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Involvement of the DnaK-DnaJ-GrpE Chaperone Team in Protein Secretion in *Escherichia coli*

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We used depletion studies designed to further investigate the role of the DnaK, DnaJ, and GrpE heat shock proteins in the SecB-dependent and SecB-independent secretion pathways. Our previous finding that SecB-deficient strains containing the *grpE280* mutation were still secretion proficient raised the possibility that GrpE was not involved in this secretory pathway. Using depletion studies, we now demonstrate a requirement for GrpE in this pathway. In addition, depletion studies demonstrate that while DnaK, DnaJ, and GrpE are involved in the secretion of the SecB-independent proteins (alkaline phosphatase, ribose-binding protein, and β-lactamase), they are not the primary chaperones in this process.

The DnaK, DnaJ, and GrpE proteins of *Escherichia coli* are heat shock proteins (Hsps) whose synthesis is induced upon exposure to high temperature. Both the structure and function of the Hsps are well conserved. The DnaK protein of *E. coli* has 50% identity with the Hsp70 family of proteins. Several eukaryotic homologs of DnaJ have been identified, and recently a homolog of GrpE was localized in yeast mitochondria (6, 26).

DnaK, DnaJ, and GrpE are involved in a large variety of cellular processes, including folding of nascent proteins (12, 43), DNA and RNA synthesis (7, 17), ribosome assembly (2), regulation of the heat shock response (30, 39), proteolysis (20, 37, 41), and λ, F, and P1 replication (16, 19, 44). The molecular role of each of these proteins has been investigated. DnaK binds to substrate molecules to influence the folding pathway, most generally by preventing off-pathway reaction but also possibly by stabilizing certain folding intermediates. DnaK has a weak ATPase activity, which is important in chaperone function. Both DnaJ and GrpE accelerate the ATPase cycle of DnaK, the former by promoting nucleotide hydrolysis and the latter by promoting nucleotide release. In addition, DnaJ binds independently to some substrates and may alter the substrate binding properties of DnaK (1, 10, 45).

There is a great deal of evidence, particularly in eukaryotes, that the cell exploits the chaperone function of the Hsps to facilitate transport of molecules across membranes. Members of the Hsp70 family maintain proteins destined for secretion in a partially folded state thought to be a collapsed folding intermediate or a "molten globule" (15), which renders them competent for translocation into the mitochondrion, through the endoplasmic reticulum, or into other cellular compartments (14, 35, 42). Likewise, DnaK and DnaJ participate in protein secretion in *E. coli* cells. In this organism, SecB is a primary chaperone involved in secretion of many periplasmic and outer membrane proteins (23). In wild-type *secB*$^+$ cells, mutations in *dnaK*, *dnaJ*, and *grpE* do not alter the secretion of SecB-dependent proteins. However, in *secB*-deficient strains, DnaK and DnaJ play a major role in secretion. In the absence of SecB, export of SecB-dependent proteins is not arrested but proceeds at a much slower rate. DnaK and DnaJ are required for this residual processing, and overproduction of DnaK and DnaJ accelerates this reaction (46, 48). Moreover, the joint overproduction of DnaK and DnaJ suppresses the growth defect of SecB-deficient strains on rich medium (24, 46). Two additional roles have been identified for DnaK in secretion. First, DnaK is involved in the secretion of β-galactosidase hybrid proteins across the cell membrane (33). Second, two *dnaK* mutant strains are defective in export of alkaline phosphatase, a SecB-independent protein, suggesting that DnaK might be a primary chaperone for this pathway (46).

In the present investigation, we used depletion studies to further examine the role of DnaK, DnaJ, and GrpE in secretion. These studies implicate GrpE in the residual secretion occurring in SecB-deficient strains. In addition, they demonstrate that DnaK, DnaJ, and GrpE participate in the secretion of three SecB-independent proteins: alkaline phosphatase (AP), ribose-binding protein (RBP), and β-lactamase (Bla). However, they are not the primary chaperones involved in the secretion of any of these proteins. We suggest that secretion of these proteins involves multiple, functionally redundant chaperones.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genetic methods. All strains used are derivatives of MC1061 (8). The original *secB*::Tn*5* insertion was MM152 (24). Strains used in experiments on AP processing contain the *phoR69* mutation, which causes constitutive synthesis of AP. The following strains were used: CAG12422 (*secB*::Tn*5* Δ*grpE* pPJR14), CAG12424 (*secB*$^+$ Δ*grpE* pPJR14), CAG13469 (*secB*::Tn*5* pB10a), CAG13522 (*secB*::Tn*5* Δ*dnaKJ thr*::Tn*10* pAKL1), CAG13582 (*phoR69 proC*::Tn*10*Kan); CAG13597 (Δ*dnaKJ phoR69*-Tn*10* pAKL1), CAG13617 (Δ*dnaKJ* Δ*grpE phoR69*-Tn*10* pAKL1 pPJR15), CAG13619 (Δ*grpE phoR69*-Tn*10* pPJR14), CAG13642 (*secB*::Tn*5* pPJR16), and CAG13633 (*secB*::Tn*5* Δ*grpE* pPJR15). A chromosomal *grpE* deletion was obtained by using a marker exchange strategy as described previously (3). Chromosomal *grpE* was replaced with a ΩCam^r cassette from pHP45ΩCam^r.

