The Periplasmic Chaperone PpiD Interacts with Secretory Proteins Exiting from the SecYEG Translocon†

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ABSTRACT: The Sec translocon of *Escherichia coli* mediates the export of numerous secretory and membrane proteins. To dissect the passage of an exported protein across the Sec translocon into consecutive steps, we generated in vitro translocation intermediates of a polypeptide chain, which by its N-terminus is anchored in the membrane and by its C-terminus tethered to the ribosome. We find that in this situation, the motor protein SecA propagates translocation of a peptide loop across SecYEG prior to the removal of ribosomes. Upon SecA-driven exit from the translocon, this loop is brought into the immediate vicinity of the membrane-anchored, periplasmic chaperone PpiD. Consistent with a coupling between translocation across the SecYEG translocon and folding by periplasmic chaperones, a lack of PpiD retards the release of a translocating outer membrane protein into the periplasm.

The inner and outer membranes of Gram-negative bacteria host a multitude of different transport machineries that export proteins to the cell envelope and to the extracellular milieu. Most exported proteins leave the cytoplasm as unfolded polypeptides through the Sec translocon, the core of which consists of the evolutionarily conserved SecY, SecE, and SecG subunits. Exported proteins are targeted to the Sec translocon either as ribosome-associated nascent chains (RNCs)[1] by the bacterial signal recognition particle (SRP) and its receptor FtsY or as full-length translation products mediated by the export-specific chaperone SecB in concert with ATPase SecA. In accordance with the X-ray structure determined for the Sec translocon of *Methanococcus jannaschii*, lateral opening of the Sec channel occurs in response to signal (anchor) sequences intercalating between two helices of the SecY subunit (*1*), whereas its vertical gating is achieved by the removal of a molecular plug that follows the insertion of the signal sequence (*2–4*).

To dissect the passage of an exported protein across the Sec translocon into consecutive steps, we made use of inner membrane proteins with long periplasmic domains. These proteins are first recognized by SRP/FtsY, leading to the cotranslational anchorage of their N-terminal transmembrane domains in the lipid bilayer. Subsequently, SecA is required for translocation of hydrophilic domains distal to the membrane anchors across the SecYEG translocon (*5–10*). By employing RNCs of such a protein, we could generate translocation intermediates that were fixed at either end, while spanning the SecYEG channel. With this approach, we show here that SecA promotes a partial translocation across the SecYEG translocon of nascent polypeptides, although they still carry the ribosome at their C-termini. This in situ action of SecA opens the transmembrane channel for loops of the translocating polypeptide chain that upon exiting from the SecYEG translocon interact with the membrane-anchored periplasmic chaperone PpiD. Interaction with PpiD kinetically favors the release of a newly translocated outer membrane protein from the membrane.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. The components of the reconstituted transcription−translation system were prepared from *Escherichia coli* strain MC4100 (*11*). INVs were prepared according to the method in ref *12* from wild-type strains MC4100 and BL21(DE3) pLysS (*13*), *secY* mutant strain TY1 (*ompT::kan, secY205*) (*14*), *secG* null mutants KN553 (Δ*uncB-C::Tn10* Δ*secG::kan*) and EK414 (MC4100 *ara*$^+$ Δ*secG::kan*) (*15*), and *ppiD* null mutant CD247 (*ppiD::kan*).

Plasmid pMomp2 (*7*) was used for the in vitro expression of Momp2 and served as a template for the introduction of TAG stop codons into positions 55, 121, and 165 by the one-step PCR method described in ref *16* using the following pairs of forward and reverse primers each: 5′-GGT GCT AAA TAG GGC TGG TCC-3′ and 5′-GGA CCA GCC CTA TTT AGC ACC-3′, 5′-GGG CGT TCA ATA GAC CGC

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[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; INVs, inverted vesicles; PK, proteinase K; RNC, ribosome-associated nascent chain; SRP, signal recognition particle; TF, trigger factor; TM, transmembrane domain; Tmd-Phe, L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine.

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TAA ACT GGG-3′ and 5′-CCC AGT TTA GCG GTC TAT TGA ACG CCC-3′, and 5′-GGC GTT TCT CCG GTC TAG GCT GGC GGT GTT GAG-3′ and 5′-CTC AAC ACC GCC AGC CTA GAC CGG AGA AAC GCC-3′. To construct Momp2 derivatives carrying TAG codons at positions 65, 82, and 93, derivatives of plasmid p717OmpA (*16*) encoding the corresponding stop codon mutants in ompA at positions 45, 62, and 73 (*16*) were cut with SpeI and EcoRI. The isolated fragments encoding most of the ompA gene were ligated onto isolated SpeI−EcoRI fragments of p717MtlA-Spe2 (*7*) encoding the N-terminus of MtlA to create Momp2 derivatives in the same manner as detailed previously (*7*). Plasmid pTompA (*17*) was used for the overexpression of OmpA in spheroplasts. All constructs that were made were verified by DNA sequence analysis.

