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Mutations That Improve Export of Maltose-Binding Protein in SecB$^-$ Cells of *Escherichia coli*

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It previously has been proposed that the *Escherichia coli* SecB protein promotes the export of the maltose-binding protein (MBP) from the cytoplasm by preventing the folding of the precursor MBP (preMBP) into a translocation-incompetent conformation. The export of wild-type MBP is only partially blocked in SecB$^-$ cells. In contrast, the export of MBP16-1, an MBP species with a defective signal peptide, is totally dependent on SecB; hence, SecB$^-$ cells that synthesize MBP16-1 are unable to utilize maltose as a sole carbon source. The selection of Mal$^+$ revertants primarily yielded mutants with alterations in the MBP16-1 signal peptide that permitted SecB-independent MBP export to the periplasm to various extents. Although each of these alterations increased the overall hydrophobicity of the signal peptide, it was not possible to strictly equate changes in hydrophobicity with the degree of SecB-independent export. Somewhat unexpectedly, two mutants were obtained in which MBP export in SecB$^-$ cells was markedly superior to that of the wild-type MBP. Although wild-type MBP is not cotranslationally translocated in SecB$^-$ cells, the two mutant proteins designated MBP172 and MBP173 exhibited significant cotranslational export in the absence of SecB. Thus, the role of SecB was partially supplanted by a signal peptide that promoted more rapid movement of MBP through the export pathway. When preMBP included the MBP172 signal peptide as well as an alteration in the mature moiety that slows folding, the SecB requirement for maximal MBP export efficiency was almost totally eliminated. These results provide additional strong support for the proposed antifolding role of SecB in MBP export.

Various studies have shown that the translocation of proteins into the endoplasmic reticulum and mitochondria of eucaryotic cells, or across the cytoplasmic membrane of bacterial cells, can be accomplished late in translation or entirely posttranslationally as long as the polypeptide chain exists in a translocation-competent conformation. Such a conformation is thought to represent a largely unfolded or loosely folded state, i.e., one that lacks a stable, tertiary structure that is inhibitory to the membrane translocation process (for reviews, see references 26 and 36). Several cytoplasmic factors have been identified in both eucaryotic and procaryotic cells whose primary function is thought to maintain the translocation-competent state of precursor proteins (6, 7, 9–11).

The export of a subset of *Escherichia coli* envelope proteins, including the maltose-binding protein (MBP), is adversely affected by mutations in *secB*, a nonessential gene encoding a soluble, multimeric protein composed of identical 16.4-kilodalton subunits (7, 19, 20, 21, 39). Collier et al. (7) found that the defect in MBP export in SecB$^-$ cells was partially suppressed by mutational alterations affecting MBP folding and that the rate of folding of wild-type precursor MBP (preMBP) synthesized in vitro into a stable, protease-resistant conformation was affected by SecB availability. On the basis of these and other findings, it was proposed that SecB functions as an antifolding factor that specifically interacts with a site within the mature moiety of preMBP to prevent its premature folding in the cytoplasm into a translocation-incompetent form. More recent studies have provided additional support for this proposal. Kumamoto and Gannon (22) reported that preMBP that accumulated in SecB$^-$ cells rapidly acquired a protease-resistant conformation. Weiss et al. (39) demonstrated that purified SecB quantitatively retarded folding of preMBP and that the presence of excess purified SecB in an in vitro assay significantly prolonged the time in which newly synthesized preMBP remained competent for posttranslational import into membrane vesicles.

Approximately 60% of wild-type MBP synthesized in SecB$^-$ cells is secreted into the periplasm, and such cells can still use maltose as a carbon source (7, 19, 20). However, the relatively slow posttranslational export exhibited by certain MBP species with mutationally altered signal peptides is completely blocked in SecB$^-$ cells (7); hence, these cells are unable to utilize maltose. Beginning with such a strain, a selection for Mal$^+$ revertants yielded mutants in which MBP export is restored in the absence of SecB. The study of these mutants provided additional insight into the role of SecB in promoting MBP export from the cytoplasm.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are derivatives of *E. coli* MC4100 (5). Strain BAR1091 (30) is MC4100 Δ*malE312 lac*$^+$ *lacI*^{q}(F′ *lacI*^{q} Tn*5*), strain DNC324 is MC4100 Δ*malE444* (33) *secB*::Tn*5* (from strain CK1953 [20])(F′ *lacI*^{q} Tn*5*) (constructed for this study), and strain DNC325 is DNC324 harboring the *lacUV5 malE16-1* plasmid pJF27 (38). Both plasmids pJF27 and pJF2 (*lacUV5 malE*$^+$) (15) harbor the intergenic region of bacteriophage M13. Plasmid pDNC215 (*lacUV5 malE172 malE2261*) was constructed by ligating the appropriate *Eco*RI-*Bgl*II fragments from pDNC172 (this study) and pJW13 (*lacUV5 malE2261*) (38).

Media and chemicals. Preparation of TYE agar, maltose-tetrazolium indicator agar, TB agar, and M63 minimal medium supplemented with thiamine (2 μg/ml) and carbon

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source (0.2%) has been described previously (27). When appropriate, ampicillin was included in complex and minimal media at concentrations of 50 and 25 μg/ml, respectively, while kanamycin and chloramphenicol were included in both media at concentrations of 30 and 12.5 μg/ml, respectively. Expression of genes under *lacUV5* promoter-operator transcriptional control was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 2 mM in liquid media or 1 mM on agar plates. IPTG was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), [^{35}S]methionine (Trans^{35}S-label; 1,162 Ci/mmol) was from ICN Radiochemicals (Irvine, Calif.), and V8 protease was from Sigma Chemical Co. (St. Louis, Mo.). T4 DNA ligase, Klenow fragment, restriction enzymes, and electrophoresis reagents were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Kodak XAR film was purchased from Eastman Kodak Co. (Rochester, N.Y.). Rabbit anti-MBP (12) and anti-SecB (39) sera have been previously described. Rabbit anti-ribose-binding protein (RBP) serum was kindly provided by Linda Randall, Washington State University, Pullman.

