Skp, a Molecular Chaperone of Gram-negative Bacteria, Is Required for the Formation of Soluble Periplasmic Intermediates of Outer Membrane Proteins*

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Using a cross-linking approach, we have analyzed the function of Skp, a presumed molecular chaperone of the periplasmic space of *Escherichia coli*, during the biogenesis of an outer membrane protein (OmpA). Following its transmembrane translocation, OmpA interacts with Skp in close vicinity to the plasma membrane. In *vitro*, Skp was also found to bind strongly and specifically to pOmpA nascent chains after their release from the ribosome suggesting the ability of Skp to recognize early folding intermediates of outer membrane proteins. Pulse labeling of OmpA in spheroplasts prepared from an *skp* null mutant revealed a specific requirement of Skp for the release of newly translocated outer membrane proteins from the plasma membrane. Δskp mutant cells are viable and show only slight changes in the physiology of their outer membranes. In contrast, double mutants deficient both in Skp and the periplasmic protease DegP (HtrA) do not grow at 37 °C in rich medium. We show that in the absence of an active DegP, a lack of Skp leads to the accumulation of protein aggregates in the periplasm. Collectively, our data demonstrate that Skp is a molecular chaperone involved in generating and maintaining the solubility of early folding intermediates of outer membrane proteins in the periplasmic space of Gram-negative bacteria.

Gram-negative bacteria are characterized by the existence of an outer membrane, which together with the inner membrane (also termed plasma membrane) delimits the intervening periplasmic space. Outer membranes harbor two unique features, (i) the outer leaflet of the lipid bilayer is composed of a specific glycolipid, called lipopolysaccharide (LPS)1, which plays a crucial role in maintaining the permeability barrier of a Gram-negative cell (1); (ii) outer membrane proteins, many of which function as porins, usually form trimers that are inserted into the lipid bilayer as antiparallel β-barrels (2). Like the soluble periplasmic proteins, outer membrane proteins are synthesized as preproteins in the cytoplasm. They are translocated in a SecA- as well as SecB-dependent manner (3) across the inner membrane into the periplasm where an ill-defined sequence of folding events must occur, leading to soluble intermediates, to the conversion of monomers into trimers, and usually to the association with LPS allowing insertion into the outer membrane (4).

A picture of how the distinct folding events are catalyzed by individual chaperones is only now emerging (recently reviewed in Ref. 5). Most of the periplasmic chaperones thus far identified belong to two major groups, the Dsb proteins catalyzing thiol-disulfide exchange reactions, and peptidyl prolyl isomerases (PPIases) catalyzing the cis-trans isomerization around Xaa-Pro peptidyl bonds. Representatives of all major families of PPIases have been detected in the periplasm of Gram-negative bacteria. RotA (PpiA) is a cyclophilin-type PPIase; FkpA, a FK506-binding protein type PPIase; and SurA and PpiD belong to the parvulin type. The latter two have recently been analyzed in more detail as to an involvement in the biogenesis of outer membrane proteins (6–9).

In addition, Skp has been suggested to function as a periplasmic chaperone. This 16-kDa, basic protein was first purified 20 years ago as a LPS-associated protein of *Salmonella minnesota* (10). In *Escherichia coli*, however, it was initially ascribed the function of a histone-like protein (HLP1; hlpA) (11) and for some time was mistaken as the product of the downstream *firA* gene (12). Kleppe and co-workers (13, 14) purified it as a 17-kDa, basic DNA-binding protein from *E. coli*, cloned its gene, and coined the name Skp for “17-kilodalton protein.” Vaara and co-workers (15, 16) identified a homologue in *S. typhimurium*, which they characterized as outer membrane protein OmpH. Thome et al. (17) reported a SecA-like activity of *E. coli* Skp, which was used to purify the protein to homogeneity. SecA is a cytosolic translocation ATPase that targets unfolded preproteins to, and energizes their transport across, the bacterial plasma membrane (3). Since Skp is a periplasmic protein, its SecA-like activity was interpreted as that of a molecular chaperone preventing premature folding of preproteins in *vitro* and thereby alleviating the need for SecA (18).