In Vitro Synthesis. The composition of the reconstituted *E. coli* transcription−translation system and the purification of its components have been described previously (*16*). To generate elongation-arrested nascent chains, mRNAs were truncated by the addition of complementary oligodeoxynucleotides and cleavage by RNaseH (*12*). The oligodeoxynucleotide used for the synthesis of Momp2−146 has been described previously (*7*). Nascent chains of Momp2−220 and Momp2−330 were generated by 5′-CGG AGC TGG AGC CGG AGC AAC-3′ and 5′-CTG TTT CAC GTT GTC ACA GGT-3′, respectively, at concentrations of 3−4 μg/25 μL of reaction mixture.

Sodium azide was added from a 1 M stock solution prepared in H_2O. DCCD was dissolved in tetrahydrofuran and diluted into DMSO (dimethyl sulfoxide) as described previously (*18*). Puromycin was added from a neutralized 11 mM stock solution to a final concentration of 0.8 mM and incubated with the samples for 15 min at 20 °C. Incorporation of the site-specific cross-linker Tmd-Phe and immunoprecipitation using 6-fold scaled-up reactions were performed as described previously (*16, 19*). Translation products were resolved by SDS−PAGE using 17% linear acrylamide gels and 7 to 17% gradient gels for the resolution of cross-linked products. Radiolabeled proteins were visualized by phosphorimaging using a Molecular Dynamics PhosphorImager.

Miscellaneous. Preparation of spheroplasts, induction for OmpA synthesis, and pulse labeling were performed as described previously (*17*). The truncated form of *ppiD* encoding amino acids 36−623 was cloned into pACYCDuet-1, thereby providing the protein N-terminally with a six-His tag. The construct was overexpressed by incubating BL21(DE3) cells, grown to an OD of 2, for 2 h with 0.5 mM IPTG. Cells were harvested and broken in a French pressure cell. After centrifugation at 15500 rpm in an SS34 rotor, the supernatant was loaded onto a Talon matrix and the bound material was eluted with 50 mM imidazole. Polyclonal antibodies directed against TF, SecY, and PpiD were raised in rabbits. Western blots were developed using an HPR-conjugated goat anti-rabbit antibody, followed by enhanced chemiluminescence (Amersham).

RESULTS

SecA-Dependent Translocation Intermediates of a Membrane-Anchored Secretory Protein. To anchor the N-terminus of a secretory protein in the cytoplasmic membrane of *E. coli*, the model protein Momp2 in which the signal sequence of the outer membrane protein A (OmpA) is replaced with 47 amino acids comprising the cytosolic N-terminus and the first signal anchor sequence of the inner membrane protein mannitol permease (MtlA) was constructed (cf. diagram in Figure 1). An *E. coli* cell free transcription−translation system was used to synthesize 330-amino acid RNCs of Momp2 (Figure 1, lane 1, white arrowhead). As expected, these RNCs could be completely digested with proteinase K (PK) except for a prominent 4 kDa fragment (lane 2, arrow) which disappeared only when ribosomes were removed (lanes 4−6). This fragment most likely represents the stretch of 30 amino acids that span the ribosomal exit tunnel. When the RNCs were incubated with inside-out cytoplasmic membrane vesicles [inverted vesicles (INVs)], larger protease-protected species of Momp2−330 were obtained (lanes 3−6). These vesicles having both SRP/FtsY and SecA bound should allow targeting and insertion of the transmembrane domain (TM) of Momp2 as well as the

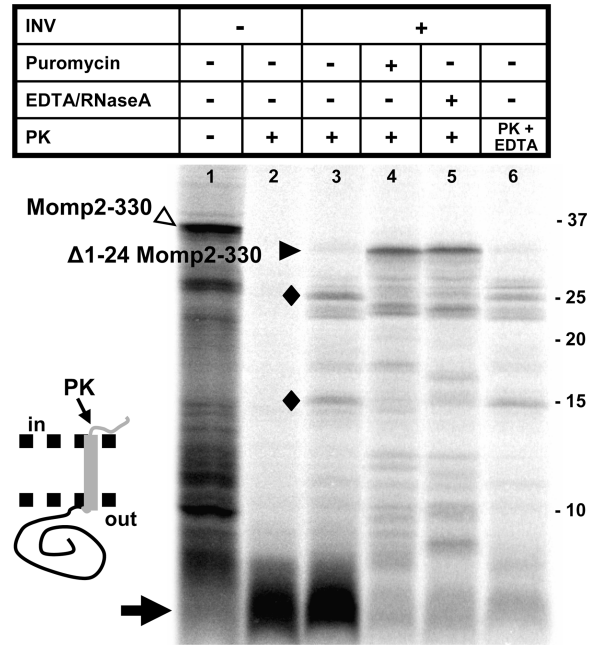

FIGURE 1: Partial translocation of a ribosome-associated nascent secretory protein that is anchored in the lipid bilayer of membrane vesicles by virtue of an N-terminal signal anchor sequence. RNCs (ribosome-associated nascent chains) of Momp2, 330 amino acids in length, were synthesized for 25 min at 37 °C by a reconstituted transcription−translation system prepared from *E. coli*. Radiolabeled translation products were separated by SDS−PAGE and visualized by phosphorimaging. The white arrowhead marks the position of the full size nascent chains. The molecular masses of marker proteins are given in kilodaltons. Where indicated, nascent chains were post-translationally incubated for 15 min at 20 °C with membrane vesicles (INVs) and subsequently with puromycin or as otherwise indicated. Samples were precipitated either directly with 5% TCA or only after digestion with 0.5 mg/mL proteinase K (PK) for 20 min at 25 °C. The black arrow marks a ribosome-protected 4 kDa fragment. Diamonds label unique PK-resistant fragments of RNCs obtained in the simultaneous presence of INVs and ribosomes. Full-size translocation products of Momp2-bound RNCs shortened through the action of PK at their cytosolic N-terminus (Δ1−24 Momp2−330) were obtained only after dissociation of the ribosomes. The diagram depicts membrane-integrated Momp2 consisting of the N-terminal part of MtlA (gray), including the signal anchor sequence (bold line) and the OmpA moiety (black line). Dotted lines represent the phospholipid bilayer. The presumed PK cleavage site is indicated.

