Characterization of the LolA-LolB System as the General Lipoprotein Localization Mechanism of *Escherichia coli*<sup>*</sup>

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The major outer membrane lipoprotein (Lpp) of *Escherichia coli* requires LolA for its release from the cytoplasmic membrane, and LolB for its localization to the outer membrane. We examined the significance of the LolA-LolB system as to the outer membrane localization of other lipoproteins. All lipoproteins possessing an outer membrane-directed signal at the N-terminal second position were efficiently released from the inner membrane in the presence of LolA. Some lipoproteins were released in the absence of externally added LolA, albeit at a slower rate and to a lesser extent. This LolA-independent release was also strictly dependent on the outer membrane sorting signal. A lipoprotein-LolA complex was formed when the release took place in the presence of LolA, whereas lipoproteins released in the absence of LolA existed as heterogeneous complexes, suggesting that the release and the formation of a complex with LolA are distinct events. The release of LolB, an outer membrane lipoprotein functioning as the receptor for a lipoprotein-LolA complex, occurred with a trace amount of LolA, and therefore was extremely efficient. The LolA-dependent release of lipoproteins was found to be crucial for the specific incorporation of lipoproteins into the outer membrane, whereas lipoproteins released in the absence of LolA were nonspecifically and inefficiently incorporated into the membrane. The outer membrane incorporation of lipoproteins including LolB *per se* was dependent on LolB in the outer membrane. From these results, we conclude that lipoproteins in *E. coli* generally utilize the LolA-LolB system for efficient release from the inner membrane and specific localization to the outer membrane.

Lipoproteins in prokaryotes are synthesized with a signal peptide at their N termini and are then translocated across the inner (cytoplasmic) membrane in a Sec machinery-dependent manner (1, 2). Lipid modification and processing to yield a mature lipoprotein take place in the inner membrane, and then the localization of the 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 is assumed to direct lipoproteins to the outer membrane.

We have investigated the mechanism underlying the sorting signal-dependent localization of the major outer membrane lipoprotein (Lpp)<sup>1</sup> of *Escherichia coli* and found two factors, LolA (4) and LolB (5), involved in this mechanism. LolA, a periplasmic chaperone, forms a water-soluble complex with Lpp, the stoichiometry being 1:1 (4). Since Lpp is water-insoluble owing to its N-terminal lipid moiety, the complex formation is critical for crossing of the periplasm. LolB, an outer membrane lipoprotein, is a receptor for the LolA-Lpp complex and mediates the outer membrane incorporation of Lpp via the transient formation of a LolB-Lpp complex (5). Recognition of the sorting signal takes place at the release step since an Lpp derivative possessing an Asp residue at position 2 remains in the inner membrane and does not form a complex with LolA (4). We recently established an *in vitro* system and found that the release of Lpp from right-side-out membrane vesicles requires ATP (6). The *in vitro* release of Lpp strictly depends on the lipoprotein sorting signal, suggesting the possibility that the sorting signal is recognized by the putative ATPase in the cytoplasmic membrane.

The complete genome sequence of *E. coli* suggests that more than 80 lipoproteins are possibly encoded on its chromosome (7). On the other hand, the functions of LolA and LolB are characterized mainly with Lpp and its derivatives. It is therefore important to determine whether or not the outer membrane localization of other lipoproteins involves the LolA-LolB system. To address this, release from the cytoplasmic membrane and localization to the outer membrane were examined for eight other lipoproteins.

We report here that the LolA-LolB system is the general mechanism by which outer membrane-directed lipoproteins are efficiently released from the cytoplasmic membrane and specifically localized to the outer membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tran<sup>35</sup>S-label (a mixture of 70% [35S]methionine and 20% [35S]cysteine, 1,000 Ci/mmol) was obtained from ICN. Anti-Lpp (8), anti-LolA (4), and anti-LolB (5) antisera were prepared as described. The anti-Pal antibody was a generous gift from T. Mizuno. IPTG was purchased from Nacalai Tesque. Restriction enzymes were from Takara Shuzo. LolA was purified from *Escherichia coli* strain J2382 (8). The specified lipoprotein gene was cloned into pTTQ18 (Amersham Pharmacia Biotech) carrying the tac promoter-lac operator and the lac<sup>β</sup> gene. A 1.6-kb HindIII-<sup>35</sup>Sl DNA fragment carrying the entire *rlpA* gene was isolated from pTT101 (11) and then inserted into the Smal-HindIII site of pTTQ18 to construct pYKT80.

