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A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane

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Lipoproteins are localized in the outer or inner membrane of *Escherichia coli*, depending on the species of amino acid located next to the N-terminal fatty acylated Cys. The major outer membrane lipoprotein (Lpp) expressed in spheroplasts was, however, retained in the inner membrane as a mature form. A novel protein that is essential for the release of Lpp from the inner membrane was discovered in the periplasm and purified. The partial amino acid sequence of this 20 kDa protein (p20) was determined and used to clone a gene for p20. Sequencing of the gene revealed that p20 is synthesized as a precursor with a signal sequence. p20 formed a soluble complex only with outer membrane-directed lipoproteins such as Lpp, indicating that p20 plays a critical role in the sorting of lipoproteins. Lpp released from the inner membrane in the presence of p20 was specifically assembled into the outer membrane *in vitro*. These results indicate that p20 is a periplasmic carrier protein involved in the translocation of lipoproteins from the inner to the outer membrane.

Key words: carrier protein/lipoproteins/membrane localization/outer membrane/sorting signal

Introduction

Proteins have to reach their final destinations to exhibit their physiological functions. Cells of Gram-negative bacteria, such as *Escherichia coli*, consist of four compartments: the cytoplasm, the inner (cytoplasmic) membrane, the periplasm and the outer membrane. Periplasmic and outer membrane proteins are synthesized in the cytoplasm and then translocated across the inner membrane to reach their final destinations. The mechanism underlying the protein translocation across the inner membrane has been studied extensively both *in vivo* (Bieker *et al.*, 1990; Schatz and Beckwith, 1990) and *in vitro* (Wickner *et al.*, 1991; Tokuda, 1994). A machinery comprising SecA (Oliver and Beckwith, 1981; Cabelli *et al.*, 1988; Kawasaki *et al.*, 1989; Lill *et al.*, 1989), SecE (Riggs *et al.*, 1988; Schatz *et al.*, 1989; Brundage *et al.*, 1990; Akimaru *et al.*, 1991; Tokuda *et al.*, 1991), SecY (Emr *et al.*, 1981; Ito *et al.*, 1983; Brundage *et al.*, 1990; Akimaru *et al.*, 1991), SecG (Nishiyama *et al.*, 1993, 1994), SecD and SecF (Gardel *et al.*, 1990; Matsuyama *et al.*, 1992, 1993; Pogliano and Beckwith, 1994) catalyzes protein translocation. The periplasmic proteins reach their final destination, the periplasm, after translocation across the inner membrane. On the other hand, very little is known about the mechanism whereby proteins are localized in the outer membrane.

The membrane localization of lipoproteins has been studied extensively (Pugsley, 1993). Some lipoproteins have been found to be specific to the outer membrane and others to the inner membrane (Ichihara *et al.*, 1981). The major outer membrane lipoprotein (Lpp) contains an N-terminal Cys which is modified with three fatty acids (Braun, 1975; Hayashi and Wu, 1990). Other lipoproteins are also thought to have a modified Cys at their N-termini. These fatty acids presumably anchor the lipoproteins in the inner or outer membrane. Irrespective of their final localization, all lipoproteins so far known are synthesized as precursors with a signal peptide at their N-termini. Translocation of lipoproteins across the inner membrane takes place in a Sec-dependent manner (Hayashi and Wu, 1985; Watanabe *et al.*, 1988; Sugai and Wu, 1992). Signal peptidase II (Innis *et al.*, 1984; Yu *et al.*, 1984), which is specific to lipoproteins, cleaves the signal peptide after the Cys residue has been modified with two fatty acids (Hussain *et al.*, 1980; Tokunaga *et al.*, 1982). This enables the further fatty acylation of the Cys residue (Hussain *et al.*, 1982). This fatty acylation is important for membrane anchoring but is not the determinant of the lipoprotein destination (Yamaguchi *et al.*, 1988). Inouye and co-workers have revealed that both Lpp and inner membrane lipoprotein-28 (NlpA) contain this determinant among their N-terminal nine and 12 amino acid residues respectively (Ghrayeb and Inouye, 1984; Yamaguchi *et al.*, 1988). Furthermore, they have demonstrated that the replacement of Ser by Asp immediately after the N-terminal Cys of a Lpp derivative caused its localization in the inner membrane, whereas the substitution of Asp for Ser at the same position of an NlpA derivative changed its final location from the inner to the outer membrane. This suggests that the amino acid residue at the N-terminal second position of lipoproteins functions as a sorting signal for the final destination (Yamaguchi *et al.*, 1988). Although the structural requirements of lipoproteins for their localization have thus been elucidated, the mechanism underlying lipoprotein localization, including the role of the sorting signal, has not been clarified.

We report here the discovery of a novel periplasmic carrier protein which is required for the release of Lpp from the inner membrane, the formation of a soluble complex with Lpp, the recognition of the lipoprotein sorting signal and the assembly of Lpp into the outer membrane.

