In vitro membrane integration of leader peptidase depends on the Sec machinery and anionic phospholipids and can occur post-translationally

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Abstract A cell-free system based on a lysis and membrane vesicles from \textit{Escherichia coli} is used to study characteristics of the membrane integration reaction of the polytopic membrane protein leader peptidase (Lep). Integration into inverted inner membrane vesicles was detected by partial protection against externally added protease. Integration is most efficient when coupled to translation but can also occur post-translationally and depends on the action of the proteinaceous Sec machinery and availability of anionic phospholipids. Lep is the first example of a membrane protein without cleavable signal sequence which requires anionic lipids for integration in vitro.

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Key words: Leader peptidase; In vitro translocation; Membrane protein; \textit{Escherichia coli}

1. Introduction

Leader peptidase (Lep) from \textit{Escherichia coli} is an integral inner membrane protein which spans the membrane with two hydrophobic segments which are connected by a short cytoplasmic loop [1]. The second transmembrane segment precedes a large periplasmic domain which during assembly has to pass the inner membrane [2]. In recent years Lep has been extensively used as a model to study membrane protein insertion in vivo and it appeared that the mechanism for translocation of periplasmic loops depends on loop length. Loops smaller than 60 amino acid residues pass the membrane bilayer in an apparently spontaneous process, while translocation of longer loops like the periplasmic domain of Lep requires the action of the so-called Sec machinery [3,4].

Originally, Sec machinery was found to be essential for translocation of periplasmic and outer membrane proteins across the inner membrane (for review see [5]). Unlike Lep, these periplasmic and outer membrane proteins are synthesized as precursors with N-terminal extensions called signal sequences. They are often maintained in a translocation competent state by the tetrameric SecB protein [6–8]. These SecB precursor complexes have a high affinity for SecA which is found in the cytosol and in multiple conformations in the inner membrane where it couples ATP hydrolysis to the progress of translocation [9–11]. Together with SecA, Sec-Y, -E and -G proteins constitute the basic machinery for transloca-

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2. Materials and methods

2.1. Materials

Trinucleotides (sodium salts), T4 ligase and the restriction endonucleases \textit{Smal} and \textit{SalI} were obtained from Pharmacia. Folic acid (calcium salt), phosphoethanolamine (diThr) polysymx B sulfate, phenyl methyl sulphonyl fluoride (PMSF) and amino acids were from Sigma (USA). SP6 RNA polymerase, creatine phosphokinase, rRNA, puromycin, dihydrochloride and protease K were purchased from Boehringer (Germany). Lep(His) was purified as described before [21]. SecA and \textit{\beta}-lactamase antibodies were a gift from Dr. H. de Cock, Utrecht University.

2.2. Strains and growth conditions

\textit{E. coli} strain MRE 600 [22] was used for the production of S-135 lysate and grown in Gotten broth at 37°C until early exponential phase. Reference vesicles were isolated from strain SD 12 [23] which was grown at 37°C in LB, PC 2977 [24] was used as a source of SecY\textsuperscript{v} vesicles. Cells were grown overnight at 30°C in LB with 10 μg/ml tetracycline and at 42°C, 3 h prior to vesicle isolation. Vesicles with low contents of anionic lipids were isolated from HDL11 [25], which was grown in LB supplemented with 10 μg/ml tetracycline, 25 μg/ml chloramphenicol and 50 μg/ml kanamycin.

2.3. In vitro translocation, translation and assembly

S-135 cell extracts and inverted inner membrane vesicles were prepared from \textit{E. coli} according to published procedures [26]. Plasmid pSPLep was created by cloning the \textit{SalI-Smal} fragment containing \textit{lepB} (from a pMMI1 derived in vivo expression vector to the in vitro
expression vector pSP64. DNA was purified using wizard minipreps DNA purification resin (Promega, USA) and used to direct the transcription of the LepB gene by SP6 RNA polymerase during a 30 min incubation at 40°C following the manufacturer’s instructions. Translations were carried out at 37°C as described [26]. Five minutes after initiation of translation, inverted inner membrane vesicles were added to a final concentration of 4 mg/ml (protein) and the incubation was continued for 25 min at 37°C. Correct assembly of Lep was demonstrated by protection of the P2 domain against the action of externally added protease K (200 μg/ml final concentration). The protease treatment was carried out for 20 min at room temperature after which 2 mM PMSF was added. The samples were analysed by SDS-PAGE and exposure of the gel in a Phosphor Imager. To assess the influence of additions (antibodies or polymyxin), these were mixed with the vesicles prior to incubation in the translocation mixture.

2.4. Immuno precipitation

Immunoprecipitations using antiserum directed against Lep were performed as described [27] using Protein A-coupled Sepharose CL4B (Pharmacia, LKB, Sweden).