1 The abbreviations used are: Lpp, major outer membrane lipoprotein; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; kb, kilobase pair(s).
The acaR, nlpA, nlpB, and slp genes on the chromosome were amplified by PCR with a pair of primers, (5'-GTACCCTCGGGATCCCTATGACGATCTGACAGCTTACATCAG-3') and (5'-CTGAGGCACAGGTTCAGTGACGATCTGACAGCTTACATCAG-3'). The PCR products were digested with SmaI and NotI, and then ligated into the same site of pTPTQ18 to construct pYKT10, pYKT20, pYKT40, and pYKT70 carrying acaR, nlpA, nlpB, and slp, respectively.

0.6-kb EcoRI-SmaI fragment carrying the pal gene was obtained from pTAN20 (12) and then inserted into the same site of pTPTQ18 to construct pTAN21. The rlpB gene on the chromosome was amplified by PCR using primers 5'-AATCCGCGGCGAGCGCGGAGGACGCTTCGAGTCGACTCTCATCTGACGGG-3' and 5'-AAAATCCGCGGGAGGACGCTTCGAGTCGACTCTCATCTGACGGAATTC-3', and then cloned into the same site of pTTQ18 to construct pYKT10, pYKT20, pYKT40, and pYKT70 carrying acaR, nlpA, nlpB, and slp, respectively.

The construction of pTAN26 carrying the inner membrane sorting signal of Lpp and the mature region of lolB was achieved as follows: to improve the yield of lolB, an ideal ribosome-binding sequence was added upstream of lolB (SD-lolB) or the signal peptide was replaced by that of lpp (lpp-lolB). To construct pYKT100 carrying SD-lolB, a synthetic DNA fragment containing the ribosome-binding sequence and the wild type signal peptide region was ligated with a 1.6-kb Bsp128I-HindIII fragment of pMAN650, which encodes the mature region of Lpp, and pTPTQ18 digested with EcoRI-HindIII. The level of LolB in MC4100 cells harboring pYKT100 increased severalfold on the addition of IPTG. To construct the lpp-lolB gene, a 1.3-kb EcoRI fragment of pYKT100 containing the SD-lolB region was ligated with a 2.6-kb EcoRI fragment of pTAN20 to construct pKR100. To create new PalI sites around the region corresponding to the N-terminal Cys of mature Lpp and LloB, pKR100 and pYJS11 (13) carrying the lpp gene were mutagenized with a QuickChange site-directed mutagenesis kit (Stratagene) using primers 5'-CTTGGCCGACCGGAGCTTTGCACTTACGCTTCGAGTCGACTCTCATCTGACGGG-3' and 5'-CTTTGGCCGACCGGAGCTTTGCACTTACGCTTCGAGTCGACTCTCATCTGACGGAATTC-3', and then cloned into the same site of pTTQ18 to construct pYKT10, pYKT121, and pYKT111, respectively. These mutant genes did not alter any amino acid residues. Digestion with NheI and XhoI generated 24- and 2.8-kb fragments from the mutated gene pKR100 and pYJS11, respectively. The two fragments were then ligated together to construct pKR103 encoding a chimeric protein possessing the signal peptide of Lpp and the mature region of LolB. The level of LolB expressed in cells was 10 times higher with pKR103 than with pMAN650.

