Effects of Lipoprotein Overproduction on the Induction of DegP (HtrA) Involved in Quality Control in the Escherichia coli Periplasm*

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Recent biochemical examination has revealed the presence of at least 90 different lipoproteins in Escherichia coli. Among previously identified lipoproteins, only an outer membrane lipoprotein, NlpE, is known to induce expression of the degP gene upon its overproduction. The degP gene encodes a periplasmic protease, which is thought to be involved in the digestion of unfolded proteins, and is essential for growth at high temperatures. However, it is not completely clear why NlpE overproduction causes degP expression. Moreover, among newly confirmed lipoproteins, there may be others that also induce degP expression. Therefore, we overproduced each of the 90 lipoproteins and examined the level of degP expression as β-galactosidase activity by using a degP promoter-lacZ fusion. The extent of degP expression caused by NlpE overproduction was dependent on the mode of degP-lacZ fusion. On the other hand, new inner membrane lipoprotein YafY strongly induced degP expression irrespective of the mode of fusion even though the level of overproduced YafY was lower than that of NlpE. The induction of degP expression by YafY overproduction was dependent on the Cpx two-component system. Alteration of the lipoprotein-sorting signals of NlpE and YafY did not abolish the degP induction. However, a YafY derivative possessing the outer membrane signal remained on inner membranes. The non-lipidated derivative of NlpE did not induce degP expression, indicating that membrane anchoring is essential for degP induction. The amino acid sequences of YafY and YfS, another inner membrane lipoprotein, are highly identical, but overproduction of the latter did not induce degP expression. Construction of various YafY-YfS chimeric lipoproteins revealed that only a few residues located in the N- and C-terminal regions were important for the induction of DegP.

Bacterial lipoproteins are synthesized as precursors in the cytoplasm and then translocated to the periplasmic side of the inner membrane where they are processed to mature forms. Mature lipoproteins have an N-terminal Cys that is modified by thioether-linked diacylglycerol and amino-linked acyl chains (1). In Escherichia coli, lipoproteins are anchored to the periplasmic leaflet of the inner or outer membrane through N-terminal lipids. When lipoproteins have an Asp at position 2 and Asp, Glu, Gln, or Asn at position 3 (2) they are retained in the inner membrane presumably through an interaction between Asp at position 2 and phospholipids (3).

The Lol system comprising five Lol proteins is required for the sorting and outer membrane localization of lipoproteins. The LolCDE complex, an ATP binding cassette transporter, releases outer membrane-directed lipoproteins from the inner membrane (4) leading to the formation of a water-soluble complex comprising one molecule each of a lipoprotein and LolA in the periplasm (6, 7). The LolA-lipoprotein complex then interacts with the outer membrane receptor LolB, which catalyzes the anchoring of lipoproteins to the outer membrane (8, 9). The crystal structures of LolA and LolB are very similar to each other despite the fact that their amino acid sequences are dissimilar (10).

More than 100 lipoproteins are predicted to be present in E. coli (11, 12). We recently cloned almost all putative lipoprotein genes and examined whether or not the proteins encoded by these genes are modified with lipids. These analyses revealed that E. coli possesses at least 90 lipoproteins. Although most lipoproteins have no known functions, they are expected to play important roles in various activities in the periplasm, because the protein moieties of lipoproteins are generally soluble and presumably exposed to the periplasm. Among previously confirmed lipoproteins, only NlpE is known to induce expression of the degP gene upon overproduction, which encodes a periplasmic protease (13, 14). Overproduction of NlpE activates a two-component signal transduction system comprising CpxA and CpxR and then stimulates the expression of degP (13, 14), which is also positively regulated by σE. This stress response system involving the Cpx two-component system and σE is thought to represent the quality control mechanism of the periplasm (15, 16). When unfolded proteins are accumulated in the periplasm, DegP is induced to clean the periplasm. DegP has also been reported to exhibit chaperone activity (17). However, it is not clear why only NlpE among the lipoproteins induces DegP. Moreover, it is possible that some newly confirmed lipoproteins may also induce DegP. Here we report that the inner membrane lipoprotein YafY also induces DegP.