Mutant selection, linkage determinations, and DNA sequencing. To select spontaneous Mal$^+$ revertants of strain DNC325, we streaked individual colonies onto maltose minimal agar plates containing IPTG. Following incubation at 37°C for 3 to 5 days, individual colonies appeared which were subsequently repurified on the same medium. Only one colony was picked from each streak, thus ensuring that all mutants characterized were independently obtained. It was necessary to determine whether the mutation responsible for the Mal$^+$ phenotype was genetically linked to the *malE16-1* gene harbored on plasmid pJF27. Plasmids packaged as phage M13 particles were prepared from each revertant strain by the method of Zagursky and Berman (41), except that M13K07 (23) was used as the helper phage and SecB$^-$ cells were grown in TB broth. These phage M13 lysates were used to transduce cells of strain DNC324 to ampicillin resistance (Amp^r) on TB-ampicillin agar. Transductants were subsequently tested for growth on maltose minimal agar containing ampicillin and IPTG. Transfer of the ability to utilize maltose to the recipient strain by transduction to Amp^r indicated that the mutation of interest was linked to plasmid pJF27. DNA sequencing of mutant derivatives of plasmid pJF27 was as described by Bankier et al. (3).

Radiolabeling, immunoprecipitation, SDS-PAGE, and autoradiography. Pulse-chase experiments were performed essentially as described previously (31), except that MBP synthesis was induced 30 min prior to labeling by the addition of IPTG. Immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography were as previously described (1, 2). The amount of radioactivity present as preMBP and mature MBP was determined, when indicated, by excising the corresponding areas of the dried gels and then rehydrating and solubilizing the gel pieces with a mixture of NCS Tissue Solubilizer (Amersham Corp., Arlington Heights, Ill.) and water (9:1) and counting the radioactivity in ScintiVerse II (Fisher Scientific Co., Pittsburgh, Pa.). The counts were adjusted for the loss of methionine when preMBP is processed to mature MBP.

Analysis of cotranslational versus posttranslational processing of MBP. The processing of nascent chains was analyzed as previously described by Josefsson and Randall (18).

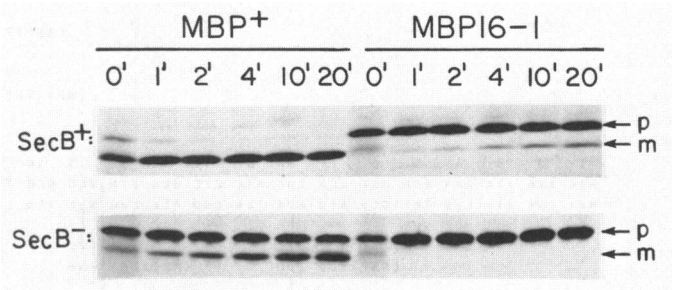

FIG. 1. SecB requirement for MBP$^+$ and MBP16-1 export. Glycerol-grown, IPTG-induced cells of strains BAR1091 (*secB*$^+$) and DNC324 (*secB*::Tn*5*) harboring either plasmid pJF2 (*malE*$^+$) or pJF27 (*malE16-1*) were pulse-radiolabeled with [^{35}S]methionine for 15 s and chased with excess unlabeled methionine. At the indicated time points (minutes), samples were removed, the chase was terminated, and the protein products were immunoprecipitated with rabbit anti-MBP serum and analyzed by SDS-PAGE and autoradiography. The positions of precursor (p) and mature (m) MBP are indicated by arrows.

RESULTS

Export of MBP16-1 is completely blocked in SecB$^-$ cells. The export kinetics of wild-type MBP and MBP16-1 in both SecB$^+$ and SecB$^-$ cells are shown in Fig. 1. Fully induced log-phase cells were pulse-radiolabeled with [^{35}S]methionine for 15 s, and MBP was immunoprecipitated at various times after the addition of excess unlabeled methionine. The precipitates were subsequently analyzed by SDS-PAGE and autoradiography. As previously reported (2, 7, 14, 31, 32), wild-type MBP was rapidly and efficiently exported in SecB$^+$ cells, while the export of MBP16-1 (Lys substituted for Ala at residue 16 of the signal peptide) was much less efficient and accomplished in a relatively slow, entirely posttranslational manner. (Note that signal peptide processing has been demonstrated in previous studies [1, 2, 12, 31] to be a reliable indicator of MBP export to the periplasm.) The results obtained with SecB$^-$ cells were markedly different. In this case, the export efficiency of wild-type MBP was considerably reduced. By the 20-min chase point, only about 60% of the MBP had been exported and processed. The export of MBP16-1 was completely blocked in SecB$^-$ cells such that, even after 20 min of chase, mature MBP could not be detected. This latter result was consistent with the previous finding that MBP19-1-R8, an MBP species that is exported in a slow, entirely posttranslational manner in SecB$^+$ cells (32), is totally export defective in SecB$^-$ cells (7).

