OmpF-Lpp Signal Sequence Mutants with Varying Charge Hydrophobicity Ratios Provide Evidence for a Phosphatidylglycerol-Signal Sequence Interaction during Protein Translocation across the Escherichia coli Inner Membrane*

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Using inverted Escherichia coli inner membrane vesicles we have analyzed the phosphatidylglycerol dependence of translocation of an OmpF-Lpp fusion protein carrying a signal sequence with varying positive charge at the N terminus and a hydrophobic core of varying length.

It is shown that there is a direct relationship between the phosphatidylglycerol requirement of translocation and the requirement within the translocation process for positive charges on the signal sequence. This provides further evidence that the negative head group of the lipid is required for functional interaction with the positively charged N terminus of the signal sequence.

Proteins which are destined for translocation across the Escherichia coli inner membrane are synthesized as precursors with an N-terminal extension. There is no conserved sequence among these N-terminal signal sequences although comparative sequence analysis suggests that they tend to be 16–26 amino acids in length with a basic N terminus, a central hydrophobic core, and a polar C terminus (1). Signal sequences are reviewed in Ref. 2. In addition to the signal sequence a number of proteins have been shown to be essential for translocation (reviewed in Ref. 3), and these include the integral membrane proteins SecE, SecY, and the peripheral membrane protein SecA. SecA has been shown to possess ATPase activity and is responsible for the hydrolysis of ATP during the translocation reaction (4). It has been postulated that the integral membrane proteins may form an aqueous channel (5) through which the protein can translocate, although as yet there is no direct evidence for this and it is possible that these proteins act as the translocation machinery (6) by allowing translocation to initiate at a protein lipid interface.

In addition the translocation process has been shown to be dependent upon the presence of anionic phospholipids. In E. coli mutants containing reduced levels of PG* and cardiolipin the translocation of outer membrane proteins is inhibited (7–9). If the anionic lipids are reintroduced then translocation is restored (10). The exact role of PG is unclear although it has been shown that SecA can interact with anionic phospholipids and indeed shows translocation ATPase activity only in the presence of anionic lipid, precursor protein, and SecY (11). A further role for PG is suggested by the lipid model for translocation which postulates that PG interacts directly with the positively charged N terminus of the signal sequence thus initiating insertion of the signal into the bilayer at the start of the translocation process (12, 13), and there is much indirect evidence which supports this view. A negatively charged lipid-specific insertion of signal peptides into model membranes has been demonstrated for the synthetic signal peptides of M13 coat protein (14), LamB (15), and the outer membrane protein PhoE (14). In addition the affinity of signal peptides for an anionic lipid interface has been seen to correlate with the efficiency with which precursor proteins containing these signal sequences are able to translocate (16). It has also been observed that increasing the level of lipid order within the membrane by the formation of a gel phase inhibits translocation (17, 18), and under monolayer packing conditions equivalent to the ordered gel state the penetration of the PhoE signal peptide is inhibited (14). Both protein and lipid elements therefore have a crucial role within the translocation process, yet the basic question underlying the role of lipid within translocation still remains unanswered. In this paper we have investigated the requirement for PG within translocation by using the model secretory protein OmpF-Lpp (19). The cleavable signal sequence has been mutated such that it contains a hydrophobic core of either 8 or 9 leucine residues and in addition the N terminus of the signal has been altered such that it contains either 0, 2, or 4 positively charged lysine residues (20). This produced two series of proteins (0K8L, 2K8L, 4K8L and 0K9L, 2K9L, 4K9L) both of which are able to undergo in vitro translocation. It has previously been shown that the translocation efficiency of the Leu8 series is strongly dependent on the charge present on the signal sequence, whereas the Leu8 series is able to translocate independently of the charge on the signal sequence (20). By performing in vitro translocation reactions with inverted inner membrane vesicles containing varying levels of PG we have been able to gain insight into the PG dependence of the translocation process with respect to the require-

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The abbreviations used are: PG, phosphatidylglycerol; IPTG, isopropyl β-D-thiogalactopyranoside; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.

