The EMBO Journal vol.13 no.14 pp.3272–3277, 1994

Disruption of the gene encoding p12 (SecG) reveals the direct involvement and important function of SecG in the protein translocation of *Escherichia coli* at low temperature

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Communicated by G.Schatz

The *Escherichia coli* cytoplasmic membrane protein, p12, stimulates the protein translocation activity reconstituted with SecY, SecE and SecA. The gene encoding p12, which is located at 69 min on the *E.coli* chromosome, was deleted to examine the role of p12 in protein translocation *in vivo*. The deletion strain exhibited cold-sensitive growth. Pulse–chase experiments revealed that precursors of outer membrane protein A, maltose binding protein and β-lactamase accumulated at 20°C but not at 37°C. The deletion strain harboring a plasmid which carries the gene encoding p12 under the control of the *araBAD* promoter was able to grow in the cold when p12 was expressed with the addition of arabinose. Furthermore, the accumulated precursors were rapidly processed to the mature forms upon the expression of p12. Immunoblot analysis revealed the steady-state accumulation of precursor proteins at 20°C, whereas the accumulation was only marginal at 37°C, indicating that the function of p12 is more critical at 20°C than at 37°C. Finally, proteoliposomes were reconstituted with or without p12 to demonstrate that the stimulation of the activity by p12 increases with a decrease in temperature. From these results, we concluded that p12 is directly involved in protein translocation in *E.coli* and plays a critical role in the cold. We propose the more systematic name, SecG, for p12.

Key words: Escherichia coli/gene disruption/p12/protein translocation/SecG

Introduction

Extensive genetic studies have revealed that several genes are involved in protein translocation across the cytoplasmic membrane of *Escherichia coli*. They are *secA* (Oliver and Beckwith, 1981), *secB* (Kumamoto and Beckwith, 1983), *secD* (Gardel *et al.*, 1987, 1990), *secE* (Riggs *et al.*, 1988; Schatz *et al.*, 1989), *secF* (Gardel *et al.*, 1990) and *secY* (Emr *et al.*, 1981; Ito *et al.*,1983). The Sec proteins encoded by these genes have been overproduced and purified (Cabelli *et al.*, 1988; Weiss *et al.*, 1988; Kawasaki *et al.*, 1989; Matsuyama *et al.*, 1990, 1992), and subjected to biochemical studies. SecB is a molecular chaperone and maintains the presecretory proteins in a translocation-competent state (Kumamoto, 1990). SecA, a peripheral membrane protein (Oliver and Beckwith, 1982), is essential for protein translocation (Cunningham *et al.*, 1989; Kawasaki *et al.*, 1989) and exhibits ATPase activity, which is coupled to the translocation (Lill *et al.*, 1989). Other Sec proteins are integral membrane proteins (Akiyama and Ito, 1985; Schatz *et al.*, 1989; Gardel *et al.*, 1990). Protein translocation activity has been reconstituted with independently purified SecY, SecE and SecA (Akimaru *et al.*, 1991) or the purified SecY–SecE complex and SecA (Brundage *et al.*, 1990), indicating the direct involvement of the three proteins in the translocation reaction. The translocation activity reconstituted with independently purified component is, however, considerably lower than that of intact everted membrane vesicles (Akimaru *et al.*, 1991). On the other hand, the activity reconstituted with the SecY–SecE complex and SecA has been reported to be comparable to that of membrane vesicles (Bassilana and Wickner, 1993). Although participation of SecD in a late step of the protein translocation reaction has been demonstrated (Matsuyama *et al.*, 1993), neither SecD nor SecF enhances the reconstituted activity (Matsuyama *et al.*, 1992). From these observations, the involvement of some unknown factors in protein translocation has been suggested. Although the SecY–SecE complex, whether purified chromatographically (Brundage *et al.*, 1990) or immunoprecipitated with an anti-SecY antibody (Brundage *et al.*, 1992), contained a protein, termed band 1, the role of this protein in the translocation reaction has not been clarified.

We previously discovered and characterized a cytoplasmic membrane protein, p12, which strongly stimulated the reconstituted activity (Nishiyama *et al.*, 1993). Sequencing of the gene encoding p12 revealed that this protein could span the cytoplasmic membrane two or three times (Nishiyama *et al.*, 1993). An anti-p12 antibody strongly inhibited the translocation into intact everted membrane vesicles (Nishiyama *et al.*, 1993). Furthermore, the anti-p12 antibody was recently found to cross-react with band 1, indicating the identity between these two proteins (K.Douville, M.Leonard, K.Nishiyama, H.Tokuda, S.Mizushima and W.Wickner, submitted). The involvement of p12 in protein translocation was thus demonstrated *in vitro*, whereas the gene encoding p12 was not identified as the *sec* or *prl* gene in previous genetic studies. The involvement of p12 in protein translocation *in vivo* remained to be clarified.