translocation of the OmpA moiety (*7, 9*). In fact when after the incubation of Momp2−330 RNCs with INVs, the ribosomes were released by puromycin or EDTA and RNaseA, almost the entire Momp2−330 polypeptide became protected against PK (lanes 4 and 5, Δ1−24 Momp2−330). The PK-mediated reduction in size stems from the cleavage of the cytosolic N-terminus preceding the TM of Momp2, which would be the only part of the protein that remained accessible to protease after transport into the vesicles (Figure 1, cartoon). These results therefore indicate that translocation of the OmpA moiety of Momp2−330 into the lumen of INVs occurred following release of the ribosome.

Because the Δ1−24 fragment is a read-out of complete translocation of Momp2, it was not observed when PK treatment was performed without prior removal of the ribosomes (compare lanes 3 and 4). Instead, treatment of Momp2−330 RNCs with PK in the absence of puromycin gave rise to two prominent protease-protected fragments of approximately 16 and 26 kDa (lane 3, diamonds). These fragments were absent when ribosomes had been removed before PK was added (compare lane 3 with lanes 4 and 5) but were retained if destabilization of the ribosomes by EDTA was performed simultaneously with the PK treatment (lane 6).

Hence, the generation of the 16 and 26 kDa fragments of Momp2−330 RNCs by PK required the presence of INVs and ribosomes, suggesting that they are partially translocated portions of the OmpA moiety shielded against the protease by the membrane and the ribosomes. This assumption was verified by the experiments shown in Figure 2A. Preformed RNCs of Momp2−330 were again incubated with INVs. Samples were now withdrawn after different times of incubation, and reactions were stopped on ice and mixtures treated with PK. The 16 and 26 kDa PK-resistant fragments appeared with time and accumulated within 15 min (lanes 4−6, diamonds). Like in Figure 1, they were chased to the Δ1−24 full-size translocation product of Momp2−330 by removal of the ribosome (lane 7, black arrowhead). Formation of the Δ1−24 translocation product was, however, largely prevented, if the SecA inhibitor sodium azide was added (lanes 12 and 17). Noticeably, sodium azide also interfered with the accumulation of the 16 and 26 kDa PK-resistant fragments when added during the incubation of RNCs with INVs (cf. lanes 9−11) or abolished their formation when present from the beginning (lanes 14−16). Likewise, virtually no 16 and 26 kDa PK-resistant fragments were detected when INVs carrying the *secY205* mutation were used (lanes 19−21). This mutation impairs the SecA-dependent translocation by interfering with a functional interaction between SecA and SecY (*14*). The results shown in Figure 2A therefore suggest that the distinct PK-resistant species of INV-bound Momp2−330 RNCs in fact represent translocation intermediates that are formed only in the presence of active SecA.

According to previous reports (*7, 9, 10*), translocation of the entire hydrophilic domains distal of type II TMs requires SecA and release of the ribosome. The novel finding here is that SecA seems to be able to functionally interact even with a nascent chain that is still attached to the ribosome and in this way mediates partial translocation across SecYEG. Consistent with this notion, INVs prepared from a *secG* deletion mutant as well as dissipation of the H^+-motive force