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ment for a positively charged signal sequence. This therefore provides strong evidence for a direct interaction between the signal sequence and anionic lipids within a functional translocation pathway.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids—**E. coli strain MRE600 (21) was used to prepare inverted inner membrane vesicles with a wild-type lipid composition. In addition, this strain was used for the isolation of an S-135 extract (22). Inverted inner membrane vesicles with differing levels of acidic phospholipid were isolated from strain HDL11 as previously described (10).

Plasmids O8K, 2K8, 4K8, O9K, 2K9, and 4K9 were used for the in vitro expression of the OmpF-Lpp mutants O8K, 2K8, 4K8, O9K, 2K9, and 4K9 respectively (20).

**Isolation of Assay Components—**SecA (23), nonspecific lipid transfer protein (24), and SecB (25) were purified as described elsewhere. 1,2-Dioleoyl-sn-glycero-3-phosphoglycerol was prepared and purified according to the published method (26). [35S]methionine (1000 Ci/mmol) was obtained from Amersham Corp., isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from Sigma.

**In Vitro Transcription-Translation and Translocation Reactions—**The in vitro reactions were basically performed as described previously (27). SP6 polymerase (Pharmacia LKB Biotechnology Inc.) was used for transcription of the plasmids at 37 °C for 45 min. The S-135 extract was from MRE600. Inverted inner membrane vesicles with varying PG content were prepared from HDL11 grown with 0, 10, 30, or 50 µM IPTG (10). Wild-type vesicles were prepared from MRE600. The phospholipid content was determined after phospholipid extraction (28) using thin layer chromatography and perchloric acid destruction (29). The chromatography plates were impregnated with 1.2% boric acid in ethanol/water (1:1) and dried before use. The solvent system used was chloroform, methanol, water and ammonium (25%) (65:37.5:3:1) v/v (30).

After transcription, translation was allowed to continue for 10 min after which the mixture was diluted 1:1 with 12 mM sodium phosphate buffer, pH 7.4. The diluted mixture was sonicated for 3 min in a sonicating water bath to prevent aggregation of the precursor proteins (20).

2.5 µl of the transcription-translation mixture were used in a translocation reaction with a final volume of 25 µl. The translocation reactions were performed at 37 °C as previously described (27) but the reaction was only allowed to proceed for 5 min after which proteinase K was added to digest all of the nontranslocated protein. After a further incubation of 10 min at 37 °C the protease treatment was stopped by the addition of trichloroacetic acid to a final concentration of 15% (w/v). After precipitating on ice the samples were rehydrated, and quantified. The percentage of added precursor which underwent translocation is shown. The average of the data from six experiments along with the standard deviations are shown.

**RESULTS AND DISCUSSION**

The relative levels to which the mutant proteins were able to translocate across wild-type vesicles (MRE600) was tested. The signal sequence which contains no charge and 8 leucines (Fig. 1A) translocates at a very low rate with the translocated protein only representing 4–5% of the available precursor. The addition of charged lysine residues at the N terminus of the signal greatly enhances the translocation efficiency of the Leu8 series, as can be seen for the 2K8L and 4K8L proteins (Fig. 1A). These data are in agreement with the results previously obtained (20) and indicate that, for this series of proteins, a positively charged signal sequence plays an essential role if efficient translocation is to be obtained, although a charged signal is not an absolute requirement for the translocation reaction since some degree of translocation occurs in the case of the 0K8L OmpF-Lpp. This would imply that the charged signals are undergoing some form of electrostatic interaction which enhances the translocation efficiency. Increasing the level of charge from 2 to 4 Lys residues causes a decrease in the level of translocation. This could be explained if the strength of the electrostatic interaction reached such a level that the following stage in the translocation process is hindered or if the increased charge interferes with the role of the signal's hydrophobic core.

In contrast to the Leu8 series the Leu4 series can be seen to
undergo levels of translocation which are independent of the charge present on the signal sequence (Fig. 1B). This would indicate that by increasing the signal sequence core hydrophobicity and length by the addition of an extra Leu residue it is possible to overcome the need for a charged signal. This suggests that the Leu$_8$ series is undergoing a translocation related interaction which is dominated by hydrophobic rather than electrostatic forces.