In this study, we constructed a chromosomal deletion of the gene encoding p12 and analysed protein translocation *in vivo*. Deletion of the gene was found to impair protein translocation and to cause the accumulation of precursor proteins, especially in the cold. Reconstitution of proteoliposomes with or without p12 revealed the special importance of p12 for protein translocation in the cold. Based on the results shown here, we propose the more systematic name, SecG, for p12.

Results

secG is essential for growth in the cold

To examine the role of SecG *in vivo*, we constructed a chromosomal deletion of *secG*. A null allele of *secG* was constructed on a plasmid by replacing the gene encoding amino acids 4−107 of the SecG protein with the *kan* gene, which confers resistance to kanamycin. This deletion should abolish the function of *secG* completely because SecG consists of 110 amino acids. Plasmid pDG41, which carries the *tet* gene and this null allele of *secG*, was linearized and introduced into FS1576 carrying the *recD* mutation (Russel *et al.*, 1989) to recombine this deletion of *secG* into the chromosome in the presence of pAG3, which carries *secG* and *leuU* under the control of the *araBAD* promoter. Kanamycin-resistant and tetracycline-sensitive mutants were screened in the presence of arabinose to supply SecG and Leu_2-tRNA, the product of *leuU* (Komine *et al.*, 1990). These mutants should have lost plasmid pDG41 as a result of the recombination into the chromosome (Figure 1). The isolated strain, KN370/pAG3, expressed a reduced amount of SecG in the absence of arabinose and exhibited arabinose-dependent growth in the cold, but not at 37°C (data not shown). After KN370/pAG3 had been cultivated for 14 h at 37°C in the absence of ampicillin, to which pAG3 confers resistance, ~3% of the cells had lost pAG3. Immunoblot analysis revealed that this strain, KN370, did not express a detectable amount of SecG (see below). From these results, we concluded that KN370 is a deletion strain of *secG*. The deletion was confirmed by sequencing of a PCR-amplified DNA fragment (data not shown).

KN370 was transformed with pAG5, which carries *secG* but not *leuU* under the control of the *araBAD* promoter, and then spread onto LB/ampicillin plates supplemented with arabinose or not supplemented (Figure 2). When these plates were incubated at 37°C, colonies formed in the presence and absence of arabinose (Figure 2A and B). On the other hand, when they were incubated at 20°C, colony formation was observed only in the presence of arabinose (Figure 2C and D). Since pAG5 carries only *secG* (and not *leuU*) the cold-sensitive growth of KN370 should be caused by the absence of the SecG function. It is unclear whether or not the expression of *leuU*, which is very close to *secG*, was perturbed upon the introduction of the Δ*secG::kan* allele. We suggest that a sufficient level of Leu_2-tRNA is expressed through the possible read-through of the *kan* gene. There were no sequences in the *kan* gene which could encode the ρ-independent terminator (data not shown). Taking the results together, we concluded that *secG* is essential for growth in the cold. The deletion strain of *secG*, however, grew normally at 37°C (Figure 2A) and 42°C (data not shown), like the $secG^+$ cells.

Expression of SecG from pAG5 was found to be tightly controlled by the level of arabinose; a detectable amount of SecG was not expressed in the absence of arabinose, whereas the addition of >0.1% arabinose caused the expression of SecG to essentially the same level as wild type cells (see Figure 6).

Deletion of secG causes proOmpA to accumulate at a non-permissive temperature

The Δ*secG::kan* allele of KN370 was introduced into W3110 M25, which was used in our previous *in vitro* studies

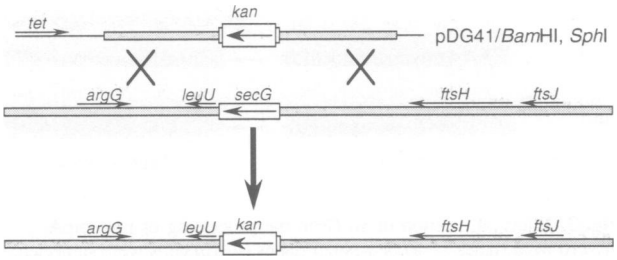

Fig. 1. Construction of a chromosomal deletion of *secG*. pDG41 was linearized with *Bam*HI and *Sph*I, and then introduced into FS1576 cells to recombine the deletion allele of *secG* into the chromosome. Dotted boxes represent the chromosomal DNA around *secG*. Each gene is indicated by an arrow with its name. The small and large open boxes represent *secG* and *kan*, respectively. The horizontal lines are regions derived from pBR322. 'X' represents homologous recombination.

