prl Mutations in the Escherichia coli secG Gene

Sandrine Bost‡ and Dominique Belin§
From the Département de Pathologie, Université de Genève, CH 1211 Geneva, Switzerland

SecG, an integral membrane component of the Escherichia coli preprotein translocase, contributes to the efficiency of the export process by undergoing cycles of topology inversion in the membrane, coupled with the insertion-deinsertion cycles of SecA. We have previously identified sec alleles of secG that cause a generalized secretion defect. In this study, by screening mutagenized secG libraries for suppressors of a malE signal sequence mutation, we isolated prl alleles of secG. By analogy with secY/prlA, secE/prlD, and secE/prlG, secG could therefore be called secG/prlH. The prlH mutations affect 13 codons distributed along the secG sequence, and none map to the codons affected by sec mutations. prlH suppressors suppress a variety of signal sequence mutations and they allow export of alkaline phosphatase lacking its entire signal sequence. Although secG was not identified in previous selections for prl mutants, several prlH alleles are as strong as the strongest known prlG alleles of secE. Some prlH alleles can also promote the export of alkaline phosphatase fused to predicted cytoplasmic domains of UhpT, an integral membrane protein. These results support the notion that SecG contributes to signal sequence recognition, and suggest that it may also contribute to the topology of integral membrane proteins.

Proteins exported across the cytoplasmic membrane to the periplasm or to the outer membrane of Escherichia coli are synthesized as precursors containing an amino-terminal signal sequence. Signal sequences have been proposed to assume several functions in the process of protein secretion, including maintenance of the preprotein in an unfolded conformation, interaction with membrane phospholipids, binding to SecB, binding to SecA, and interaction with other components of the translocation machinery. Signal sequence mutations prevent proper targeting and lead to the accumulation of precursors in the cytoplasm, although proteins completely devoid of signal sequences can be exported at very low level in wild-type strains. Mutations in prl1 genes that encode components of the translocation machinery suppress signal sequence mutations. Furthermore, both periplasmic and outer membrane proteins completely devoid of signal sequences are exported quite efficiently in certain prl suppressor strains.

Suppressors of signal sequence mutations have been identified in three genes: prlA, prlD, and prlG. These genes are allelic to secY, secA, and secE, respectively. Three genes essential for protein export in vivo; conditional lethal mutations in each of these genes confer a generalized secretion defect (sec mutations). In vitro protein translocation has been achieved with proteoliposomes reconstituted with purified SecA, SecY, and SecE. SecA is a peripheral membrane ATPase, which promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion/deinsertion. SecY and SecE form a membrane-embedded complex that supports preprotein translocation across the plasma membrane.

SecG, an integral membrane protein that copurifies with the Sec-Y-E complex, stimulates protein translocation both in vivo and in vitro. Disruption of the gene encoding SecG causes an accumulation of the precursors of exported proteins and confers a cold-sensitive phenotype, in some genetic backgrounds. Recently, Nishiyama et al. have proposed that SecG undergoes a topological inversion in the membrane, which is coupled to the SecA insertion-deinsertion cycles, and may thereby ensure a maximal efficiency of protein translocation. Thus, SecG together with SecA, SecY, and SecE constitute the central components of the E. coli translocation machinery.

Both sec and prl alleles have been identified in secA/prlD, secY/prlA, and secE/prlG. However, the only known mutations in secG are sec alleles; they cause a weak generalized secretion defect and were not isolated in the classic selections for recessive conditional lethal sec mutants that repeatedly identified secA, secY, and secE alleles. Similarly, previous selections for suppressors of signal sequence mutations, which identified a large number of prlD and prlA alleles, and a more limited number of prlG alleles, did not reveal any prl mutations in secG.

