A Dual Role for Phosphatidylglycerol in Protein Translocation across the *Escherichia coli* Inner Membrane*

(Received for publication, July 26, 1993, and in revised form, September 20, 1993)

Ron Kusters†‡, Eefjan Breukink†, Andreas Gallusser§, Andreas Kuhn‡, and Ben de Kruijf‡**

From the †Department of Biochemistry of Membranes and Lipid Enzymology and the **Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands and the ¶Department of Applied Microbiology, University of Karlsruhe, Engesserstrasse 7, 75001 Karlsruhe, Germany

The involvement of phosphatidylglycerol in the SecA-independent translocation of M13 procoat in *Escherichia coli* was demonstrated. Processing of procoat to mature coat protein was retarded when the level of phosphatidylglycerol was reduced. In *vitro* translocation experiments using inner membrane vesicles isolated from a strain with inducible synthesis of phosphatidylglycerol, showed that translocation of procoat and of a SecA-dependent procoat analog was proportional to the content of phosphatidylglycerol. Moreover, introduction of phosphatidylglycerol by means of a lipid transfer method into phosphatidylglycerol-depleted inner membrane vesicles, efficiently restored procoat translocation.

The phosphatidylglycerol dependence in both the SecA-dependent and -independent translocation pathway indicates that phosphatidylglycerol plays a dual role in translocation. We suggest that besides membrane binding of SecA this lipid has a direct interaction with the M13 procoat in translocation across the inner membrane.

Newly synthesized proteins often have to move across membranes in order to reach their final destination. The role of (specific) membrane phospholipids in this process has been subject of several studies. Models which evolved from this work (1–5) are based on thermodynamical consequences of the interaction between the targeting signals and membrane lipids, the structural flexibility of membrane lipids, and the analysis of specific and more simple translocation pathways. Direct demonstration of the involvement of the acidic lipid phosphatidylglycerol (PG) in *in vivo* and *in vitro* translocation in the prokaryotic secretion pathway was obtained using *E. coli* mutants with a reduced PG content (6). These studies were confirmed and extended (7–9) and it was demonstrated that the negative charge on the lipid headgroup was directly responsible for efficient translocation across the cytoplasmic membrane. In addition it was shown that SecA, a key enzyme in this pathway, requires PG for membrane binding and ATPase activity (8–10).

Subsequent studies on model systems demonstrated that membrane insertion and conformation of SecA was PG-dependent and modulated by nucleotides (11, 12).

However, these studies did not give insight into the important mechanistic question on the functional interaction between the precursor and, in particular, the signal sequence and acidic membrane lipids proposed in many translocation models (5, 13, 14) and based on both theoretical arguments and studies on isolated signal peptides and membrane systems (15–17).

To get insight into this question we analyzed in this study the role of PG in inner membrane translocation of M13 procoat, a precursor protein which inserts and partially moves across the E. coli inner membrane in a signal sequence-dependent manner (18, 19) without the help of the SecA protein (20). By making use of *E. coli* strains with controlled levels of PG we demonstrate in *vitro* and in *vivo* that PG is involved in this SecA-independent pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**

*Bacterial Strains and Plasmids—*From *Escherichia coli* MRE600 (21) inverted inner membrane vesicles with wild type phospholipid composition and an S-135 cellular extract were isolated. *E. coli* MM52, a derivative of MC4100 (F− Δ lacI*U169 araD136 rpsL1 thi 1 relA) carrying a SecA451 mutation (22) was used for the isolation of a SecA-deficient S-135 cell extract. Inverted inner membrane vesicles with different levels of acidic phospholipids were isolated from *E. coli* HDL11 (pgsA::kan Φ (lac OP pgaA*1 lacA2::lac Y SYN9 pp2 zdg::Tn10) (7). Plasmids pR66-5 and pQN-8 (18) were used for *in vitro* and *in vivo* expression of wild type M13 procoat, respectively. Plasmid pQ-840 ΔE-GDD encoding an M13 procoat-leader peptide fusion protein with deletion of amino acids 27–48 of the procoat sequence was described in Ref. 23.

