LolA-dependent Release of a Lipid-modified Protein from the Inner Membrane of Escherichia coli Requires Nucleoside Triphosphate*

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The outer membrane-directed lipoproteins are released from the inner membrane of Escherichia coli as a complex with LolA, a periplasmic chaperone. The LolA-dependent release of lipoproteins is critical for lipoprotein sorting as it depends on the outer membrane-specific sorting signal. To clarify molecular events involved in the LolA-dependent lipoprotein release, we attempted to establish an in vitro assay system. The major outer membrane lipoprotein (Lpp) was found to lose its release competence soon after being processed to mature Lpp in the inner membrane and therefore could not be used as a substrate for an in vitro system. An Lpp derivative, L10P, was constructed and found to retain the release competence long after its maturation. L10P was synthesized and radiolabeled in spheroplasts in the absence of LolA; therefore, it remained anchored to the inner membrane of spheroplasts. Right-side out membrane vesicles containing L10P were then prepared and used to examine the release of L10P. In addition to LolA, L10P release absolutely required nucleoside triphosphate (NTP). A non-hydrolyzable NTP analogue strongly inhibited the NTP-dependent release. The outer membrane-specific sorting signal was essential for the in vitro release of L10P. Furthermore, L10P released in vitro was specifically incorporated into the outer membrane. These results indicate that the in vitro release of L10P represents an in vivo reaction and requires energy.

Lipid-modified proteins such as Rab in eukaryotes (1) and lipoproteins in bacteria (2, 3) have been studied with respect to post-translational modification pathways, physiological significance, and membrane targeting mechanisms. Cys residues at the N termini of lipoproteins and in the C-terminal region of Rab are modified with lipids, which anchor these proteins to the target membranes. Vesicular transport requires correct membrane targeting of Rab (1), and the mislocation of lipoprotein is lethal to Escherichia coli (4).

More lipoproteins than known are predicted by the complete genome sequence to be present in E. coli (5). All lipoproteins are thought to be synthesized with a signal peptide at the N terminus and then to be translocated across the inner (cytoplasmic) membrane in a Sec machinery-dependent manner (3). Lipid modification and processing to mature lipoproteins take place in the inner membrane, and the localization of a mature lipoprotein to either the inner or outer membrane follows. The amino acid residue next to the lipid-modified Cys at the N terminus of a mature lipoprotein functions as a sorting signal (6). An Asp residue at this position makes lipoproteins specific to the inner membrane, whereas other residues direct lipoproteins to the outer membrane.

A periplasmic chaperone, LolA (7), mediates the release of the outer membrane-directed lipoproteins from the inner membrane by forming a soluble lipoprotein-LolA complex. Inner membrane-specific lipoproteins remain anchored to the inner membrane in the presence of LolA, indicating that the formation of the lipoprotein-LolA complex represents a critical step of the lipoprotein sorting (7). When the complex interacts with an outer membrane receptor, LolB, the lipoprotein is transferred from the complex to LolB and then incorporated into the outer membrane (8). LolB is also a lipoprotein possessing the outer membrane sorting signal and is essential for E. coli (8). An in vitro assay system has been constructed to examine the interaction between the lipoprotein-LolA complex and LolB (8). The LolA-dependent release of lipoproteins was examined in spheroplasts (7). Although a proton motive force was found to be unnecessary for LolA release (7), a possible energy requirement for the release step has not been ruled out. Furthermore, since LolA forms a complex with a lipoprotein existing in the inner membrane but not with an outer membrane-localized lipoprotein, it seems highly likely that a factor involved in the lipoprotein-LolA complex formation is present in the inner membrane.

We report here the establishment of an in vitro assay system for lipoprotein release. By using this system, we found that the release of lipoprotein from the inner membrane requires nucleoside triphosphate.

EXPERIMENTAL PROCEDURES

Materials—Tran35S-label (a mixture of 70% [35S]methionine and 20% [35S]cysteine, 1,000 Ci/mmol) was obtained from ICN. Anti-Lpp (9), anti-LolB (8), and anti-SecD (10) antisera were prepared as described. IgGs were purified from the antiserum by means of immobilized protein A column chromatography (Pierce). ATP, GTP, CTP, UTP, deoxy-ATP, AMP-CPP,1 and AMP-PCP were purchased from Sigma, and AMP-PNP, GMP-PNP, and creatine kinase were from Boehringer Mannheim. DNase I was obtained from Worthington. LolA was purified from an LolA-overproducing strain as described (7).