Cells were grown at 30°C in rich Luria-Bertani medium or in M9 minimal medium (32) supplemented with 0.5% glycerol or 0.2% glucose and all amino acids except L-methionine and L-cysteine. When indicated, minimal medium was supplemented with 0.2% maltose and/or 0.2 mM isopropylthiogalactopyranoside (IPTG). Antibiotics were added at the following concentrations: ampicillin, 50 mg/ml; kanamycin, 50 mg/ml; tetracycline, 50 mg/ml; and chloramphenicol, 10 mg/ml. Transformations and P1 transductions were performed as described previously (37).

Plasmids. pB10 is a pBR322 derivative containing Amp^r, *lacI*^{q}, and the IPTG-inducible *lacUV5* promoter. It was used for construction of pNRK416, pAKL1, pPJR14, and pPJR16. pNRK416 carries *dnaK* fused to the *lacUV5* promoter (*PlacUV5*) and was kindly sent to us by T. Yura. Plasmid pAKL1 plasmid (47) carries the *dnaK* and *dnaJ* structural genes fused to *PlacUV5*.

To construct pPJR14, pDA1 DNA was digested with *Bsm*I and blunt ended

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with T4 DNA polymerase. The 656-bp *Bsm*I fragment, containing the entire *grpE* coding sequence and its ribosome-binding site, was ligated to *Xho*I-linearized and T4 DNA polymerase blunt-ended pB10a. Transformants of strain CAG9253 carrying the *grpE280* mutation were screened for the ability to grow at 42°C in the presence but not the absence of IPTG. Plasmid DNA was isolated from several candidates and restricted with *Hin*dIII, to verify the presence of a 495-bp fragment that contains 84% of the *grpE* coding sequence. The *grpE280* mutation (Gly-122→Asp) was verified by sequencing. Plasmid pPJR15 is a version of pPJR14 containing the p15A replicon and Tet^r and Spc^r drug markers. It was obtained by cloning a *Bsm*I-*Nde*I fragment after T4 DNA polymerase treatment into *Pvu*II-digested pPJR10. Spc^r CAG9253 (*grpE280 tyr*::Tn*10*) transformants were screened for IPTG-dependent viability at 42°C. Plasmid pPJR16 is identical to pPJR14 except that it encodes wild-type *grpE*. Its construction strategy differed from that for pPJR14 in two respects. First, the GrpE-encoding sequence was subcloned from λ*grpE22* DNA on a *Bsm*HI fragment. Second, products of ligation were transformed into a strain having a $grpE^+$ wild-type gene. Plasmid DNA was extracted from several transformants, retransformed into a *grpE280* mutant, and tested for growth at 42°C in the presence or absence of IPTG. Unlike pPJR14, pPJR16 complements the temperature-sensitive growth of *grpE280* even in the absence of IPTG. The wild-type *grpE* sequence on pPJR16 was confirmed by sequencing. Plasmid pPJR9 was used in a marker exchange strategy to delete *grpE* from the chromosome and insert a Cam^r marker in its place. pPJR9 is identical to pDA4, and the cloning strategy has been described elsewhere (3).

Creating a conditional deficiency of GrpE or DnaK and DnaJ. Strains to be depleted for GrpE or DnaK and DnaJ were grown at 30°C to an A_{450} of 0.2 in minimal medium containing the appropriate supplements and 0.2 mM IPTG. To shut off the plasmid-encoded synthesis of GrpE or DnaK and DnaJ proteins, IPTG was removed by filtering and washing cells as described previously (46). Washed cells were resuspended in prewarmed IPTG-free medium and grown for three doublings to dilute GrpE or DnaK and DnaJ synthesized before IPTG removal.

Labeling and immunoprecipitation. One-milliliter samples of cells growing exponentially at 30°C in minimal medium were pulse-labeled with 40 μCi of Tran^{35}S-Label (1,000 Ci/mmol; ICN) per ml, chased with a nonradioactive mixture of L-methionine and L-cysteine (200 mg/ml), and precipitated with trichloroacetic acid as described previously (13). Specific proteins were detected on autoradiograms after immunoprecipitation with antibodies and resolution by electrophoresis on sodium dodecyl sulfate–10% polyacrylamide gels. Radioactivity was quantified with an Ambis radioanalytic imaging system (AMBIS Systems, San Diego, Calif.) interfaced with an IBM computer. The level of GrpE was determined by Western blot (immunoblot) analysis as described previously for DnaK (40).

RESULTS

GrpE is required for growth and secretion in strains lacking SecB. Our previous studies indicated that both DnaK and DnaJ were required for viability and secretion in a *secB* null mutant strain. This conclusion was based on the findings that some *dnaK* mutant alleles could not be transduced into a *secB* null mutant strain and that growth and secretion of a *secB* null mutant strain ceased upon depletion of DnaK and DnaJ (46). In contrast, a *grpE280 secB* double mutant strain was viable and did not have an enhanced secretion deficiency, raising the possibility that GrpE function was not necessary in this strain. However, the *grpE280* allele must retain some function, since strains lacking GrpE are inviable. Lack of a $GrpE^-$ phenotype in secretion could simply indicate that sufficient GrpE was present in the mutant strain to carry out function.