Other Materials—SecA(12) and nonspecific lipid transfer protein (24) were isolated, purified, and stored as described. 1,2-Dioleoyl-sn-glycerol-3-phosphoglycerol was chemically synthesized and purified according to established methods (25). [35S]Methionine (1000 Ci/mmol) was from Amersham. Isopropylthiogalactoside (IPTG), 9-amino-7-chloro-2-methoxyxycine, and carbonyl cyanide trifuoromethoxy phenyl hydrazide (FCCP) were obtained from Sigma.

**Methods**

*Pulse-chase Experiments—*For radioactive labeling studies, HDL11 cells bearing plasmid pQN-8 were grown in M9 medium to an early log phase at 37 °C in the presence and absence of 100 µM IPTG. The synthesis of procoat protein was induced by the addition of 0.5% l-arabinose for 30 min and the cells were subsequently labeled for 30 s with 100 µCi/ml [35S]methionine. The chase was initiated by the addition of 1 mg/ml unlabelled methionine. After 6, 20, and 40 s samples were withdrawn from the incubation mixture, immunoprecipitated, and analyzed by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and fluorography (26).

*In Vitro Transcription-Translation-Translocation Reactions—*The *in vitro* reactions were basically performed as described in Ref. 27. SP6...
tracts used in the translation-translocation reactions were prepared from *E. coli* MRE600 grown at 37 °C or MM52 grown at 30 °C as indicated. To obtain an S-135 cell extract which is deficient for SecA, the MM52 extract was incubated at 42 °C for 90 min and depleted for SecA by means of immunoprecipitation (28), before storage at −80 °C. Inverted inner membrane vesicles with wild type phospholipid composition were isolated from strain MRE600. Inner membrane vesicles with different levels of acidic phospholipids were prepared from strain HDL11 grown without or with 10, 20, 30, or 50 μM IPTG and had the expected phospholipid composition (7). Inner membrane vesicles from strain MM52 used in the SecA-translation system were preincubated for 1 h at 42 °C in the presence of 50 μM NaN₃. The translation reactions were initiated 5 min after the start of translation by the addition of inverted inner membrane vesicles (0.4 mg of protein/ml).

Due to dilution of the preincubated MM52 membrane suspension, the final azide concentration in the SecA-translocation mixture was 2 mm. After an incubation of 20 min at 37 °C (in the translation-translocation reaction with MRE600 cell extract) or 42 °C (in the translation-translocation reaction with MM52 cell extract) proteinase K (500 μg/ml) was added to degrade all nontranslocated proteins. This reaction was carried out at room temperature and stopped after 30 min by the addition of trichloroacetic acid to a final concentration of 20% (w/v). After precipitation of the proteins, the samples were analyzed by Tricine-SDS-PAGE (29), followed by fluorography. In *in vitro* protein translocation is defined by the amount of precursor and mature proteins remaining after the proteinase K treatment. Where indicated, translocation was determined by liquid scintillation counting of rehydrated excised protein bands (precursor and mature together) of the dried gels. Radioactivity of the protein bands was corrected for background radioactivity present in the gel. For calculation of the translocation efficiency, in each sample the amount of translocated protein, relative to the amount of synthesized precursor was determined. The maximal value in each experiment was taken as 100%.

*Introduction of Phosphatidylglycerol into Inner Membrane Vesicles*—A nonspecific lipid transfer protein was used to introduce 1,2-dioleoyl-sn-glycero-3-phosphoglycerol from small unilamellar vesicles into PG-depleted inner membranes (from *E. coli* HDL11) essentially as described in Ref. 7. The concentration of specifically incorporated PG is expressed as mole percentage of the total phospholipid content of the inverted inner membrane vesicles.

*Determination of the Relative Proton Motive Force*—The relative levels of the proton motive force of inner membrane vesicles from strain HDL11 with wild type or low levels of PG were determined by measuring the fluorescence quenching of 9-amino-7-chloro-2-methoxyacridine and OXONOL-6 largely as described in Ref. 27.