Notably, the *in vitro* substrates of Skp thus identified were the outer membrane proteins LamB and OmpA. A specific interaction of Skp with outer membrane proteins was later confirmed (19, 20). A *skp* null mutation leads to a moderate reduction in outer membrane proteins (19), suggesting that Skp is involved in the assembly process of outer membrane proteins. Lately, Skp was also identified as a periplasmic protein improving folding of recombinant proteins (21). By analyzing early steps of the biogenesis of OmpA in *vitro* in combination with characterizing a *skp* null phenotype we now show that Skp in fact is a molecular chaperone whose function is the generation and maintenance of early soluble folding intermediates of outer
membrane proteins in the periplasmic space of Gram-negative bacteria.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—**If not stated otherwise *E. coli* K12 strain MC4100 (22) was used. Strains CAG18515 (proA::B::Tn10 containing the kanamycin resistance gene) (22), CJC198 (degP::Tn10) (24), D194 (ompA::Tn10) (25), and ME600 (17) have been described. For the lac-dependent expression of fira and skp, plasmids pSLF3 and pTRST7 were used (26). Plasmids pDMB (27) and p1710omaA were used for T7 promoter-dependent in vitro expression of ompA. Similarly, mtdA encoding mannitol permease of *E. coli* was subcloned into vector pKSM717 (28) yielding p17710omaB. In plasmid pTompA, ompA excision was facilitated by the PstI-EcoRI fragment and linker sequence added. The tetracycline resistance of *E. coli* strains MC4100 and MRE600 were prepared and extracted as described previously (27). Cross-linking—A 25 ml stock solution of DSS (Pierce) was diluted 10-fold into samples and incubated at 25 °C for 30 min. The reaction was stopped by adding Tris-HCl, pH 7.5, to 50 mM and incubating at 25 °C for 15 min.

**Construction of a Mutant Carrying an In-frame Deletion in skp—**The skp gene was excised from plasmid pGAH317 (13) by KpnI and BamHII yielding a fragment with about 300 base pairs on either side of skp. This fragment was ligated into pGEM3Z (Promega) cut with KpnI and HindIII. By using primers that hybridized to the insert upstream of nucleotide 12 and downstream of nucleotide 376 of skp (cf. Fig. 3A) an inverse PCR product of this plasmid was obtained that lacked 363 nucleotides of skp (5'-primer, CGG GAT CCC CAC TTT TTC ACA ATA AAC TCC; 3'-primer, CGC GGA TCC TCC GTT GGC AAC AGC CAG GAT ATC). Both primers contained BamHII linkers by which the PCR product was religated. These linker sequences introduced a Gly and a Ser between the remaining flanking regions of skp. The inverse PCR product of this plasmid was obtained that lacked 363 nucleotides of *skp* (5'-primer, CGG GAT CCC CAC TTT TTC ACA ATA AAC TCC; 3'-primer, CGC GGA TCC TCC GTT GGC AAC AGC CAG GAT ATC). Both primers contained BamHII linkers by which the PCR product was religated. These linker sequences introduced a Gly and a Ser between the remaining flanking regions of *skp*. The inverse PCR product of this plasmid was obtained that lacked 363 nucleotides of *skp* (5'-primer, CGG GAT CCC CAC TTT TTC ACA ATA AAC TCC; 3'-primer, CGC GGA TCC TCC GTT GGC AAC AGC CAG GAT ATC). Both primers contained BamHII linkers by which the PCR product was religated. These linker sequences introduced a Gly and a Ser between the remaining flanking regions of skp.

**RESULTS**

**Skp Interacts with Newly Translocated Non-native Outer Membrane Proteins—**To demonstrate interaction of Skp with its substrates, we synthesized outer membrane proteins by coupled transcription/translation and analyzed their translocation into inside-out plasma membrane vesicles (INV). As shown in Fig. 1A, the precursor of the outer membrane protein A (pOmpA) synthesized in vitro was partially converted to its signal sequence-free form (OmpA) by the cotranslational addition of INV (lane 3). Translocation into the lumen of INV is indicated by the accumulation of proteinase K-resistant material (lane 4), which was not obtained in the absence of INV (lane 2). Treatment with the membrane-permeable cross-linker DSS gave rise to an approximately 50-kDa cross-linking product (lane 5, asterisk), which was recognized by antisera raised against OmpA (lane 6) and Skp (lane 7). The 50-kDa material corresponds in size to one molecule of OmpA (35 kDa) and Skp (16 kDa) each. As expected for an OmpA-Skp complex formed after translocation of OmpA into INV, it was resistant to digestion with proteinase K as long as the vesicles were not disrupted with Triton X-100 (lanes 8 and 9). In addition, the intensity of the cross-link decreased when translocation was reduced by a lowered reaction temperature or by blockage of SecA with a nonhydrolyzable analogue of ATP (Fig. 1B, lanes 1–12; see lanes 2, 6, and 10 for the different extents of translocation). The specificity of the interaction of Skp with OmpA thus detected is demonstrated by the absence of this 50-kDa cross-linking product from assays containing INV of a skp null mutant (Fig. 1A, compare lanes 7 and 13). Similar results were obtained when the outer membrane protein LamB was synthesized in vitro (not shown).