Results

A periplasmic protein involved in the release of Lpp from spheroplasts

E.coli spheroplasts secrete various protein species which are localized in the periplasm or outer membrane of intact

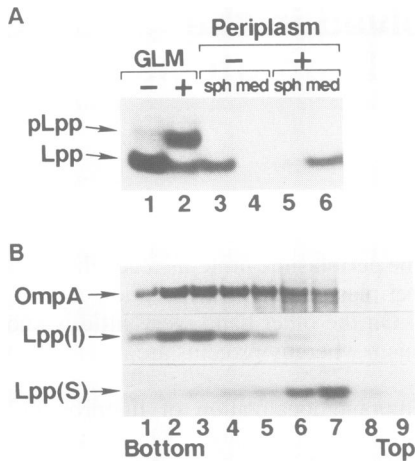

Fig. 1. A periplasmic factor is involved in the release of Lpp from spheroplasts. (**A**) Spheroplasts were labeled with Tran^{35}S-label for 3 min at 30°C in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of the periplasmic fraction, followed by a chase for 3 min with cold methionine and cysteine. The culture was fractionated into spheroplasts (lanes 3 and 5) and the spheroplast medium (lanes 4 and 6). As a control, intact cells were labeled for 2 min at 30°C in the absence (lane 1) or presence (lane 2) of globomycin (GLM), and then chased for 1 min. All samples were immunoprecipitated with an anti-Lpp antibody, and then analyzed by SDS–PAGE and fluorography. The modified precursor (pLpp) and mature (Lpp) forms of Lpp are indicated. (**B**) Membrane fractions were prepared from intact cells [OmpA and Lpp(I)] or spheroplasts [Lpp(S)], which had been labeled with Tran^{35}S-label, and then subjected to sucrose density gradient centrifugation, followed by fractionation into fractions 1–9, from the bottom to the top of the gradient. Each fraction was analyzed by SDS–PAGE and fluorography. When cells or spheroplasts were labeled, both OmpA and Lpp were identified as major proteins.

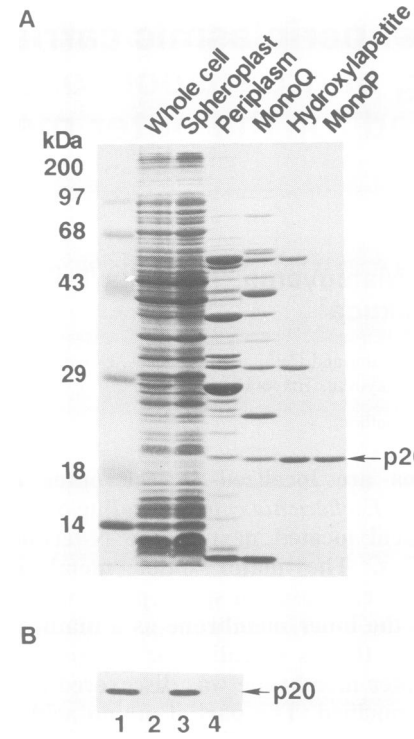

Fig. 2. Purification and cellular localization of p20. (**A**) Purification of p20 was carried out as described in Materials and methods. Samples at each purification step were analyzed by SDS–PAGE, and then stained with Coomassie brilliant blue. The amounts of proteins applied to the lanes of the gel were, from the left (whole-cell) to the right (MonoP), 75, 75, 22, 14, 4 and 2 μg respectively. The migration positions of molecular weight marker proteins are shown to the left of the gel. The position of p20 is indicated. (**B**) MC4100 cells (lane 1) were converted to spheroplasts as described in Materials and methods. The spheroplasts (lane 2) were collected by centrifugation at 16 000 *g* for 2 min. The resulting supernatant was fractionated further by centrifugation at 100 000 *g* for 30 min into the periplasmic fraction (lane 3) and the insoluble fraction (lane 4). Equivalent amounts of samples were analyzed by SDS–PAGE and immunoblotting with the anti-p20 antibody.

cells. OmpF and OmpA, for example, are each secreted into the spheroplast medium in a soluble form (Sen and Nikaido, 1990; Matsuyama *et al.*, 1993), although both are hydrophobic outer membrane proteins. The N-terminal Cys residue of mature Lpp is modified with three fatty acid moieties (Braun, 1975; Hayashi and Wu, 1990). The signal peptide is cleaved after the thio-linked glyceryl residue has been modified with two fatty acid moieties, and further fatty acylation takes place at the amino group of the Cys residue (Hussain *et al.*, 1982). Globomycin inhibits the cleavage of the signal peptide after modification with the two fatty acid moieties (Hussain *et al.*, 1980; Tokunaga *et al.*, 1982). Because of these modifications, mature Lpp is extremely water-insoluble. To examine the secretion of Lpp, spheroplasts were labeled with Tran^{35}S-label in M63 minimal medium containing 0.25 M sucrose, and then fractionated into spheroplasts and the medium. Lpp material was recovered in the spheroplast fraction and migrated to the same position as that labeled in intact cells on SDS–PAGE (Figure 1A, lanes 3 and 4). Since Lpp possessing a free amino group that is liberated upon signal peptide cleavage migrates faster than fully modified Lpp on the SDS–PAGE employed (Hussain *et al.*, 1982), these results indicate that the Lpp recovered with spheroplasts had undergone complete processing. The subcellular localization of Lpp remaining in spheroplasts was examined by sucrose density gradient centrifugation (Figure 1B). Lpp labeled in spheroplasts was found in the lower density fractions, whereas mature Lpp and OmpA labeled in cells and localized in the outer membrane were recovered in the higher density fractions, indicating that mature Lpp in spheroplasts remains in the inner membrane.

Spheroplasts are devoid of the periplasmic fraction. When the labeling of spheroplasts was carried out in the presence of externally added periplasmic fraction, Lpp was almost completely secreted into the medium (Figure 1A, lanes 5 and 6). Secretion was not due to the lysis of spheroplasts because elongation factor Tu, a cytosolic protein, was not detected in the medium by immunoprecipitation with an anti-Tu antibody. The addition of periplasmic fraction to spheroplasts, which had been pulse–chased, also caused the secretion of radiolabeled Lpp into the medium, although the secretion was not complete (data not shown). These observations strongly suggest that a periplasmic factor is involved in the release of mature Lpp from the inner membrane of spheroplasts. It has been reported that the proton motive force is required for the release of the maltose binding protein from the inner membrane (Geller, 1990; Ueguchi and Ito, 1990). The release of Lpp, however, did not require the proton motive force because it was resistant to a proton conductor, tetrachlorosalicylanilide (data not shown).