We wished to observe whether translocation of the charge dependent OmpF-Lpp Leu$_n$ series of mutants was dependent on PG. The *E. coli* strain HDL11 has the *pgsA* gene encoding for the enzyme phosphatidylglycerol phosphate synthase regulated by the lac operon hence by growing the cells in the presence of varying IPTG concentrations it is possible to control *pgsA* expression and therefore PG synthesis (10). In the following experiments vesicles with varying levels of PG were produced from HDL11, and the level of translocation across these vesicles was compared to the level of translocation across wild-type membrane vesicles which were assumed to be 100% efficient for this system. The 0K8L protein which contained an uncharged signal sequence appeared to undergo translocation in a manner which was independent of the level of PG present (Fig. 2A). In contrast, the translocation efficiencies of the charged proteins, 2K8L (Fig. 2B) and 4K8L (Fig. 2C) show a strong dependence on the level of PG present within the membrane vesicles. Importantly wild-type levels of PG (19 mol% of total lipid) allowed translocation to proceed at 100% efficiency. Included in Fig. 2B is an inset showing an autoradiograph which clearly demonstrates the increase in translocation efficiency of the 2K8L protein with increasing PG.

This data set implies that the Leu$_n$ series not only needs a charged signal sequence for efficient translocation but also requires PG. In addition the PG requirement seems to be directly related to the charge present on the signal with the uncharged signal showing zero PG dependence. These results therefore give the first indication that there is a direct interaction between the positively charged signal sequences and the anionic PG.

The Leu$_n$ series has been seen to undergo translocation in a manner which is independent of the charge on the signal sequence (Fig. 1B) thus raising the question of whether these mutants have a PG requirement for translocation.

Translocation of the Leu$_n$ series across vesicles with varying PG content was compared to translocation across wild-type vesicles which were assumed to be 100% efficient. As can be seen in Fig. 3 all three of the Leu$_n$ mutants are able to translocate at wild-type levels in a manner which is independent of the amount of PG present. Included in Fig. 3B is an inset showing an autoradiograph which clearly demonstrates that the translocation efficiency of the 2K9L protein is independent of the PG present. When compared to the Leu$_n$ series it appears that the addition of an extra Leu residue within the signal’s hydrophobic core has overcome the requirement for PG.

In the above experiments translocation reactions were performed using inverted inner membrane vesicles containing different levels of PG. To ensure that the varying levels of translocation which were observed were directly due to the levels of PG present PG was reintroduced into PG depleted vesicles (HDL11 grown without IPTG induction) via the use of a nonspecific lipid transfer protein (10). The translocation efficiencies of the 2K8L and 2K9L mutants across these membrane vesicles were then observed in an *in vitro* translocation reaction (Fig. 4). If the data are compared to those obtained in Figs. 2B and 3B it can be seen that over the range of PG tested the data obtained by *in vitro* and *in vivo* incorporation of PG into the membrane are comparable. Fig. 4 shows that the addition of PG to PG depleted vesicles restores translocation of the 2K8L protein whereas the 2K9L mutant shows wild-type translocation efficiency independent of the level of PG present. The restoration of translocation efficiency solely by the introduction of PG into the vesicles confirms that the above data were obtained due to varying
levels of PG and were not secondary effects.

These data indicate that there is a direct relationship between the PG required for translocation and the dependence of translocation efficiency on the charge present on the signal sequence. Since the signal sequence is positively charged and PG is anionic, the simplest interpretation which fits these data is that the signal sequence directly interacts with PG at some stage within the translocation process.

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Fig. 3. PG dependence of translocation for the mutants containing a signal sequence with a Leu\(_w\) core. Translocation assays were performed on OmpF-Lpp signal sequence mutants which contained a Leu\(_w\) hydrophobic core and 0 (A), 2 (B), or 4 (C) Lys residues at the N terminus. The samples were analyzed as in Fig. 1. Translocation across PG-depleted membranes was related to translocation across wild-type vesicles (18.5 mol% PG), which was assumed to be 100% efficient for this system. The inset shows the results of a translocation experiment with the 2K9L mutant where 1 represents translation product added to the system, L a leader peptidase digest, W shows translocation across wild-type vesicles (100% translocation), and the remaining lanes show translocation across E. coli HDL11 vesicles containing 3.8 (1), 8.3 (2), 14.2 (3), and 19.1 (4) mol% PG. Results from five data sets are shown along with their standard deviations.