EXPERIMENTAL PROCEDURES

Bacteria and Plasmids—E. coli K12 strains MC4100 (18), PAD280 (MC4100 Δrps8 [degP-lacZ]) (19), and PAD299 (PAD280 cpxR::spc) were grown on L broth at 37 °C. The last two strains were kindly supplied by Dr. Tom Silhavy. When required, the medium was supple-

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DegP Induction Expressed by Lipoproteins

**Table I**

| Lipoprotein | Oligonucleotide |
|-------------|-----------------|
| NlpE(D)     | 5'-TTACTCTGATGGAGTGTATGACCCGGGGCCGAAGTCATAC-3' |
|             | 5'-GTGACTCTGCGCCGCTGTCGTCATCCATCAGGTAATC-3' |
|             | 5'-TGGTCTCTCCACCGTCCGTCATCCATCAGGTAATC-3' |
| YafY        | 5'-CACACTCAGCAGATGAGAAGGTGACTGACGAGACGAA-3' |
|             | 5'-ACAGAAGCTTAATAGTAGATGATGATTCTCTGATTGAGG-3' |
|             | 5'-ACAGAAGCTTAATAGTAGATGATGATTCTCTGATTGAGG-3' |

Oligonucleotides used for the construction of lipoprotein derivatives

Underlined letters in the NlpE(DD) and YafY(SS) primers correspond to the second and third residues. Underlined bold letters and bold letters represent the reaction time (min) and culture volume (OD420)/(OD550), respectively.

| Derivative | Oligonucleotide |
|------------|-----------------|
| NlpE(D)    | 5'-TTACTCTGATGGAGTGTATGACCCGGGGCCGAAGTCATAC-3' |
|            | 5'-GTGACTCTGCGCCGCTGTCGTCATCCATCAGGTAATC-3' |
|            | 5'-TGGTCTCTCCACCGTCCGTCATCCATCAGGTAATC-3' |
| YafY       | 5'-CACACTCAGCAGATGAGAAGGTGACTGACGAGACGAA-3' |
|            | 5'-ACAGAAGCTTAATAGTAGATGATGATTCTCTGATTGAGG-3' |
|            | 5'-ACAGAAGCTTAATAGTAGATGATGATTCTCTGATTGAGG-3' |

| mYafY | 5'-CACACTCAGCAGATGAGAAGGTGACTGACGAGACGAA-3' |
| mNlpE | 5'-ACAGAAGCTTAATAGTAGATGATGATTCTCTGATTGAGG-3' |

Lipidated derivatives of NlpE (mNlpE) and YafY (mYafY), a Cys to Ala mutation was introduced at position 1 of the mature region, as described for the construction of mOmpA (8). The oligonucleotide primers used are indicated in Table I. Mutations were confirmed by sequencing.

**Construction of pDegP-LacZ**—To construct pDegP-LacZ encoding LacZ under the control of the degP promoter, the 480-bp upstream region of degP was amplified by PCR using oligonucleotide primers 5'-CAGCACCGGTTGAGCGCAATGTCGTCATCCATCAGGTAATC-3' (the HindIII site is underlined) and 5'-CCGGCCGATCCGATGTCGTCATCCATCAGGTAATC-3' (the BamHI site is underlined) with MC4100 chromosomal DNA as a template. The amplified DNA was digested with HindIII and BamHI and then inserted into the same site of pCB192 (20) carrying lacZ and bla. The plasmid thus constructed was digested with ScaI and HindIII, and the resultant fragment carrying degP-lacZ was cloned at the Smal-HindIII site of pSTV28 (Takara), which carries a chloramphenicol resistance gene.

**Construction of Lipoprotein Derivatives**—To construct NlpE(D) and YafY(SS) having Asp and Ser, respectively, as the second and third residues, pKT-NlpE and pKT-YafY were mutagenized using a QuikChange site-directed mutagenesis kit (Stratagene) with the respective primers. Underlined letters in the NlpE(DD) and YafY(SS) primers correspond to the second and third residues. Underlined bold letters and bold letters represent the reaction time (min) and culture volume (OD420)/(OD550), respectively.