The Lpp-releasing activity was purified to near homogeneity from the periplasmic fraction (Figure 2A). The

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                                          -22                  -1
                                          MM KKIAITCALL SSLVASSVWA
+1                                                              50
DAASDLKSRL DKVSSFHASF TQKVTDGSGA AVQEGQGDLW VKRPNLFNWH
                                                               100
MTQPDESILV SDGKTLWFYN PFVEQATATW LKDATGNTPF MLIARNQSSD
                                                               150
WQQYNIKQNG DDFVLTPKAS NGNLKQFTIN VGRDGTIHQF SAVEQDDQRS
                                182
SYQLKSQQNG AVDAAKFTFT PPQGVTVDDQ RK
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Fig. 3. Amino acid sequence deduced from the nucleotide sequence of the *lplA* gene. The boxed sequence represents a signal sequence. The N-terminal sequence of p20 and the partial sequences of p20 fragments coincided with those underlined. The complete nucleotide sequence of the *lplA* gene has been registered in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession no. D49398.

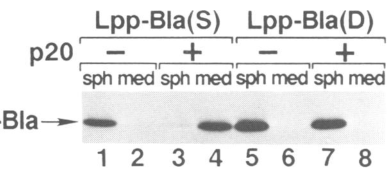

Fig. 4. Recognition of the lipoprotein sorting signal by p20. MC4100 cells harboring pMAN955 [Lpp-Bla(S)] (lanes 1–4) or pMAN960 [Lpp-Bla(D)] (lanes 5–8) were grown in M63 medium containing 0.4% maltose instead of glucose, and then converted into spheroplasts. The spheroplasts were incubated in M63 medium containing 0.4% maltose and 0.05% L-arabinose with (lanes 3, 4, 7 and 8) or without (lanes 1, 2, 5 and 6) p20 at 30°C for 2 min. Tran^{35}S-label was then added to initiate 2 min labeling. After a 2 min chase, the culture was fractionated into spheroplasts (lanes 1, 3, 5 and 7) and the spheroplast medium (lanes 2, 4, 6 and 8). Samples were immunoprecipitated with the anti-Bla antibody, and then analyzed by SDS–PAGE and fluorography. The position of Lpp-Bla is indicated.

apparent molecular mass of the purified protein was found to be 20 kDa on SDS–PAGE and 27 kDa on gel filtration chromatography (Superdex 75), indicating that the protein exists as a monomer. Hereafter, the protein is designated as p20. An antibody was raised against the purified p20. The number of p20 molecules in a single cell was estimated to be 200–400 by quantitative immunoblotting with the anti-p20 antibody using purified p20 as a standard. Immunoblotting analysis with the anti-p20 antibody also revealed that p20 is specifically localized in the periplasm (Figure 2B).

Amino acid sequencing was carried out with the purified p20 and peptide fragments obtained after treatment of p20 with a limited amount of lysyl endopeptidase. The N-terminal sequence of DAASDLKXRLDKVXSFHA was determined with p20. Sequences of QEGQGD and MTQPDE were determined with the peptide fragments. Mixed oligonucleotide probes that encode the two sequences of the peptide fragments were synthesized and used for Southern hybridization with *E.coli* chromosomal DNA digested with *Pst*I. A 5.1 kb *Pst*I fragment hybridized to both probes and was cloned. From the sequence of the cloned DNA, an open reading frame that encodes a protein of 204 amino acid residues was found (Figure 3). The protein was found to possess a putative signal sequence comprising 22 amino acid residues, followed by the sequence of mature p20, confirming the periplasmic localization of this water-soluble protein. The gene encoding p20 was located at 20 min on the *E.coli* chromosome and was named *lplA* (**lip**oprotein **l**ocalization).

The amino acid sequence of p20 did not exhibit similarity to those of any proteins registered in the Swiss Protein Data Bank.

p20 recognizes the lipoprotein sorting signal

Some lipoproteins of *E.coli* exist in the outer membrane, whereas others exist in the inner membrane (Ichihara *et al.*, 1981; Pugsley, 1993). Yamaguchi *et al.* (1988) have shown that the second amino acid residue from the N-terminal end of lipoproteins plays an important role in the determination of their final destinations, namely lipoproteins with amino acids other than Asp at this position are localized in the outer membrane, whereas those with Asp remain in the inner membrane. Thus, the amino acid residue next to the N-terminal Cys of lipoproteins functions as a sorting signal. An Lpp-β-lactamase hybrid protein possessing either Ser [Lpp-Bla(S)] or Asp [Lpp-Bla(D)] at the second position has been designed by Yamaguchi *et al.* (1988). We examined the release of these proteins from spheroplasts. Spheroplasts harboring *lpp-bla* hybrid genes on plasmids were labeled in the presence and absence of p20, and then fractionated into spheroplasts and the medium (Figure 4). Lpp-Bla(S) was exported into the medium in a p20-dependent manner, as Lpp was. On the other hand, Lpp-Bla(D) was not secreted into the medium at all, remaining in spheroplasts as a mature form (Figure 4). These results strongly indicate that p20 recognizes the outer membrane-directed signal and releases only lipoproteins possessing this signal.

Lpp forms a soluble complex with p20

Lpp isolated from the outer membrane is water-insoluble. In contrast, Lpp secreted into the spheroplast medium containing p20 was not precipitated even by ultracentrifugation (see Figure 6A). These results suggest that Lpp exists as a soluble complex with p20 in the spheroplast medium. To examine this, Lpp secreted into the spheroplast medium was subjected to chemical cross-linking and gel filtration chromatography.