In this study, we describe the isolation and characterization of prl alleles of secG, which we propose to call prlH alleles. Using PCR-mutagenized secG plasmid libraries, we screened for mutant alleles that improved MBP export mediated by the mutant signal sequence encoded by the malE18-1 gene. This sensitive screening allowed us to isolate a large number of prlH alleles, representing mutations scattered along the secG sequence. The prlH alleles suppress a variety of signal sequence mutations and the effect of several of them is as strong as that of the strongest known prlG alleles of secE. Finally, our results indicate that prl mutations in secG may also influence the topology of integral membrane proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—All bacterial strains are derivatives of E. coli K12 described in Table I. They were constructed by P1-mediated transduction or transformation. The plasmid encoding prlH+ (pH+) is identical to psecG+, a pACYC184 derivative described previously. Plasmids encoding prlH mutants (pH1 to pH6) are...
derivatives of pH−, and are listed in Table I. Plasmids encoding prlG+, prlG1 (L108R), prlG2 (S105P), prlG3 (S120P), and prlG4 (D112Q) are derivatives of pJS51 (41) and were a kind gift of Dr. M. Pohlschroder (Harvard Medical School, Boston, MA). pKJ1, which contains the secDF operon, was a kind gift of Dr. K. Pogliano (Harvard Medical School). pBAD22/TnphoA contains a signal sequence less derivative of phoA constructed by subcloning a KpnI-XbaI fragment from pSWFI (42) into the cognate sites of pBAD22 (43). pBAD22 contains the promoter of the arabinose BAD operon, a Shine-Dalgarno sequence upstream of a multiple cloning site, the gene encoding the regulator AraC, and an ampicillin resistance cassette. Plasmids encoding the UhpT:PhoA fusions 57 and 141 (44) were a kind gift of Dr. R. K. Jendrzejewski (University of Virginia, Charlottesville, VA). Plasmid encoding MBP18−1 and yields white colonies on McConkey plates supplemented with 1% maltose (Mal− phenotype). On these plates, increased MBP export is detected by the red color of the colonies. SB70 cells were electroporated (25 microfarads, 200 ohms, 2.5 kV) with six different PCR-mutagenized secG inserts that conferred both a Mal− and a PhoA− phenotype on 5-bromo-4-chloro-3-indolyl phosphate indicator plates to a strain expressing a signal sequence less derivative of alkaline phosphatase (SB81). Only the secG inserts that conferred both a Mal− and a PhoA− phenotype to SB81 were retained. 41 secG inserts were sequenced, and all contained mutations in secG.

**Alkaline Phosphatase Assay**—AP activity was measured by determining the rate of p-nitrophenyl phosphate (Sigma) hydrolysis in permeabilized cells and was normalized to the A0.6 of the cell suspension (36).

**Pulse Labeling and Immunoprecipitation**—Cell cultures were grown and pulse-labeled at 37 °C as described previously (32). Quantification was performed by scanning the gels with a Molecular Dynamics PhosphorImager, and using the ImageQuant version 3.22 software.

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**FIG. 1. Localization of secG mutations exhibiting a Prl phenotype.** The sequence of SecG (SwissProt accession no. P33582) is represented in single-letter code. Transmembrane domains (33) are indicated by solid boxes. Wild-type SecG amino acids affected by the prl mutations are represented in **bold** characters below the sequence and the observed substitutions are aligned underneath. All the substitutions represented are due to single mutations. Amino acids 41−43 marked with **asterisks** represent the TLF domain, where most sec mutations map (32).
RESULTS

Isolation of prl Alleles of secG—We have used as a reporter a well characterized periplasmic protein of E. coli, MBP, whose export is required for maltose uptake (46). Signal sequence mutations in the malE gene cause the accumulation of the precursor form of MBP (preMBP) in the cytoplasm and affect the ability of E. coli to use maltose as a carbon source (38). The malE18-1 mutation encodes an M18R change in the hydrophobic core of the preMBP signal sequence. Strains carrying this mutation have a Mal− phenotype on McConkey maltose plates.