The levels of overproduced lipoproteins were examined in membranes prepared from cells grown in the presence of IPTG (Fig. 2). The overproduction levels differed depending on the lipoprotein species and were normalized as to molar amount by means of the molar amount of OmpA. The extent of degP induction determined with lipoprotein overproduction was plotted as a function of the normalized level of the lipoprotein (Fig. 3A). The extent of degP induction was found to be independent of the molar amount of a lipoprotein, for example, YegR was overproduced more than NlpE but did not induce degP. Many lipoproteins were overproduced to levels similar to that of OmpA and caused only marginal degP induction. Strikingly, YafY strongly induced degP, although the level of its overproduction was similar to the molar amount of OmpA. YafY has no known function but is predicted to be located in the inner membrane, because it has Asp at both the second and third positions. This dipetide function as a Lol-avoidance signal and causes the retention of the inner membrane (2, 3, 25).

**Induction of DegP by YafY Is Dependent on the Cpx Two-component System**—It has been shown that overproduction of NlpE induces DegP through activation of the two-component system comprising CpxA and CpxR (13, 14). CpxA is the sensor kinase in the inner membrane, is autophosphorylated upon detection of envelope stress, and then transfers the phosphate to the response regulator CpxR. The phosphorylated CpxR then induces the expression of genes encoding factors involved in the holding and digestion of envelope proteins. DegP induction caused by the overproduction of 11 lipoproteins, which include both DegP-induction positive and negative lipoproteins in MC4100 cells, was examined in PAD280 (cpxA::Km) and its derivative PAD299 (cpxA::Km::cpxB). Both strains carry the degP-lacZ fusion gene, which was inserted into the chromosome by means of Φ phage transduction (19). The levels of overproduced lipoproteins were essentially the same in PAD280 and PAD299 (Fig. 3B) and did not significantly differ from those determined in MC4100 (Fig. 3A), except for OsmB. However, the β-galactosidase activity determined in PAD280 was significantly higher than that determined in MC4100 harboring pDegP-

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2 The abbreviation used is: IPTG, isopropyl-β-D-thiogalactopyranoside.

3 H. Miyadai, K. Tanaka-Masuda, S. Matsuyama, and H. Tokuda, unpublished observation.
The difference in \( \beta \)-galactosidase activity between the two strains is presumably caused by differences in the location and structure of the \( \text{degP-lacZ} \) fusion genes. The \( \text{lacZ} \) gene in \( \text{PAD280} \) was fused to a truncated \( \text{degP} \) and located on the chromosome, whereas that in \( \text{MC4100} \) harboring \( \text{pDegP-LacZ} \) was fused to the upstream region of \( \text{degP} \) carried by a plasmid. Although the mRNA length is different, only \( \text{lacZ} \) is expressed upon induction of the \( \text{degP} \) promoter in either strain. The difference in the structures of the transcripts might have caused the difference in \( \text{DegP} \) expression. The copy numbers of the \( \text{degP-lacZ} \) fusion genes were also different between the two strains. The number of phosphorylated \( \text{CpxR} \) molecules might be insufficient for the full induction of \( \text{lacZ} \) in \( \text{MC4100} \) harboring \( \text{pDegP-LacZ} \). Despite the very high \( \beta \)-galactosidase activity in the \( \text{PAD280} \) strain, the induction of \( \text{DegP} \) on the overproduction of most lipoproteins was nearly completely abolished in the \( \text{cpxA} \) null strain \( \text{PAD299} \) (Fig. 3B). \( \text{DegP} \) induction by \( \text{NlpE} \) was remarkable in \( \text{PAD280} \), as reported previously (15). Furthermore, the overproduction of \( \text{YafY} \) strongly induced \( \text{DegP} \) in a \( \text{Cpx} \)-dependent manner even in \( \text{PAD280} \). \( \text{Pal} \), \( \text{OsmB} \), and \( \text{NlpA} \) also caused \( \text{DegP} \) induction in a \( \text{Cpx} \)-dependent manner, albeit to a lesser extent.