To construct a lolB gene possessing the inner membrane sorting signal, pYKT100 was mutagenized with the site-directed mutagenesis kit using primers 5'-GTGTCTACGCTGCTGTACGCTGCTCGACGCAGCTTACATCAG-3' and 5'-ACCTTTGGCCGACCGGAGCTTTGCACTTACGCTTCGAGTCGACTCTCATCTGACGGG-3'. The plasmid, pYKT110, thus constructed encodes LolB(D), in which the residue Ser required to the N-terminal Cys is replaced by Asp. 1.3-kb EcoRI fragments of pYKT100 and pYKT110 were inserted into the EcoRI site of pMAN995 (6) to construct pYKT121 and pYKT111, respectively. These plasmids encode LolB and LolB(D) under the control of P\text{BAD}, respectively.

The construction of pTAN26 carrying the gene for Pal(D) under the control of the tac promoter-lac operon will be reported elsewhere.

Release of Lipoproteins from Spheroplasts—The release of lipoproteins from spheroplasts was examined as described previously (4). Briefly, cells harboring a specified plasmid were grown at 37°C on M63 minimal medium (14) supplemented with 10 μg/ml thiamine and 20 μg/ml each of all amino acids except methionine and cysteine. Where specified, M63 (NaCl)-maltose (13) was also used. When the OD_{600} of the culture reached 1.0, lipoprotein synthesis was induced for 5 min in the presence of 1 mM IPTG or 0.2% arabinose. Lpp was constitutively expressed from the chromosomal copy. Spheroplasts were released from the spheroplasts as described (15). A suspension (300 μl) containing about 5 × 10^9 spheroplasts was kept on ice for 3 min in the presence or absence of purified LolA (20 μg). M63 medium (750 μl) containing 0.25 m sucrose and 10 μCi of Tran^{35}S-label was then added for 2-min labeling at 30°C. The labeling was chased with non-radioactive methionine and cysteine (each at 12 μM). The release of lipoprotein was terminated by chilling of the reaction mixture in ice water, and analyzed after fractionation into spheroplasts and medium by centrifugation at 16,000 × g for 2 min.

Analysis of Lipoproteins by SDS-PAGE—35S-Labeled Lpp, Pal, and LolB in the membrane and supernatant fractions were immunoprecipitated with antibodies raised against the respective proteins, and then analyzed by SDS-PAGE and fluorography as described (4). Other lipoproteins were directly analyzed by SDS-PAGE and fluorography. The medium fraction containing lipoproteins released from spheroplasts was centrifuged at 100,000 × g for 30 min to remove insoluble materials. The supernatant (200 μl) was applied on a Superdex 75 column (1 × 30 cm, Amersham Pharmacia Biotech), which had been equilibrated with 50 mM potassium phosphate (pH 7.5) and then layered over a linear gradient of 50–35% (v/v) sucrose dissolved in 50 mM potassium phosphate (pH 7.5), centrifuged at 60,000 × g for 15 h at 4°C, and fractionated into the inner and outer membranes.

The medium (200 μl) containing lipoproteins released from spheroplasts was centrifuged at 100,000 × g for 30 min to remove insoluble materials and then incubated with a specified amount of membrane at 30°C for 30 min. The membrane incorporation of lipoprotein was terminated by chilling of the reaction mixture in ice water, and analyzed after fractionation into a supernatant and pellet by centrifugation at 100,000 × g for 30 min.