[^{35}S]Lpp in the spheroplast medium was treated with bis-(sulfosuccinimidyl) suberate (BS_3) and then immunoprecipitated with an anti-Lpp antibody, followed by SDS–PAGE (Figure 5A). A unique smear band corresponding to 25–29 kDa was detected as a cross-linked product of Lpp (compare lane 1 with lane 2). Since the molecular mass of Lpp is 7.2 kDa, the molecule cross-linked with Lpp should have a mass of 18–22 kDa, most probably indicating that Lpp forms a complex with p20. In another experiment, the spheroplast medium was obtained after incubation of unlabeled spheroplasts with ^{125}I-labeled p20, and then subjected to chemical cross-linking. After immunoprecipitation with the anti-Lpp antibody, the cross-linked product was analyzed by SDS–PAGE (Figure 5A, lanes 3 and 4). The product cross-linked with [^{125}I]p20 exhibited the same molecular mass as that of the product cross-linked with [^{35}S]Lpp. No other cross-linked products >20 kDa were observed except for possible aggregated materials at the top of the gel. These results indicate that Lpp and p20 interact directly and form a soluble complex comprising these proteins in a 1:1 stoichiometry. Chemical

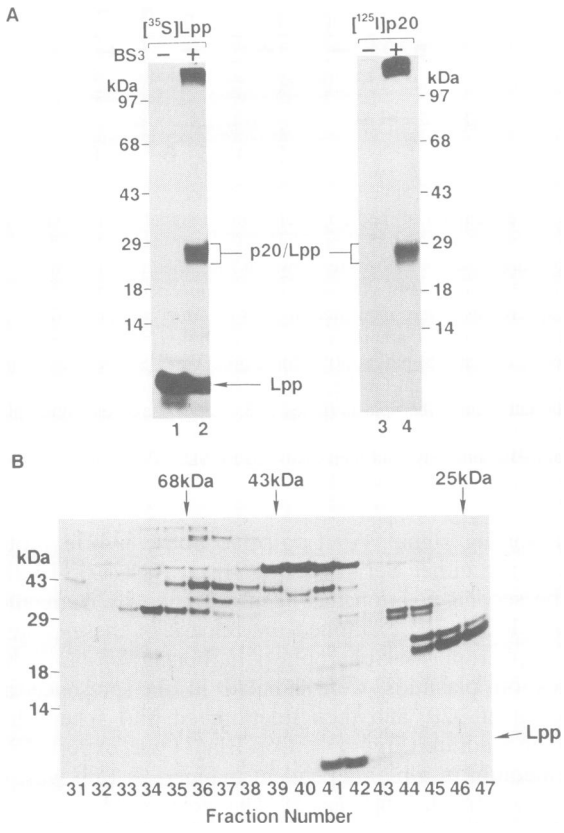

Fig. 5. Lpp forms a soluble complex with p20. (**A**) Spheroplasts were preincubated at 30°C for 2 min in the presence of p20 and labeled with Tran^{35}S-label for 2 min, followed by a 2 min chase (lanes 1 and 2). In another experiment, spheroplasts were incubated for 6 min at 30°C with ^{125}I-labeled p20 (lanes 3 and 4). The labeled culture was then centrifuged to obtain the spheroplast medium. The spheroplast medium (1 ml) containing the soluble Lpp was centrifuged further at 100 000 *g* for 30 min, and then subjected to gel filtration on a Sephadex G75 column. The void volume fraction was treated without (lanes 1 and 3) or with (lanes 2 and 4) a chemical cross-linker, BS_3, at 4°C for 50 min. Each sample was immunoprecipitated with an anti-Lpp antibody, and then analyzed by SDS–PAGE and fluorography (lanes 1 and 2) or autoradiography (lanes 3 and 4). The positions of Lpp and the p20–Lpp complex are indicated. The migration positions of molecular weight marker proteins are also indicated. (**B**) Spheroplasts were labeled with Tran^{35}S-label in the presence of p20. The spheroplast medium containing Lpp was centrifuged at 100 000 *g* for 30 min, and the resulting supernatant was subjected to gel filtration chromatography on a Superdex 75 column (1×30 cm; Pharmacia), which had been equilibrated with 50 mM K-phosphate (pH 7.5). The column was eluted with the same buffer at a flow rate of 0.5 ml/min. Each fraction (0.25 ml) was analyzed by SDS–PAGE and fluorography. The elution positions of bovine serum albumin (68 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) under the same conditions are indicated above the fluorogram. The position of Lpp is also indicated.

cross-linking studies carried out with [^{35}S]Lpp-Bla(S) or [^{125}I]p20 also revealed that Lpp-Bla(S) interacts directly and forms a complex with p20 (data not shown).

[^{35}S]Lpp exported into the spheroplast medium in the presence of unlabeled p20 was fractionated by gel filtration chromatography. Fractions containing Lpp were then analyzed by SDS–PAGE and fluorography (Figure 5B). Lpp was eluted in fractions corresponding to an apparent molecular mass of 34–37 kDa (fractions 41 and 42). This also supports the formation of a heterodimer complex from one molecule each of [^{35}S]Lpp and