**Membrane Anchoring of NlpE Is Required for DegP Induction**—Lipoproteins are sorted to either the inner or outer membrane depending on the sorting signal located at position 2 (2, 25–27). Asp at this position with Asp, Glu, Asn, or Gln at position 3 causes the strong retention of lipoproteins on the inner membrane (2). In contrast, residues other than Asp at position 2 direct lipoproteins to the outer membrane. \( \text{NlpE} \) has Ser at positions 2 and 3 and is located on the outer membrane (14), whereas \( \text{YafY} \) having Asp at positions 2 and 3 is expected to be located on the inner membrane. The second and third residues were mutated to mislocate \( \text{NlpE} \) and \( \text{YafY} \) to the inner

**Fig. 1. Effect of lipoprotein overproduction on the expression of degP.** \( \text{E. coli} \) MC4100 cells harboring \( \text{pDegP-LacZ} \) were transformed with plasmids (pRT-lipoprotein) encoding the indicated lipoproteins and then either induced at \( A_{600} = 0.3 \) by the addition of 1 mM IPTG for 1 h or not induced. \( \beta \)-Galactosidase activity was examined as described under “Experimental Procedures.” \( \text{NlpE} \) and \( \text{YafY} \) are indicated by arrows.

**Fig. 2. Identification of overproduced lipoproteins.** The indicated lipoproteins were induced as described in the legend to Fig. 1. Membrane fractions were prepared from cells overproducing lipoproteins and then analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. Closed and open arrowheads indicate an outer membrane protein, \( \text{OmpA} \), and overproduced lipoproteins, respectively. The SDS-PAGE reported by Hussain et al. (22) was employed for the analysis of \( \text{OsmB} \). Other lipoproteins were analyzed according to Laemmli (21).

\( \text{LacZ} \). The difference in \( \beta \)-galactosidase activity between the two strains is presumably caused by differences in the location and structure of the \( \text{degP-lacZ} \) fusion genes. The \( \text{lacZ} \) gene in \( \text{PAD280} \) was fused to a truncated \( \text{degP} \) and located on the chromosome, whereas that in \( \text{MC4100} \) harboring \( \text{pDegP-LacZ} \) was fused to the upstream region of \( \text{degP} \) carried by a plasmid. Although the mRNA length is different, only \( \text{lacZ} \) is expressed upon induction of the \( \text{degP} \) promoter in either strain. The difference in the structures of the transcripts might have caused the difference in \( \text{DegP} \) expression. The copy numbers of the \( \text{degP-lacZ} \) fusion genes were also different between the two strains. The number of phosphorylated \( \text{CpxR} \) molecules might be insufficient for the full induction of \( \text{lacZ} \) in \( \text{MC4100} \) harboring \( \text{pDegP-LacZ} \). Despite the very high \( \beta \)-galactosidase activity in the \( \text{PAD280} \) strain, the induction of \( \text{DegP} \) on the overproduction of most lipoproteins was nearly completely abolished in the \( \text{cpxA} \) null strain \( \text{PAD299} \) (Fig. 3B). \( \text{DegP} \) induction by \( \text{NlpE} \) was remarkable in \( \text{PAD280} \), as reported previously (15). Furthermore, the overproduction of \( \text{YafY} \) strongly induced \( \text{DegP} \) in a \( \text{Cpx} \)-dependent manner even in \( \text{PAD280} \). \( \text{Pal} \), \( \text{OsmB} \), and \( \text{NlpA} \) also caused \( \text{DegP} \) induction in a \( \text{Cpx} \)-dependent manner, albeit to a lesser extent.

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and outer membranes, respectively. Moreover, the N-terminal Cys was changed to Ala to express them as soluble periplasmic proteins. These derivatives were induced in MC4100 harboring pDegP-LacZ, and the levels of their overproduction and their subcellular location were examined (Fig. 4). The level of NlpE(DD) was lower than that of NlpE (Fig. 4A), whereas DegP induction was stronger with the former than with the latter (Fig. 4C). The level of overproduced YafY(SS) was also significantly lower than that of the wild-type YafY, but it still induced DegP. Both derivatives remained associated with spheroplasts (Fig. 4B). Non-lipidated mNlpE molecules were mostly found in the periplasm (Fig. 4B). Interestingly, the level of overproduced mNlpE was higher than that of NlpE(DD) (Fig. 4A), but mNlpE did not induce DegP (Fig. 4C), indicating that NlpE must be membrane-anchored for the induction of DegP. In contrast to that of mNlpE, the expression of mYafY was very low, and it remained in the spheroplasts for an unknown reason. Therefore, it remains to be clarified whether or not membrane anchoring is also essential for DegP induction by YafY.