To analyze a large number of secG mutations, we have generated mutant secG plasmid libraries by random PCR mutagenesis (32). Upon electroporation of these libraries into SB70 (Table I), we have screened for secG mutations that confer a Mal− phenotype. 41 Mal− isolates were analyzed by sequencing the secG insert carried on their plasmids. 25 secG inserts encoded mutant proteins with a single amino acid substitution (17 had a single mutation, and 8 had one or two additional silent mutations); 16 inserts encoded proteins with two or more amino acid substitutions, one of which was identical to those shown in Fig. 1. In contrast to the sec alleles of secG identified previously (32), most of which mapped to three contiguous codons defining the TLF domain at positions 41–43 of SecG, the 25 prl alleles of secG encoding a single amino acid substitution affect 13 different codons distributed along the secG sequence (Fig. 1). These mutations specify 16 different prl alleles, which we named prlH1 to prlH16. None of the prlH mutations map in the codons encoding the TLF domain affected by sec mutations. The most frequent mutation recovered (n = 7) encodes the K26E substitution, which possibly represents a hot spot for PCR mutagenesis. We further characterized the prlH1 to prlH6 alleles, which represent mutations distributed along the secG sequence (Table 1).

PrlH Mutants Improve Protein Export Mediated by Several Signal Sequence-defective PreMBP—MM4 strain, which carries the malE18-1 signal sequence mutation that severely affects preMBP export, is able to grow on minimal maltose plates at 37 °C, since only small amounts of MBP are required in the periplasm to ensure sufficient transport of maltose into the cell. However, the export of MBP18-1 is cold-sensitive (47), and MM4 grows extremely slowly on minimal maltose plates at 23 °C. Plasmids carrying different mutant prlH alleles also suppressed the export defect caused by the malE14-1 and malE16-1 mutations, as scored on indicator plates (data not shown). These two signal sequence mutations, like the malE18-1 mutation, map to the hydrophobic core of the signal sequence but they result in milder export defects. To quantitate the extent of prlH suppression of these signal sequence mutations, we performed pulse labeling experiments in strains containing the malE14-1 or the malE16-1 mutation. In the case of MBP14-1, the efficiency of MBP export
to the periplasm, as indicated by the fraction of processed mature MBP, was significantly improved, from 2- to 3-fold, in cells harboring anyone of the four mutant prlH alleles tested (Fig. 3). The amount of MBP exported in these cells was not further improved after a 10-min chase period (data not shown). For MBP16-1, the fraction of preMBP that was processed to the mature form was higher at all time points studied in cells harboring either the prlH3 or the prlH6 allele, when compared with cells harboring the prlH1 allele. In this case, both the kinetics of MBP export and the total amount of preMBP processed after a 9.5-min chase period were increased by the mutant prlH alleles (Fig. 4). It should be noted that the overexpression of prlH- itself also caused a slight improvement in MBP16-1 processing (Fig. 4), an effect similar to that observed for MBP18-1 at 37 °C (32) and at 23 °C (Fig. 2). In conclusion, these results demonstrate that prlH mutations facilitate the export of several mutant preMBP proteins.

PrlH Alleles Improve the Export of PreAP with Several Signal Sequence Mutations as Well as That of Signal Sequence Less AP—To assess whether the prlH alleles also suppress the export defect of other signal sequence mutations, we used a collection of strains carrying different signal sequence mutations in phoA (Ref. 36; Table I). These mutations map to the hydrophobic core of the signal sequence and interfere to various extents with alkaline phosphatase export (36). The AP activity of these strains is a quantitative measure of the efficiency of AP export, since this enzyme only becomes active after being exported to the periplasm (49). AP activity was determined in each of these strains carrying either a plasmid containing the prlH1 to prlH6 alleles, or the control plasmid containing the prlH- gene (Fig. 5). Although prlH1 and prlH2 exhibited a very weak effect on AP export, the prlH3 to prlH6 alleles all caused a 2-4-fold increase in AP enzymatic activity. As expected, none of the prlH suppressors affected the enzymatic activity of the Mph42 strain, which encodes a wild-type preAP. The suppressor activity of the prlH alleles was also compared with that of four well characterized prlG alleles of secE, prlG1 to prlG4, which represent the strongest prlG alleles isolated so far. Similarly to prlH1 and prlH2, prlG1 and prlG4 had a very weak effect on the AP activity of these strains. The strongest effect was that observed with prlG2, which caused a 3- and 4-fold increase in the AP activity of Mph53 and Mph55, respectively; this Prl effect is practically identical with that of the prlH3, prlH4, and prlH6 alleles. Thus, at least some of the prlH alleles, isolated on the basis of improved export of mutant preMBP signal sequences, significantly improve the export of various signal sequence-defective preAP. Furthermore, the suppressor activity of several of these alleles is as strong as that of the strongest known prlG alleles of secE.