These results suggest that the used INV, which had been washed with 1 M salt to remove contaminating Skp from the outside of the vesicles, retained Skp sequestered within their lumen. This portion of Skp must tightly be associated with the periplasmic side of the membrane since it is unpurified with the INV. Consequently, the interaction between Skp and newly translocated outer membrane proteins demonstrated to occur in these vesicles must reflect an event taking place in close proximity to the outer leaflet of the plasma membrane.

Proteins translocated across the plasma membrane before

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Fig. 1. Newly translocated OmpA binds to Skp on the periplasmic side of the membrane. A. pOmpA was synthesized by an S-135 mutant in the presence of INV as indicated. Wild type INV had been freed of Skp on the cytosolic side by high salt washing (K INV wt). For cross-linking, samples were incubated post-translationally with DSS. Samples were either directly precipitated with trichloroacetic acid or first treated with proteinase K or immunoprecipitated with antibodies against OmpA and Skp (OmpA, αSkp). Shown are radioactively labeled proteins separated by SDS-PAGE and visualized by phosphorimaging. The asterisk marks the cross-link between OmpA and Skp. The electrophoretic mobility of marker proteins is indicated on the right in kilodaltons. B, same as described for A except that INV were added post-translationally and incubated at the indicated temperatures either in the presence of 2.5 mM ATP or 25 mM AMP-PNP.

Fig. 2. Skp efficiently and specifically interacts with nascent chains of an outer membrane protein after release from the ribosome. A, in vitro synthesis of a 125-amino acid-long NH2-terminal fragment of pOmpA (pOmpAβ5). RANCs were isolated by sucrose gradient centrifugation and subsequently incubated with purified SecA and Skp (approximately 1 µM each) prior to cross-linking with DSS where indicated. Some full-length pOmpA cosynthesized with the nascent chains was usually also found in the ribosomal pellet. Cross-linking products of pOmpAβ5 with SecA (asterisk) and Skp (double arrows) are indicated. B, same as described for A except that RANCs were incubated with a total cytosolic extract containing Skp. In addition, a 189-amino acid-long nascent chain of mannitol permease (MtlA189) was analyzed. Where indicated, samples had been treated with 0.8 mM puromycin prior to cross-linking.
paralleled by an altered permeability of the outer membrane. ∆skp cells exhibited a higher sensitivity than wild type cells toward antibiotics that cross the lipids of the outer membrane, such as rifampicin, vancomycin, and novobiocin (Fig. 4). The increase in sensitivity was more pronounced when cells were grown on minimal medium and at lower temperature (20 °C). It was reversed by expressing Skp in the ∆skp mutant from an extrachromosomal copy.

Because skp is cotranscribed with firA, whose gene product is involved in lipid A biosynthesis, we compared the LPS content of the ∆skp mutant to that of wild type cells (data not shown). In contrast to the described LPS deficiency of the firA200 mutant (39), deletion of skp resulted in an about 2-fold higher amount of LPS, which became evident only when cells were grown on minimal medium.

Collectively, the phenotypic features of the ∆skp mutant are rather moderate and are more pronounced upon growth in minimal medium and at low temperature. To rule out the possibility that the lack of a strong phenotype of the ∆skp mutant was caused by the acquisition of a second site suppressor mutation the skp deletion was moved by P1 transduction into wild type cells. No change in phenotype was obtained (not shown).