The defect in MBP16-1 export imparted by the loss of SecB was so severe that cells of strain DNC325 were unable to utilize maltose as a carbon source. Beginning with this strain in which the *malE16-1* gene is located on a multicopy plasmid (pJF27), a number of independently isolated maltose-utilizing revertants were obtained. The responsible mutation was linked to pJF27 for the great majority of the isolates tested (see Materials and Methods), indicating that the ability to utilize maltose resulted from an alteration in the MBP. For the few remaining isolates, the mutation responsible for the Mal$^+$ phenotype was genetically linked to the chromosomal *secB* gene (see below).

Mutational alterations in MBP16-1 signal peptide restore export in SecB$^-$ cells. The nucleotide sequence of the *malE* signal peptide-coding region was determined for a number of plasmid-linked Mal$^+$ isolates. In each instance, a mutation was encountered in this region of the gene. Altogether, nine

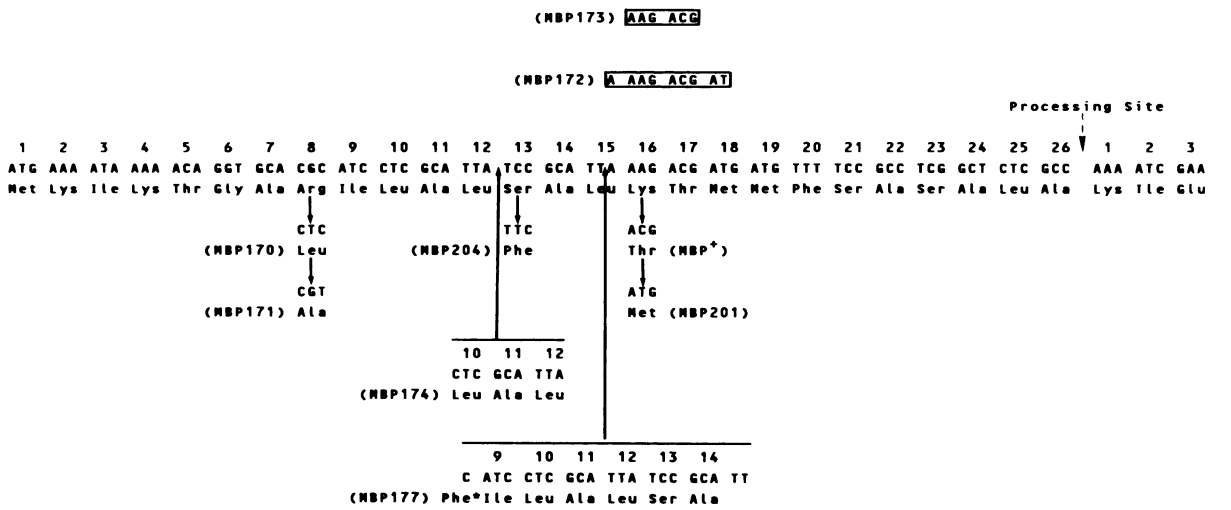

FIG. 2. Mutational alterations in the MBP16-1 signal peptide that restore export in SecB cells. The DNA and predicted amino acid sequences for the amino-terminal 29 residues of MBP16-1 are shown, including the entire signal peptide and the processing site. Single amino acid substitutions that suppress the SecB$^-$ export defect, as well as the DNA alterations, are indicated by downward-pointing arrows. An upward-pointing arrow indicates a duplication of genetic information, whereas nucleotides deleted from the *malE16-1* gene are boxed. The corresponding designations for each mutant protein are also provided. Note that for MBP172, the deletion of nine base pairs from the signal sequence-coding region removes residues 16 through 18 from the hydrophobic core. For MBP177, Phe* denotes the addition of a novel Phe codon.

unique mutational alterations in the MBP16-1 signal peptide that restored MBP export in SecB$^-$ cells were identified (Fig. 2). Each of the new *malE* signal sequence mutations resulted in an increase in the overall hydrophobicity of the signal peptide compared with that of MBP16-1 (24). Hydrophobicity was increased in four different ways. (i) Point mutations in codon 16 resulted in the substitution of a Thr (MBP$^+$, i.e., a true *malE*$^+$ revertant) or a Met (designated MBP201) for the offending Lys residue at position 16 of the signal peptide. (ii) Deletions of six (MBP173) or nine (MBP172) base pairs also resulted in removal of the offending Lys residue. (iii) Point mutations resulted in the substitution of a Leu (MBP170) or Ala (MBP171) residue for the Arg residue at position 8 and a Phe residue for the Ser residue at position 13 (MBP204) of the signal peptide. (iv) Duplications in the signal sequence-coding region extended the hydrophobic core by the introduction of three (MBP174) or seven (MBP177) hydrophobic or neutral amino acid residues.

The plasmid-linked mutants fell into two classes based on their growth rates on maltose minimal agar plates. One class grew with a rate approximately equal to that of *secB*::Tn*5* *malE*$^+$ control cells, while growth of the second class of mutants was demonstrably slower. For cells to utilize maltose, MBP must be translocated to the periplasm; hence, cells better able to utilize maltose for growth would be expected to export MBP with greater efficiency. MBP export in mutants of the slow-growing class was examined by pulse-chase labeling with a single 20-min chase point. The export of MBP170, MBP171, MBP177, MBP201, and MBP204 was compared with that of MBP$^+$ and MBP16-1 in both SecB$^+$ and SecB$^-$ cells (Fig. 3). MBP170, MBP171, MBP201, and MBP204 were all exported to approximately the same extent in SecB$^-$ cells. However, in SecB$^+$ cells, the export of MBP171 (approximately 34% of the total synthesized) was significantly less than that observed for MBP170, MBP201, and MBP204 (all greater than 95%). Processing of MBP177 in SecB$^-$ cells was just barely detectable and could not be discerned at all in SecB$^+$ cells. Fractionation experiments indicated that this MBP species was unusual in that it was translocated without proteolytic cleavage of its signal peptide (data not shown). Mutant MBP species that are translocated without processing have been previously described (15).