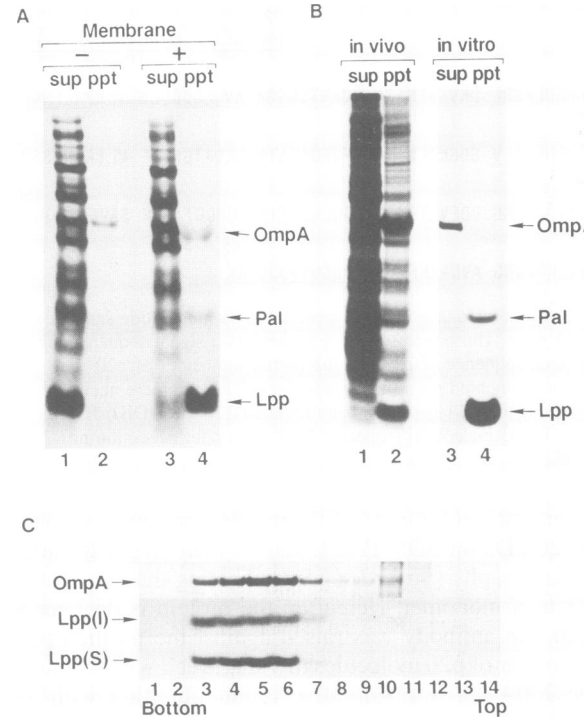

Fig. 6. *In vitro* assembly of Lpp into the outer membrane. (**A**) Spheroplasts were labeled in the presence of p20. The spheroplast medium containing the p20–Lpp complex was incubated at 30°C for 30 min in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the total membrane fraction prepared from Q13Lpp$^-$ cells. After centrifugation at 100 000 *g* for 30 min, the supernatant (lanes 1 and 3) and precipitate (lanes 2 and 4) were analyzed by SDS–PAGE and fluorography. The positions of Lpp, Pal and OmpA are indicated. (**B**) The membrane fraction prepared from ^{35}S-labeled intact cells (lanes 1 and 2) and the precipitate containing [^{35}S]Lpp obtained in (A) (lanes 3 and 4) were treated with 6 M urea at room temperature for 30 min, and then centrifuged at 200 000 *g* for 30 min. The supernatant (lanes 1 and 3) and precipitate (lanes 2 and 4) were analyzed by SDS–PAGE and fluorography. The positions of Lpp, Pal and OmpA are indicated. (**C**) The precipitate containing [^{35}S]Lpp obtained in (A) was subjected to sucrose density gradient centrifugation, being fractionated into fractions 1–14, from the bottom to the top of the gradient. Each fraction was analyzed by SDS–PAGE and fluorography [Lpp(S)]. The membrane fraction was also prepared from ^{35}S-labeled intact cells and analyzed as a control [OmpA and Lpp(I)].

unlabeled p20, since p20 behaves as a 27 kDa protein on gel filtration chromatography. These results also indicate that p20-dependent secretion of Lpp does not involve the formation of micelles or membrane vesicles.

p20 presumably interacts with Lpp at its hydrophobic N-terminal region containing the sorting signal, thus forming a stable water-soluble complex.

In vitro assembly of Lpp into the outer membrane

A soluble complex formed from p20 and lipoproteins destined for the outer membrane seemed to be a periplasmic intermediate of the lipoprotein localization pathway. To examine this, the spheroplast medium containing the p20–Lpp complex was incubated at 30°C with or without total membrane fractions and then centrifuged. Soluble Lpp was quantitatively converted into a membrane-associated form upon incubation with the membrane (Figure 6A). The peptidoglycan-associated lipoprotein (Pal; Chen and Henning, 1987), which is located in the outer membrane in cells, was also recovered with the

membrane. The secretion of Pal into the spheroplast medium also required p20 (data not shown), whereas that of OmpA did not (Matsuyama *et al.*, 1993). A portion of OmpA was precipitated not only in the presence but also in the absence of the membrane, suggesting that this precipitation is non-specific. In contrast, many other protein species were recovered in the supernatant, irrespective of the presence or absence of the membrane. Taken together, these results suggest the preferential association of the two outer membrane-directed lipoproteins with the membrane in the presence of p20. We examined next whether or not the lipoproteins associated with the membrane *in vitro* are solubilized with 6 M urea (Figure 6B). Lpp, which was labeled and localized in the outer membrane *in vivo*, was resistant to urea extraction (lanes 1 and 2). Both lipoproteins associated with the membrane *in vitro* were also resistant to urea treatment, whereas non-specifically precipitated OmpA was solubilized (lanes 3 and 4). These results suggest that the *in vitro* association of the two lipoproteins with the membrane takes place in a physiological manner, but is not caused by non-specific aggregation.

When the membrane fractions incubated with Lpp *in vitro* were fractionated by sucrose density gradient centrifugation, *in vitro*-incorporated Lpp was recovered in the outer membrane as well as OmpA and Lpp labeled *in vivo* (Figure 6C), whereas Lpp remaining in the spheroplasts in the absence of p20 was found in the inner membrane (Figure 1B). Taken together, these results indicate that Lpp released from the inner membrane through the formation of a complex with p20 is subsequently incorporated into the outer membrane.

Lpp localized in the outer membrane *in vivo* is resistant to trypsin and exists as a trimer (Choi *et al.*, 1986). In contrast, Lpp secreted into the spheroplast medium in the presence of p20 was completely digested with trypsin (Figure 7A, lanes 1 and 2). The accumulation of trypsin-sensitive Lpp has also been observed in the periplasm of phenetyl alcohol-treated cells (Halegoua and Inouye, 1979). Once Lpp was incorporated into the outer membrane, however, ~50% of it gained trypsin resistance (Figure 7A, lanes 3 and 4). To examine the Lpp trimer, the total membrane fractions prepared from ^{35}S-labeled cells were subjected to chemical cross-linking, and then analyzed by SDS–PAGE before and after trypsin treatment (Figure 7B, lanes 2 and 3). Since the cross-linking reagent is bifunctional, the Lpp dimer was predominant over the Lpp trimer as the cross-linked product. The dimer and trimer of Lpp gave heterogeneous bands before trypsin treatment. Upon trypsin treatment, these bands became more prominent and migrated slightly faster. These results are essentially the same as those reported previously (Choi *et al.*, 1986), and most probably suggest that the dimer and trimer contain Lpp molecules to which peptidoglycan fragments are covalently linked, and that the C-terminal region of Lpp carrying the heterogeneous peptidoglycan fragments is cut out by trypsin. When the total membrane fractions containing the *in vitro*-incorporated [^{35}S]Lpp were subjected to chemical cross-linking and subsequent trypsinization, essentially the same results were obtained, although the bands corresponding to the dimer and trimer were more homogeneous even before trypsin treatment (Figure 7B, lanes 5 and 6). These results indicate that the