2.5. ProOmpA translocation

[^35]SproOmpA was purified as described [28] and stored in 8 M urea. [^35]SproOmpA was diluted 50-fold into 25 μl of translocation buffer (40 mM Tris-acetate, pH 8.0, 10.8 mM magnesium acetate, 28 mM potassium acetate, 2 mM DTT, 0.5 mg/ml BSA) containing inverted inner membrane vesicles (0.4 mg protein/ml), 5 μl S-135 cell extract and 4 mM ATP [8]. When indicated 1 μl of polyclonal antiserum directed against SecA or β-lactamase was added. Translocation was allowed to proceed for 20 min at 37°C, followed by analysis as described for the assembly of Lep.

3. Results

To allow in vitro synthesis of mRNA by SP6 RNA polymerase, the LepB gene was cloned into an in vitro expression vector. Most expression plasmids carry the gene coding for the selectable marker β-lactamase in the same orientation as the SP6 promoter which gives rise to substantial amounts of expression of this protein. Because of similar molecular weights of Lep and β-lactamase, this would complicate the interpretation of the results. Therefore we made use of pSP64 to clone the LepB gene in the opposite orientation as the β-lactamase gene to result in pSPLep (Fig. 1A). After translation of the RNA by a membrane-free lysate of E. coli MRE 600 in the presence of [^35]S-methionine, the products were resolved by SDS-PAGE and visualised by autoradiography. Plasmid pSP64 without insert did not direct the synthesis of any radio-labelled protein (Fig. 1B, lane 1), but the plasmid carrying the LepB insert (lane 2) gave rise to one radio-labelled band. A comparable construct with the gene coding for β-lactamase reading in the same direction as Lep gave rise to a second band directly under the band of Lep (results not shown). The band in lane 2 has an apparent molecular weight of 36 kDa and runs at the same position as purified unlabelled His-tagged Lep (position indicated). Moreover, after proteinase K treatment of the sample, no radio-labelled protein was observed (lane 3) showing that there is no intrinsic protease resistance in this protein. The identity was confirmed by immunoprecipitation with Lep antibody (lane 4). It was therefore concluded that pSPLep specifically directs the synthesis of Lep.

To study membrane integration of Lep in the same direction as in whole cells, inverted inner membrane vesicles from strain SD12 were added during translation. Proteinase K was added afterwards to investigate putative luminal localisation of parts of Lep. This revealed one single band of 32 kDa (Fig.

![Fig. 1. Plasmid directed in vitro synthesis and identification of Lep. A: Plasmid map of pSPLep. Restriction sites, genes (LepB and Bla), the SP6 promoter and the origin of replication (ori) are indicated. B: In vitro protein synthesis from pSP64 without (lane 1) or with an insert containing the LepB gene (lanes 2-4). After synthesis samples were put on ice (lane 2) or treated with 200 μg/ml proteinase K (lane 3) or immunoprecipitated using Lep antiserum (lane 4). All samples were after their respective treatments analysed by SDS-PAGE followed by autoradiography. The position of non-labelled purified Lep on the gel is indicated.](image-url)
proOmpA and OmpA. In the absence of antibodies (Fig. 3A, lane 2) 20% translocation was observed, while the presence of SecA antibody prevented translocation of proOmpA completely (Fig. 3A, lane 3). As a control, translocation was studied in the presence of the β-lactamase antibodies (Fig. 3A, lane 4) which did not influence translocation efficiency. Since the SecA antibodies block Sec-dependent translocation, this procedure was used to establish whether SecA is required in the in vitro assembly reaction of Lep. In the absence of antibodies Lep assemblies into IMVs with an efficiency of 20 ± 1% (n = 4) (Fig. 3B, lanes 1 and 2). In the presence of SecA antibody this efficiency drops below 1% (lanes 3 and 4) while no influence of the addition of β-lactamase antibodies was measurable (lane 5 and 6). The requirements for SecY were tested using vesicles from a temperature sensitive strain as described before for preproteins. Lep assembly into these vesicles was reduced to 1.5 ± 1% (n = 3) (lanes 7 and 8). This very minor integration activity could be due to residual functional SecY molecules. It is therefore concluded that the in vitro integration reaction of Lep requires a functional Sec machinery.