To further pursue this possibility, we constructed a strain in which we could create a conditional deficiency of GrpE. In this strain, the chromosomal *grpE* gene is deleted and all synthesis of GrpE is directed from a plasmid, pPJR14, whose synthesis of GrpE280 is driven by *PlacUV5*. In the presence of IPTG, synthesis from *PlacUV5* is induced, whereas upon its removal, most *PlacUV5*-directed synthesis is shut off. The Δ*grpE* strain carrying pPJR14 is viable only in the presence of IPTG, indicating that the uninduced level of expression of *grpE280* is insufficient for function. Thus, plasmid pPJR14 carrying *PlacUV5-grpE280* could be used for creating a conditional deficiency of GrpE. In contrast, a similar plasmid construct carrying wild-type *grpE* was able to complement the $GrpE^-$ phenotype even in the absence of IPTG and thus could not be used

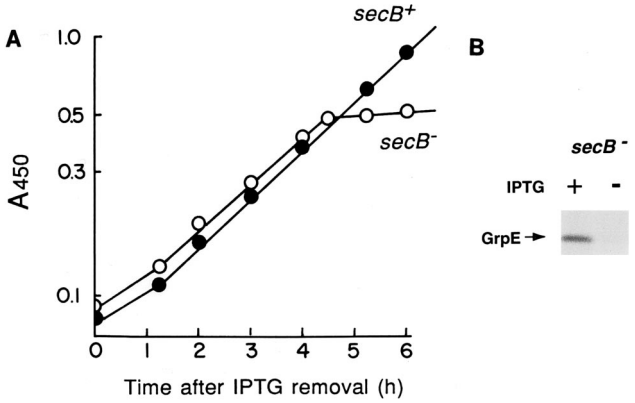

FIG. 1. Effect of GrpE depletion on growth of the *secB* null and *secB* wild-type strains. (A) Growth curves of strains CAG12422 (*secB*::Tn*5* Δ*grpE* pPJR14) (○) and CAG12424 ($secB^+$ Δ*grpE* pPJR14) (●) grown at 30°C in minimal medium supplemented with glycerol, maltose, ampicillin, and IPTG to induce transcription of *grpE280* from *PlacUV5* carried on pPJR14. At an A_{450} of 0.2 mM, IPTG was removed as described in Materials and Methods and cells were resuspended in a fresh medium lacking IPTG (time zero) for further growth. (B) Synthesis of GrpE is shut off after IPTG removal. Cells were grown as described above. Samples were withdrawn before IPTG removal (+) and at three doublings of growth after IPTG removal (−). To detect the GrpE protein, samples of cell extracts were immunoprecipitated with an anti-GrpE antibody.

to create a GrpE deficiency. The fact that GrpE280 is about 10-fold less active than wild-type GrpE (37a) probably explains this difference in phenotype.

To determine whether *secB* strains have an enhanced requirement for GrpE, we compared the growth of a Δ*grpE secB* null mutant strain with that of the isogenic Δ*grpE* $secB^+$ strain after cessation of GrpE280 synthesis from pPJR14. After three doublings of growth in the absence of IPTG, growth of the Δ*grpE secB* null mutant was arrested, whereas that of the Δ*grpE* $secB^+$ strain continued for at least six doublings (Fig. 1A). Eventually growth of the $secB^+$ strain was also arrested, as colony formation requires IPTG. Immunoprecipitation experiments confirmed that synthesis of GrpE was very low in the absence of IPTG (Fig. 1B). The more severe effect of depleting GrpE from the *secB* null mutant strain than from $secB^+$ wild-type strain indicates an additional role for GrpE in strains lacking SecB.

The enhanced requirement for GrpE in *secB* null strains most likely involves protein secretion. We therefore examined whether depletion of GrpE (by removing IPTG) impaired secretion. As an indicator of secretion, we examined the processing of two SecB-dependent proteins, pre-LamB and pre-maltose-binding protein (pre-MBP), whose processing had been previously determined to be dependent on DnaK and DnaJ. Whereas depletion of GrpE in a $secB^+$ strain had no effect on pre-LamB and pre-MBP processing, it had profound effects in the *secB* strain (Fig. 2). Retardation of pre-LamB and pre-MBP processing was already visible one doubling after IPTG removal, and by three doublings, no processing of LamB and MBP was evident during a 5-min chase (Fig. 2). Even when the chase time was increased to 20 min, essentially no processing of either LamB or MBP occurred (Fig. 3). Thus, the residual secretion of LamB or MBP observed in *secB* deficient strains is absolutely dependent on GrpE. GrpE together with DnaK and DnaJ provides the chaperone function necessary for secretion of these proteins in the absence of SecB.

OmpA is another SecB-dependent protein. Our previous studies had indicated that depletion of DnaK and DnaJ had only a minor effect on OmpA processing in *secB* strains. How-

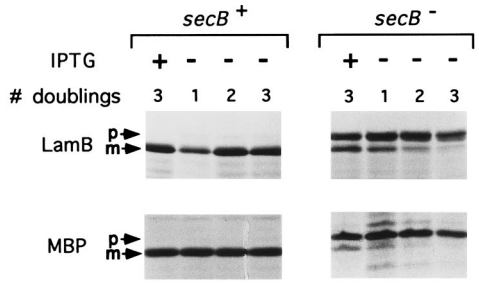

FIG. 2. Signal sequence processing of pre-LamB and pre-MBP following GrpE depletion. The *secB*::Tn*10* (CAG12422) and *secB*$^+$ (CAG12424) strains in which all of the synthesis of GrpE is directed from the IPTG-inducible *PlacUV5* were grown in minimal medium supplemented with glycerol, maltose, ampicillin, and IPTG. After IPTG removal (see Materials and Methods), cells were resuspended in fresh medium lacking IPTG and grown at 30°C. Cell samples withdrawn at time corresponding to the indicated number of growth doublings were labeled with Tran^{35}S-Label for 30 s and chased for 5 min with nonradioactive amino acids. To detect precursor (p) and mature (m) forms of LamB and MBP proteins, samples of cell extracts were immunoprecipitated with a specific antibody and electrophoresed.