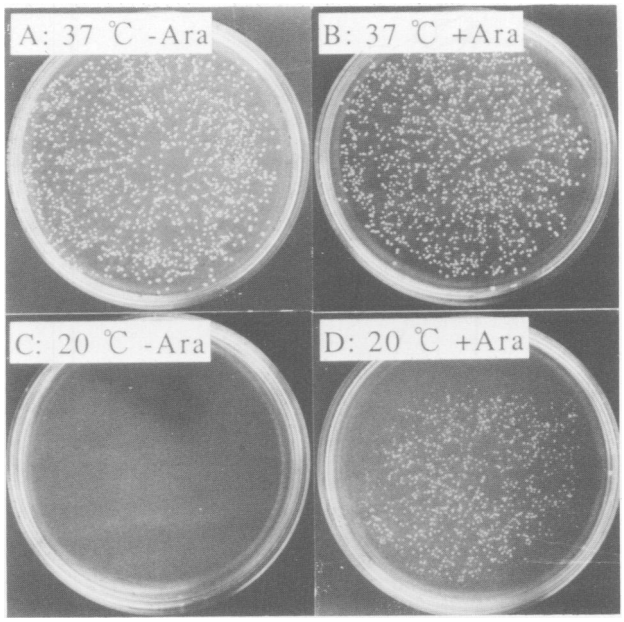

Fig. 2. *secG* is essential for growth in the cold. Equal amounts of KN370 (FS1576 Δ*secG::kan*) cells which harboured pAG5 were streaked onto LB/ampicillin (50 μg/ml) plates supplemented with 0.2% arabinose (**B** and **D**) or not supplemented (**A** and **C**). They were incubated at 37°C (A and B) for 14 h or at 20°C (C and D) for 2.5 days.

(Akimaru *et al.*, 1991; Nishiyama *et al.*, 1993), by P1 transduction to construct KN425. This strain also exhibited cold-sensitive growth (data not shown). Pulse−chase experiments were then carried out to examine the processing of outer membrane protein A (OmpA) by the W3110 M25 strain and its Δ*secG* derivative, KN425 (Figure 3). At 37°C, only the mature form of OmpA was detected in both types of cells (Figure 3, upper panel). The pulse−chase experiment was also carried out after the cells had been incubated for 4 h at 20°C (Figure 3, lower panel). Only low levels of the precursor form of OmpA (proOmpA) were detectable in the wild type cells. On the other hand, accumulation of proOmpA was observed even after a 4 min chase in Δ*secG* cells. These results indicate that SecG is involved in OmpA translocation in the cold. This cold-sensitive processing of proOmpA observed in the Δ*secG* strain coincided with its cold-sensitive growth (Figures 2 and 3).

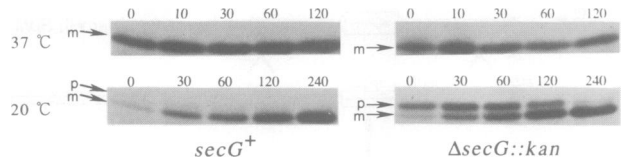

Fig. 3. Effect of deletion of *secG* on the processing of proOmpA. W3110 M25 (lanes 1–5) or KN425 (lanes 6–10) cells were subjected to the pulse–chase experiment at 37°C (upper panel) or 20°C (lower panel). The cells were labelled with Tran^{35}S-label for 1 min at 37°C. The cells were preincubated at 20°C for 4 h and labelled for 1.5 min. The labelling was subsequently chased with non-radioactive methionine and cysteine for the specified time (in seconds). OmpA and proOmpA were immunoprecipitated and subjected to SDS–PAGE, followed by fluorography. The positions of the mature (m) and precursor (p) forms of OmpA are indicated by arrows.

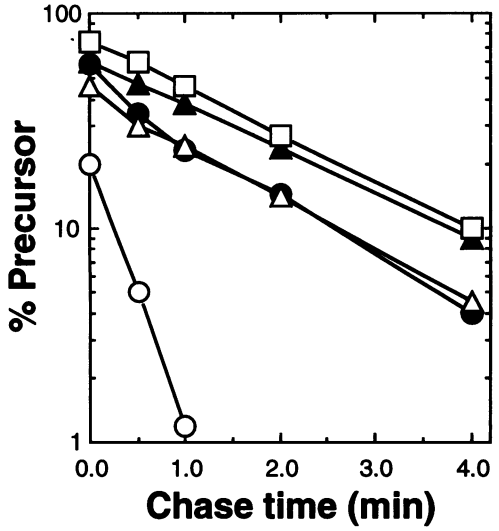

Fig. 4. Immediate perturbation of proOmpA translocation in Δ*secG* cells at 20°C. The KN425 strain was grown at 37°C to 4×10^8 cells/ml and then transferred to 20°C. After incubation for 3 min (●), 1 h (△), 2 h (▲) or 4 h (□) at 20°C, the cells were subjected to the pulse–chase experiment. The parent strain, W3110 M25, was also examined after 4 h incubation at 20°C (○). The cells were labelled for 1.5 min and then chased for the indicated period. The amounts of proOmpA and OmpA on the fluorograms were determined by densitometric scanning. The percentage of proOmpA against the total amount of OmpA materials was calculated. The numbers of methionine plus cysteine residues in proOmpA and mature OmpA, which are 8 and 7, respectively, were used in the calculation.