Mutants with growth rates similar to those of *secB*::Tn*5* *malE*$^+$ cells were predicted to export MBP with efficiencies close to that achieved by MBP$^+$ in the absence of SecB. The export kinetics of MBP172, MBP173, and MBP174 in both SecB$^+$ and SecB$^-$ cells were determined (Fig. 4). The export of each of these MBP species in SecB$^+$ cells was considerably improved over that exhibited by MBP16-1. In fact, both MBP172 and MBP173 having signal peptides truncated by three or two residues, respectively, in the hydrophobic core region were exported at a rate and efficiency indistinguishable from that of MBP$^+$. MBP174, which still retained the Lys residue in the hydrophobic core that is responsible for the export defect of MBP16-1, was exported at a slower rate and with a somewhat reduced efficiency, although by 20 min

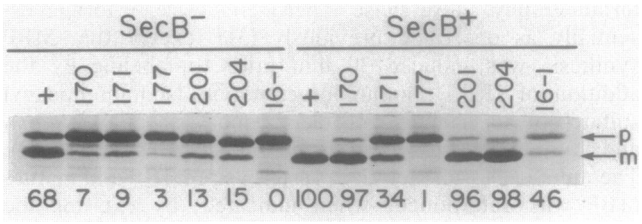

FIG. 3. Export of mutant MBP species in SecB$^-$ and SecB$^+$ cells. Glycerol-grown, IPTG-induced cells of strain DNC324 (*secB*::Tn*5*) and BAR1091 (*secB*$^+$) harboring plasmids encoding various MBP species were pulse-radiolabeled with [^{35}S]methionine for 15 s and chased with excess unlabeled methionine for 20 min. The protein products were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The percentage of total radiolabeled MBP precipitated in the mature form (see Materials and Methods) is shown below each lane. The positions of precursor (p) and mature (m) MBP are indicated by arrows. The corresponding designations for each MBP species are provided atop each lane.

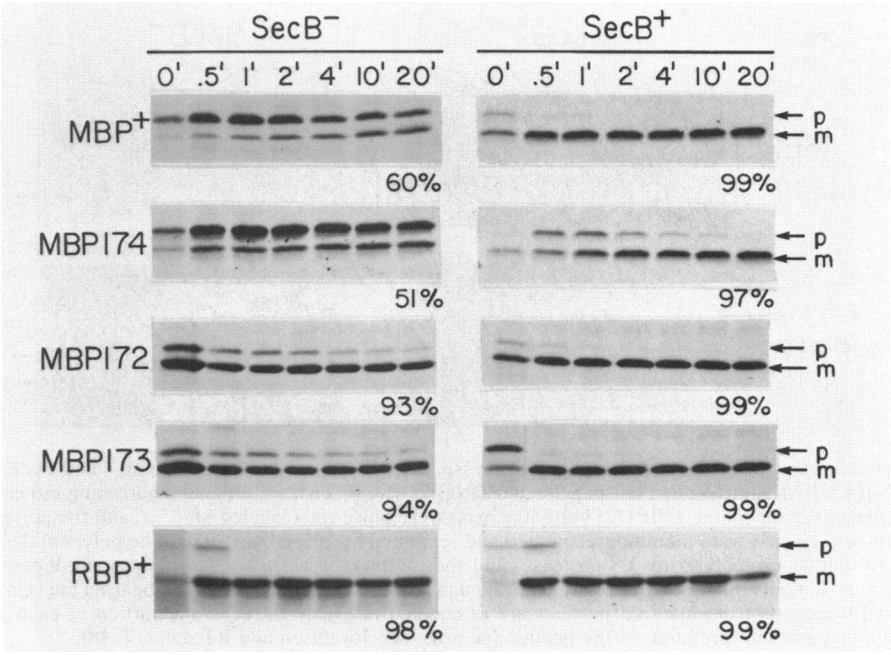

FIG. 4. Kinetics of protein export in SecB⁻ and SecB⁺ cells. Glycerol-grown, IPTG-induced cells of the indicated SecB phenotype harboring plasmids encoding the indicated MBP species or wild-type RBP were pulse-radiolabeled with [^{35}S]methionine for 15 s and chased with excess unlabeled methionine. At the indicated time points (minutes), samples were removed, the chase was terminated, and the protein products were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The positions of precursor (p) and mature (m) MBP or RBP are indicated by arrows. The percentage of total radiolabeled MBP and RBP precipitated in mature form at the 20-min chase point is indicated (see Materials and Methods).

of chase, approximately 97% had been exported. In SecB⁻ cells, MBP174 export was also less efficient than MBP⁺ export but clearly more efficient than that of the MBP species shown in Fig. 3. However, it was surprising to find that both MBP172 and MBP173 were exported significantly better than MBP⁺ in the absence of SecB. At the 20-min chase point, greater than 90% of the MBP synthesized had been exported and processed, compared with only 60% of MBP⁺. This difference was even more striking at earlier chase points. For example, after 30 s of chase, 73% of MBP172 was exported compared with just 24% of MBP⁺.