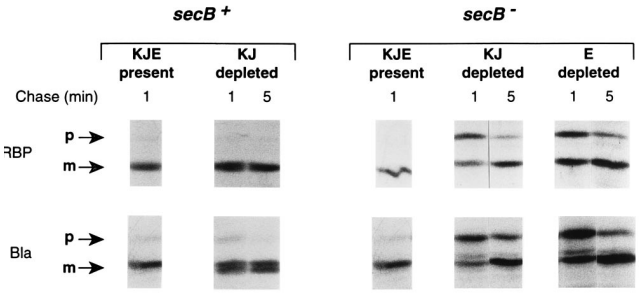

FIG. 4. Processing of pre-RBP and pre-Bla is retarded upon depletion of DnaK and DnaJ or GrpE. Strains CAG13533 (*secB*$^+$ Δ*dnaKJ* pAKL1), CAG13522 (*secB*::Tn*5* Δ*dnaKJ* pAKL1), and CAG12422 (*secB*::Tn*5* Δ*grpE* pPJR14) were grown in minimal medium containing glycerol and ampicillin according to the protocol for creating conditional deficiency of DnaK and DnaJ or GrpE (see Materials and Methods). In the control cultures (DnaK, DnaJ, and GrpE present), 0.2 mM IPTG was added to the growth medium to induce the synthesis of DnaK and DnaJ. After three doublings of growth, cells were labeled for 30 s, chased with nonradioactive amino acids for the time periods indicated, and processed to detect precursor (p) and mature (m) forms of RBP or Bla.

ever, those studies used a 5-min chase time. As a more sensitive test, we examined the kinetics of OmpA processing following GrpE depletion in a *secB* null strain. These studies clearly indicate that OmpA processing is retarded upon GrpE depletion (Fig. 3). Similar effects on DnaK and DnaJ depletion were seen (data not shown). Although DnaK, DnaJ, and GrpE are involved in the residual processing of OmpA in *secB* strains, clearly additional chaperones also facilitate secretion of this protein.

Overproduction of GrpE does not suppress SecB phenotypes. We previously showed that many of the phenotypes of a *secB* null mutation, including the inability to grow on rich medium and retardation of preprotein processing, were suppressed by overproduction of the DnaK and DnaJ Hsps (46). We examined whether overproduction of GrpE also suppresses SecB phenotypes. To achieve overproduction, we used a *secB* null strain that retained the chromosomal copy of the *grpE* gene and also carried plasmid pPJR16, which allows IPTG-inducible synthesis of wild-type GrpE. In contrast to DnaK-DnaJ overproduction, an increased amount of GrpE neither permitted growth of *secB* strains on rich medium nor improved the rate of pre-MBP processing (data not shown). Thus, overproduction of GrpE alone is not sufficient to compensate for a SecB deficiency.

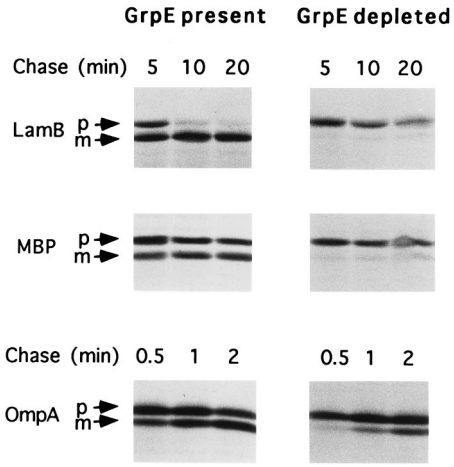

FIG. 3. Kinetics of signal sequence processing of SecB-dependent LamB, MBP, and OmpA secretory proteins following GrpE depletion. Strains CAG13469 (*secB*::Tn*5* *grpE*$^+$ pB10a) and CAG12422 (*secB*::Tn*5* Δ*grpE* pPJR14) were grown in minimal medium containing glycerol, maltose, and ampicillin but lacking IPTG to create a deficiency of GrpE (see Materials and Methods). At three doublings of growth following IPTG removal, cells were labeled for 30 s and chased for the indicated time periods. Precursor (p) and mature (m) forms of proteins were detected following immunoprecipitation with a specific antibody and electrophoresis.

PrlA and PrlF do not relieve the export block imposed by the GrpE deficiency. The *prl* suppressor mutations were identified because they allow proteins with defective signal sequences to be secreted (9). Bieker-Brady and Silhavy (5) have used some of the *prl* mutations as well as the overproduction of their respective wild-type products to try to order the secretion process. The *prlA666* suppressor improves secretion of signal sequence-defective pre-MBP. Overproduction of either PrlA (SecY) or PrlF (21) relieved the secretion block caused by synthesis of the LamB-LacZ hybrid protein, which is believed to occur after the hybrid protein enters the membrane, and affects a late step in secretion, occurring just prior to signal sequence cleavage. We examined whether the same conditions could ameliorate the secretion block imposed by the GrpE depletion. We examined the processing of MBP following GrpE depletion in strains overproducing PrlF or PrlA or carrying the *prlA666* allele. None of these conditions relieved the secretion defect caused by the GrpE deficiency (data not shown).