After the wild type cells and Δ*secG* cells had been incubated at 20°C for various periods, the pulse–chase experiment was carried out to determine the rate of pro-OmpA processing. The amounts of proOmpA relative to the total amount of OmpA material, which was determined by densitometric scanning of the fluorograms, were plotted as a function of the chase time (Figure 4). The half life of proOmpA in *secG*$^+$ cells, which were incubated for 4 h at 20°C, was ~15 s. Essentially the same rate of processing was obtained with cells incubated for 3 min at 20°C (data not shown). On the other hand, the half life of proOmpA in the Δ*secG* cells was ~90 s irrespective of the incubation period of 20°C. The defect in proOmpA translocation was observed as early as 3 min after the transfer of the Δ*secG* cells to the non-permissive temperature, indicating that the translocation was immediately perturbed by the lack of SecG at 20°C.

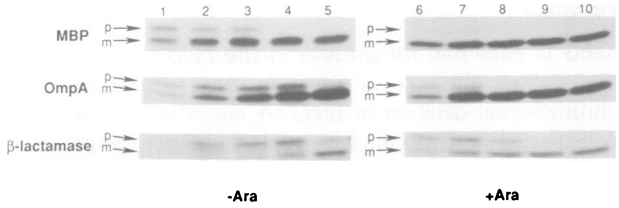

Fig. 5. Expression of SecG stimulates the processing of MBP, OmpA and β-lactamase. KN425 cells harbouring pAG5 were cultivated at 20°C for 4 h in the presence (lanes 6–10) or absence (lanes 1–5) of 0.2% arabinose. They were then pulse-labelled for 1.5 min and chased for 0 min (lanes 1 and 6), 0.5 min (2 and 7), 1 min (3 and 8), 2 min (4 and 9) or 4 min (5 and 10). MBP, OmpA and β-lactamase were visualized by fluorography after immunoprecipitation. The positions of the precursor (p) and mature (m) forms of each protein are indicated by arrows.

SecG is a general component of the protein translocation machinery

The effects of SecG expression on the translocation of maltose binding protein (MBP), OmpA and β-lactamase were examined by means of pulse–chase experiments at 20°C in strain KN425 harbouring pAG5 (Figure 5). The precursor forms of all three proteins accumulated in the absence of arabinose. The rate of the processing in the absence of arabinose differed depending on the precursor species. Expression of SecG on the addition of arabinose significantly stimulated the processing of the precursors of all the proteins. The half lives of each precursor protein were estimated after densitometric quantification of the fluorograms. The estimated values in the presence and absence of arabinose, respectively, were ~15 s and ~90 s for proOmpA, <10 s and ~35 s for MBP, and ~45 s and ~180 s for β-lactamase. Expression of SecG, therefore, caused a 4- to 6-fold increase in the processing rate for all the proteins examined. The half lives of all the proteins in the presence of arabinose were essentially the same as those observed for the parent cells, W3110 M25 (for example, compare the OmpA processing shown in Figures 3 and 5).

The accumulation of precursor proteins revealed by the pulse–chase experiment in the absence of SecG was rather transient and observed only in the cold. We next examined whether or not the absence of SecG causes the steady-state accumulation of proOmpA. KN425/pAG5 cells were incubated at either 37°C or 20°C with or without arabinose. Total cellular proteins were analysed by SDS–PAGE, followed by immunoblotting (Figure 6). The level of SecG in the Δ*secG* cells with arabinose was essentially the same as that in the wild type cells at both 37°C and 20°C, whereas a detectable amount of SecG was not expressed in the Δ*secG* cells without arabinose. No precursor form of OmpA was detected in the wild type cells or in the Δ*secG* cells with arabinose at either temperature. In contrast, accumulation of proOmpA was detected in the Δ*secG* cells at 20°C without arabinose. Although the accumulation of proOmpA was constantly observed at 37°C in the Δ*secG* cells without arabinose, its level was much lower than that at 20°C. Perturbation of protein translocation resulting from the absence of SecG is, therefore, only marginal at 37°C, so growth seems to be unaffected.