Efficient translocation of precursor proteins depends on anionic phospholipids in the membrane [13,25]. To investigate the influence of anionic phospholipids on the assembly of Lep, vesicles were isolated from a lipid biosynthetic mutant strain, HDL11. In this strain, the pgaA gene is placed under control of the lac operon. This gene is responsible for the synthesis of the major negatively charged phospholipid in E. coli, phosphatidylglycerol. In the absence of the inducer IPTG, the anionic phospholipid content of the inner membrane is below 10% [25], which is 2.5 times lower than in wild-type cells. Lep assembles into vesicles isolated from HDL11, which was grown in the absence of IPTG, with efficiencies below 1% (lanes 9 and 10). The importance of anionic phospholipids for the integration process was also tested by shielding the negative charges by a positively charged agent. The poly cationic antibiotic Polymyxin B specifically interacts with anionic phospholipids [29]. Vesicles with wild-type lipid composition were incubated with different concentrations of polymyxin B prior to the integration experiment. Fig. 4 shows that integration efficiencies decrease with increasing polymyxin concentrations. Polymyxin B (20 μM) causes a half maximal block of the integration reaction.

For precursor proteins it was shown that translocation could occur after completion of translation. Using the in vitro system the possibility of post-translational integration of the integral membrane protein Lep could be investigated. In our in vitro system, synthesis of Lep reaches a maximum within 25-30 min (results not shown) and to ensure arrest of synthesis, puromycin was added to dissociate the ribosomes. Post-translational integration was assayed as follows: 28 min after start of translation, 20 μM puromycin was added. After 2 min vesicles were added (Fig. 5, lanes 2–5) or not (lane 1). ATP was added to one incubation mixture (lane 4) to make sure that energy was not in short supply. After 20 min PK was added (lanes 3 and 4). Both in absence and presence of extra ATP, 3 ± 1% integration was observed, which is 5-10 times less than in the cotranslational experiment. When Triton X-100 was added before protease treatment, all protein was degraded (lane 5). It was observed that under the integration conditions no protein synthesis takes place. For this purpose, translation was carried out in the presence of unlabelled methionine for 28 min. Subsequently the translation mixture was incubated with 20 μM puromycin for 2 min, after which radioactively labelled methionine was added and the incubation prolonged for 20 min. No radioactive material was observed at the position of the full-length Lep protein (Fig. 5, lane 6). It should therefore be concluded that under the conditions described above, integration can occur post-translationally albeit very inefficient.

4. Discussion

In this paper a cell-free transcription-translation system is described to synthesize leader peptidase which can be used to study membrane integration. In the present system Lep is produced in a S-135 lysate to study co- and post-translational
membrane integration. During a cotranslational incubation with wild-type vesicles 20% of the synthesized Lep became integrated in the vesicles. This seems reasonable for in vitro systems since translocation of precursor proteins in similar systems is usually in the same range, typically 25-40% [26,30]. In a previous paper [21], the native population of Lep molecules in inverted membrane vesicles was studied. Protease K treatment of inverted membrane vesicles, followed by SDS-PAGE and Western blotting with Lep antibody yielded also a band of 32 kDa [21]. This not only corroborates the assignment of the observed 32 kDa fragment in the present study, but also shows that the in vitro synthesized Lep resides in the same orientation in inverted inner membrane vesicles as the native population.

On the basis of in vivo experiments the integration of membrane proteins is thought to follow one of two possible pathways. Proteins with large periplasmic loops, such as Lep, depend fully on the activity of the Sec machinery, while smaller periplasmic loops can pass the inner membrane independent of the Sec machinery. However, Sec-independence is most often only operationally defined as the ability to successfully integrate in the membranes of mutant strains in which the Sec-dependent precursor translocation is impaired [4]. Only

![Fig. 3](image)

**Fig. 3.** Requirements for membrane assembly of Lep. **A:** Translocation of purified proOmpA can be blocked by antibodies against SecA. The amount of translocated OmpA and proOmpA were compared to 20% of the added precursor (lane 1). Translocation was carried out in the presence of SecA antibodies (lane 3), β-lactamase antibodies (lane 4) or in the absence of antibodies (lane 2). **B:** Assembly of Lep in wild-type vesicles (lanes 1 and 2), in vesicles treated with antibodies against SecA (lanes 3 and 4) or with antibodies against β-lactamase (lanes 5 and 6), vesicles depleted of functional SecY (lanes 7 and 8) or vesicles reduced in anionic phospholipid contents (lanes 9 and 10). The amount of protease protected Lep (even numbered lanes) was compared to 20% of the Lep synthesized (odd numbered lanes) in presence of vesicles.

![Fig. 4](image)

**Fig. 4.** Effects of polymyxin B on integration efficiency. Prior to the integration reaction vesicles were incubated with the indicated concentrations polymyxin B sulfate. Translocation was carried out and analysed as described under Section 2. Mean values of translocation efficiencies are depicted and the error bars indicate standard deviation.
in vitro systems allow to firmly address this point. In our system the integration of Lep was dependent on the Sec machinery just as was observed in vivo. Previously, another in vitro system was described to study membrane integration of Lep, which was focussed on identifying segments within Lep that were required for assembly [31]. The in vitro system from literature differs in two points from the present system. Our system does not require purified AraC protein and it makes use of a S-135 lysate instead of a S-30 lysate.