The export kinetics of RBP in SecB⁺ and SecB⁻ cells also are included in Fig. 4. As previously reported (7, 20), the efficient export of this protein exhibited no SecB dependence.

Cotranslational export of MBP172 in SecB⁻ cells. The finding that MBP172 and MBP173 were more efficiently exported in SecB⁻ cells than MBP⁺ was unexpected. It was not immediately obvious how an alteration in the signal peptide could suppress the requirement for SecB, a factor which had been previously proposed to serve primarily an antifolding role in maintaining preMBP in an export-competent state (7, 39). Earlier studies demonstrated that the presence of the signal peptide significantly retards the refolding of purified preMBP (25, 28) and the folding of newly synthesized preMBP in vitro (38). Thus, the possibility was considered that the mutant signal peptide of MBP172 and MBP173 further modulated preMBP folding, extending the period that preMBP remained export competent in the absence of SecB. However, by using the acquisition of protease resistance as an assay for folding (7, 28, 29, 38, 39), no difference could be discerned in the rate of folding of MBP⁺ and MBP172 synthesized in vitro (data not presented).

Kumamoto and Gannon (22) had previously reported that cotranslational processing (and hence translocation) of MBP⁺ was totally blocked in SecB⁻ cells. The relatively large amounts of mMBP172 and mMBP173 detected at the early chase points in SecB⁻ cells (Fig. 4) strongly suggested that some processing of these two MBP species was occurring cotranslationally, despite the absence of SecB. This suggested that the higher level of SecB-independent export of these mutant MBP species was the result of their more rapid movement through the export pathway. The technique developed by Josefsson and Randall (17, 18) employing two-dimensional SDS-PAGE was used to determine the mode of processing of MBP⁺ and MBP172 in both SecB⁺ and SecB⁻ cells (Fig. 5). As previously reported (17, 18, 22), cotranslational processing of MBP⁺ was indicated by the immunoprecipitation of processed nascent chains from pulse-labeled SecB⁺ cells, as shown by the horizontal streak designated m′ in the top left panel of Fig. 5. The horizontal streak designated p′ consists of the amino-terminal proteolytic fragment that is present in all peptides retaining the signal peptide and sufficiently elongated to carry the first staphylococcal V8 protease cleavage site, while m′ consists of the corresponding amino-terminal proteolytic fragment of incomplete nascent chains from which the signal peptide has been removed. The finding that incomplete chains compose the m′ streak indicates that processing is occurring before translation is complete, i.e., cotranslationally. As expected (22), cotranslational processing of MBP⁺ was completely abrogated in SecB⁻ cells (Fig. 5, top right panel). In comparison with MBP⁺, a much greater proportion of MBP172 was cotranslationally processed in SecB⁺ cells (Fig. 5, bottom left panel), as judged by the relative intensities of the p′ and m′ streaks. Remarkably, the cotranslational processing of MBP172 appeared to be largely refractory to the loss

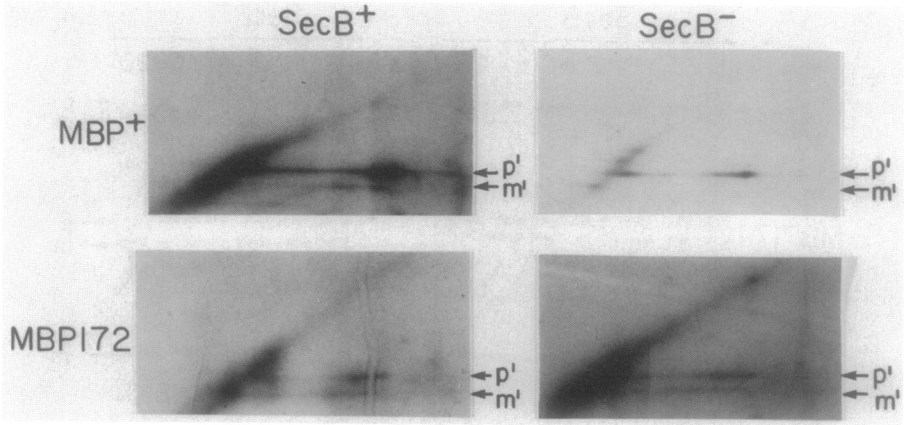

FIG. 5. Two-dimensional analysis of the cotranslational processing of MBP$^+$ and MBP172 in SecB$^+$ and SecB$^-$ cells. Glycerol-grown, IPTG-induced cells of strain BAR1091 (*secB*$^+$; left panels) and DNC324 (*secB*::Tn*5*; right panels) harboring either plasmid pJF2 (encoding MBP$^+$; top panels) or pDNC172 (encoding MBP172; bottom panels) were pulse-radiolabeled for 15 s, and the polypeptides related to MBP, including incomplete nascent chains, were immunoprecipitated and separated by 10% SDS-PAGE. The polypeptides contained in excised gel slices were subjected to limited proteolysis by V8 protease, and the products were resolved by a second-dimension 15% SDS-PAGE as described by Josefsson and Randall (18). The amino-terminal fragments derived from molecules still bearing the signal peptide are designated p', while amino-terminal fragments from matured proteins are designated m'. Only the relevant portion of each gel is shown. For useful discussions of this technique and interpretation of the peptide patterns, see Josefsson and Randall (17, 18).

of SecB. In SecB$^-$ cells, nascent MBP172 chains were clearly processed (bottom right panel), and there was no discernable alteration in the ratios of p' and m' compared to that obtained for SecB$^+$ cells.