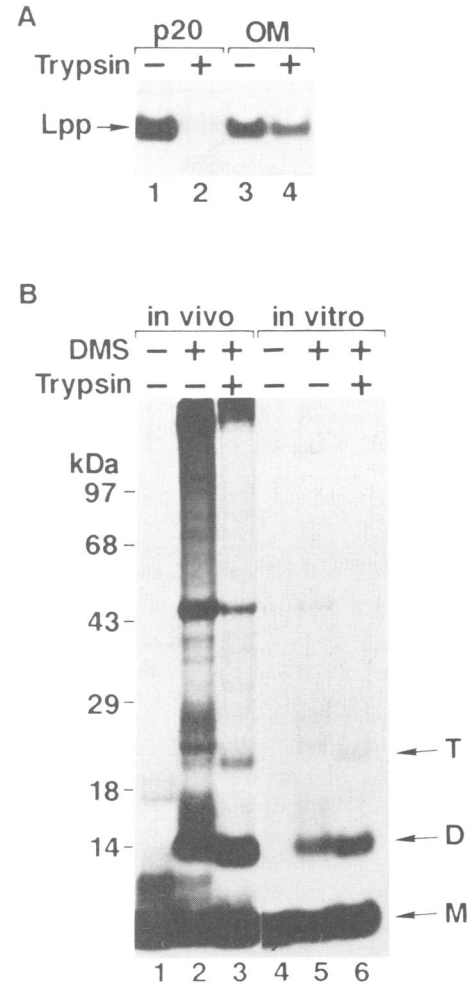

Fig. 7. Characterization of Lpp assembled into the outer membrane *in vitro*. (**A**) Lpp secreted into the spheroplast medium in the presence of p20 (lanes 1 and 2), and Lpp incorporated *in vitro* into the outer membrane (OM) of Q13Lpp$^-$ cells (lanes 3 and 4), were treated with 50 μg/ml trypsin at 4°C for 30 min, before (lanes 2 and 4) or after (lanes 1 and 3) the addition of 100 μg/ml trypsin inhibitor. Samples were analyzed by SDS–PAGE and fluorography. (**B**) *In vitro* assembly of Lpp was carried out with the total membrane fraction prepared from Q13 cells (lanes 4–6). As a control, the total membrane fraction was prepared from ^{35}S-labeled MC4100 cells (lanes 1–3). These membrane fractions containing ^{35}S-labeled Lpp were treated with (lanes 2, 3, 5 and 6) or without (lanes 1 and 4) dimethyl suberimidate (DMS) at 25°C for 60 min. The cross-linking reaction was quenched by the addition of 200 mM Tris–HCl (pH 8.5). Trypsin treatment of the cross-linked samples was carried out in 50 mM Tris–HCl (pH 7.5) containing 50 μg/ml of trypsin at 37°C for 1 h (lanes 3 and 6). All samples were immunoprecipitated with an anti-Lpp antibody, and then analyzed by SDS–PAGE and fluorography. The migration positions of molecular weight markers are indicated. The positions of the monomer (M), dimer (D) and trimer (T) of Lpp are also indicated.

in vitro-incorporated Lpp underwent trimerization. A band corresponding to ~45 kDa most probably represents the Lpp–OmpA complex because Lpp interacts with OmpA in the outer membrane (Choi *et al.*, 1986).

When the total membrane fractions were treated with trypsin and then used for the *in vitro* assembly of Lpp, the incorporation of Lpp was inhibited significantly (unpublished observation), suggesting that a proteinaceous component in the outer membrane is involved in the specific assembly of Lpp.

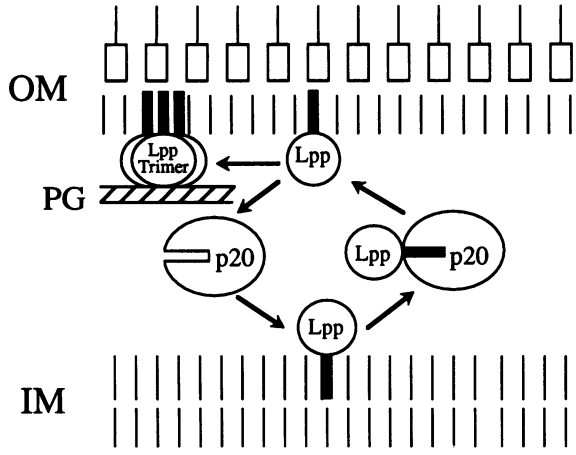

Fig. 8. Model for the localization of Lpp from the inner to the outer membrane. IM, inner membrane; OM, outer membrane; PG, peptidoglycan. (■) Hydrophobic N-terminal region of Lpp. (—) and (□—) represent phospholipid and lipopolysaccharide respectively. For details, see the text.

Discussion

In this study, we have discovered and characterized a novel periplasmic protein, p20, as a carrier for Lpp. p20 caused the release of Lpp from the inner membrane by forming a complex with Lpp in a 1:1 stoichiometry. The complex formation was specific to lipoproteins which are destined for the outer membrane. Furthermore, Lpp was specifically incorporated into the outer membrane when the complex was subsequently incubated with the total membrane fractions. The incorporated Lpp existed as a trimer, which represents its physiological structure, in the outer membrane. Taken together, these results indicate that p20 plays an essential role in the localization of lipoproteins in the outer membrane. Based on the observations reported here, we propose the model depicted in Figure 8 for the p20-dependent localization of Lpp in the outer membrane.