RESULTS

Release of Lipoproteins from the Cytoplasmic Membrane—We compared the release of six outer membrane-specific lipoproteins, Pal(S) (18), NlpB(S) (19), RlpA(T) (11), RlpB(S) (11), Slp(S) (20), and LolB(S) (5), and two inner membrane-specific lipoproteins, Pal(A) (21) and AcrA(D) (22), with the release of Lpp(S). Each lipoprotein possesses the N-terminal second residue indicated in parenthesis following the name of the lipoprotein. They were labeled in spheroplasts with [35S]methionine and [35S]cysteine for 2 min in the presence and absence of LolA, and then chased with non-radioactive methionine and cysteine for 2 min, followed by fractionation into spheroplasts and medium. As reported previously, about 20 labeled proteins were secreted into the spheroplast medium (see Fig. 6 of Ref. 4). LolA had no effect on the secretion of most proteins, suggesting that these secreted proteins are mainly non-lipoproteins in the periplasm and outer membranes. On the other hand, all the outer membrane-specific lipoproteins as well as Lpp were released into the spheroplast medium as the exported form. This agreement, however, for RlpA and AcrA remained in the spheroplasts even in the presence of LolA (Fig. 1), indicating that the LolA-dependent release represents secretion but not lysis and is specific to the outer membrane-directed lipoproteins. These results, taken together, suggest that LolA functions as a general chaperone for outer membrane lipoproteins. The release of Pal, NlpB, RlpB, and LolB also
Expression of lipoproteins was induced for 5 min by the addition of 1 mM promoter-carrying the gene for the indicated lipoprotein under the control of the lac operator were grown at 37 °C on M63 minimal medium. Expression of lipoproteins was induced for 5 min by the addition of 1 mM IPTG prior to spheroplast formation. LolB was expressed from pYKT100 carrying SD-lolB. Lpp was encoded on the chromosome and expressed in the absence of IPTG. Spheroplasts were labeled at 30 °C with Tran35S-label for 2 min in the presence or absence of 20 μg/ml purified LolA, and then chased for 2 min with non-radioactive methionine and cysteine. The labeling mixture was fractionated into spheroplasts and a supernatant by centrifugation, followed by analysis by SDS-PAGE and fluorography. The amounts of lipoprotein in the pellet and supernatant fractions were determined. The relative amount of lipoprotein released into the supernatant was calculated by taking the total amount of lipoprotein as 100% and is indicated in the figure. OM and IM represent outer and inner membrane lipoproteins, respectively.

LolA-independent release did not occur with NlpA and AcrA, suggesting that the release in the absence of LolA also depends on the outer membrane sorting signal. To test this, we constructed Pal(D) and LolB(D) possessing the inner membrane sorting signal. Both derivatives were localized in the inner membrane in vivo. Moreover, expression of LolB(D) and Pal(D) caused severe growth arrest, suggesting that the mislocalization of LolB and Pal as well as Lpp (13) is toxic.3 Both Pal(D) and LolB(D) remained in spheroplasts irrespective of the presence or absence of LolA, whereas the wild type Pal and LolB were released to some extent even in the absence of LolA (Fig. 3), indicating that the LolA-independent release also depends on the outer membrane sorting signal. Not only the mature form but also the precursor form of LolB(D) was accumulated in spheroplasts, suggesting that the translocation of LolB(D) by the Sec machinery is perturbed by the mutation.

Pal, NlpB, and LolB released in the presence and absence of LolA were analyzed by size exclusion chromatography (Fig. 4A). The elution profiles of Pal and NlpB significantly differed between LolA-dependent and -independent release. Pal (19 kDa) and NlpB (36 kDa) released in the presence of LolA were each eluted as a sharp peak in fractions corresponding to apparent molecular masses of 46 and 68kDa, respectively (Fig. 4B). Since the LolA monomer was eluted as a 27-kDa protein on size exclusion chromatography (4), these results indicate that both lipoproteins are released as a 1:1 complex with LolA. In marked contrast, Pal and NlpB released in the absence of LolA were each eluted as a broad peak in fractions corresponding to higher molecular masses, suggesting the formation of heterogeneous complexes. In the presence and absence of LolA, LolB (23 kDa) was eluted in similar fractions corresponding to molecular masses of 40–200 kDa (Fig. 4B). However, the amount of LolB eluted as an ~50-kDa protein markedly increased when LolB was released in the presence of LolA. As described above, a considerable fraction of LolB was released in the absence of LolA, whereas LolB released in the absence of LolA had a tendency to be adsorbed to the resin, causing less efficient recovery. Thus, the release of LolB appeared to be somewhat different from that of other lipoproteins.