Bacterial Strains and Media—E. coli K12 strains, JM83 (F− lac−proAB) rpsL (800 lacZA15) (11), JE5505 (F− pp pps his proA argE thi gal lac xyl mtl tex) (12), and SM704 (JE5505 lac−lolB) were used. The last strain was constructed by introducing the chromosomal lac−lolB allele of SM602 (8) by P1 transduction. Cells were grown on LB broth or M9 (NaCl)-maltose (4). Where specified, chloramphenicol was added to the medium at the concentration of 25 μg/ml.

Construction of Plasmids—To construct a LolA-overproducer,

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1 The abbreviations used are: AMP-CPP, α,β-methylene adenine 5′-triphosphate; AMP-PCP, β,γ-methylene adenine 5′-triphosphate; AMP-PNP, β,γ-imido adenine 5′-triphosphate; GMP-PNP, β,γ-imido guanosine 5′-triphosphate; Lpp, the major outer membrane lipoprotein; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair.
pMAN994 (7) was digested with PstI, treated with T4 DNA polymerase, and then digested with KpnI to generate a 0.8-kb fragment containing lolaA. This fragment was ligated with a large KpnI-Smal fragment of pMAN885 (4), which carries a chloramphenicol resistance gene, araC, and Plac. pMAN995 thus constructed carries lolaA under Plac.

To construct various Lpp derivatives, a 3.9-kb KpnI-Smal fragment lacking lpp and a 4.1-kb NcoI-SmaI fragment carrying PstAad and araC of pYJ811 (4) were mixed with one of the following synthetic oligonucleotides, in which the specified restriction site was either disrupted or created (underlined), and the specified amino acid residue was substituted:

- "-CGTGCT-3 for Arg.
- "-CTAAAATCGATCAG-3" for Pro.
- "-AGATGACGCAGCTCGG AAC-3" for Asp.
- "-TCTGACGTTCAGACT-3" for Ser.
- "-TTTCTGACGTTCA-3" for Thr.

pJYL10P was created (underlined), and the specified amino acid residue was substituted, in which the specified restriction site was either disrupted or created (underlined).

pJY811 (4) were mixed with one of the following synthetic oligonucleotides, in which the specified restriction site was either disrupted or created (underlined), and the specified amino acid residue was substituted:

- "-AGG-9" was converted to Pro. After transformation into JM83, plasmid pMAN995 thus constructed carries lolaA under Pbad.
- "-AGCAAC-9" was converted to Arg.
- "-CTTCTGACGTTCA-3" for Ala.

In Vitro Membrane Incorporation of L10P—SM704, which carries the wild type Lpp, was grown on M63 (NaCl)-maltose at 37 °C. When the OD660 of the culture reached 1.0, the culture was kept on ice for 30 min. Lpp was then labeled with [35S]methionine for 2 min in the absence of LolA, followed by a chase with non-radioactive methionine and cysteine (each at 100 μg/ml) for 2 min. The labeling was chased for 2 min.

To construct pYJ10LIP(D) encoding an L10P derivative, which possesses Asp instead of Ser at position 2, a 0.1-kb XbaI-Smal fragment of pKY702 (6) and a 0.24-kb EcoRI-Smal fragment of pJY10LIP were ligated and inserted into the EcoRI-XbaI site of pMAN885. pYJ10LIP(D) encodes L10P(D) under the control of Pbad.

Energy-dependent Release of Lipoproteins from Membranes

mRNA encoding Lpp was expressed for 5 min in the presence of 0.2% arabinose. The amount of LolA was under the detection limit when 28 μg of protein of the LolB-deleted membranes was analyzed by SDS-PAGE and immunoblotting with an anti-LolB antibody (3). The ATP-dependent release of 35S-L10P from membranes was examined in the presence of LolA as described above. The supernatant containing the 35S-L10P-LolA complex was incubated at 30 °C for 30 min with 0.4 mg/ml LolB-containing or -depleted membrane fraction as described (8). The incorporation of L10P into the membrane was determined by SDS-PAGE and fluorography after centrifugation.

Co- and Post-processing Release of Lpp from Spheroplasts—The co-processing release of Lpp from spheroplasts was examined as described previously (7). Briefly, JM5505 cells harboring a plasmid, which carries the wild type or mutant lpp gene under Plac, were grown on M63 (NaCl)-maltose at 37 °C. When the OD600 of the culture reached 1.0, the lpp gene was expressed for 5 min in the presence of 0.2% arabinose. The cells were then centrifuged to separate the membrane-associated Lpp from the soluble fraction of Lpp. M63 (NaCl)-maltose containing 0.25% sucrose and 10 μCi of Tran35S-labeled Lpp was then added for 2-min labeling at 30 °C. The labeling was chased for 2 min after the addition of non-radioactive methionine and cysteine (each at 20 μg/ml). To examine the post-processing release of Lpp, labeling of spheroplasts was started and terminated as described above but in the absence of LolA. The spheroplast suspension in the absence of LolA was kept on ice for 30 min, followed by the addition of 7 μl of fresh Lpp-containing lpp derivative released into the supernatant or incorporated into the membranes was calculated by taking the total amount of 35S-Lpp or its derivative as 100%.