The number of p20 molecules in a single cell was estimated to be 200–400. This value is several orders of magnitude lower than the number (10^5–10^6) of Lpp molecules in a single cell (Braun, 1975; Mizushima, 1984). Furthermore, p20 was localized specifically in the periplasm but was not detected in the outer or inner membrane. It seems likely, therefore, that p20 functions catalytically as a shuttle between the inner and outer membranes.

Inouye and co-workers have revealed that the amino acid residue next to the modified N-terminal Cys functions as a sorting signal for the localization of lipoproteins in the inner or outer membrane. Lipoproteins with an amino acid other than Asp at this position have been shown to be destined for the outer membrane, whereas those having Asp are destined for the inner membrane (Yamaguchi *et al.*, 1988). We have shown that a lipoprotein derivative with Asp at this position was retained in the inner membrane and was not released by p20, indicating that the p20-dependent release, or complex formation with p20, is a critical step in the lipoprotein sorting process. The inner membrane localization of lipoproteins having Asp is not, therefore, caused by the prevention of their retention in the outer membrane due to the inner membrane sorting signal (Pugsley, 1993). It is not completely clear, however, whether p20 recognizes the sorting signal directly or indirectly. A factor which directly recognizes the inner membrane sorting signal, and prevents complex formation between p20 and inner membrane lipoproteins, may exist in the inner membrane.

The *in vitro* incorporation of Lpp into the outer membrane took place almost quantitatively, indicating that Lpp localization is unidirectional. Factors other than p20 seem to be required for this unidirectional reaction. A receptor for the p20–Lpp complex should be present in the outer membrane as the determinant of the membrane specificity. Lpp was incorporated *in vitro* into the outer membrane prepared not only from Q13 (Figure 7B) but also from its Lpp^- derivative (Figures 6 and 7A), indicating that pre-existing Lpp is not the receptor. The resolubilization of Lpp by p20 from the outer membrane needs to be prevented for unidirectional localization. The trimerization or interaction with other outer membrane components may be responsible for this. Because of the finding of p20, we are now able to examine in detail the molecular mechanism underlying Lpp localization *in vitro*. The putative outer membrane receptor is currently under examination, with proteoliposomes reconstituted from outer membrane proteins. Furthermore, we are currently constructing an *E.coli* Δ*lplA* strain to clarify the *in vivo* role of p20.

Eukaryotic cells contain various proteins which are post-translationally modified through fatty acylation (Glomset *et al.*, 1990). The modification has been thought to be important for their membrane anchoring and functions (Hancock *et al.*, 1990; Jackson *et al.*, 1990). For example, small GTP binding proteins are localized in specific cellular organelles and play essential roles in polarized vesicle-mediated protein transport. However, little is known about how these proteins are destined for the respective organelles (Pryer *et al.*, 1992). A factor similar to p20 may be involved in the specific localization of these proteins.

Materials and methods

Bacterial strains and media

E.coli strain MC4100 (F^- Δ*lacU169 araD rpsL relA thi fibB*; Casadaban, 1976) was used in all labeling experiments. The cells were grown in M63 minimal medium supplemented with 10 μg/ml thiamine and 20 μg/ml each of all amino acids except methionine and cysteine. Glucose (0.4%) was used as a carbon source. KH87 (MC4100 *malQ apeA ptr pepN sppA*; a gift from Dr S.Ichihara) grown in L broth was used for the preparation of the periplasmic fraction. Q13 (HfrH *pnp-13 tyr met* $RNaseI^-$; Reiner, 1969) and $Q13Lpp^-$ (Yamane *et al.*, 1987) cells were used for the preparation of total membrane fractions.

Construction of plasmids

pMAN950 carries the P_{BAD} promoter and the *araC* gene at the *Eco*RI site of pBR322. The construction of this plasmid will be reported elsewhere. The *Xba*I and *Sca*I sites of pMAN950 are located downstream from the P_{BAD} promoter and in the *bla* gene respectively. A large *Xba*I–*Sca*I fragment derived from pMAN950 was ligated with a 0.35 kb *Xba*I–*Sca*I fragment from pJG311 (Ghrayeb and Inouye, 1984) and pKY702 (Yamaguchi *et al.*, 1988) to construct pMAN955 and pMAN 960 respectively. Both plasmids carry the P_{BAD}-controlled *lpp-bla* fusion genes, *araC* and *tet*, although *lpp-bla* of pMAN960 possesses a mutation which converts the Ser immediately after the modified Cys in Lpp to Asp.

Release of Lpp from spheroplasts

The experiments involving spheroplasts were performed essentially as described previously (Matsuyama *et al.*, 1993). MC4100 cells were converted to spheroplasts, and 300 μl of the spheroplast suspension containing 5×10^8 spheroplasts were mixed with 50 μl of 10 mg/ml periplasmic fraction or 15 μl of 0.2 mg/ml purified p20 on ice. M63 (0.75 ml) medium containing 0.25 M sucrose and 10 μCi Tran^{35}S-label were added to initiate labeling at 30°C. After 3 min, the labeling was chased with 12 mM cold methionine and cysteine for 3 min. The labeled culture was chilled immediately in ice-water, and then centrifuged at 16 000 *g* for 2 min to obtain the spheroplast and medium fractions.

Immunoprecipitation and SDS–PAGE

Samples were subjected to trichloroacetic acid precipitation, followed by immunoprecipitation with anti-Lpp, anti-Pal and anti-Bla antibodies, as described (Matsuyama *et al.*, 1993). SDS–PAGE was carried out as described (Hussain *et al.*, 1980).