Expression of SecA is known to be derepressed by defects of protein secretion (Bieker *et al.*, 1990; Schmidt *et al.*, 1991). Quantitative immunoblotting revealed that the level

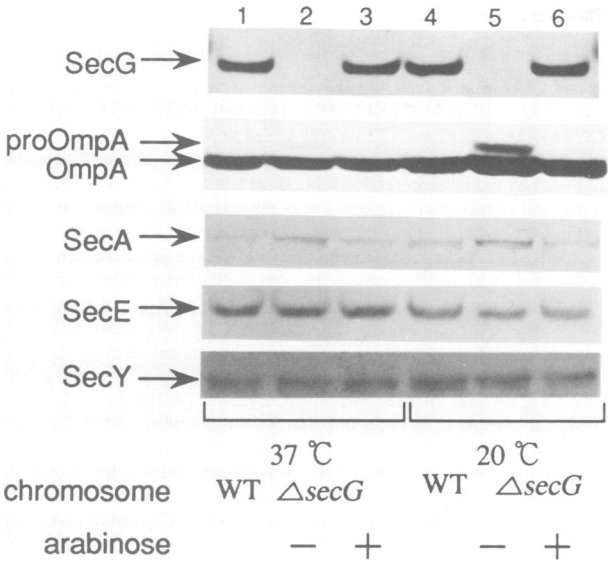

Fig. 6. Immunoblot analysis of OmpA materials and Sec proteins in the presence and absence of SecG expression. W3110 M25/pKQ2 (lanes 1 and 4) and KN425/pAG5 (lanes 2, 3, 5 and 6) cells were cultivated at 37°C (lanes 1−3) or 20°C (lanes 4−6) for 4 h, in the presence (lanes 3 and 6) or absence (lanes 1, 2, 4 and 5) of 0.2% arabinose. Equal amounts of total cellular proteins (10 μg for SecG, OmpA and SecE; 2 μg for SecA; and 20 μg for SecY) were analysed by immunoblotting using anti-SecG, anti-OmpA, anti-SecA, anti-SecE or anti-SecY antiserum as described in Materials and methods.

of SecA in the Δ*secG* cells at 20°C without arabinose was ~5-fold higher than that in the wild type cells at 37°C, whereas the levels of SecY and SecE were similar (Figure 6). These results indicate that the lack of SecG directly impairs protein translocation and causes the derepression of SecA. The level of SecA in the Δ*secC* cells at 37°C without arabinose was slightly higher than that in the wild type cells. There may be a slight secretion defect at 37°C.

From the results shown in Figures 5 and 6, we concluded that SecG is directly involved in the protein translocation in *E.coli*.

Stimulatory activity of SecG in reconstituted proteoliposomes increases with a decrease in temperature

The *in vivo* studies described above revealed that the SecG function is important for protein translocation, especially at low temperature. We next examined whether or not SecG-dependent stimulation of the reconstituted activity differs depending on the temperature. Proteoliposomes were reconstituted with SecY and SecE in the presence and absence of SecG, then assayed for the translocation of proOmpA D26, a model secretory protein, at various temperatures in the presence of SecA and ATP. SecG stimulated the translocation activity over the entire range of temperature examined (Figure 7A). The translocation activity decreased with a decrease in temperature, irrespective of the presence or absence of SecG, however. When SecG-dependent stimulation of the activity was estimated at each temperature, the stimulatory effect of SecG was found to increase markedly with a decrease in temperature (Figure 7B). The results indicate that the importance of the SecG function increases with a decrease in temperature not only in cells but also in reconstituted proteoliposomes.

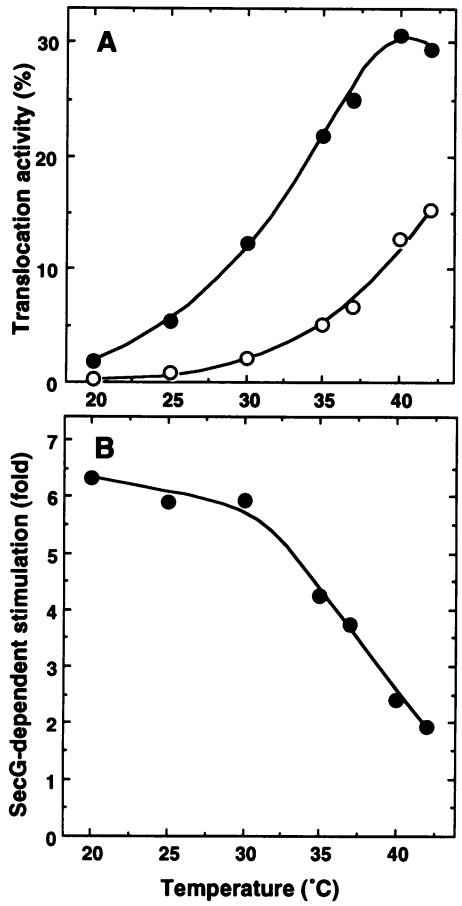

Fig. 7. Stimulation of the reconstituted activity by SecG increases with a decrease in temperature. (**A**) SecE (80 pmol) and SecY (25 pmol) were supplemented with SecG (300 pmol) (●) or not supplemented (○), followed by reconstitution into proteoliposomes. The translocation activity was determined in the presence of SecA and ATP at the indicated temperatures for 20 min, using proOmpA D26 as the substrate. (**B**) The ratio of the reconstituted activity obtained with SecG to that without SecG was estimated at each temperature.