It was conceivable that in whole cells, a lack of Skp remained cryptic because of compensatory periplasmic proteins. We therefore employed spheroplasts as a periplasm-free semi-in vitro model. Wild type and mutant cells each transformed with a plasmid carrying ompA under tac promoter control were converted to spheroplasts which were then induced for OmpA synthesis and labeled with [35S]methionine/cysteine (Fig. 5A). Spheroplasts (S) were separated from proteins secreted during pulse labeling (P, periplasmic fraction) either by centrifugation at 16,000 × g (lanes 1 and 2, 7, and 8) or by low speed centrifugation at 6000 × g. In the latter case, the supernatant was subsequently resolved into soluble (sP) and aggregated (aP) periplasmic material by centrifugation at 16,000 × g (lanes 5 and 6, 11, and 12). Fractions thus obtained were displayed by SDS-PAGE and phosphorimaging. Upon induction with IPTG, OmpA was the major labeled protein in both spheroplasts and

Fig. 3. Characterization of a skp null mutant. A depicts the organization of the E. coli chromosome in the 4-min region. Numbers reflect nucleotides beginning with the start codon of skp. The deletion is indicated by the hatched area. Arrows represent the two primers used for the PCR shown in B. B, PCR products separated on agarose gel electrophoresis and stained with ethidium bromide. The right lane contains DNA standards of the indicated sizes in nucleotides. The expected length of the PCR product derived from wild type DNA was 500, that from the ∆skp mutant 136 plus an additional 6 nucleotides of a BamHI cleavage site introduced for ligation of the two skp flanking regions. The two other panels are immunoblots of total cells decorated with polyclonal antibodies directed against Skp and FirA. FirA was also identified by overproduction from plasmid pSLF3 (p firA).
the periplasm. In these overproducing conditions, the sphero-
plasts even retained some precursor of OmpA (pOmpA) as indicated by its resistance toward proteinase K (lanes 3 and 9).

Whereas 68% of newly synthesized mature OmpA was secreted from wild type spheroplasts into the medium (lanes 1 and 2), only 13% was released from the Δskp mutant spheroplasts (lanes 7 and 8). Despite the fact that 87% remained associated with the mutant spheroplasts, this OmpA material had been translocated across the plasma membrane, because it was completely digested by proteinase K (lane 9). Cosedimentation of OmpA with the mutant spheroplasts was not due to aggregates of OmpA formed in the absence of Skp because the same high amount of OmpA was pelletable upon low speed centrifugation (compare lanes 7 and 10). Rather, OmpA was not released from the mutant spheroplasts because of the absence of Skp. Accordingly, induction of Skp synthesis from plasmid pTRS7 during pulse labeling of Δskp mutant spheroplasts restored secretion of OmpA (Fig. 5B). Attempts to achieve release by adding purified Skp to spheroplasts were, however, unsuccessful (not shown). Possibly, the active conformation of Skp requires its biosynthetic association with the plasma membrane. These results clearly demonstrate an involvement of Skp in the acquirement of a soluble periplasmic conformation of newly translocated outer membrane proteins. A similar experiment was performed with cells expressing either a wild type copy of maltose binding protein or a mutant allele lacking the authentic C terminus (Fig. 5C, MalE and MalE*). With both species, the distribution between spheroplast-associated and soluble, periplasmic material was identical for wild type and Δskp mutant cells, indicating a specificity of Skp for outer membrane proteins as suggested previously (17, 20).

Skp Is a Chaperone Maintaining the Soluble State of Periplasmic Proteins—DegP is a periplasmic protease whose
function is to degrade misfolded proteins in the periplasm (Ref. 40 and references therein). It was conceivable that the effect of an inactivation of Skp was obscured by the proteolytic activity of DegP. We therefore combined the skp deletion with a degP allele, which had been inactivated by Tn10 insertion (24), by transducing tetracycline resistance and deletion of degP into the Dskp mutant using P1 (Fig. 6A). Two double mutants independently obtained (DD1 and DD2) grew normally at 30 °C (Fig. 6B). In contrast to both single mutants, Dskp and degP::tet, double mutant cells, however, stopped growing in Luria broth medium about 3 h after shifting them to 37 °C (Fig. 6C). In other words, the near normal growth behavior of cells lacking an active DegP protease is strictly dependent on an active Skp and vice versa, a lack of Skp is tolerated as long as DegP is functional.