**RESULTS**

*Reduced Levels of Phosphatidylglycerol Retard in Vivo Processing of M13 Procoat Protein*—The M13 coat protein is synthesized as a precursor protein with an N-terminal signal sequence. Upon translocation across the *E. coli* inner membrane the protein is processed to its mature size by cleavage of the signal sequence. In order to test whether acidic phospholipids in the inner membrane influence the SecA-independent membrane translocation of this precursor, we made use of strain HDL11 in which the levels of anionic phospholipids can easily be controlled. Wild type procoat protein was expressed in *E. coli* HDL11, grown either in the presence or in the absence of IPTG, which induces the synthesis of PG. After a 30-s pulse with [35S]methionine and a subsequent chase of 5, 20, and 40 s, coat and procoat proteins were immunoprecipitated and analyzed by SDS-PAGE and fluorography. It was found that procoat is very rapidly converted to mature coat protein when high levels (see Ref. 7 for inner membrane lipid composition) of acidic phospholipids are present, whereas processing is retarded when these levels are reduced (Fig. 1, compare lanes 1 and 4). Both in the presence and in the absence of IPTG the precursor disappeared during the chase as can be seen in lanes 2, 3 and 5, 6, respectively. This result implies that the rate of procoat translocation depends on the concentration of acidic phospholipids in the membrane.

**Acidic Phospholipids Efficiently Stimulate M13 Procoat Translocation**—To get more direct insight into the role of PG in SecA-independent translocation of M13 procoat we developed an *in vitro* translocation system for this protein. Such a system in general allows a more direct analysis of the translocation process. In the *in vitro* studies we compared the translocation of two precursor proteins which follow either the SecA-independent or the SecA-dependent pathway, respectively, wild type procoat and a procoat analog, Δprocoat-lep. The latter protein consists of procoat, C terminally extended with 103 amino acids, derived from leader peptidase and having a deletion of the membrane spanning residues +27 to +48 of the coat sequence, which makes the protein dependent on SecA for *in vivo* translocation (23). Initial membrane insertion of both precursors is expected to be comparable, since the N-terminal 40 amino acids (including the signal sequence) are identical. Later stages are probably more distinct, since procoat contains a membrane spanning hydrophobic region and translocates only a small part of the sequence across the membrane, whereas the complete mature part of Δprocoat-lep moves across the membrane (23). Fig. 2 shows the *in vitro* synthesis of, respectively, wild type procoat (lane 1) and Δprocoat-lep (lane 5). The precursors are partly processed to their mature size when inverted inner membrane vesicles are added to the translation reaction (lanes 2 and 6). Lanes 3 and 7 show that part of both precursor proteins translocate across the membrane, since precursor and mature proteins become protected from degradation by externally added protease. Upon addition of a detergent the membrane vesicles are disrupted and the protease completely digests the precursor and mature proteins (lanes 4 and 8).

Next, we prepared inner membrane vesicles from strain HDL11 with different levels of acidic phospholipids and assayed translocation of both precursors. It appeared that increasing PG levels in the inner membrane resulted in an enhanced translocation efficiency of procoat. Fig. 3 shows this result both quantitatively in a translocation-PG plot and as a fluorogram (inset, lanes 1–5). The translocation efficiency of Δprocoat-lep was found to parallel closely the data obtained for wild type procoat. The reduction in translocation of cells grown at low IPTG levels could be the direct result of the reduced PG content or be due to another, unknown, factor. To get insight into this question, we applied a lipid-transfer protein based method (described in Ref. 7) to incorporate PG from small unilamellar lipid vesicles into inner membrane vesicles with low PG content. Fig. 4 shows that incorporation of PG into PG-depleted inner membrane vesicles from strain HDL11 efficiently stimulates translocation (lanes 2–6) of both wild type procoat (upper panel) and Δprocoat-lep (lower panel). The amounts of protected precursors as well as mature proteins increase proportional to the amount of incorporated PG. The translocation efficiencies increased for Δprocoat-lep from 21% to the maximal value (set to 100%) and for wild type procoat from 28 to 100%. For comparison, lanes 1 show translocation across wild type inner membranes (from strain MRE600). We
A Dual Role for PG in Protein Translocation