Alteration in the mature moiety can further reduce SecB dependence of MBP export. MBP2261 has a single amino acid substitution (Asp for Tyr) at residue 283 of the mature MBP (8) that has been shown to slow significantly the refolding kinetics of purified mMBP (25). It was found previously that MBP2261 is exported in SecB$^-$ cells with an efficiency considerably higher than that of MBP$^+$, a result that strongly supported the proposed antifolding role of SecB (7). It was suspected that this same alteration in the mature MBP might further enhance the export of MBP172 in SecB$^-$ cells. In this study, a *malE172 malE2261* double mutant was constructed and designated *malE215*. The export kinetics of MBP215 and MBP2261 in SecB$^-$ cells were determined (Fig. 6, top) and graphically compared with those previously obtained for MBP$^+$ and MBP172 (Fig. 6, bottom). Compared with MBP2261, a much greater percentage of MBP215 was exported at the early chase points, but by 10 min postchase, greater than 90% of the total synthesized of each MBP species had been translocated and processed. There was also a clear difference between the export kinetics exhibited by MBP215 compared with MBP172 during the early chase points, indicating that the MBP species with the additional alteration in its mature moiety was exported at a somewhat faster rate. The alteration in the mature moiety had no noticeable effect on the amount of MBP exported at the 0 min chase point when in *cis* to either the wild-type or mutant signal peptide (compare MBP$^+$ with MBP2261, and MBP172 with MBP215). Likewise, there was no noticeable effect on the cotranslational processing of these MBP species (data not shown). Thus, this alteration in the mature moiety only affected the posttranslational export kinetics of MBP species synthesized in the absence of SecB.

Mal$^+$ revertants resulting from a mutation in chromosomal *secB* gene. As previously mentioned, a few Mal$^+$ revertants were obtained in which the responsible mutation was not linked to the *malE16-1* plasmid. Unlike the parental *secB*::Tn*5* strain which is unable to grow on a nutrient-rich medium such as TYE agar (20), 9 of the 10 unlinked revertants were able to grow on such a medium. In addition, each of these nine isolates was kanamycin sensitive (Km^s), indicating that the ability to utilize maltose had resulted from excision of the Tn*5* insertion in the chromosomal *secB* gene. To demonstrate that SecB synthesis had been restored in these Mal$^+$ revertants, cells were radiolabeled for 20 min with [^{35}S]methionine, and the protein products were analyzed by immunoprecipitation with anti-SecB sera (39), SDS-PAGE, and autoradiography (Fig. 7). All 10 revertant strains produced some SecB protein, in comparison with *secB*::Tn*5* parental cells, from which SecB could not be precipitated. Some heterogeneity was noted with respect to both the apparent size of SecB and the amount synthesized by the various isolates. The one revertant which remained Km^r and unable to grow on TYE agar produced the smallest amount of SecB (Fig. 7, lane J) and also exhibited the least efficient export of MBP16-1 (data not shown).

DISCUSSION

Beginning with an *E. coli* strain, DNC325, that was SecB$^-$ and that synthesized an MBP species (MBP16-1) whose export was totally SecB dependent, a selection for Mal$^+$ revertants was expected to yield at least three different kinds of mutants. First, mutational alterations in the MBP16-1 signal peptide were anticipated that suppressed the export defect sufficiently to permit some MBP export in a SecB-independent manner. Included in this class would be those that had reverted to *malE*$^+$, since approximately 60% of MBP$^+$ synthesized is exported to the periplasm in the absence of SecB (7, 19, 20). Second, mutants synthesizing preMBP with alterations in the mature moiety that affected its folding properties were expected. MBP16-1 is exported in a slow, entirely posttranslational manner in SecB$^+$ cells (31). If the primary role of SecB is to maintain the preMBP in the cytoplasm in an unfolded, export-competent conformation, then it was thought that the absence of this antifolding factor could also be overcome by alterations that slowed the