We next assessed whether the prlH suppressors were potent enough to suppress a complete deletion of the AP signal sequence. For this purpose, we transformed plasmids carrying prlH mutants or prlH- into SB81, which expresses a signal sequence less derivative of AP (42) from the arabinose-inducible pBAD22 expression vector (Ref. 43; Table I). In the presence of arabinose, this strain accumulates AP in the cytoplasm and has a PhoA- phenotype on plates supplemented with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate. All the prlH alleles isolated were able to improve phenotypically the secretion efficiency of signal sequence less AP (data not shown). To quantitate their effect, we measured the level of AP activity of SB81 derivatives containing a plasmid-encoded prlH+ or the prlH1 to prlH6 alleles (Fig. 6). Although prlH1 had no detectable effect on the AP activity of this strain, the prlH2 to prlH6 alleles all increased AP activity 2-3-fold in comparison to prlH-. This effect was comparable to that conferred by secDF overexpression, but was much weaker than the 20-fold increase conferred by the strong prlA4 allele (data not

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very low AP activity in AP activity of two UhpT::PhoA fusion proteins, which exhibit alleles in these strains. We found that indicates the S.D. represents the average from three independent cultures. Error bars indicate the average from three independent cultures. Error bars indicate the S.D.

Thus, the suppressor activity of several prlH alleles of secG is potent enough to promote, at least to some extent, the export of a protein completely devoid of its signal sequence.

Effect of prlH Alleles on the Enzymatic Activity of AP Fused to an Integral Membrane Protein—It has been shown that several prlA alleles can facilitate translocation of AP fused either to the cytoplasmic side of transmembrane domains of integral membrane proteins or to transmembrane domains of integral membrane proteins that are unable to function as efficient export signals (45, 50, 51). Indeed, Puziss et al. (45) reported that the strong prlA666 allele was able to enhance the AP activity of two UhpT::PhoA fusion proteins, which exhibit very low AP activity in prl+ cells (44, 45). The E. coli UhpT protein is an integral membrane protein predicted to contain 12 membrane-spanning regions with cytoplasmic amino and carboxyl termini (44). In the fusion protein 141, the AP moiety is fused to the cytoplasmic end of the fourth membrane-spanning region of UhpT. In the fusion protein 57, the AP moiety is fused downstream of the first membrane-spanning region, within a predicted periplasmic loop of UhpT. In this fusion, the unexpectedly low enzymatic activity was attributed to the moderate hydrophobic character of the first transmembrane segment; furthermore, the presence of three charged residues immediately preceding the AP fusion joint renders the first transmembrane domain of UhpT unlikely to function as an effective signal sequence (44). It was therefore of interest to determine whether the prlH mutants could increase the activity of such fusion proteins. We analyzed the effect of the prlH1 to prlH6 alleles in these strains. We found that prlH3 and prlH6 promote a 2-fold increase in the AP activity of fusion 141, while prlH2, prlH3 and prlH6 increase to a similar extent the AP activity of fusion 57 (Fig. 7). These effects are comparable to that of prlA666, which was shown to cause a 3- and 2-fold increase in the AP activity of fusions 141 and 57, respectively (45). The remaining three prlH mutants tested had no significant effect on the AP activity of these hybrid proteins (data not shown). These results indicate that at least three of the prlH mutants analyzed can influence the orientation of the hydrophobic domains of these UhpT::PhoA fusion proteins so that the AP moiety is now translocated to some extent into the periplasm.