RESULTS

Co- and Post-processing Release of Lpp—We previously showed that the release of Lpp from the inner membrane of spheroplasts requires LolA (7). As shown in Fig. 1, when LolA was present during the synthesis and maturation of Lpp, almost all 35S-labeled Lpp molecules were recovered in the medium after centrifugation, whereas essentially all Lpp molecules remained in spheroplasts in the absence of LolA. LolA thus causes very efficient release of Lpp in a co-processing manner. Lpp released into the spheroplast medium sometimes migrated on SDS-PAGE slightly faster than Lpp remaining in spheroplasts for an unknown reason. When spheroplasts were kept on ice for 15 min after the labeling of Lpp in the absence of LolA, the subsequent addition of LolA caused the release of only a marginal amount of Lpp (Fig. 1), suggesting that Lpp loses the release competence soon after its maturation. To examine Lpp release that is independent of its maturation in vitro, Lpp derivatives competent in post-processing release were required.

Other outer membrane lipoproteins such as Slp (14) and Pal (15) retained their release competence for at least 15 min after their maturation (data not shown). However, most studies on lipoprotein localization have been performed with Lpp, and a number of Lpp derivatives have been constructed. Furthermore, the Lpp release absolutely requires LolA, whereas about 50% of Pal labeled in spheroplasts was released when spheroplasts were incubated for a long time in the absence of LolA (data not shown). The physiological significance of this LolA-independent release was not clear, but a strict LolA dependence seemed to be critical for in vitro examination of the release. We therefore tried to construct Lpp derivatives that retain their release competence after their maturation.
**Fig. 2.** Lpp derivatives competent as to post-processing release. A, the secondary structure of Lpp is depicted according to the models proposed by Inouye (19) and Braun (17). Closed and hatched circles represent hydrophobic and charged residues, respectively. Two potential α-helical regions are indicated by vertical lines. B, Lpp derivatives were constructed as described under "Experimental Procedures." The co- and post-processing release of the Lpp derivatives was examined as in Fig. 1 except that the immunoprecipitation with the anti-Lpp antibody was omitted. The release of the wild type Lpp was also examined as a control. Arrowheads indicate the positions of Lpp and its derivatives. The amount of each Lpp material released in a post-processing manner is expressed as a percentage of that released in a co-processing manner and is indicated at the right.

**Lpp Derivatives Competent as to Post-processing Release**—Lpp is released into the spheroplast medium as a 1:1 complex with LolA and then transported to the outer membrane, where Lpp exists as a trypsin-resistant trimer (7, 16). We found that mature Lpp accumulated in the inner membrane of spheroplasts in the absence of LolA is partly resistant to trypsin, presumably due to trimerization (data not shown). The trimerization of Lpp is postulated to cause the loss of ability to interact with LolA, thereby leading to the loss of release competence. The α-helix content of Lpp was estimated to be 80–90% (17, 18). According to the structure model of Lpp proposed by Inouye (19), most hydrophobic amino acid residues are located on the same side of the α-helix (Fig. 2A). Lpp lacking the N-terminally modified Cys still forms a water-soluble trimer (16, 20). From these observations, it seemed likely that the introduction of a charged residue or an α-helix breaker into the hydrophobic face perturbs the trimer formation and thus increases the competence as to post-processing release. Seven Lpp derivatives possessing either Pro or Arg in place of Leu or Ala at various positions were constructed and expressed in JE5505 (Δlpp) cells. The replacement was expected to have no effect on the outer membrane localization, since the sorting signal at position 2 (4, 6) is intact. Indeed, we confirmed the outer membrane localization of L10P, L17R, and A37P in _vivo_ (data not shown). Co- and post-processing release of the seven Lpp derivatives and the wild type Lpp were examined in spheroplasts (Fig. 2B). Since immunoprecipitation with the anti-Lpp antibody was omitted, Lpp and some other labeled proteins in spheroplasts migrated to the same position on the gel. When LolA was not added, the spheroplast medium contained no labeled protein that migrated to the position of Lpp (lanes 2 and 6). The co-processing release of all the Lpp derivatives as well as the wild type Lpp took place in the presence of LolA (lane 4), indicating that the amino acid replacement did not inhibit the interaction with LolA. The post-processing release of the wild type Lpp was only marginal, whereas that of all the derivatives except A37P was more than 50% relative to co-processing release (lane 8), suggesting that a structural change in the N-terminal half of Lpp increases the competence as to post-processing release. It is not known, however, whether the trimerization of these derivatives is stable or not. The post-processing release of L17R was highly efficient, but this derivative was unstable.