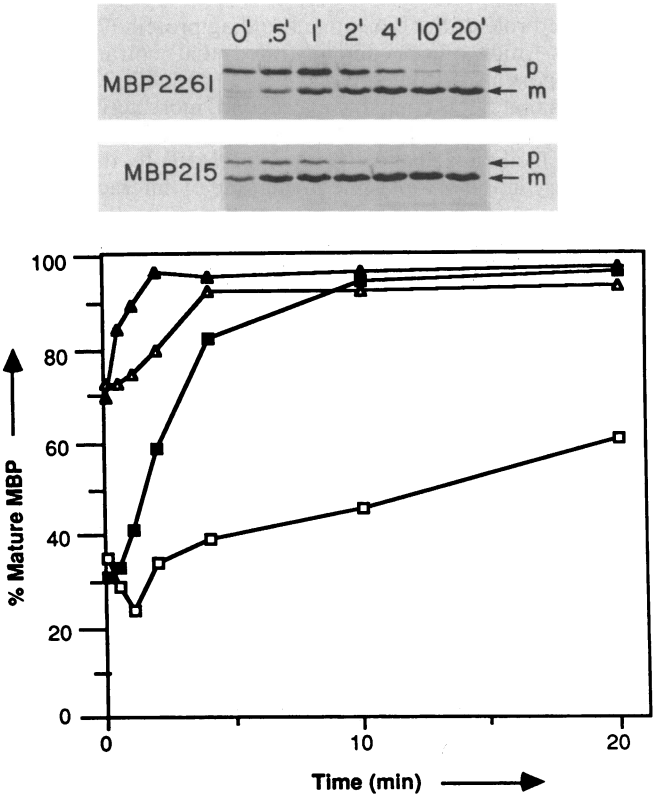

FIG. 6. SecB-independent export of MBP2261 and MBP215. Top panel: Glycerol-grown, IPTG-induced cells of strain DNC324 (*secB*::Tn*5*) harboring plasmid pJW13 (encoding MBP2261) or pDNC215 (encoding MBP215) were pulse-radiolabeled with [^{35}S]methionine for 15 s and chased with excess unlabeled methionine. At the indicated time points (minutes), samples were removed, the chase was terminated, and the protein products were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The positions of precursor (p) and mature (m) MBP are indicated by arrows. Bottom panel: The percentage of total radiolabeled MBP precipitated as the mature form was determined for each time point (see Materials and Methods). Symbols: △, MBP172; ▲, MBP215; □, MBP$^+$; ■, MBP2261. Note that the data for MBP$^+$ and MBP172 are derived from the experiment shown in Fig. 4.

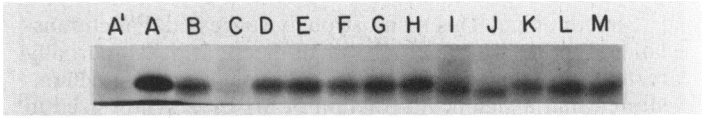

FIG. 7. Analysis of SecB synthesis in SecB$^+$ revertants. Glycerol-grown cultures were radiolabeled with [^{35}S]methionine for 10 min, and the protein products were immunoprecipitated with rabbit anti-SecB serum and analyzed by SDS-PAGE and autoradiography. Lanes: A, DNC445 (harbors plasmid pDC2 which carries *secB*$^+$); B, MC4100 (haploid *secB*$^+$); C, CK1953 (*secB*::Tn*5*); D through M, the 10 SecB$^+$ revertants obtained in this study (see text). Note: The first lane designated A' represents the precipitate obtained from the extract of strain DNC445 cells with preimmune rabbit serum.

folding of preMBP16-1, thus providing more time for this export-defective MBP species to engage the export machinery productively in the cytoplasmic membrane. The final class of expected revertants were those in which either the synthesis of functional SecB had been restored or possibly another cell protein had been recruited that could serve in the same capacity.

When Mal$^+$ revertants of strain DNC325 were analyzed, it was found that the responsible mutation was linked to the plasmid-encoded *malE16-1* gene in most cases. None of the mutations was found to reside in the coding region for the mature MBP; rather, DNA sequencing of a number of such revertants revealed that each one harbored a mutation in the signal peptide-coding region. It was not surprising to find that the effect of these mutations was to increase the overall hydrophobicity of the signal peptide compared with that of the parental MBP16-1. The stronger revertants were generally those that removed the offending Lys residue from the middle of the core, either by substituting another residue such as Thr (thus regenerating the wild-type signal) or by deleting the Lys along with one or two adjacent residues. The remainder were second-site revertants that retained the Lys at residue 16 but suppressed the export defect imparted by the presence of this charged residue in the hydrophobic core by one of three different mechanisms: (i) lengthening the core into the amino-terminal hydrophilic segment by substituting a nonpolar residue for the Arg at position 8; (ii) lengthening the core by duplicating base pairs in the core-coding region; and (iii) substituting a more hydrophobic residue, Phe, for the Ser at position 13. Similar mutations have been described previously among revertants of stronger *malE* signal sequence mutations obtained with SecB$^+$ cells (2, 32).

The finding that mutational alterations in the MBP16-1 signal peptide could restore MBP export efficiency to the level exhibited by MBP$^+$ in SecB$^-$ cells was not unexpected. However, it was surprising to encounter mutant signal peptides that promoted MBP export in SecB$^-$ cells at a rate and efficiency markedly superior to that of the wild-type signal peptide. Such mutant signal peptides resulted from the deletion of either two (MBP173) or three (MBP172) residues from the hydrophobic core. The hydropathy profile of these two signals determined by the method of Kyte and Doolittle (24) indicated that the deletion of the two slightly hydrophilic Thr residues (positions 16 and 17) from the core increases to a small extent the overall hydrophobicity of the core region compared with the wild-type MBP signal peptide (data not presented). This hydrophobicity difference may be important, but it cannot entirely explain the high degree of SecB-independence exhibited by MBP172 and MBP173. MBP201 also presents a more hydrophobic profile than MBP$^+$, but export of this MBP species in SecB$^-$ cells was considerably less efficient than that of the wild-type protein.

A good correlation between overall hydrophobicity of the signal peptide and efficiency of MBP export in SecB$^+$ cells has previously been demonstrated (2, 14, 31, 32). This was further illustrated in this study by the difference in export efficiencies exhibited by MBP170 and MBP171. MBP170 was exported much better than MBP171 in SecB$^+$ cells, a result which was consistent with the substitution of a more hydrophobic residue, Leu, for the Arg at position 8 (MBP170), compared with the substitution of an Ala residue at the same position (MBP171). In contrast, in SecB$^-$ cells, the export of these two MBP species was nearly indistinguishable. Hence, mutational alterations in the MBP signal peptide that suppress the requirement for SecB seem to augment some property of the signal peptide that cannot be strictly equated with hydrophobicity. This may be related to the multiple roles proposed for the signal peptide in the export process (13, 31, 34, 35).