Our results indicate that the integral membrane protein Lep can integrate post-translationally in inverted membrane vesicles using an in vitro system. The obvious question is then whether this can also happen in living cells. Andersson and von Heijne performed in vivo integration experiments in which the membrane passage of P2 of Lep is blocked by addition of the uncoupler CCCP. After inactivation of this compound, translocation of the P2 domain could proceed [32]. In addition, genetically engineered membrane proteins with four membrane-spanning segments inserted in vivo in the membrane in a fashion which was not compatible with a simple linear N- to C-integration process. Such a linear integration was expected if insertion was to happen completely cotranslationally [33]. It should therefore be concluded that integration of membrane proteins can occur post-translationally. For precursor proteins it was shown that their translocation could also occur after completion of translation. 8% of the prePSbE which was synthesized in an in vitro system translocated into the lumen of a vesicle in a post-translational translocation experiment while during cotranslational incubations; 25% of the synthesized material was translocated [26]. In our experiments, post-translational membrane integration of Lep is even less efficient (3%) when compared to cotranslational experiments in which 20% was integrated. The relatively low efficiency of post-translational integration of Lep may be explained by properties of the protein. Lep contains three hydrophobic stretches, of which two assume a transmembrane configuration. It can be envisaged that these segments have a strong tendency to aggregate and require a chaperone for post-translational insertion. Recently, it was reported that post-translational membrane insertion of the hydrophobic membrane protein lactose permease was possible in vitro systems and stimulated by the presence of the molecular chaperone GroEL [34].

In this paper two methods were used to study the influence of anionic lipids on integration of Lep. Vesicles from the lipid biosynthetic mutant strain HDL1.1 which contain less than 10% anionic phospholipids were employed and the polycationic antibiotic polymyxin B was used to shield negative charges on vesicles with wild-type lipid composition. In both experiments, the integration efficiencies were decreased in comparison to the control situation. Strain HDL1.1 was used before in several studies aimed at the effects of reduced anionic lipid on protein translocation and colicin action. [25, 35] It was shown there, that in this particular strain low anionic lipid concentrations did not affect internal ATP concentration nor the membrane potential [35]. It was therefore concluded that the effects of anionic lipids are not caused by a de-energized membrane. Polymyxin which carries five positive charges, was used previously to study the anionic lipid dependency of precursor proteins in vitro [36]. It was reported that polymyxin did not affect the membrane potential or cause aggregation of vesicles [36]. In our studies a half-maximum inhibitory effect was found at approximately 20 μM which corresponds to the presence of 1 polymyxin molecule in the assay per 4–5 anionic phospholipid molecules which is close to a charge-stoichiometric complex. It is concluded that in vitro membrane insertion of Lep depends on anionic lipids. This is the first example of a membrane protein without degradable signal sequence which requires anionic phospholipids for integration.

There are at least two different ways in which anionic phospholipids could affect the integration of Lep. One possibility is that the decreased translocation ATPase activity of SecA upon lowering the amount of anionic phospholipids [14] accounts for hampered precursor translocation or membrane protein integration at decreased anionic lipid contents. On the other hand, also direct interactions between anionic lipids and Lep are feasible.

There are a few examples illustrating that hampered SecA function is at least not the only factor explaining decreased translocation efficiencies at low anionic lipid levels. Experiments with dimeric proOmpF-Lpp molecules with artificial signal sequences revealed that effects of anionic phospholipids on translocation depended on the amino acid composition of the signal sequence, but not on the SecA dependency of translocation. Translocation of ProOmpF-Lpp molecules with long hydrophobic polyethylene stretches in their signal sequence was independent of anionic phospholipids but still required SecA for translocation [37, 38]. In addition it was shown that SecA independent integration of the M13 procoat membrane protein had a similar requirement for anionic phospholipids as a construct in which the periplasmic domain was enlarged to become SecA dependent [39]. These studies demonstrate that anionic lipids could also interact directly with precursors and membrane proteins. For the M13 procoat protein it was observed that positively charged amino acyl residues in the cytoplasmic regions flanking the two hydrophobic segments were required for insertion [40] and the results of binding studies with lipid vesicles indicated that electrostatic interactions between these positively charged residues and anionic phospholipids are involved in initiation of insertion [40]. It is feasible that binding of positive charges to the negative surface of the membrane facilitates the insertion of hydrophobic
segments into the hydrophobic core of the membrane. Charge interactions between anionic phospholipids and positively charged amino acids can play a general role in membrane protein insertion.

References