Discussion

To examine the *in vivo* role of SecG in protein translocation in *E.coli*, we disrupted the *secG* gene. The Δ*secG* strain thus constructed was transformed with a plasmid carrying *secG* under a tightly controllable promoter. The results obtained with these cells support our proposal that p12 be named SecG.

The phenotype of the Δ*secG* strain can be summarized as follows. The deletion of *secG* was lethal for the cells in the cold. Expression of SecG rescued the cells from this cold-sensitive phenotype (Figure 2). A decrease in temperature caused precursor proteins to accumulate in the Δ*secG* cells (Figures 3 and 5). Expression of SecG enhanced the processing of the precursor proteins to the mature forms (Figure 5). The Δ*secG* cells exhibited steady-state accumulation of proOmpA at 20°C (Figure 6). The level of SecA in these cells was derepressed, whereas the levels of SecY and SecE were not affected (Figure 6). Taken together, these results indicate that SecG is a general component of the translocation machinery.

SecG, discovered in reconstitution studies, was found to be directly involved in protein translocation *in vivo*. This

indicates that reconstituted proteoliposomes accurately represent at least part of the translocation events that occur *in vivo*. On the other hand, reconstitution studies failed to demonstrate the function of SecD, which participates in a late step of protein translocation (Matsuyama *et al.*, 1993). These results indicate that SecG partipicates in an early event of protein translocation, which is correctly reproduced in proteoliposomes. It has been postulated that the protein translocation reaction involves a cold-sensitive step, possibly the step when the signal peptide is inserted into the lipid bilayer (Pogliano and Beckwith, 1993). A *secD* and *secF* deletion strain was recently reported to exhibit cold-sensitive growth (Pogliano and Beckwith, 1994). However, the secretion defect was severe even at the permissive temperature in this strain. Moreover, protein translocation into everted membrane vesicles lacking SecD and SecF was not cold-sensitive (Arkowitz and Wickner, 1994). On the other hand, both *in vivo* and *in vitro* studies revealed the importance of the SecG function for protein translocation in the cold. It seems likely, therefore, that SecG plays a role in the postulated early step of the translocation reaction.

Although extensive genetic studies revealed many components involved in protein translocation (Bieker *et al.*, 1990; Gardel *et al.*, 1990), SecG was not identified in those genetic studies. The reason for this seems to be the fact that the SecG function is dispensable at 37°C; however, a new mutation, *prlZ1*, which suppresses a signal sequence mutation, was found to be mapped to 69 min, where *secG* is located, on the *E.coli* chromosome (J.Stader, personal communication). Whether or not *prlZ* and *secG* are allelic remains to be clarified.

SecG was recently found to be identical to band 1 (K.Douville, M.Leonard, K.Nishiyama, H.Tokuda, S.Mizushima and W.Wickner, submitted), which was co-purified with SecY and SecE (Brundage *et al.*, 1990). It has been reported that the protein translocation machinery, the Sec61p complex, in the endoplasmic reticulum membrane also comprises three protein subunits (Görlich and Rapoport, 1993). The sequences of α and γ subunits exhibit some similarity to those of SecY (Görlich *et al.*, 1992) and SecE (Hartmann *et al.*, 1994), respectively. Although similarity between the β subunit and SecG is not clear at present, the fundamental structure of the machinery seems to be conserved from prokaryotes to mammals.

Detailed examinations of the function of SecG are currently under way, in membrane vesicles prepared from the Δ*secG* cells.

Materials and methods

Chemicals

n-Octyl-β-D-glucopyranoside was purchased from Dojindo Laboratories. ATP and creatine kinase were from Boehringer Mannheim, proteinase K from Merck, and IgGsorb from Enzyme Center Inc. Endonucleases, T4 DNA ligase, Klenow enzyme and SP6 RNA polymerase were obtained from Takara Shuzo Co., Ltd. Tran^{35}S-label, a mixture of 70% [^{35}S]methionine and 20% [^{35}S]cysteine, 1000 Ci/mmol, was obtained from ICN.