Based on these observations, the co- and post-processing release of L10P were examined in more detail (Fig. 3). Immunoprecipitation with the anti-Lpp antibody revealed that the L10P derivative was nearly quantitatively released into the spheroplast medium not only in a co-processing manner but also in a post-processing manner (Fig. 3A). Furthermore, LolA was essential for the release of L10P in both cases. To examine the loss of release competence of L10P and the wild type Lpp after their maturation, spheroplasts containing mature L10P or Lpp were incubated on ice in the absence of LolA for various periods, and then post-processing release was induced by the addition of LolA at 30 °C (Fig. 3B). The loss of release competence was only marginal with L10P, whereas the post-processing release of the wild type Lpp decreased after its maturation. About 70% of L10P was released within 5 min from spheroplasts kept on ice for 15 min on the addition of LolA, whereas the release of the wild type Lpp was less than 20% (Fig. 3C). From these results, it was assumed that L10P could be used as a substrate for _in vitro_ release experiments.

**In Vitro Release of L10P Involves a Vanadate-sensitive Nucleoside Triphosphatase**—To construct an _in vitro_ assay system, L10P was expressed and labeled with Tran^35S-label in...
spheroplasts in the absence of LolA. Right-side out membrane vesicles containing \(^{35}S\)-L10P were then prepared after lysis of the spheroplasts by osmotic shock. Release of L10P from the membrane vesicles was examined by the addition of LolA. LolA alone did not induce the release of L10P into the supernatant (Fig. 4A, upper panel). However, when membrane vesicles were prepared in the presence of ATP, about 50% of the L10P remained in the membrane vesicles was released into the supernatant on the addition of LolA, indicating that the LolA-dependent release of L10P is energy-dependent. The addition of NADH instead of ATP or with ATP did not induce or further enhance the L10P release, indicating that a proton motive force has little effect on the release, as previously observed with spheroplasts (7). The non-quantitative release of L10P may be caused by incompletely broken cells. These assays were also carried out with membrane vesicles containing the wild type Lpp (Fig. 4A, lower panel). Release of the wild type Lpp did not take place under any conditions examined, indicating that the wild type Lpp completely lost its competence as to post-processing release.

The addition of an ATP-generating system consisting of creatine kinase and creatine phosphate (ATP-generating system); dATP, deoxy-ATP. The concentrations (mM) of AMP-CPP, AMP-PCP, and ATP are indicated above the lower panel. C, the release of L10P was examined in the presence of 2 mM ATP and one of the following: 1.2 mg/ml anti-SecD IgG, 30 mM sodium azide (Na\(\text{N}_3\)), 1 mM orthovanadate (VO\(\text{O}_4^{2-}\)), 10 mM sodium arsenate (As\(\text{O}_4^{3-}\)), 10 mM Na\(F\), or 1 mM N-ethylmaleimide (NEM).
deoxy-ATP. Neither AMP-PCP nor AMP-CPP induced the L10P release (Fig. 4B, lower panel). Moreover, both analogues inhibited the ATP-dependent L10P release.

Protein translocation machinery comprising Sec factors such as SecA requires ATP as an energy source and mediates the translocation of secretory proteins including lipoproteins across the inner membrane (3, 21). The nucleotide specificity is significantly different between protein translocation (22) and L10P release (Fig. 4B). Furthermore, an anti-SecD IgG, which has been reported to inhibit the release of mature OmpA and maltose-binding protein from membranes (10), had no effect on the L10P release. These results indicate that the translocation machinery is not involved in the release of L10P. In marked contrast, 1 mM sodium vanadate completely inhibited the release. N-Ethylmaleimide, arsenate, or sodium fluoride did not inhibit the release. Taken together, these results indicate that the L10P release involves a certain nucleoside triphosphatase, which has a rather broad nucleoside specificity and is sensitive to vanadate. Both α-β and β-γ phosphoester bonds seem to be important for the enzyme activity.