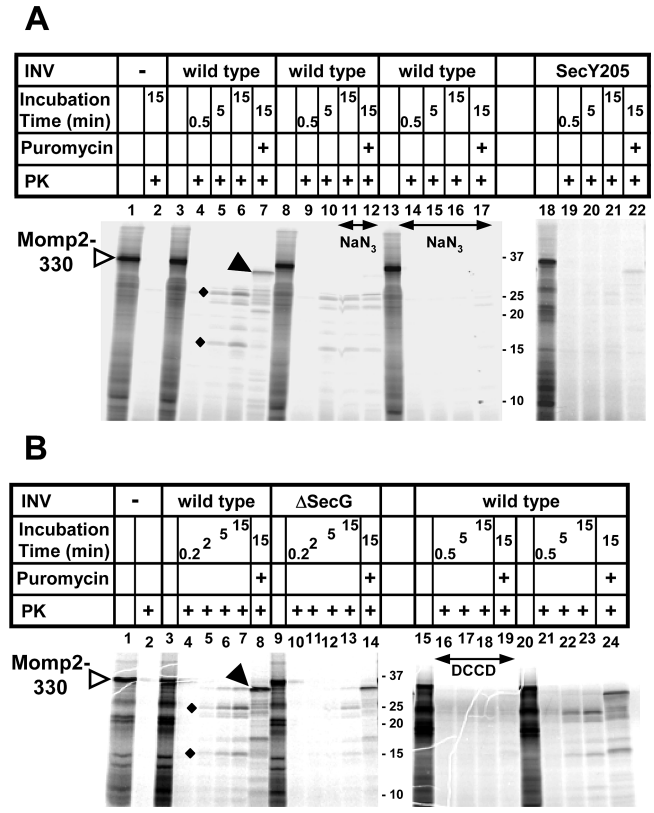

FIGURE 2: Active SecA is required to partially translocate Momp2 while it is still tethered to the ribosome. (A) Momp2 nascent chains 330 amino acids in length were synthesized as described in the legend of Figure 1, except that during incubation with INVs samples were withdrawn at the indicated times. NaN_3 (final concentration of 20 mM) was added either after incubation for 5 min or from the beginning. INVs were prepared from wild-type *E. coli* or the *secY205* mutant. Azide and the *secY205* mutation block formation of PK-resistant species of Momp2−330. (B) As in panel A, wild-type INVs are compared with those derived from *secG* deletion strain EK414, in which the simultaneous lack of a functional *unc* operon reinforces the *secG* phenotype; similar results were obtained with INV prepared from a single *secG* deletion mutant (not shown). The inhibitor of the F_1F_0-ATPase, DCCD (0.5 mM dissolved in DMSO), was added to dissipate the H^+-motive force. The control sample (shown in lanes 21−24) received an equivalent volume of solvent. The absence of SecG and dissipation of the H^+-motive force interfere with the accumulation of the unique PK-resistant fragments of Momp2−330.

of wild-type INVs by DCCD also severely affected the appearance of the translocation intermediates (Figure 2B). Collectively, these results indicate that partial translocation of an otherwise membrane-anchored secretory protein across the Sec translocon is promoted by SecA while the ribosome is still attached at the C-terminus of the polypeptide chain.

SecA-Dependent Insertion of a Membrane-Anchored Nascent Secretory Protein into SecY. Next we employed site-specific cross-linking by introducing a photoreactive cross-linker into the OmpA domain of Momp2 at the sites indicated in Figure 3A. This was achieved by engineering stop codons into the *momp2* DNA and suppressing them in vitro by a suppressor tRNA charged with the photoprobe L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine (Tmd-Phe).

Figure 3B depicts the results obtained with Tmd-Phe placed at positions 93, 82, and 65 of a 146-residue nascent chain of Momp2. In the absence of membranes, UV irradiation of these RNCs yielded distinct adducts of 75 kDa (asterisk), whereas in the presence of wild-type (wt) INVs,

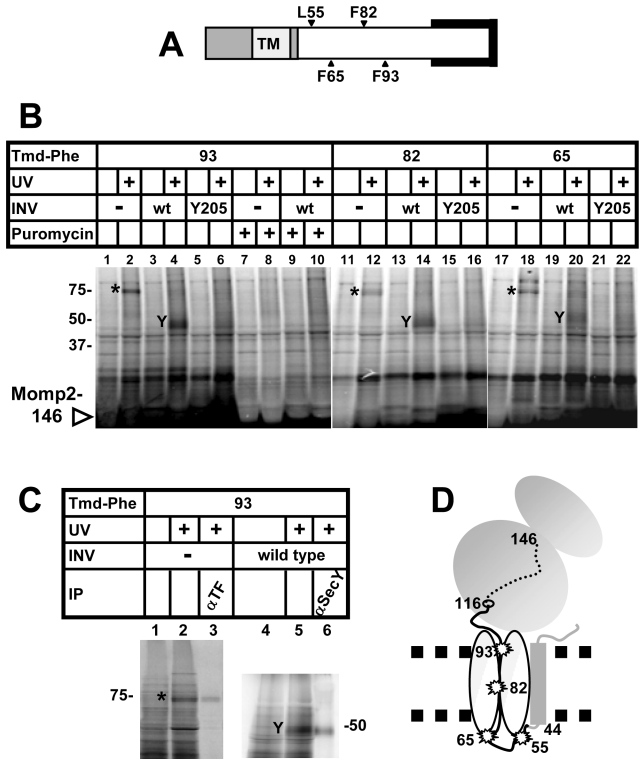

FIGURE 3: Even short Momp2 nascent chains are driven into the SecY channel by SecA despite their linkage to ribosomes. (A) Schematic representation of Momp2−146. Gray boxes represent the 47-residue MtlA-derived sequence with the predicted TM which extends from amino acid 25 to 44 (*39*). The OmpA moiety of Momp2 is depicted in white, and the positions and amino acids at which stop codons had been introduced to allow for the incorporation of the photoreactive cross-linker Tmd-Phe are marked. Numbering starts with the most N-terminal amino acid of Momp2. The black clamp illustrates the ribosomal exit tunnel. (B) Momp2−146 RNCs harboring stop codons at the three indicated positions were synthesized in vitro by means of $tRNA^{sup}$ charged with Tmd-Phe. Where indicated, INVs derived from a wild-type strain (wt) or the *secY205* mutant (Y205) were added 15 min after synthesis had started, and incubation was continued for an additional 15 min at 37 °C. Irradiation of samples with UV light at 365 nm resulted in the appearance of unique adducts in the absence (asterisk) and presence (Y) of wild-type membrane vesicles. (C) As in panel B, with analysis of Momp2−146 RNCs harboring the stop codon at position 93. Obtained cross-links were immunoprecipitated (IP) by antisera directed against trigger factor (αTF) and SecY. (D) Diagram depicting a Momp2−146 RNC engaged with the Sec translocon. Integration into the lipid bilayer (bold dotted lines) has occurred via the signal anchor sequence (bold gray line ending with amino acid 44). The dotted line represents the stretch of approximately 30 amino acids burried in the exit tunnel of the large ribosomal subunit, while the solid black line illustrates the OmpA moiety that has been partially translocated into the Sec translocon (upright ellipses). The flash symbols mark the positions of the Momp2−146 chain that were found cross-linked to SecY. The SecA required to proceed up to this step of translocation is not shown.