Since the chromosomal lolA gene was intact, we could not completely exclude the involvement of endogenous LolA, which was expressed during the assay or contaminated the spheroplasts, in the apparent LolA-independent release. We therefore immunoprecipitated Pal and LolB released in the presence and absence of LolA with the respective antibodies. The precipitates were analyzed by SDS-PAGE and immunoblotting with anti-LolA antibodies (Fig. 5). Pal released in the absence of LolA did not contain a detectable amount of LolA, whereas the anti-Pal antibody co-immunoprecipitated Pal and LolA in the case of the LolA-dependent release. The anti-LolB antibody also co-immunoprecipitated LolB and LolA when LolB was released in the presence of LolA, indicating that LolB is released as a complex with LolA. In contrast to Pal, a small amount of LolA was co-immunoprecipitated with LolB even

3 N. Yokota, T. Yakushi, S. Matsuyama, and H. Tokuda, unpublished observation.
thoL A was not added externally, suggesting that the assay mixture contained a trace amount of endogenous L oLA which could induce LolB release. However, when the LolA/LolB density ratio was compared between the LolA-dependent and -independent release, it was estimated that about 45% of the LolB was released without forming a complex with LolA.

Specific Outer Membrane Localization of Lipoproteins—Outer membrane localization was examined with lipoproteins released in the presence or absence of LolA. When Pal and NlpB were released in the absence of LolA and then incubated with the inner or outer membrane, a significant amount of the two lipoproteins remained in the supernatant. Moreover, the amounts of lipoproteins recovered in the pellet fractions were similar for the inner and outer membranes. On the other hand, when Pal and NlpB released in the presence of LolA were incubated with the two membranes, only marginal amounts of Pal and NlpB were precipitated with the inner membrane, indicating that the formation of the LolA-lipoprotein complex prevents the nonspecific incorporation of lipoprotein into the inner membrane.

FIG. 4. Gel filtration analysis of lipoproteins released in the presence and absence of LolA. Pal and NlpB were expressed in MC4100 cells harboring pTAN21 and pYST100, respectively. LolB was expressed in JM505 cells harboring pKR103. The cells were converted to spheroplasts, labeled with Traps-1-S-label for 2 min in the presence or absence of 20 μg/ml LolA, and then chased for 2 min with non-radioactive methionine and cysteine. A, the spheroplast supernatant (200 μl) was analyzed on a Superdex 75 column as described under “Experimental Procedures.” Each fraction was analyzed by SDS-PAGE and fluorography. The molecular mass markers used were catalase (232 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). B, the amounts of the specified lipoproteins in each fraction were determined by densitometric quantitation of the fluorogram shown in A. Closed and open circles represent the results with and without LolA, respectively.

FIG. 5. Immunohistochemical analysis of Pal and LolB released in the presence and absence of external LolA. Pal and LolB were expressed in spheroplasts prepared from MC4100 cells harboring pTAN21 and pYST100, respectively, in the presence or absence of 20 μg/ml LolA. The spheroplast medium (5.25 ml) was obtained by centrifugation and then subjected to immunoprecipitation with anti-Pal or anti-LolB antibodies. The precipitates were analyzed by SDS-PAGE and immunoblotting with anti-LolA, anti-Pal, or anti-LolB antibodies.

The membrane incorporation of lipoproteins released in the presence and absence of LolA was examined with LolB-containing or -depleted membranes (Fig. 6B). Lp p was only released in the presence of LolA. Pal and NlpB released in the absence of LolA did not exhibit LolB-dependent membrane incorporation. In contrast, Pal, NlpB, and Lpp released in the presence of LolA absolutely required LolB for their incorporation into the membrane.

We also examined the membrane incorporation of LolB. However, the incorporation of LolB into membranes containing a normal level of LolB was inefficient whether LolA was present or not at the release step (Fig. 7A). Moreover, it was neither dependent on LolB nor specific to the outer membrane (data not shown). We therefore used membranes prepared from LolB-overproducing cells. The amount of LolB in these membranes was about 10-fold higher than that in normal membranes. LolB released with LolA was rapidly and completely incorporated into LolB-overproduced membranes (Fig. 7A). When LolB released in the absence of LolA was used, the rate and extent of membrane incorporation were also significantly enhanced by LolB overproduction, indicating that LolB in the outer membrane functions as a receptor for LolB itself even in the absence of LolA. The membrane incorporation of Pal and NlpB released in the absence of LolA was not affected by LolB overproduction, whereas the overproduction substantially stimulated the membrane incorporation of these lipoproteins released in the presence of LolA (Fig. 7B).