When NlpE, YafY, and their lipidated derivatives were overproduced, inner and outer membranes could not be separated by means of sucrose density gradient centrifugation (data not shown). Therefore, membranes were prepared from cells grown in the presence of a low concentration of IPTG and then fractionated (Fig. 4D). NlpE(DD) was located in the inner membrane as expected suggesting the inner membrane location of the overproduced NlpE(DD). Smear bands appeared for the outer membrane fractions after prolonged incubation with anti-His-tag antibodies. These bands migrated slightly lower than that of NlpE(DD) and seemed to be nonspecific ones. YafY(SS) remained in the inner membrane even though it had an outer membrane-directed signal. It is likely that YafY is retained in the inner membrane through an interaction with other inner membrane components. If this is the case, the Asp residues at positions 2 and 3 of the wild-type YafY may not function as a lipoprotein-sorting signal.

**Residues of YafY Important for DegP Induction**—The amino acid sequence of YafY is highly homologous to that of YfjS (Fig. 5). Furthermore, the levels of overproduced YafY and YfjS were similar (Fig. 3). On the other hand, YfjS did not induce DegP in MC4100 or PAD280 (Fig. 3). To determine the critical regions or residues for DegP induction, a number of YafY-YfjS chimeras were constructed (Fig. 6). The expression levels of these derivatives did not differ significantly (data not shown). Both the yafY and yfjS genes have unique sites for SmaI and AccI inside the coding region. In addition, plasmids carrying the lipoprotein genes have a unique site for MluI outside the coding region. By taking advantage of these restriction sites, the mature regions of the two lipoproteins were separated into three parts, i.e. the N-terminal, central, and C-terminal regions (Fig. 5). Six YafY-YfjS chimeric lipoproteins were constructed by exchanging these regions (Fig. 6, chimeras A–F). Replacement of the N- or C-terminal region of YafY by the corresponding region of YfjS significantly reduced DegP induction (Fig. 6, chimeras A and C). In contrast, replacement of the central region of YafY by that of YfjS only slightly reduced DegP induction (Fig. 6, chimera B). The N- or C-terminal region of YafY alone did not cause the maximum induction of DegP (Fig. 6, chimeras D and F), whereas the level of induction was higher than that caused by the central region alone (chimera E). These results indicate that the N- and C-terminal regions are both important for DegP induction.

To determine the most important residues for DegP induction in the N- and C-terminal regions of YafY, eight residues present in YafY but not in YfjS were variably exchanged with those in YfjS (Fig. 6, chimeras G–N). This revealed that Asp at position 40, Tyr at position 41, and Gln at position 116 are critical residues for DegP induction. Finally, chimera N thus constructed was a derivative of YfjS possessing these three residues of YafY. Induction of DegP caused by the overproduction of chimera N was significantly higher than that by the overproduction of YfjS, although the DegP induction by chimera N was still lower than that by the wild-type YafY.
The protein moieties of most, if not all, lipoproteins of *E. coli* are expected to be exposed to the periplasm. Lipoproteins are therefore expected to be responsible for various functions in the periplasm. In this study, we focused on the periplasmic stress response and found that not only NlpE but also a new inner membrane lipoprotein, YafY, causes DegP induction upon overproduction. The role of NlpE in up-regulation of the Cpx two-component system has been extensively studied (15). It has been revealed that the Cpx system is generally required for a response to various envelope stresses and induces the expression of genes such as *dsbA*, *degP*, *ppiA*, and *spy* (13, 28, 29). On the other hand, NlpE is specifically required for the activation of the Cpx system caused by cell adhesion to a hydrophobic surface (30). It is speculated that NlpE is located upstream of the Cpx pathway and senses cell adhesion (30). However, it is not completely clear why overproduction of NlpE also activates the Cpx system. Overproduction of outer membrane proteins causes the accumulation of an unfolded β-barrel structure and then activates σE, which is negatively regulated by anti-σ factor RseA and a periplasmic protein, RseB. Unfolded proteins are speculated to bind to the PDZ domain of a periplasmic protease DegS, which eventually causes the digestion of RseA (16). Expression of *degP* is under the regulation of both the Cpx pathway and σE.