Bacterial strains

FS1576 (C600 *recD1009*) (Stahl *et al.*, 1986; Ogura *et al.*, 1989), and W3110 M25 (W3110 *ompT*) (Sugimura, 1988) were used. KN370 (FS1576 Δ*secG::kan*), and KN425 (W3110 M25 Δ*secG::kan*) were the deletion strains of *secG* used. The latter strain was constructed by introducing the Δ*secG::kan* alelle of KN370 into W3110 M25 by P1 transduction.

Plasmid construction

A 250 bp *Alu*I−*Apa*LI fragment, that contains codons 5−89 of SecG, a 530 bp *Apa*LI−*Bam*HI fragment of λ clone 521 (Kohara *et al.*, 1987; Noda *et al.*, 1991) (the *Bam*HI site was derived from the λ vector), that carries codons 90−110 of SecG and *leuU*, and synthetic oligonucleotides (5′-GATCCTAGGAGGTTTAAATTT*ATG*TATGAAG-3′, and 3′-GATCCTCCAAATTTAAATACATACTTC-5′), that contain the consensus SD sequences followed by the initiation codon (italicized) and codons 2−4 of SecG, were mixed and cloned into the *Bam*HI site of plasmid pUSI2 (Shibui *et al.*, 1988). pTG3 thus constructed contains *secG−leuU*, under the control of the *tac* promoter, *lacI* and *bla*. pTG3 overproduced SecG upon the addition of IPTG (data not shown), confirming that the open reading frame which we reported (Nishiyama *et al.*, 1993) really corresponds to SecG. A 810 bp *Bam*HI fragment of pTG3 carrying *secG−leuU* was then cloned into the *Bgl*II site of pKQ2 to construct pAG3. pKQ2 carries the replication origin of the low copy plasmid pSC101 (Cabello *et al.*, 1976), *bla*, *araC* and the *araBAD* promoter, which is followed by a unique *Bgl*II site (A.Honda, S.Matsuyama, S.Mizushima and H.Tokuda, unpublished observation). pAG3 thus carries *secG−leuU* under the control of the *araBAD* promoter.

pAG5, in which *leuU* was deleted, was constructed as follows. A 340 bp *Bam*HI−*Eco*RV fragment of pTG3, which contains the first 107 codons of SecG, and synthetic oligonucleotides (5′-ATCCCGAAC*TAA*AAAGGTAC-3′, and 3′-TAGGGCTTGATTTTTC-5′) which contain the last three codons and the termination codon (italicized) of SecG, were recloned into pUSI2 digested with *Bam*HI and *Kpn*I to construct pTG5 and pTG5 thus carries only *secG* under the *tac* promoter. A 380 bp *Bam*HI−*Bgl*II fragment of pTG5 carrying *secG* (the *Bgl*II site lies immediately downstream of the *Kpn*I site) was inserted into the *Bgl*II site of pKQ2 to construct pAG5. pAG5 carries only *secG* under the control of the *araBAD* promoter.

pDG41 employed for the disruption of *secG* was constructed as follows. A 1.4 kbp *Eco*RI−*Bam*HI fragment of λ clone 521 (Kohara *et al.*, 1987; Noda *et al.*, 1991) carrying *secG* and *leuU* was cloned into pBR322 to construct pGE1. Digestion of pGE1 with *Mlu*I and *Eco*RV resulted in two fragments. The small one (670 bp), which contains the first 107 codons of SecG, was further digested with *Alu*I to obtain a 360 bp *Mlu*I−*Alu*I fragment. A *kan* gene cassette (Pharmacia) digested with *Hinc*II to make the ends blunt and the 360 bp *Mlu*I−*Alu*I fragment that contains the first three codons of SecG were ligated into the large *Mlu*I−*Eco*RV fragment of pGE1. pDG1 thus constructed carries the *secGΔ4-107::kan* allele and *leuU*. The directions of the inserted *kan* gene and *leuU* were the same. A 3 kbp *Pst*I−*Pvu*II fragment of λ clone 520, which carries the downstream region of *secG*, was cloned into the *Pst*I and *Ssp*I sites of pBR322 to construct pDGP2. pDGP2 thus carries the last 12 codons of SecG and the following 3 kbp downstream region. An *Eco*RI−*Acc*I fragment of pDG1 (the *Acc*I site lies in *leuU*) carrying Δ*secG::kan* was cloned between the *Eco*RI and *Acc*I sites of pDGP2 to construct pDG21. pGB1 contains a 3 kbp *Bam*HI fragment of λ clone 521 carrying *secG*, *leuU* and 2.5 kbp of the upstream region of *secG* at the *Bam*HI site of pBR322. A 2.1 kbp *Spl*I−*Bam*HI fragment carrying the upstream region of *secG* was obtained from pGB1, and then cloned into the *Spl*I and *Bam*HI sites of pDG21 to construct pDG31. Therefore, pDG31 carries a 2.5 kbp fragment of the upstream region, and a 3.0 kbp fragment of the downstream region of *secG* around the Δ*secG::kan* allele. However, this subcloning step resulted in disruption of the *tet* gene. To regenerate the *tet* gene, a *Ssp*I−*Sph*I fragment of pBR322 was introduced into the Klenow enzyme-treated *Bam*HI−*Sph*I fragment of pDG31. The tetracycline-resistant derivative of pDG31 was called pDG41. pDG41 linearized with *Sph*I and *Bam*HI, both of which cut only once in pDG41, was used to construct a chromosomal deletion of *secG*.