DISCUSSION

The isolation of suppressors of signal sequence mutations to characterize the genes that encode components of the E. coli translocation machinery has long been validated (52). Using a sensitive phenotypic screening and a collection of PCR-mutagenized secG plasmid libraries, we have now identified prl alleles of secG, which could therefore be named prlHsecG, by analogy with prlA/secY, prlD/secA, and prlG/secE. Considering the large extent to which this approach has been applied, one could have assumed that all the target genes had already been identified. secG may have escaped previous selections for Prl mutants because of its small size or because of its rather weak suppression efficiency compared with that of most prlA and prlD alleles. However, the effect of several prlH alleles is as strong as that of the strongest known prlG alleles of secE, and prlH alleles should therefore appear in the selections that identified these prlG alleles.

In general, prlG alleles are considered as weak suppressors of signal sequence mutations (22, 41), although they are efficient enough to suppress a mutation as severe as the deletion of the lamB signal sequence (16). Furthermore, prlG1 and prlG2 restore maltose sensitivity to a lamB17D-lacZ fusion strain to a degree comparable to that of the strong prlA4 allele (53). Similarly, several prlH alleles are potent enough to suppress a complete deletion of the AP signal sequence, and the effect of some of them on the export of the UhpT::PhoA fusions is equivalent to that of the strong prlA666 allele. In any event, prlH suppressors were easily detected by several phenotypic assays, and their phenotype could also be detected by biochemical pulse-chase assays, which are in general less sensitive to reveal small changes in export efficiency.
The collection of point mutations in secG that result in a Prl phenotype affect 13 different amino acids and are distributed along the NH2-terminal 2/3 portion of SecG, up to the end of the second transmembrane domain of the protein (Fig. 1). We have previously characterized several mutations in secG that confer a Sec phenotype. These mutant secG were identified in a selection for suppressors of the toxicity of a chimeric protein, in which a mammalian signal sequence is fused to AP (32). All single point mutations isolated mapped to amino acids 41–43, defining a TLF domain between the two transmembrane regions of the protein, and one double mutation mapped to the second transmembrane region. Additional sec mutations were isolated by screening for multicopy dominant sec alleles of secG conferring a Mal phenotype to a strain carrying the malE19-1 signal sequence mutation (38). Among six mutations isolated, four mapped after the second transmembrane domain of the protein and the two remaining mutations flanked the TLF domain. None of the prl mutations identified in this study map to the codons affected by the sec mutations previously identified, although prl and sec mutations are interspersed in the second transmembrane domain of the protein. The localization of the sec mutations suggested that the normal activity of SecG involves at least three domains: the central TLF domain, the second transmembrane domain, and the COOH-terminal tail (Fig. 1). The distribution of prlH mutations now indicates that other residues in SecG also contributes to its function. Overall, 22 amino acids among 110 residues of SecG can be changed to generate a large spectrum of different phenotypes.

Overexpression of components of the translocation machinery such as SecDF and SecA has been shown to result in a Prl phenotype (20, 48). In the case of SecD and SecF, these proteins are present in lower amount than the SecY-E complex, suggesting that they may be limiting factors, particularly for protein export mediated by inefficient signal sequences. We have previously shown that SecG overexpression accelerates the kinetics of export of wild-type proteins. We have now extended this observation and found that SecG overexpression also slightly improved the export of signal sequence-defective preMBP and of signal sequence less AP (data not shown). This reinforces the notion that two types of complexes, with and without SecG, may exist in the cell, and that SecG overexpression accelerates overall protein export by titrating SecY-E complexes to provide fully active complexes. It seems worth emphasizing that the Prl phenotype conferred by the plasmid-encoded prlH suppressors is not simply due to SecG overexpression. Indeed, their Prl phenotype was stronger than that of a plasmid-encoded prlH+ . We have verified by Western blot that the level of SecG in strains overexpressing prlH+ or the mutant prlH alleles are indistinguishable (data not shown). Thus, the amino acid substitutions are responsible for the Prl phenotype.