Sucrose density gradient centrifugation

The inner and outer membranes were separated by sucrose density gradient centrifugation. The membrane fractions were suspended in 50 mM K-phosphate (pH 7.5), and then layered over 2.2 ml of a linear gradient of 25–55% (w/w) sucrose dissolved in 50 mM K-phosphate (pH 7.5), centrifuged at 60 000 *g* for 15 h at 4°C, and fractionated into several fractions.

Purification of p20

To avoid possible proteolysis, KH87 lacking the activity of four kinds of protease was used. Wet KH87 cells (20 g) were converted to spheroplasts as described (Osborn and Munson, 1974). Spheroplasts were removed by centrifugation at 10 000 *g* for 10 min, and the resulting supernatant was subjected to further centrifugation at 100 000 *g* for 30 min to remove insoluble materials. Periplasmic fraction (500 ml) thus obtained was concentrated with an Amicon concentrator, and then dialyzed against 20 mM Tris–HCl (pH 8.0). The concentrated fraction (25 ml) was applied to a MonoQ column which had been equilibrated with 20 mM Tris–HCl (pH 8.0). The column was then eluted at a flow rate of 4 ml/min with a linear gradient of 0.0–0.5 M NaCl in 20 mM Tris–HCl (pH 8.0). Each fraction was assayed for the release of Lpp from spheroplasts. The active fractions were collected and dialyzed against 25 mM K-phosphate (pH 7.5). A hydroxylapatite column, which had been equilibrated with 25 mM K-phosphate (pH 7.5), was employed for further purification. The column was eluted at a flow rate of 1 ml/min with a linear gradient of 25–500 mM K-phosphate (pH 7.5). The active fractions were collected and dialyzed against 20 mM Tris–HCl (pH 8.0). Finally, p20 was purified on a MonoP column, which had been equilibrated with 20 mM Tris–HCl (pH 8.0). The column was eluted at a flow rate of 1 ml/min with a linear gradient of 0.0–0.2 M NaCl in 20 mM Tris–HCl (pH 8.0). Active fractions (0.5 ml) containing 100 μg of p20 were dialyzed against 50 mM K-phosphate (pH 7.5), and then stored at –80°C.

Amino acid sequencing of p20

Purified p20 (27 μg) was digested with 1.3 μg of lysylendopeptidase in 400 μl of 0.1 M sodium bicarbonate (pH 7.5) at 37°C for 12 h, and then subjected to fractionation by reverse-phase column chromatography. The amino acid sequences of peptide fragments and the intact p20 were determined with an Applied Biosystems gas phase protein sequencer 477A.

Cloning and DNA sequencing of the lplA gene

Oligonucleotide probes 5′-CA(AG)GA(AG)GG(TC)CA(AG)GG(TC)-GA-3′ and 3′-TACTG(AG)GT(TC)GG(TCA)CT(AG)CT-5′ were synthesized based on the partial amino acid sequences of p20, QEGQGD and MTQPDE respectively. A 5.1 kb *Pst*I fragment of KH87 chromosomal DNA hybridized to both probes and was cloned into pSTV29 (Takara Shuzo Co.) derived from pACYC184. After the transformation of MC4100 cells with pSTV29, recombinant plasmids were isolated from 200 transformants and examined for hybridization with the probes. Three transformants were found to be carrying the DNA fragment on a plasmid. A plasmid, pMAN994, was isolated from one of the three transformants and used to determine the location of the *lplA* gene. The 1.05 kb *Bam*HI–*Kpn*I fragment of pMAN994 hybridized to both probes. The entire nucleotide sequence of this fragment was determined directly with pMAN994 and appropriate synthetic primers.

Chemical cross-linking

Spheroplast medium was applied on a Sephadex G-75 column which had been equilibrated with 50 mM K-phosphate (pH 7.5). The void volume fraction was collected and incubated in the presence of 1 mM BS_3 at 4°C for 50 min. The cross-linking reaction was then quenched for 5 min by the addition of 10 mM lysine. Cross-linking with dimethyl suberimidate was carried out as described (Choi *et al.*, 1986). The total membrane fraction (40 μg) was incubated with 0.5 mg of dimethyl suberimidate in 200 μl of 0.2 M triethanolamine–HCl (pH 8.5) at 25°C for 60 min. The reaction was then quenched for 5 min after the addition of 200 mM Tris–HCl (pH 8.5).

In vitro assembly of Lpp into the outer membrane

Spheroplast medium containing the p20–Lpp complex was centrifuged at 100 000 *g* for 30 min to remove insoluble materials. The supernatant (200 μl) was incubated at 30°C for 30 min in the presence of the total membrane fraction (80 μg as protein) containing the outer membrane and right side-out cytoplasmic membrane vesicles. The total membrane fraction was prepared from Q13 or Q13Lpp$^-$ cells according to the reported method (Kaback, 1971). After centrifugation at 100 000 *g* for 30 min, the supernatant and precipitate were analyzed by SDS–PAGE. Where specified, the precipitate was treated with 50 mM K-phosphate (pH 7.5)–6 M urea at room temperature for 30 min, and then centrifuged at 200 000 *g* for 30 min. The supernatant and precipitate thus obtained were analyzed by SDS–PAGE.

Chemicals

Tran^{35}S-label (a mixture of 70% [^{35}S]methionine and 20% [^{35}S]cysteine, 1000 Ci/mmol) and [^{125}I]Bolton-Hunter reagent (2800 Ci/mmol) were obtained from ICN. Bis-(sulfosuccinimidyl) suberate was obtained from Pierce. Trypsin and soybean trypsin inhibitor were purchased from Funakoshi Co. Ltd. The anti-Bla antibody was obtained from 5 Prime → 3 Prime, Inc. The anti-p20 antibody was raised against the purified p20 in rats.

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