DnaK, DnaJ, and GrpE are involved in the export of RBP and Bla. RBP and Bla secretion still occurs rapidly in a *secB* null mutant strain, suggesting that their secretion might be SecB independent. To determine whether the DnaK, DnaJ, and GrpE Hsps play a role in RBP secretion, we previously measured pre-RBP processing in *dnaK*, *dnaJ*, and *grpE* mutants. None of these mutations affected RBP export (46). However, all these mutants retained some activity, since they grew well at permissive temperature. To examine this question more rigorously, we monitored pre-RBP and pre-Bla processing after depletion of DnaK, DnaJ, or GrpE (Fig. 4). As expected, depletion of DnaK-DnaJ or GrpE in a *secB*$^+$ strain did not have a pronounced effect on processing of these preproteins. However, in the *secB* null strain, pre-RBP and pre-Bla were still present even after a 5-min chase. The fact that both chaperone systems must be inactivated in order to observe a significant secretion defect suggests that SecB and the DnaK-DnaJ-

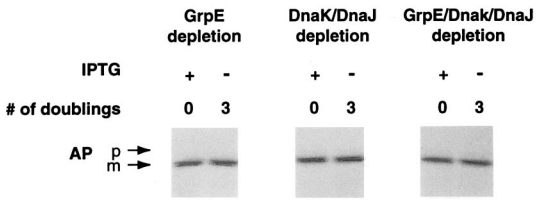

FIG. 5. Depletion of DnaK and DnaJ or GrpE has no effect on pre-AP processing. Strains CAG13597 (Δ*dnaKJ phoR69*-Tn*10* pAKL1), CAG13619 (Δ*grpE phoR69*-Tn*10* pPRJ14), and CAG13617 (Δ*dnaKJ* Δ*grpE phoR69*-Tn*10* pAKL1 pPJR15) were grown in minimal medium supplemented with glucose and ampicillin. To create a deficiency of DnaK and DnaJ or GrpE (see Materials and Methods), cells were grown in medium lacking IPTG. To provide synthesis of DnaK and DnaJ or GrpE, 0.2 mM IPTG was added to the growth medium. Cell samples were labeled for 30 s, chased for 30 s with nonradioactive amino acids, and processed to detect precursor (p) and mature (m) forms of AP.

GrpE chaperone group are functionally redundant for secretion of these two proteins.

Roles of DnaK, DnaJ, and GrpE in AP secretion. Periplasmic AP is believed to be exported predominantly independently of SecB (11, 24), although some dependence on SecB function can be demonstrated under particular growth conditions (25). The fact that some *dnaK* and *dnaJ* mutations decreased the rate of pre-AP processing led us to suggest that DnaK and DnaJ might be the primary chaperone group involved in AP secretion (46). To further examine this point, we monitored AP processing after depletion of DnaK and DnaJ or GrpE. These cells contained the *phoR69* allele to ensure a high level of AP synthesis. Surprisingly, depleting either DnaK-DnaJ or GrpE did not delay AP processing (Fig. 5). These data suggest that DnaK, DnaJ, and GrpE do not constitute the primary chaperone for AP. We tried to determine whether depletion of SecB together with DnaK-DnaJ-GrpE might be synergistic as was the case for RBP and Bla processing. However, we were unable to construct a *phoR69 secB* strain in which the chromosomal copy of either *grpE* or *dnaK-dnaJ* was deleted, even when these strains carried plasmids producing appropriate proteins to cover the deletions. The primary transductants grew very slowly and exhibited a plate phenotype indicating that AP synthesis (presumably transport) was impaired. These transductants could not be studied because they rapidly reverted to fast-growing cells that were proficient in synthesis of AP.

DISCUSSION

This report extends our understanding of the role of chaperones in protein secretion in *E. coli*. We show that GrpE, as well as DnaK and DnaJ (46), is required for both the residual secretion and survival of SecB-deficient strains. In addition, our examination of the role of the DnaK-DnaJ-GrpE chaperone machine in the secretion of a variety of proteins has convinced us that many preproteins utilize multiple, functionally redundant chaperones, including DnaK, DnaJ, and GrpE, to facilitate their export.

Role of GrpE in SecB-deficient strains. By creating strains that exhibit a conditional deficiency of GrpE, we have been able to determine whether GrpE functions along with DnaK and DnaJ in the secretion process. Upon depletion of GrpE, SecB-deficient strains cease growth and LamB and MBP processing (Fig. 1 and 2). The fact that this phenotype is equivalent to that observed upon DnaK and DnaJ depletion indicates that GrpE is required along with DnaK and DnaJ for this chaperone team to function in protein secretion. Our previous observation that *secB grpE280* cells are viable and secretion competent most likely indicates that the residual GrpE activity in this mutant strain is sufficient to promote secretion. In contrast to this congruence of phenotypes upon chaperone depletion, overproduction of chaperones does not give equivalent phenotypes. Whereas overproduction of DnaK and DnaJ partially compensates for SecB, overproduction of GrpE does not.