prlH suppressors suppress a variety of signal sequence mutations in preMBP and preAP, and some of them also facilitate export of signal sequence less AP. This is similar to the suppression spectrum of several prlA and prlG suppressors, which suppress mutations in the hydrophobic core of signal sequences as well as signal sequence deletions. A variety of prlA alleles improve the signal sequence processing of MBP +3/+4 (45), a preMBP species with an altered charge distribution around the intact hydrophobic core, resulting from the substitution of 2 Glu residues by 2 Arg residues in the amino terminus of the mature portion of MBP. PrlH2 was able to improve by at least 2-fold the processing of MBP +3/+4 (data not shown). This result indicates that the suppressor activity of prlH alleles might not be limited to alterations in the hydrophobic core of signal sequences. Finally, three of the six prlH suppressors assayed were able to increase the translocation efficiency of AP fused to two different transmembrane domains of UhpT, an integral membrane protein. This result suggests that some prlH suppressors that affect the export of signal sequence-defective proteins can also affect the interaction of the translocon with membrane-spanning segments of integral membrane proteins such as UhpT. Alternatively, the Prl phenotype could simply reflect an interaction with the mature portion of AP, since signal sequence less AP is also exported to some extent in prlH suppressors. However, this interpretation is not supported by the observation that some of the prlH alleles, such as prlH4 and prlH5, have a significant effect on signal sequence less AP but not on the UhpT::PhoA fusions. Thus, prl mutations in secG may influence the topology of integral membrane proteins.

Different prlH mutations exhibit different pattern and strength of suppression. Whereas prlH3 to prlH6 improve export of signal sequence less AP, as well as that of preMBP and preAP altered in the hydrophobic core of their signal sequence, prlH2 does not suppress the signal sequence mutants of preAP significantly and prlH1 apparently only suppresses MBP +3/+4 detectably. This probably simply reflects a difference in the effectiveness of suppression, prlH3 to prlH6 being stronger than prlH2, which is itself stronger than prlH1. In any event, there is no evidence for allele specificity in prlH suppression.

We have shown previously that sec mutations in secG can affect differently the export efficiency of AP containing either its wild-type signal sequence or a mammalian signal sequence, suggesting that secG contributes to the functional activity of signal sequences. The isolation of prl mutations in secG further support this notion. Taken together, these observations suggest a participation of SecG in signal sequence recognition. However, as repeatedly observed for prlA and prlG suppressors, the lack of allele specificity and the suppression of a complete signal sequence deletion indicate that prlH suppressors may prevent the rejection of defective preproteins from the export pathway, rather than restoring the recognition of altered signal sequences. Thus, prlH suppression would work like prlA and prlG suppression, by loss of the proposed proofreading activity of the translocon (15, 16, 18, 54). Interestingly, 5 among the 16 prlH mutants are charge mutants, all resulting in the introduction of negatively charged residues in between the two transmembrane domains of the protein (Fig. 1). These charge substitutions could modify the dynamics of SecG topology inversion in the membrane (33) and thus alter the contribution of SecG to the postulated proofreading activity of the translocon. This mechanism of suppression, by loss of a proofreading activity, appears different than that of several prlD suppressors, which exhibit a markedly different pattern of suppression (signal sequences altered in their hydrophilic segment), and do not suppress a complete deletion of the lamB signal sequence (16). Huie and Silhavy (21) proposed that at least a subset of prlD suppressors affect an activity of SecA related to ATP hydrolysis. However, an independent set of prlD alleles have been shown to suppress signal sequence mutations in the hydrophobic core of preMBP, and some of them also allow export of signal sequence less AP.2 More recently, it has been shown that prlA suppressor mutations, but not prlG or prlD mutations, were able to relieve the proton motive force dependence of the translocation of a wild-type precursor protein (55). Furthermore, a translocation defect due to a stably folded structure in the mature domain of proOmpA was suppressed by some prlA suppressor mutations, suggesting that prlA suppression is not restricted to signal sequence defects but extends to defects in mature sequences of precursor proteins. Thus, the mechanism

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2 M. Stoll and D. Oliver, personal communication.
of prl suppression could be more complex and diverse than initially anticipated. The function of SecG in the export process is far from being completely understood. However, the ability of SecG to stimulate protein translocation both in vitro and in vivo, and the isolation of both sec and prl mutations in this gene illustrate the importance of this component of the E. coli translocation machinery for the export process.

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