A clue about the mechanism by which MBP172 and MBP173 were efficiently exported in SecB$^-$ cells was provided by the appearance of a large amount of processed mature MBP immediately following a 15-s pulse-label with

[^{35}S]methionine. This result strongly suggested that cotranslational processing of MBP had been at least partially restored in these mutant cells. It was subsequently demonstrated that a significant fraction of MBP172 synthesized in SecB$^-$ cells was cotranslationally processed. Since processing requires translocation of the amino-terminal portion of the MBP, including the signal peptidase I cleavage site, across the cytoplasmic membrane (40), this experiment clearly indicated that translocation of MBP172 was initiated cotranslationally, despite the absence of SecB.

Previous studies have suggested that export of MBP$^+$ in SecB$^-$ cells represents a race between delivery of the newly synthesized, export-competent polypeptide to the export machinery in the cytoplasmic membrane and folding of the preMBP in the cytoplasm into an export-incompetent conformation (7, 22, 29, 38, 39). By this reasoning, MBP16-1 is extremely inefficient at engaging the membrane machinery, and hence, folding of the precursor into an export-incompetent form prior to translocation is virtually ensured. On the other hand, delivery of MBP172 to the membrane is significantly more efficient than that of even MBP$^+$. As a result, much more of this MBP species is translocated prior to the loss of export competence. MBP172 exhibits no obvious export defect in SecB$^+$ cells. Thus, it appears to represent a rare example of an altered protein that functions better than its wild-type counterpart. It is not now known how the MBP172 signal peptide promotes such rapid export. The possibility remains that the mutant signal peptide does have some effect on preMBP folding that was not discerned in this study but, nonetheless, contributes to it suppressing activity. A major goal of current studies is to understand the functional differences between the wild-type and MBP172 signal peptides.

Mutational alterations in the mature moiety of MBP that could restore MBP16-1 export in SecB$^-$ cells were anticipated but not obtained in this study. Mutations that alter the folding properties of preMBP sufficiently to suppress the requirement for SecB and yet maintain functional maltose uptake activity may be quite rare. However, it was known that such mutants were possible. It was determined that, when the *malE2261* mutation was placed *cis* to *malE16-1*, the resulting double-mutant protein was exported to a small extent in SecB$^-$ cells, permitting growth on maltose (J. B. Weiss and P. J. Bassford, Jr., unpublished observation). The *malE2261* mutation was originally obtained as an intragenic suppressor of a *malE* signal sequence mutation (8), and as previously mentioned, purified mature MBP2261 exhibits refolding kinetics that are significantly slower than those of MBP$^+$ (25). An earlier study had shown that MBP2261 is exported in SecB$^-$ cells with a greater efficiency than MBP$^+$ (7). With this in mind, an MBP species combining the MBP172 signal peptide and the MBP2261 mature moiety, designated MBP215, was constructed and found to be exported in SecB$^-$ cells at a rate and efficiency close to that of MBP$^+$ in SecB$^+$ cells. Thus, this combination of alterations in the preMBP nearly eliminated the SecB requirement for maximal efficiency of MBP export. It was interesting that MBP172 and MBP215 were exported cotranslationally to the same extent in SecB$^-$ cells, i.e., that the alteration in the mature moiety only affected the posttranslational export kinetics. This suggests that the nature of the signal peptide has a major role in determining the degree to which a protein is cotranslationally translocated.

The findings that alterations in the MBP that promote more rapid export and slow folding can significantly suppress the SecB requirement are very consistent with the postulated role of SecB as an antifolding protein (7, 22, 39). Previous studies have demonstrated that cotranslational MBP export also is defective in SecB$^-$ cells (7, 22), which indicates that SecB could have a second, more active role in MBP export. It has been suggested (7, 22, 39) that SecB actively facilitates the delivery of preMBP to the export machinery in the cytoplasmic membrane by interacting with a membrane receptor in a manner analogous to that shown for the eucaryotic signal recognition particle (reviewed in reference 37). However, since the export of only a subset of proteins is SecB dependent, and since the MBP signal peptide can be easily changed to restore cotranslational translocation in the absence of SecB, the idea of a SecB receptor in the membrane seems less likely. It has also been suggested that, since SecB has been shown to interact with the mature moiety of preMBP (7, 16), this cytoplasmic factor promotes cotranslational preMBP export simply by maintaining the nascent chain in a conformation that ensures that the signal peptide is readily accessible to the export machinery (39).

Finally, the selection for Mal$^+$ revertants of DNC325 yielded a few mutants in which the responsible mutation was unlinked to the plasmid-encoded *malE16-1* gene. It was thought that mutants might be obtained in which some new protein had supplanted the role of SecB in the MBP export process. Candidates for such an activity included the "trigger factor" that serves to maintain the export competency of proOmpA protein for in vitro translocation (9, 10) and the GroEL protein that has been suggested to maintain the unfolded state of pre-β-lactamase in the cytoplasm (4). However, all 10 nonplasmid-linked mutants obtained proved to be revertants of the chromosomal *secB*::Tn*5* locus. SecB produced by these revertants demonstrated some variation in the amount produced and in its migration pattern on SDS-PAGE. Interestingly, some of these revertant *secB* alleles have exhibited the ability to suppress *malE16-1* and certain other *malE* signal sequence mutations (data not presented) in a manner similar to that mediated by various *prl* alleles of *E. coli* (12, 31). This suppression activity may result from a small increase in SecB production over the normal haploid level, although it is known that a large overproduction of SecB is somewhat inhibitory to MBP$^+$ export (7, 20). MBP export in these new *secB* mutants is currently being investigated.

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