Fig. 4. In vitro manipulation of PG levels. PG depleted vesicles from E. coli HDL11 had increasing levels of PG incorporated into the bilayer via the use of a nonspecific lipid transfer protein (10). The vesicles were then used in a translocation assay with mutant OmpF-Lpp proteins containing a signal sequence with Lys\(_w\) and a hydrophobic core of either 8 (3) or 9 (4) leucine residues. The level of translocation was related to the 100% level of translocation seen with wild-type vesicles (MREG00, 18.5 mol% PG). Samples were analyzed as in Fig. 1.

We shall consider mechanisms of translocation in relation to the initial precursor interaction. Two scenarios have been suggested for signal sequence interactions within translocation. The first model emphasizes the role of specific phospholipids in the process of translocation (12, 13). It is postulated that the signal sequence primarily interacts with the bilayer in an electrostatic manner with the basic N terminus of the signal interacting with anionic phospholipid head groups. This interaction then stabilizes the signal in a given conformation which is probably helical in nature (32, 33) thus allowing insertion of the signal's hydrophobic core into the bilayer. This insertion may occur via the formation of a helix-turn-helix motif (12, 13) and is postulated to locally destabilize the bilayer structure thus facilitating translocation (34). Many experiments have emphasized a signal sequence lipid interaction and shown the importance of both the basic N terminus (35–38) and hydrophobic interior of the signal (32). It has also been shown that SecA ATPase activity is stimulated by anionic lipids in conjunction with SecY and the precursor protein and (11) in addition it has been demonstrated that SecA can interact and insert into negatively charged monolayers with the level of interaction being dependent on the nucleotides present (25). Anionic lipids have therefore been implicated in the functioning of both the signal sequence and SecA and postulated to be involved in the formation of a stable translocation complex. The data presented here provide some insight into the role(s) anionic lipids are allocated within the in vivo translocation reaction.

The Leu\(_w\) series of mutants show that wild-type levels of translocation are possible when the PG content of the membrane is as low as 3.8 mol%. Presumably this level of PG is therefore sufficient for the formation of translocation sites, and so at low PG concentrations this is probably not a limiting factor. Furthermore, since the Leu\(_w\) series has been shown to be SecA-dependent (20) the low levels of PG present seems to be sufficient to stimulate SecA ATPase activity and allow these mutants to translocate at wild-type levels. In the case of the Leu\(_w\) series wild-type levels of translocation are obtained only for the charged mutants when normal levels of PG (19 mol%) are present in the vesicles. Providing that 4 mol% PG is sufficient to produce enough translocation sites and ATPase activity for high levels of translocation then the
fact that this does not occur suggests that the additional PG is required for an extra step prior to translocation. It should, however, be noted that other explanations are possible, and if the variations in the tripartite structure of the signal sequence mutants cause the mutants to interact differentially with the translocation machinery (SecA/SecE/SecY) then this could in turn affect the PG requirement of the translocation machinery. If the signal sequence does interact with PG this could occur early in the translocation pathway with subsequent transfer to SecA and then to SecE/Y at the translocation site or the precursor could be targeted directly to SecE/Y by SecA (8, 39) and then encounter PG. We are unable to distinguish between these mechanisms with these data but it is interesting to note that there is evidence for an interaction between the charged N terminus of the signal sequence and SecA (8, 39). By assigning the major PG requirement to a signal sequence-membrane interaction it can be postulated that the increase in the length and hydrophobicity of the Leu9 signal sequences allows direct interaction with the hydrophobic membrane interior thus overcoming the requirement for PG. This agrees with previous work (32) where the length and mean hydrophobicity of the proOmpA signal sequence were seen to be key elements in the formation of a functional signal.

In the alternative hypothesis the signal sequence binds directly to a proteinaceous pore formed by SecE/Y (40-42) but on the basis of the data we have presented here we must discount the fact that the signal sequence interacts solely with proteinaceous components without encountering lipid. The hypothesis that the signal sequence interacts with anionic lipids during translocation does appear to fit our data since we have shown that characteristics of the signal sequence directly affect the anionic lipid dependence of the translocation reaction.

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