50 kDa adducts were obtained (Y). These cross-linking partners of Momp2−146 were identified by immunoprecipitation as trigger factor in the absence of INVs (Figure 3C, lanes 1−3) and as SecY in the presence of INVs (Figure 3C, lanes 4−6). Cross-linking to SecY (and to trigger factor) occurred only when the ribosome was still attached to the nascent chains (Figure 3B, compare lane 4 to lane 10). The signal intensity of the cross-links between Momp2−146 and SecY was highest for the photoprobe in position 93 and gradually decreased when Tmd-Phe was moved to residue 65 (Figure 3B, compare lanes 4, 14, and 20) and further to position 55 (data not shown). These results suggest the situation depicted in Figure 3D, where a stretch of approximately 40 amino acids distal of the TM (from residue 55 to 93) of Momp2−146 was brought into differently close contacts with the Sec translocon if the ribosome prevented complete translocation. Many fewer SecY adducts were formed when wild-type INVs were replaced with vesicles prepared from the *secY205* mutant (Figure 3B; compare lanes 4 and 6, 14 and 16, and 20 and 22). This was not due to a decreased level of SecY in the mutant vesicles (data not shown) and therefore rather resulted from the failure of SecA to open the SecY205 mutant protein (*14*) for a partial translocation of Momp2−146 up to residue 93. Hence, SecA functionally interacts also with the shorter Momp2−146 nascent chains and enables a partial translocation across wild-type SecYEG.

Sequestration of Nascent Translocating Domains by PpiD. If the contacts demonstrated above between SecY and the area from residue 55 to 93 of Momp2−146 indeed reflect an intermediate stage in translocation caused by the arresting ribosome, how would the molecular neighborhood of the same residues change, once translocation was allowed to proceed further, such as in Momp2−220 and Momp2−330? Whereas the photoprobe in position 93 of 146-amino acid Momp2 nascent chains yielded the clear cross-link to SecY (Figure 4A, lanes 1−3), anti-SecY antibodies no longer recognized a specific adduct of Tmd-Phe in position 93, when Momp2 nascent chains were 330 amino acids long (Figure 4A, lanes 4−6). Instead, UV irradiation yielded a major adduct that was 70 kDa larger than Momp2−330 (lane 5, white arrow). A distinct cross-link to a 70 kDa component was also observed for residue 93 in Momp2 RNCs 220 amino acids in length (Figure 4B, lane 2, white arrow). During our search for potential cross-linking partners of 70 kDa, we found that this adduct of Momp2−330 and Momp2−220 cross-reacted with an antiserum raised against the membrane-anchored, periplasmic chaperone PpiD (Figure 4B, lanes 2 and 3). The identity of the 70 kDa cross-linking partner was confirmed by the absence of the adduct from INVs of a *ppiD* knockout strain of *E. coli* (Figure 4B, lanes 4−6). The contact between Momp2−220 RNCs and PpiD was strongly reduced with INVs from the *secY205* mutant (lanes 7 and 8) and from a mutant both lacking SecG and harboring a defective F_1F_0-ATPase (*unc*) operon (lane 9). These results therefore indicate that longer Momp2 nascent chains reach out to the periplasmic PpiD when driven by SecA, SecG, and the H^+-motive force.

The contact between the OmpA moiety of Momp2 and PpiD was lost after treatment with puromycin (Figure 4B, lanes 10 and 11), indicating that it was maintained only *in statu translocandi*. The loss of Momp2−PpiD cross-links upon completion of translocation implies that the periplasmic chaperone PpiD interacts only with an unfolded Momp2 chain. We therefore examined the area in which interaction of Momp2−220 with PpiD could be demonstrated (Figure 4C). Under the experimental conditions that were employed, PpiD gave the strongest adducts with an area extending from residue 65 to 93. Amino acid 121 was still in the proximity of PpiD, whereas residue 55 yielded only marginal cross-links. The finding that up to 60 amino acids come into contact with PpiD is consistent with the chaperone recognizing the