GrpE is a nucleotide exchange factor that increases release of ADP bound to DnaK (31). The critical role of GrpE in protein export might be to promote release of the precursor protein from the complex with DnaK by exchanging bound ADP. Lack of GrpE may lead to the accumulation of precursor proteins that are unable to dissociate from the ternary complex with DnaK-DnaJ. Indeed, upon GrpE depletion in *secB* null strains, LamB and MBP are found exclusively in their precursor forms and could be bound to DnaK (Fig. 2). From this point of view, it is not surprising that overproduction of GrpE neither restores growth on rich medium nor accelerates processing of LamB and MBP in *secB* null mutant strains. As long as there is sufficient GrpE to function as a catalytic cofactor to recycle DnaK, overproduction of GrpE will not further increase the function of the DnaK-DnaJ-GrpE chaperone machine and therefore will not rescue additional SecB− phenotypes. In contrast, overproduction of DnaK and DnaJ will increase the chaperone potential of the cell and therefore provide additional compensation for the lack of SecB.

It is currently not known where the GrpE-mediated block in translocation is manifested. Preproteins could simply remain in the cytoplasm, without interacting with other members of the secretion apparatus. Alternatively, the precursor proteins could begin an interaction with the secretory apparatus but not be able to successfully complete transit of the cell membrane. Our results favor the idea that the block is imposed before entry into the membrane. First, physical jamming of the translocation apparatus should result in blockage of all secreted proteins that use the same pathway. This was not the case. For example, a combined deficiency of SecB and GrpE had a very small effect on OmpA secretion. Second, overproduction of SecY or PrlA did not ameliorate the secretion block in a GrpE-depleted strain. Overproduction of SecY or PrlA protein has suppressed secretion defects resulting from jamming of the secretory apparatus (4). Both results would be explained if DnaK, DnaJ, and GrpE, like SecB, interact with cytoplasmic preproteins. In the absence of correct interaction, these preproteins would not make appropriate contact with SecA to initiate translocation.

Multiple chaperones are involved in protein translocation. Three of secreted proteins that we examined, RBP, Bla, and AP, are generally considered to be secreted independently of the SecB protein. There has been a question as to whether these proteins require a chaperone and, if so, which chaperone is utilized. For these proteins, we find that several different chaperones must be depleted before an effect on secretion is observed. These results, together with other results in the literature, suggest that each of these preproteins utilizes multiple, functionally redundant chaperones in the secretion process.

Our results suggest that secretion of Bla and RBP involves either SecB or the DnaK-DnaJ-GrpE chaperone machine, such that either alone efficiently promotes translocation. Eliminating either SecB or depleting only the DnaK chaperone complex has very marginal effects, if any, on the processing kinetics of Bla or RBP. However, limiting simultaneously for SecB and the DnaK-DnaJ-GrpE chaperone complex significantly decreases the rate of RBP and Bla processing so that

even after a 5-min chase, a significant amount of the appropriate preprotein has not been processed (Fig. 5). This is the expected result if the two systems are functionally redundant. In this regard, it is interesting that a mutant RBP with a defective signal sequence is dependent on SecB function whereas wild-type RBP is not (22). Possibly, the mutant protein is no longer a substrate for the non-SecB chaperone pathways.

It has previously been demonstrated that Bla requires the GroEL-GroES chaperone machine for its secretion (25, 27). One role of the GroEL-GroES chaperone machine could be to complete the transfer from the DnaK chaperone machine to the secretory apparatus. The cooperation of these DnaK-DnaJ-GrpE and GroEL-GroES chaperone machines in sequential protein folding reactions has recently been demonstrated for rhodanese folding (28). However, the fact that mutations in GroEL or GroES have a more severe effect on Bla secretion than does DnaK depletion suggests an additional role for GroEL in this process. The GroEL-GroES chaperone machine may itself function independently as a chaperone for Bla, it may function in conjunction with SecB as well as DnaK-DnaJ in a sequential transfer process, or it may work with other, yet unidentified chaperones.

It is very likely that AP secretion utilizes multiple chaperones. Several different chaperones have previously been implicated in AP secretion. SecB null mutant strains are defective for AP secretion at low temperatures but not at high temperatures (25). In addition, depletion of Ffh has a small but demonstrable effect on AP secretion (34). Finally, certain *dnaK* mutants had a very deleterious effect on AP secretion, leading us to suspect that the DnaK chaperone machine had a primary role in AP secretion. However, depletion experiments do not bear out that hypothesis, as there is little effect on AP secretion after depletion either of DnaK and DnaJ or GrpE. Given these results, we suspected that SecB, the DnaK chaperone machine, and Ffh might all be involved in AP secretion (46). However, we were unable to make the appropriate strains to simultaneously restrict for either Ffh or SecB and the DnaK-DnaJ-GrpE chaperone machine when the cells were synthesizing the high levels of AP necessary to perform the secretion measurements. Our inability to construct stable strains suggests that when two of these three chaperones are restricted, cytoplasmic AP accumulates, which is toxic to the cell. Thus, all three of these chaperones probably participate in AP secretion.

Our results suggest that the cell contains two classes of secreted proteins: those dedicated to the SecB chaperone and those utilizing a wide range of functionally redundant chaperones. In the latter case, more than one chaperone must be depleted before an effect on secretion is observed. In this regard, it will be interesting to reexamine the in vivo role of trigger factor (29). Trigger factor was initially identified in vitro as a chaperone involved in OmpA secretion and more recently has been identified as a ribosome-associated peptidylprolyl isomerase (38) and implicated in proteolysis (18). However, it has never been shown to perform a chaperone role in vivo. Simultaneous depletion for multiple chaperones may reveal a chaperone role for trigger factor as well as for other, yet unidentified chaperones in the cell.