As a control experiment we measured the relative proton motive force ($\Delta\mu^+$) of the isolated inner membrane vesicles by means of a fluorescence quenching assay. Both vesicles with wild type levels of PG and PG-depleted vesicles in which PG was reintroduced were able to generate a similar $\Delta\mu^+$ upon the addition of ATP or NADH. Fluorescence quenching in the presence of inner membrane vesicles containing low PG levels was lower compared to vesicles with wild type PG levels (respectively, 41 and 71% reduction of the fluorescence level, not shown). The addition of 0.8 $\mu$m FCCP (a protonophore) reduces the $\Delta\mu^+$ of wild type vesicles to the level present in PG-depleted vesicles. In order to investigate whether the difference in the $\Delta\mu^+$ occurring between the two types of inner membrane vesicles would affect the efficiency of procoat translocation, a translocation assay was performed in the presence of 1 $\mu$m FCCP. At this concentration the protonophore hardly affects procoat translocation across membranes with wild type PG levels (the reduction of translocation in the presence of 1 $\mu$m FCCP amounted to 2.4%, not shown). Therefore the difference in energetic state between membrane vesicles containing different amounts of PG (which also in whole HDL11 cells appeared to be very small (30)) may be ignored.

In Vitro Translocation of Procoat Is Independent on SecA—In the in vitro translocation assay SecA is present and although procoat translocates in vivo in a SecA independent manner, it could be argued that SecA stimulates procoat translocation and that is why it is PG dependent. To investigate this possibility, we applied a SecA-deficient cell free system, in which both an S-135 lysate and inner membrane vesicles from E. coli MM52 (SecA51ts) were used. In order to further inhibit residual SecA activity, prior to translation the lysate was depleted for SecA by immunoprecipitation (28) and the inner membranes were pre-incubated at the nonpermissive temperature in the presence of 50 mM NaN$_5$. It has been shown that NaN$_5$ is an effective inhibitor of the ATPase activity of SecA (31). The final NaN$_5$ concentration during the translocation reaction was 2 mM. Fig. 5 clearly demonstrates that wild type procoat translocates, also in the in vitro system, in a SecA independent manner (lanes 3 and 4). Higher concentrations of azide did not influence procoat translocation (not shown). As expected, the SecA-dependent procoat analogue translocates with a very low efficiency in the SecA-deficient system (less than 1% of the synthesized precursor), which increased more than 10-fold when purified SecA was added (lanes 1 and 2).

DISCUSSION

This study shows by making use of phospholipid mutant strains that efficient translocation in the SecA-independent pathway followed by procoat requires the presence of wild type levels of phosphatidyglycerol in the inner membrane. This then demonstrates that the negatively charged lipid phosphatidyglycerol plays a dual role in protein translocation in the prokaryotic secretion pathway, i.e., that besides its role in providing efficient functioning of the SecA protein in the Sec-dependent route (8-10), it exerts another type of function in the insertion/translocation route followed by procoat. The relative simplicity of this latter pathway, and the observation that pro-

![Fig. 2. In vitro translocation of M13 procoat protein. Translation of transcripts from plasmids pSP65-8, encoding wild type procoat protein (lanes 1-4) and pJQ-840 ΔABGD, encoding Δprocoat-lep (lanes 5-8) was carried out in the absence (lanes 1 and 5) or presence (other lanes) of inner membrane vesicles from strain MRE 600. The samples in lanes 3, 4, 7, and 8 were treated with protease K (500 $\mu$g/ml). The samples in lanes 4 and 8 were before protease treatment subjected to a lysis treatment by the addition of 1% Triton X-100. To prevent overloading of lanes 1, 2, 5, and 6 only a fraction of each sample was loaded onto the gel, therefore this autoradiogram serves as a qualitative demonstration of the in vitro translocation system. p and m indicate, respectively, precursor and mature bands.](image1)