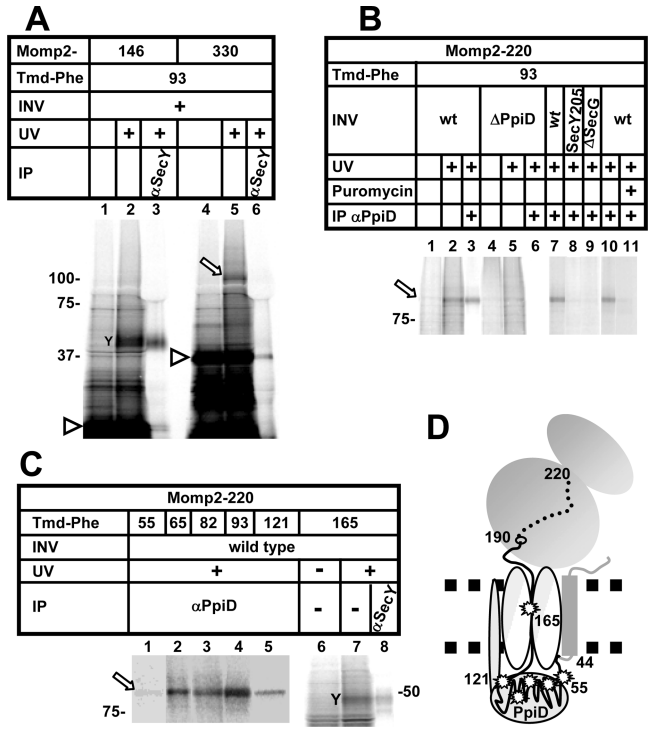

FIGURE 4: SecA-dependent translocation intermediates looping out of the Sec translocon are sequestered by the periplasmic chaperone PpiD. (A) Momp2−146 and Momp2−330 RNCs each harboring Tmd-Phe at position 93 were synthesized and UV-irradiated for cross-linking as described in the legend of Figure 3. Tmd-Phe present in the longer nascent chain does not cross-link to SecY but to a 70 kDa membrane protein (downward-pointing white arrow). (B) Tmd-Phe at position 93 of Momp2−220 RNCs also cross-links to a 70 kDa membrane protein, which is recognized by antibodies directed against PpiD. The PpiD adduct disappears when INVs from a *ppiD* deletion strain, the *secY205* mutant, and the *secG* deletion strain KN553 are used or after removal of the ribosomes by puromycin. (C) As in panel B, except that Momp2−220 RNCs carrying Tmd-Phe at the various positions indicated were cross-linked to PpiD or SecY. (D) Schematic representation of the results of panels B and C. PpiD is depicted as a periplasmic protein with a transmembrane anchor. Flash symbols mark the cross-links with PpiD and SecY. The SecA required to proceed up to this step of translocation is not shown.

non-native structure, in which Momp2 leaves the Sec translocon. Because of its membrane anchor, PpiD would be predestined to be one of the first chaperones waiting for substrates such as the OmpA moiety of Momp2 at the exit of the Sec translocon. When the photoprobe was introduced in a position distal to residue 121, UV irradiation of membrane-inserting Momp2−220 RNCs yielded adducts again with SecY (exemplified for position 165 in Figure 4C, lanes 6−8). Figure 4D depicts this intermediary stage of translocation, in which a Momp2 nascent chain on its SecA-driven passage across the Sec translocon forms a non-native loop structure at the translocon exit site which likely needs protection by the periplasmic chaperone PpiD.

Involvement of PpiD in the Release of a Secreted Protein into the Periplasm. The findings depicted in Figure 4 are consistent with a direct transfer of Momp2 RNCs from the SecYEG translocon to PpiD. We therefore asked whether PpiD was functionally involved in the translocation of proteins across the cytoplasmic membrane of *E. coli*. To this end, we made use of spheroplasts which due to the lack of a contiguous outer membrane release newly synthesized periplasmic and outer membrane proteins into the medium. Since Momp2 even after leaving the Sec translocon would remain membrane-anchored, these experiments were performed with the outer membrane protein OmpA.

Cells transformed with an OmpA-encoding plasmid were converted to spheroplasts, induced for the synthesis of OmpA, and pulse-labeled with [^{35}S]methionine/[^{35}S]cysteine. Proteins released during the pulse period were separated from spheroplasts by centrifugation. The major protein secreted under these experimental conditions was the size of the signal sequence-less OmpA (Figure 5A, lane 3). Indeed, it was recognized by anti-OmpA antibodies (lane 2) together with a somewhat smaller secreted species (asterisk), which probably represents an unusually folded species or a degradation product. Spheroplasts, on the other hand, contained substantial amounts of the precursor pOmpA (lanes 1 and 4) in addition to nonreleased mature OmpA. As expected, the precursor accumulating within the spheroplasts under these experimental conditions was totally resistant to PK (lane 5), whereas spheroplast-associated mature OmpA was not, indicating that this nonreleased material nevertheless had been translocated across the cytoplasmic membrane. Protease protection of the precursor pOmpA and of minor labeled species reflects the stability of the prepared spheroplasts, whereas the predominant protease accessibility of the spheroplast-associated mature OmpA indicates little contamination with intact cells (lanes 4 and 5).