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REFERENCES

1. **Alfano, C., and R. McMacken.** 1989. Ordered assembly of nucleoprotein structures at the bacteriophage λ replication origin during the initiation of DNA replication. J. Biol. Chem. **264:**10699–10708.
2. **Alix, J.-H., and M.-F. Guerin.** 1993. Mutant DnaK chaperons cause ribosome assembly defects in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **90:** 9725–9729.
3. **Ang, D., and C. Georgopoulos.** 1989. The heat-shock-regulated *grpE* gene of *Escherichia coli* is required for bacterial growth at all temperatures but is dispensable in certain mutant backgrounds. J. Bacteriol. **171:**2748–2755.
4. **Bieker, K. L., G. J. Phillips, and T. J. Silhavy.** 1990. The *sec* and *prl* genes of *Escherichia coli*. J. Bioenerg. Biomembr. **22:**291–310.
5. **Bieker-Brady, K., and T. J. Silhavy.** 1992. Suppressor analysis suggests a multistep, cyclic mechanism for protein secretion in *Escherichia coli*. EMBO J. **11:**3165–3174.
6. **Bolliger, L., O. Deliche, B. S. Glick, C. Georgopoulos, P. Jeno, N. Kronidou, M. Horst, N. Morishima, and G. Schatz.** 1994. A mitochondrial homolog of bacterial GrpE interacts with mitochondrial hsp70 and is essential for viability. EMBO J. **13:**1998–2006.
7. **Bukau, B., and G. C. Walker.** 1989. Δ*dnaK52* mutants of *Escherichia coli* have defects in chromosome segregation and plasmid maintenance at normal growth temperatures. J. Bacteriol. **171:**6030–6038.
8. **Casadaban, M., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
9. **Emr, S. D., S. Hanley-Way, and T. J. Silhavy.** 1981. Suppressor mutations that restore export of a protein with a defective signal peptide. Cell **23:**79–88.
10. **Gamer, J., H. Bujard, and B. Bukau.** 1992. Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor σ^{32}. Cell **69:**833–842.
11. **Gannon, P. M., P. Li, and C. A. Kumamoto.** 1989. The mature portion of *Escherichia coli* maltose-binding protein (MBP) determines the dependence of MBP on SecB for export. J. Bacteriol. **171:**813–818.
12. **Gragerov, A. E. N., N. Komissarova, G. A. Gaitanaris, M. E. Gottesman, and V. Nikiforov.** 1992. Cooperation of GroEL/GroES and DnaK/DnaJ heat shock proteins in preventing misfolding in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **89:**10341–10344.
13. **Grossman, A. D., D. B. Straus, W. A. Walter, and C. A. Gross.** 1987. σ^{32} synthesis can regulate the synthesis of heat shock proteins in *Escherichia coli*. Genes Dev. **1:**179–184.
14. **Hannavy, K., S. Rospert, and G. Schatz.** 1993. Protein import into mitochondria: a paradigm for the translocation of polypeptides across membranes. Curr. Opin. Cell Biol. **5:**694–700.
15. **Hendrick, P. J., and F.-U. Hartl.** 1993. Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem. **62:**349–384.
16. **Hoffman, H. J., S. K. Lyman, C. Lu, M.-A. Petit, and H. Echols.** 1992. Activity of the Hsp70 chaperone complex—DnaK, DnaJ, and GrpE—in initiating phage λ DNA replication by sequestering and releasing λ P protein. Proc. Natl. Acad. Sci. USA **89:**12108–12111.
17. **Hwang, D. S., E. Crooke, and A. Kornberg.** 1990. Aggregated *dnaA* protein is dissociated and activated for DNA replication by phospholipase or *dnaK* protein. J. Biol. Chem. **265:**19244–19248.
18. **Kandror, O., M. Sherman, M. Rhode, and A. L. Goldberg.** 1995. Trigger factor is involved in *groEL*-dependent protein degradation in *Escherichia coli* and promotes binding of GroEL to unfolded proteins. EMBO J. **14:**6021–6027.
19. **Kawasaki, Y., C. Wada, and T. Yura.** 1990. Roles of *Escherichia coli* heat shock proteins DnaK, DnaJ and GrpE in mini-F replication. Mol. Gen. Genet. **220:**277–282.
20. **Keller, J. A., and L. D. Simon.** 1988. Divergent effects of a *dnaK* mutation on abnormal protein degradation in *Escherichia coli*. Mol. Microbiol. **2:**31–41.
21. **Kino, D. R., G. J. Phillips, and T. J. Silhavy.** 1990. Increased expression of the bifunctional protein PrlF suppresses overproduction lethality associated with exported β-galactosidase hybrid proteins in *Escherichia coli*. J. Bacteriol. **172:**185–192.
22. **Kim, J., Y. Lee, C. H. Kim, and C. H. Park.** 1992. Involvement of SecB, a chaperone, in the export of ribose-binding protein. J. Bacteriol. **174:**5219–5227.
23. **Kumamoto, C. A.** 1989. *Escherichia coli* SecB protein associates with exported protein precursors in vivo. Proc. Natl. Acad. Sci. USA **86:**5320–5324.
24. **Kumamoto, C. A., and J. Beckwith.** 1985. Evidence for specificity at an early step in protein export in *Escherichia coli*. J. Bacteriol. **163:**267–274.
25. **Kusukawa, N., T. Yura, C. Ueguchi, Y. Akiyama, and K. Ito.** 1989. Effects of mutations in heat-shock genes *groES* and *groEL* on protein export in *Escherichia coli*. EMBO J. **8:**35–3521.
26. **Laloraya, S., B. D. Gambill, and E. A. Craig.** 1994. A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation. Proc. Natl. Acad. Sci. USA **91:**6481–6485.
27. **Laminet, A. A., T. Ziegelhoffer, C. Georgopoulos, and A. Plückthun.** 1990. The *Escherichia coli* heat shock proteins GroEL and GroES modulate folding of the β-lactamase precursor. EMBO J. **9:**2315–2319.
28. **Langer, T., C. Z. Lu, H. Echols, J. Flanagan, M. K. Hayer, and F. U. Hartl.** 1992. Successive action of DnaK, DnaJ and GroEL along the pathway of