Among the 90 lipoproteins examined, the effect of YafY overproduction on the induction of DegP was remarkable (Figs. 1 and 3). This induction was dependent on the *cpxA* gene (Fig. 3B), indicating that YafY overproduction causes an induction of the expression of *degP* through the activation of the Cpx system. Although the function of YafY is not known, it seems likely that YafY is involved in the monitoring of certain envelope stresses as speculated for the case of NlpE. Consistent with its sorting signal, YafY was located on the inner membrane. However, alteration of its signal to an outer membrane-specific one did not cause the sorting of YafY(SS) to the outer membrane (Fig. 4D). These results suggest that the location of YafY is not determined by the sorting signal. Previous examinations (2, 27) revealed that the membrane location of *E. coli* lipoproteins is basically determined by the second residue as originally proposed (26). The reason that YafY(SS) remained on the inner membrane is not known at present. However, it has been reported that the function of sorting signals is abolished depending on the structure of the lipoprotein mature region. For example, dimerization of lipoproteins inhibits the LolCDE-dependent release of outer membrane-specific lipoproteins (3), and an intramolecular disulfide bond formed in the N-terminal region abolishes the Lol avoidance function of Asp at position 2 (31). It is likely that YafY exists as a homo- or hetero-oligomer on the inner membrane thereby inhibiting the localization of YafY(SS) to the outer membrane.

In contrast to YafY, NlpE(DD) expressed at a low level was located on the inner membrane in accordance with its sorting signal (Fig. 4D). We therefore speculate that overproduced NlpE(DD) is also located on the inner membrane, although its overproduction perturbed membrane separation. We previously observed that overproduction of inner membrane-specific derivatives of the major outer membrane lipoprotein Lpp pre-
vented membrane separation (32). The induction of DegP by NlpE(DD) was stronger than that by NlpE (Fig. 4C), although the overproduction level was significantly lower with NlpE(DD) than with NlpE (Fig. 4A). This may suggest that the overproduction of NlpE generates mislocated NlpE on the inner membrane and that this species of NlpE is responsible for the activation of the Cpx system. It may be interesting to determine whether or not NlpE(DD) is also required for the response to cell adhesion.

The level of mNlpE was more than 3-fold higher than that of NlpE(DD) (Fig. 4A), whereas mNlpE did not induce DegP (Fig. 4C). Because most mNlpE molecules were present in the periplasm (Fig. 4B), the membrane anchoring of NlpE seems to be essential for the induction of DegP. Overproduction of lipoproteins possessing three fatty acyl chains may cause an alteration of the properties of membranes. However, because only a limited number of species of lipoproteins induced DegP upon overproduction (Figs. 1 and 3) changes in the membrane properties cannot be the reason for DegP induction. Instead, these results suggest that the protein moiety of NlpE should be close to the membrane for efficient induction of DegP through activation of the Cpx system. On the other hand, mYafY was expressed at a very low level (Fig. 4A) and was only detected in membrane fractions (Fig. 4B). Therefore, it is not clear at present whether or not the membrane anchoring of YafY is also required for DegP induction.

The identity of the amino acid sequences of YafY and YfjS is higher than 90% (Fig. 5), whereas DegP was strongly induced only by YafY overproduction. On the exchanging of regions and residues, only three residues of YafY were found to be important for DegP induction. However, neither YafY nor YfjS has any known function. Moreover, a motif search did not allow speculation as to their functions. To clarify the specific role of YafY in stress response mechanisms, it is essential to reveal...
the differences in structure and function between YafY and YfJS. The various YafY derivatives constructed here will be useful for the analyses of a possible interaction with the Cpx components in the inner membrane. It seems to be possible that NlpE and YafY are both components of a regulatory network, which also consists of the Cpx system, and that overproduction of one of the two lipoproteins leads to uncontrolled expression of DegP.

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