If interaction with PpiD were to have an influence on the release of mature OmpA from the Sec translocon, accumulation of OmpA in the medium should be retarded in the absence of PpiD. Figure 5B compares release of OmpA from spheroplasts prepared from a *ppiD* knockout strain and wild-type cells at 20 °C. Evidently, the liberation of newly synthesized OmpA from the spheroplasts was slowed in the mutant. To rule out the possibility that this effect was due to different rates of synthesis in wild-type and mutant strains or to any other secondary strain-specific property, we purified a His-tagged version of the soluble periplasmic domain of PpiD and compared release of OmpA from Δ*ppiD* spheroplasts in the absence and presence of purified PpiD (Figure 5C). Clearly, the presence of the periplasmic PpiD domain reversed the impairment of release and led to a faster rate of accumulation of OmpA in the medium. These experiments suggest that interaction of newly translocated OmpA with PpiD accelerates its release from the cytoplasmic membrane without affecting the overall amount of secreted OmpA. If, however, expression of OmpA was boosted by increasing the temperature during induction of the spheroplasts from 20 to 37 °C and by extending the pulse period from 1 to 5 min, a drastic steady state level reduction of release of OmpA from the Δ*ppiD* spheroplasts could be observed (Figure 5D, compare lanes 1 and 2 to lanes 4 and 5). A decrease in the level of OmpA release under these conditions could have been caused by the accumulation of misfolded OmpA at the exit site of the Sec translocon, leading to aggregation and cosedimentation of mature OmpA with the spheroplasts. Taken together, the results are consistent with an involvement of PpiD in the release of a secretory protein from the Sec translocon.

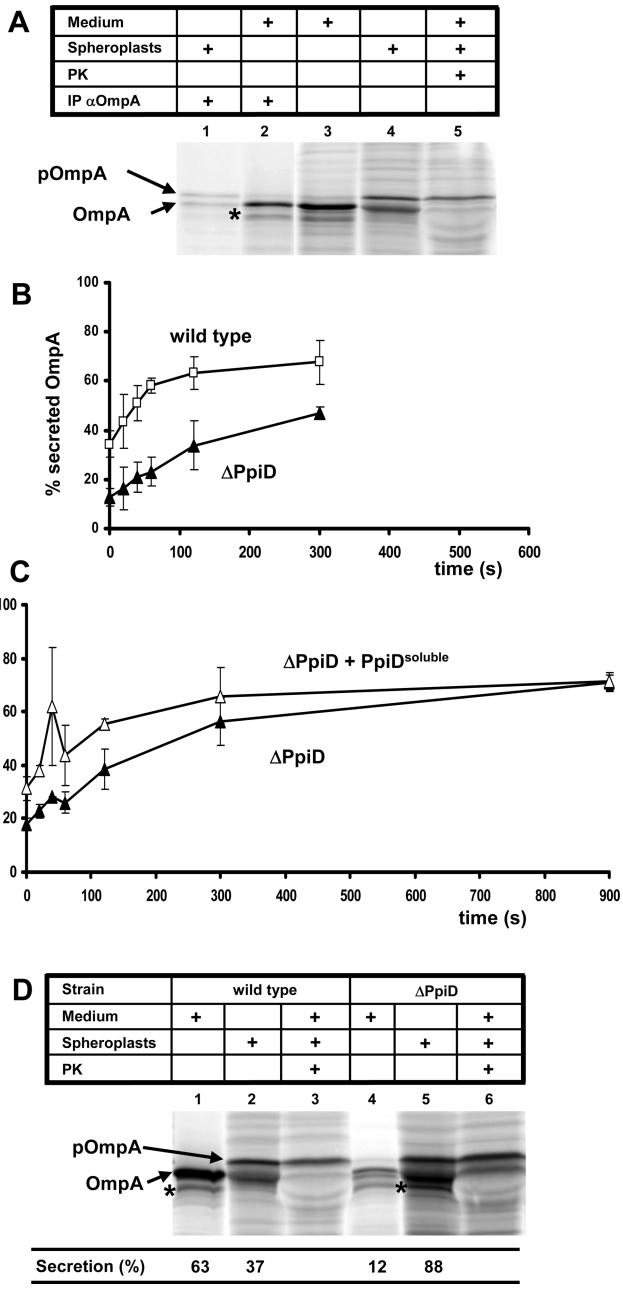

FIGURE 5: Lack of PpiD impairs the release of newly synthesized OmpA from the outside of the cytoplasmic membrane. Cells of *E. coli* strain MC4100 (wild type) and a *ppiD* deletion mutant (ΔPpiD), both transformed with plasmid pTompA, were converted to spheroplasts, induced for the expression of OmpA by IPTG, labeled with [^{35}S]methionine/[^{35}S]cysteine, and either digested with PK or spun to separate secreted proteins (Medium) from the spheroplasts. Following separation by SDS−PAGE, labeled proteins were visualized by phosphorimaging. These were quantified using Imagequant to calculate the amount of released OmpA as a percentage of total OmpA plus nonreleased pOmpA. (A) Induction was performed at 37 °C for 10 min using 2 mM IPTG followed by pulse labeling for 5 min. The asterisk labels an unusually folded species or degradation product of OmpA. This species was not considered for the calculations presented below. (B) Spheroplasts were induced at 20 °C using 5 mM IPTG, pulsed at 20 °C for 1 min, and chased with 1 mg/mL cold methionine/cysteine, whereafter samples were withdrawn at the indicated times. Data are the means of three independent experiments. (C) As in panel B, except that 30 μg/mL chloramphenicol was added to the chase medium. Of the purified soluble form of PpiD (PpiD^{soluble}), 125 μg was added to 1 mL of spheroplasts together with IPTG. The means of two independent experiments are given. (D) As in panel A.

