mail address) it is free to use [71,72]. Chimera, available at website http://www.cgl.ucsf.edu/chimera/ (accessed on 15 January 2024), was used to view the created PDB files in this study.

4.6. In Silico Protein Translocation Assay

In order to mimic the protein translocation event, the peptide–membrane interaction was checked using MCPep. The peptide of interest was elongated with additional amino acids of the mature part of the corresponding protein until no significant clusters could be found. In addition, the tendency of the peptide to form a transmembrane helix was checked using ΔG predictor, available at http://dgpred.cbr.su.se/ (accessed on 24 December 2023). This server was used to calculate the so-called ΔG_{app}, a measure for the prediction of the apparent free energy difference, for insertion of this sequence into the endoplasmic reticulum (ER) membrane by means of the Sec61 translocon [73,74].

5. Conclusions

The current paper focuses on signal peptide–phospholipid interactions in relation to protein translocation. Where possible, a comparison is made between the results as obtained from several analyses using bioinformatics and in silico experiments and the results of in vitro and in vivo experiments as described in the literature. As discussed, every single approach either in silico, in vitro and in vivo have their own benefits and limitations. Having said this, it is intriguing to see that the tendencies as seen in the presented in silico results correlate remarkably well with what is seen in the various mutation studies as described in the literature. The finding that almost all in vivo and in vitro results in the related matters can be explained solely by looking at the signal peptide–phospholipid interaction seems to stress that the current model of protein translocation needs revision. This study points towards potentially interesting mechanistic questions open for further study in this still evolving field.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/lipidology1010002/s1, Figure S1: details of prePhoE SP; Table S1: comparison WT and mutated prePhoE SP; Table S2: additional info Figure 1; Figure S2: MCPep results of b5 TMD; Figure S3: helical wheel plot of b5 AA 5-22.

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