![Fig. 3. PG-dependent translocation of M13 procoat. Translocation efficiency of wild type procoat (●) and Δprocoat-lep (○), is presented as a function of the PG content of HDL11 inner membrane vesicles. The translocation efficiency is defined as the amount of protein which is protected from degradation by protease K relative to the amount of synthesized precursor. The maximal value in each experiment is taken as 100%. The insert shows the bands of wild type procoat (p) and mature (m) coat proteins, translocated at increasing concentrations of PG in the inner membrane vesicles (lanes 1-5). Since in the different experiments similar amounts of precursor were synthesized (not shown), the autoradiogram illustrates the increase in translocation as shown quantitatively in the graph.](image2)

![Fig. 4. Restoration of procoat translocation by incorporation of PG. Translocation of wild type procoat (upper panel) and Δprocoat-lep (lower panel) was assayed at increasing concentrations of PG, incorporated into PG-depleted inner membrane vesicles from strain HDL11 by nonspecific lipid transfer protein-mediated lipid transfer (lanes 2-6). Lanes 1 show a control translocation across wild type (from strain MRE600) vesicles. The samples were treated with protease K. Precursor and mature protein bands are indicated by p and m, respectively.](image3)
coat translocation requires the presence of basic amino acids at the signal sequence (19), suggests that direct interactions between the precursor and the anionic lipids are essential for translocation of procoat. Indirect effects such as a change in energetic state of the inner membrane, or an altered composition and the anionic lipids. Studies on synthetic signal peptides interacting with model membranes revealed that anionic lipids mediate the initial membrane association and moreover are required for deep membrane penetration of the signal peptide (5, 17, 32). Second, it has been shown that procoat translocation was blocked when the basic amino acids are deleted (19) and that this is paralleled by a decreased binding of the precursor to PG-containing liposomes (19). Our studies do not exclude the possibility that besides the anionic lipids a proteinaceous signal sequence receptor is involved.

How do we reconcile the similar PG dependence of the SecA-dependent and -independent pathways? We suggest that in the SecA-dependent pathway SecA is involved in the formation of a signal sequence-PG interaction, SecA requires anionic phospholipids for functional membrane binding (10) and thus will be localized in an area rich of these molecules. Recent experiments lead to the conclusion that ATP binding causes SecA to insert more deeply into a PG-containing monolayer (12). This membrane penetration of SecA could facilitate transfer of the signal sequence from SecA to the negatively charged phospholipids. Consistent with this hypothesis, it was reported that the initial movement of the signal sequence of proOmpA across the membrane requires ATP binding to SecA (33). Direct support for the hypothesis comes from experiments on the model secretory protein OmpF-Lpp with mutated signal sequences (34). This protein follows the SecA-dependent route. When this precursor carries a signal sequence with a wild type balance between the positively charged N terminus and the hydrophobic region, its translocation requires PG (35), which can be blocked by drugs like doxorubicin which interact with PG (36). However, when the hydrophobic central region of the signal sequence is increased in size, membrane translocation becomes independent on: first, the number of charges at the N terminus (34); second, the presence of PG (35); and third, the presence of PG-interactive drugs (36). This strongly suggests that also in the SecA-dependent route, PG-signal sequence interactions play a role in determining the efficiency of translocation. Furthermore, these experiments suggest that the main function of these interactions is to facilitate efficient insertion of the signal sequence into the lipid phase, possibly close to the translocation machinery. It cannot be excluded that the anionic lipids in addition have other, still unknown effects on protein components of the translocation machinery that have not been discovered yet.

Acknowledgments—We thank M. Kleerebezem and J. van Hellemond for the generous gift of MM2 cell extract used in the initial experiments. We are grateful to R. Keller for assistance with the fluorescence experiments.

REFERENCES