ATP and GTP added at the same concentration caused essentially the same extent of L10P release (Fig. 5A). Furthermore, AMP-PNP and GMP-PNP strongly inhibited the ATP- and GTP-dependent release of L10P, respectively (Fig. 5B). These results indicate that ATP and GTP are equally effective as to the energization of L10P release. The GTP-dependent release of L10P from membrane vesicles absolutely required LolA (Fig. 5C), as was the case for the release from spheroplasts (Fig. 3A). The L10P release in the presence of 20 μg/ml LolA took place rapidly and was completed within 2–3 min (Fig. 5D).

Sorting Signal-dependent Release and LolB-dependent Outer Membrane Localization of L10P—To determine whether or not the in vitro L10P release depends on the lipoprotein sorting signal, Ser at position 2 of L10P was converted on the DNA level to Asp, an inner membrane-specific signal (4, 6). Membrane vesicles containing L10P(D) were prepared and subjected to the in vitro release assay with L10P as a control (Fig. 6A). Irrespective of the presence or absence of LolA, L10P(D) remained in the spheroplasts, indicating that the in vitro release of L10P also depends on the lipoprotein sorting signal. Gel filtration chromatography revealed that L10P released in vitro also exists as a 1:1 complex with LolA (data not shown).

The outer membrane localization of Lpp requires the outer membrane receptor, LolB, which is also a lipoprotein (8). To determine whether or not L10P released in vitro is localized to the outer membrane in a LolB-dependent manner, LolB-containing or -depleted membrane fractions were prepared and incubated with L10P released in vitro in the presence of LolA (Fig. 6B). After incubation, the reaction mixture was fractionated into membranes and a supernatant by centrifugation. When the membrane contained LolB, L10P was recovered in the membrane fraction, whereas it remained in the supernatant when the membrane lacked LolB. Taken together, these results indicate that the in vitro release of L10P represents the physiological reaction.

**DISCUSSION**

We constructed seven Lpp derivatives by substituting a hydrophobic residue with Arg or Pro. The post-processing release competence of most derivatives was found to be significantly enhanced. *In vivo* expression of all the derivatives had no effect on the growth of the cells. Furthermore, three derivatives were found to be correctly localized to the outer membrane *in vivo*. Taken together, these results indicate that all the mutations introduced into Lpp do not affect the interaction with LolA and LolB, both of which play critical roles in the outer membrane localization of lipoproteins. An *in vitro* assay system was suc-
cessfully constructed with L10P as a substrate, whereas the wild type Lpp could not be used as the substrate for it, suggesting that the stable trimer formation renders Lpp incompetent as to post-processing release. However, it is not completely clear whether or not the loss of release competence is directly caused by the trimerization, as the formation of the L10P trimer was not examined. It is noteworthy that the trimerization of Lpp in the inner membrane rarely takes place in vivo since the in vivo release most likely occurs in a co-processing manner.

The following observations strongly indicate that the in vitro release of L10P represents the physiological release: 1) the in vitro release of L10P depends on the outer membrane sorting signal; 2) L10P released in vitro is incorporated into the outer membrane in a LolB-dependent manner; and 3) not only L10P but also Pal is released in vitro in an ATP-dependent manner.

The release of L10P from right-side out membrane vesicles was found to require nucleoside triphosphate, suggesting the existence of an inner membrane factor involved in the release of lipoproteins. The inhibition of the ATP-dependent L10P release by AMP-PNP, AMP-CPP, and AMP-PCP suggests that both $\alpha$-$\beta$ and $\beta$-$\gamma$ phosphoester bonds are important for the reaction. Vanadate, which is known as an inhibitor of P-type ATPase, completely inhibited the L10P release. It is still premature to discuss, based on these observations, the type of nucleoside triphosphatase involved in the reaction, although Sec factors such as SecA are not involved in the release reaction. It is, however, noteworthy that the presence of a factor in the inner membrane is critical for the one-way transport of lipoproteins from the inner to the outer membrane, i.e. LolA never releases lipoproteins from the outer membrane.

Formation of the lipoprotein-LolA complex depends on the outer membrane sorting signal (7), which is located at the N-terminal second position (6). LolA is therefore thought to recognize the sorting signal (7). However, it is possible that an inner membrane factor recognizes the sorting signal and releases the outer membrane-specific lipoprotein upon NTP hydrolysis, thereby enabling a water-soluble lipoprotein-LolA complex to form. Hydrolysis of ATP, which is coupled to L10P release, was not detected with right-side out membrane vesicles, presumably because of the limited amount of L10P. It is therefore not clear whether the product of ATP hydrolysis is ADP or AMP. We recently succeeded in the reconstitution of a lipoprotein releasing system into proteoliposomes. Characterization of the putative inner membrane factor and its ATP hydrolyzing activity are under investigation.

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