chaperone-mediated protein folding. Nature (London) **356:**683–689.
29. **Lecker, S., R. Lill, T. Ziegelhoffer, C. Georgopoulos, P. J. Bassford, Jr., C. A. Kumamoto, and W. Wickner.** 1989. Three pure chaperone proteins in *Escherichia coli*—SecB, trigger factor and GroEL—form soluble complexes with precursor proteins in vitro. EMBO J. **8:**2703–2709.
30. **Liberek, K., T. P. Galitski, M. Zylicz, and C. Georgopoulos.** 1992. The DnaK chaperone modulates the heat shock response of *Escherichia coli* by binding to the σ^{32} transcription factor. Proc. Natl. Acad. Sci. USA **89:**3516–3520.
31. **Liberek, K., J. Marszalek, D. Ang, C. Georgopoulos, and M. Zylicz.** 1991. *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc. Natl. Acad. Sci. USA **88:**2874–2878.
32. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Phillips, G. J., and T. J. Silhavy.** 1990. Heat-shock proteins DnaK and GroEL facilitate export of LacZ hybrid proteins in *E. coli*. Nature (London) **344:**882–884.
34. **Phillips, G. J., and T. J. Silhavy.** 1992. The *E. coli ffh* gene is necessary for viability and efficient protein export. Nature (London) **359:**744–746.
35. **Sanders, S., and R. Schekman.** 1992. Polypeptide translocation across the endoplasmic reticulum membrane. J. Biol. Chem. **267:**13791–13794.
36. **Sherman, M. Y., and A. L. Goldberg.** 1992. Involvement of the chaperonin *dnaK* in the rapid degradation of a mutant protein in *E. coli*. EMBO J. **11:**71–77.
37. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37a. **Skowyra, D.** Personal communication.
38. **Stoller, G., K. P. Rucknagel, K. H. Nierhaus, F. X. Schmid, G. Fischer, and J. U. Rahfeld.** 1995. A ribosome-associated peptidyl-prolyl cis/trans isomerase identified as the trigger factor. EMBO J. **14:**4939–4948.
39. **Straus, D., W. Walter, and C. A. Gross.** 1990. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis of σ^{32}. Genes Dev. **4:**2202–2209.
40. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1987. The heat shock response of *E. coli* is regulated by changes in the concentration of σ^{32}. Nature (London) **329:**348–351.
41. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1988. *Escherichia coli* heat shock gene mutants are defective in proteolysis. Genes Dev. **2:**1851–1858.
42. **Stuart, R. A., D. M. Cyr, E. A. Craig, and W. Neupert.** 1994. Mitochondrial molecular chaperones: their role in protein translocation. Trends Biochem. Sci. **19:**87–92.
43. **Szabo, A., T. Langer, H. Schroder, J. Flanagan, B. Bukau, and F. U. Hartl.** 1994. The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system-DnaK, DnaJ, and GrpE. Proc. Natl. Acad. Sci. USA **91:**10345–10349.
44. **Wickner, S., D. Skowyra, J. Hoskins, and K. McKenney.** 1992. DnaJ, DnaK, and GrpE heat shock proteins are required in *ori*P1 DNA replication solely at the RepA monomerization step. Proc. Natl. Acad. Sci. USA **89:**10345–10349.
45. **Wickner, S. H.** 1990. Three *Escherichia coli* heat shock proteins are required for P1 plasmid replication: formation of an active complex between *E. coli* DnaJ protein and the P1 initiator protein. Proc. Natl. Acad. Sci. USA **87:**2690–2694.
46. **Wild, J., E. Altman, T. Yura, and C. A. Gross.** 1992. DnaK and DnaJ heat shock proteins participate in protein export in *Escherichia coli*. Genes Dev. **6:**1165–1172.
47. **Wild, J., A. Kamath-Loeb, E. Ziegelhoffer, M. Lonetto, Y. Kawasaki, and C. A. Gross.** 1992. Partial loss of function mutations in DnaK, the *Escherichia coli* homologue of the 70-kDa heat shock proteins, affect highly conserved amino acids implicated in ATP binding and hydrolysis. Proc. Natl. Acad. Sci. USA **89:**7139–7143.
48. **Wild, J., W. A. Walter, C. A. Gross, and E. Altman.** 1993. Accumulation of secretory protein precursors in *Escherichia coli* induces the heat shock response. J. Bacteriol. **175:**3992–3997.