DISCUSSION

The results presented here indicate that the LolA-LolB system functions as a general mechanism by which lipoproteins are sorted, released, targeted, and localized in the outer membrane. Not only the LolA-dependent release but also the LolA-independent release observed with some lipoproteins were absolutely dependent on the outer membrane sorting signal. Lipoproteins released in the presence of LolA existed as a complex with LolA, whereas Pal and NlpB released in the absence of LolA existed as heterogeneous complexes. These results most likely indicate that the release and complex formation with LolA are distinct events. It seems plausible that...
an ATPase that has been suggested to exist in the cytoplasmic membrane (6) catalyzes the release of lipoproteins in a sorting signal-dependent manner irrespective of the presence or absence of LolA in the medium. The released lipoproteins can stably remain in the medium by forming a water-soluble complex with LolA, thereby causing efficient release. The lipoprotein in this complex is then transferred to LolB upon the interaction with LolB in the outer membrane. This causes specific localization of lipoproteins to the outer membrane. On the other hand, lipoproteins remaining in the medium in the absence of LolA exist as heterogeneous complexes or micelles, in which the N-terminal lipid moiety is shielded from the aqueous environment. When such a structure comes into contact with membranes, nonspecific incorporation of lipoproteins into the membranes may take place, causing less efficient release. It is not completely clear why the release in the absence of LolA varied depending on the species of lipoproteins. Since the concentration (300 μg/ml) of LolA in vitro (4) is substantially higher than that employed for the release assay, lipoproteins are most likely released in a LolA-dependent manner in vivo.

The release and localization of LolB are specifically important since the correct localization of LolB is essential for the correct localization of other lipoproteins. Moreover, rapid exclusion of LolB from the inner membrane is also important to prevent the mislocalization of other outer membrane lipoproteins in the inner membrane. Indeed, the LolB release was extremely efficient even with a trace amount of LolA. However, LolB released into the medium existed as a heterogeneous complex even in the presence of LolA, indicating that not only the LolA-LolB complex but also larger complexes are formed. Such heterogeneous complex formation in the presence of LolA was specific to LolB and most likely resulted from the fact that LolB is a receptor for lipoproteins including LolB itself. Therefore, LolB in the LolA-LolB complex seems to be able to accommodate LolB or other lipoproteins, causing the formation of heterogeneous complexes.

The in vitro outer membrane localization of LolB required a higher amount of LolB in the outer membrane than that of other lipoproteins. The LolA-LolB complex must be dissociated for the localization of LolB in the outer membrane. These results therefore seem to suggest that a large amount of LolB is required in the outer membrane for dissociation of the tight LolA-LolB complex. The in vivo concentration of LolB was estimated to be about 300 μg/ml (5), which is more than 1000-fold higher than that used for the in vitro assay (0.12 μg/ml). Therefore, it is likely that LolB localization in vivo takes place in a LolB-dependent manner. It would be interesting to determine whether or not LolB localized in the outer membrane in vivo can function as a lipoprotein receptor.

A Blast search of the data base provided by the National Center for Biotechnology Information revealed LolA and LolB homologs in various Gram-negative microorganisms such as Yersinia pestis, Haemophilus influenzae, Actinobacillus actinomyctecomitans, Pseudomonas aeruginosa, Salmonella typhi murium, and Neisseria meningitidis, indicating that the LolA-LolB system is evolutionarily conserved in various Gram-negative bacteria. All putative lipoproteins having an outer membrane sorting signal in E. coli are hydrophilic except for their N-terminal highly hydrophobic lipid moiety. While anchored to membranes through their N-terminal lipid, most of the structures of lipoproteins therefore seem to be exposed to an aqueous environment and to be involved in various reactions occurring in the cell envelope. The LolA-LolB system may be a potential target for preventing host invasion by pathogenic bacteria.

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