The Δskp,degP::tet double mutant finally allowed us to directly demonstrate a chaperone activity of Skp. In Fig. 7, wild type (lanes 1), Δskp single mutant (lanes 2), degP::tet single mutant (lanes 3), and Δskp,degP::tet double mutant (lanes 4 and 5) cells were converted to spheroplasts and periplasmic fractions were obtained. These were subsequently divided by high speed centrifugation into soluble and pelletable, i.e. aggregated PPPs. When grown at the permissive temperature of 30 °C, all strains exhibited the same pattern of soluble PPPs (A) as revealed by SDS-PAGE and staining with Coomassie Blue. The same held true for aggregated PPPs with the exception that this fraction was consistently found diminished in the degP::tet single mutant (B). In contrast, when cells had been shifted to 37 °C for 3 h before preparing PPPs, significantly more aggregated proteins of all sizes accumulated specifically in the periplasm of the double mutant (D). This is corroborated by a Western blot of these fractions using anti-OmpA antibodies (inset of D). Aggregation was clearly a phenotypic feature of the double mutant, because it developed gradually during growth at 37 °C (G). The periplasm of the double mutant also contained more soluble proteins (C), this phenomenon too being dependent on growth at 37 °C (F). This, however, was not due to an enhanced fragility of spheroplasts obtained from the double mutant as shown by the almost complete absence of a cytosolic protein (P48 or Ffh) from all periplasmic protein fractions prepared (E), nor was it caused by an increased synthesis of outer membrane proteins (not shown). Thus, the absence of Skp leads to an accumulation of denatured proteins in the periplasm if cells lack DegP to degrade them. These results strongly support the view that Skp functions as a chaperone maintaining periplasmic intermediates of outer membrane proteins in a soluble state.

DISCUSSION

Folding of Outer Membrane Proteins in the Periplasm—After their translocation across the plasma membrane, outer membrane proteins of Gram-negative bacteria sequentially pass through several intermediate stages characterized as unfolded monomers, folded monomers, metastable trimers, and stable trimers (summarized in Ref. 4). The data now available suggest that Skp participates as molecular chaperone early in these folding events, because first, Skp physically associates with the plasma membrane, and second, it recognizes an NH2-terminal part of OmpA during or immediately after folding is initiated.

Skp Is Firmly Associated with the Plasma Membrane—Puri-
fied Skp penetrates into phospholipid bilayers and acquires a partial resistance toward proteolysis by the addition of phospholipids (20). Here, we demonstrate that during purification involving sucrose gradient centrifugation, plasma membrane vesicles retain Skp bound on their luminal membrane leaflet. These findings suggest that Skp is tightly associated with the periplasmic side of the plasma membrane also in vivo. DegP also behaves like a peripheral membrane protein, which cannot be released by osmotic shock (40) and which interacts with phospholipids (41), whereas the PPlase SurA, which is specifically involved in the conversion of unfolded to folded monomers of outer membrane proteins (8), is released from osmotically shocked cells (6).

Skp Binds to Non-native Outer Membrane Proteins—It is well known that many periplasmic proteins are synthesized in an unfolded state, and some of them have the potential to cause perturbations in the outer membrane if not correctly folded. The in vivo association of Skp with periplasmic proteins suggests that this protein could serve as a molecular chaperone.

**Skp Is Required for the Solubility of Outer Membrane Proteins in the Periplasm**—The latter notion is also consistent with the involvement of Skp in the release of newly synthesized outer membrane proteins from the plasma membrane into the periplasm as demonstrated here. Because a lack of Skp did not cause a reduction in the steady-state level of periplasmic proteins, its influence on the release of newly translocated OmpA encompassing the first five β-strands is sufficient. On the other hand, Skp does not bind to the COOH-terminal part of OmpA (19). These findings suggest that Skp recognizes its substrates soon after or even during their appearance in the periplasm.

**Toward an Elucidation of Skp’s Chaperone Activity**—In summary, the function of Skp as periplasmic folding catalyst is indicated by several criteria: (i) a lack of Skp enhances the σE-response and skp itself appears to be a member of the σE-regulon; (ii) a lack of Skp is associated with fewer outer membrane proteins and an elevated outer membrane permeability much like described for other periplasmic chaperones; (iii) Skp is required for the efficient release of newly translocated outer membrane proteins from the plasma membrane; (iv) Skp is needed to maintain periplasmic proteins in solution; (v) Skp binds to non-native outer membrane proteins, a tendency that might be facilitated by its association with the plasma membrane. These findings suggest a time point of interaction between Skp and outer membrane proteins early during their folding cascade and soon after translocation into the periplasm. It has been hypothesized that Skp, due to its genomic localization in close proximity to LPS biosynthetic genes and its especially basic nature, binds LPS species and serves as an exchange factor removing LPS molecules from folded monomers of outer membrane proteins (7). However, Skp does not appear to bind to folded monomers (20) but as shown here rather to early folding stages like the unfolded monomer. This notion would be in full agreement with the fact that like Skp, early folding intermediates of outer membrane proteins are still membrane-associated (46).

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