Pulse−chase and immunoprecipitation

Escherichia coli cells were grown to a density of ~5×10^8 cells/ml in M63 minimal medium (Miller, 1972) supplemented with 2% glycerol and 20 μg/ml each of all amino acids except methionine and cysteine. Maltose (0.5%) was used to induce MBP. For labelling, Tran^{35}S-label was added to 20 μCi/ml. Labelling was terminated by adding non-radioactive methionine and cysteine at a concentration of 12 mM each. After the specified chase time, 1 ml of cells was withdrawn and immediately mixed with 50 μl of 100% (w/v) trichloroacetic acid on ice. The cellular proteins were recovered by centrifugation (16 000 *g*, 5 min, 4°C), and washed with acetone and then with ether. Immunoprecipitation was carried out as described (Matsuyama *et al.*, 1993).

Reconstitution of proteoliposomes and assaying of protein translocation activity

SecA (Akita *et al.*, 1990), SecE (Tokuda *et al.*, 1991), SecY (Akimaru *et al.*, 1991) and SecG (Nishiyama *et al.*, 1993) were purified as described.

Reconstitution of Sec proteins into proteoliposomes was carried out as described by Akimaru *et al.* (1991). Protein translocation activity was measured with ^{35}S-labelled proOmpA D26 (Tokuda *et al.*, 1991) as the substrate, as described by Nishiyama *et al.* (1993). The ^{35}S-labelled proOmpA D26 was synthesized *in vitro* in the presence of Tran^{35}S-label (0.46 mCi/ml) (Yamane *et al.*, 1987), and partially purified as described by Tani *et al.* (1989).

Antibodies

Anti-SecA (Akita *et al.*, 1990), anti-SecE (Matsuyama *et al.*, 1993), and anti-OmpA (Tani *et al.*, 1990) antisera raised in rabbits were used. Anti-SecY (Nishiyama *et al.*, 1991) and anti-p12 (SecG) (Nishiyama *et al.*, 1993) antisera were raised against Ser426–Arg443 of SecY and Gln95–Asn110 of SecG, respectively. The anti-β-lactamase antibody was purchased from 5 Prime→3 Prime Inc. The anti-MBP antiserum was a gift from Dr Koreaki Ito, Institute of Virus Research, Kyoto University.

SDS–PAGE and immunoblotting

SDS–PAGE was carried out to analyse SecA, SecE and SecG according to Hussain *et al.* (1980), and to analyse other proteins according to Laemmli (1970). The gels used were composed of 10% acrylamide and 0.13% *N,N'*-methylenebisacrylamide for the analysis of MBP, OmpA and β-lactamase, 12.5% acrylamide and 0.33% *N,N'*-methylenebisacrylamide for SecY, and 12.5% acrylamide and 0.27% *N,N'*-methylenebisacrylamide for SecA, SecE, SecG and proOmpA D26. Quantitative immunoblotting was carried out as described by Nishiyama *et al.* (1992) to determine the cellular contents of SecA, SecE, SecY, SecG and proOmpA. The level of each protein was determined by densitometric scanning of the immunoblot using purified protein as a standard.

Acknowledgements

We wish to thank Dr Shoji Mizushima of the Tokyo College of Pharmacy for his encouragement, Dr Shin-ichi Matsuyama of this laboratory for genetic techniques and stimulating discussions, Dr Joan Stader of University of Missouri–Kansas City for communicating data prior to publication, Dr Teru Ogura of Kumamoto University of *E.coli* FS1576, Dr Koreaki Ito of Kyoto University for the anti-MBP antiserum, Dr Yuji Kohara of the National Institute of Genetics for the λ clones, Mr Eiichi Kunioka for his technical support, and Mrs Akiko Ishii for her secretarial support. This work was supported by grants to H.T. from the Ministry of Education, Science and Culture of Japan (nos 05454620 and 05268214), and by a JSPS Fellowship for Japanese Junior Scientists to K.N.

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Received on March 22, 1994; revised on April 20, 1994