DISCUSSION

Momp2, a Tool for Illuminating Intermediary Stages of the Translocation across the Sec Translocon. We have tried to characterize individual stages of translocation of a Sec-dependent *E. coli* secretory protein. To arrest translocation at defined steps, we made use of ribosome-tethered nascent chains. Nascent chains of SRP-independent bacterial preproteins, when bound to homologous bacterial ribosomes, usually do not stably interact with the Sec translocon (*7, 12*), at least not until very late in synthesis (*20*). This problem was overcome by choosing nascent chains of an N-terminally membrane-anchored protein for analysis. Our major findings are (i) that the SecYEG translocon while carrying a ribosome-associated nascent protein engages with SecA to promote transmembrane translocation, (ii) that during ongoing translocation a secretory protein is forwarded from SecYEG to the periplasmic chaperone PpiD, and (iii) that PpiD kinetically facilitates the release of the secretory protein from the periplasmic side of the membrane.

Translocation intermediates of 330-amino acid Momp2 RNCs appeared as membrane-protected, PK-resistant fragments, if active SecA was present. These fragments were visible only with ribosome-associated nascent chains, indicating that SecA must have been operative while the ribosome was still in place. The major populations of PK-resistant Momp2−330 fragments exhibited discrete sizes of 16 and 26 kDa. They appeared independently of the total length of Momp2 RNCs (not shown), provided that those were longer than 26 and 16 kDa, respectively. These 16 and 26 kDa PK-resistant fragments are very similar to translocation intermediates of full-size pOmpA previously described to accumulate in the absence of the H^+-motive force and at reduced levels of ATP (*21, 22*). The occurrence of distinct translocation intermediates of OmpA, which are only partially sequestered by membrane vesicles, is explained by the finding of Sato et al. (*23*) that distinct hydrophobic segments in the OmpA moiety cause some translocation arrest.

Simultaneous Cooperation of the Sec Translocon with Ribosomes and SecA. Some of the mapped interaction sites of SecA with SecY overlap with those for ribosomes (*24, 25*), but simply for reasons of size, a simultaneous association of both ligands with the translocon is difficult to conceive. Nevertheless, ribosomes were coisolated with SecY−SecA complexes, and SecA was demonstrated to bind to a Sec translocon engaged in translocation without leading to the dissociation of prebound ribosomes (*26*). Therefore, it is not clear whether the SecYEG translocon at the time when it is targeted by an SRP−Momp2−ribosome complex retains prebound SecA which is known to associate with inactive SecYEG complexes (*27*). Moreover, as the degree of oligomerization of an active Sec translocon currently is an unsettled issue, with models ranging from monomers to tetramers (*1, 28–32*), it is open if SecA and ribosome when acting on the same polypeptide chain do so by using the same SecYEG monomer. While SecA might interact with a translocating nascent chain via cytosolic loops protruding between ribosomes and the translocon, its physical interaction with SecY would always require release of the ribosome from the translocon. For unknown reasons, we were unable to demonstrate by cross-linking a direct molecular contact between SecA and SecYEG-targeted Momp2 RNCs, even

if SecA-mediated translocation was prevented by sodium azide (not shown).

PpiD, a Periplasmic Gatekeeper of the SecYEG Translocon. PpiD is one of several peptidyl prolyl isomerases (PPIases) found in the periplasm of Gram-negative bacteria. Its gene was isolated as a multicopy suppressor of *surA* (*33*) that encodes another periplasmic PPIase involved in the biogenesis of outer membrane proteins (*34, 35*). PpiD is anchored in the cytoplasmic membrane by a single N-terminal transmembrane domain. A null mutation in *ppiD* was reported to result in an overall reduction in the level and folding of outer membrane proteins and in the induction of the periplasmic stress response but does not confer lethality (*33*).

We demonstrate here that PpiD has an influence on the release of a newly synthesized outer membrane protein from the cytoplasmic membrane into the periplasm. A similar property was previously described for Skp, another periplasmic chaperone involved in the biogenesis of outer membrane proteins (*17, 36*). Different from PpiD and consistent with the lack of a membrane anchor, Skp was, however, not found to interact with its substrate before this was released from the translocon (*17*). Thus, it is conceivable that if PpiD and Skp act together in folding newly delivered outer membrane proteins, PpiD would first interact with the substrate protein as soon as this exposes binding sites at the periplasmic opening of the Sec translocon. Skp could then take over once release from the Sec translocon has been completed. Consistent with the fact that PpiD is not essential (*33, 37*), its deficiency manifested itself not as a complete block but as retardation in OmpA secretion. A partial block in secretion was observed when the substrate load was increased and presumably could be detected only because spheroplasts are likely to lack most of the other periplasmic chaperones, which in whole cells would compensate for a lack of PpiD. Similarly, the cytosolic chaperones trigger factor, DnaK/DnaJ, and SecB of *E. coli* are individually dispensable because they display functional redundancy (*38*). We propose that at the periplasmic exit site of the Sec translocon PpiD accepts newly translocated outer membrane proteins and by promoting their folding helps to release them into the periplasm.

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