Insertion of the Polytopic Membrane Protein MalF Is Dependent on the Bacterial Secretion Machinery*

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We examined the dependence of protein export and membrane protein insertion on SecE and SecA, two components of the secretion (Sec) apparatus of Escherichia coli. The magnitude of the secretion defect observed for signal sequence-containing proteins in cells depleted of SecE is larger and more general than that in many temperature- or cold-sensitive Sec mutants. In addition, we show that the proper insertion of the polytopic MalF protein (synthesized without a signal sequence) into the cytoplasmic membrane is also SecE-dependent. In contrast to an earlier study (McGovern, K., and Beckwith, J. (1991) J. Biol. Chem. 266, 20870–20876), the membrane insertion of MalF also is inhibited by treatment of cells with sodium azide, a potent inhibitor of SecA. Therefore, our data strongly suggest that the cytoplasmic membrane insertion of MalF is dependent on the same cellular machinery as is involved in the export of signal sequence-containing proteins. We propose that the mechanism of export from the cytoplasm is related for both signal sequence-containing and cytoplasmic membrane proteins, but hydrophobic membrane proteins such as MalF may have a higher affinity for the Sec apparatus.

Translocation of proteins across the cytoplasmic membrane in Escherichia coli (2, 3) is catalyzed by a machinery whose components have been identified both genetically and biochemically. These components include the SecA, SecE, and SecY proteins, which are essential for protein export, and at least four other proteins (SecB, SecD, SecF, and SecG) that greatly enhance protein translocation, especially at lower temperatures. Proteins localized by the Sec apparatus to the periplasm or outer membrane are synthesized with an N-terminal signal sequence that is proteolytically cleaved from the mature portion of the protein during export by the action of leader peptidase.

In vivo studies of the dependence of a particular protein on the Sec machinery for proper cellular localization have relied largely on the use of conditional Sec missense mutants. These mutant strains accumulate untranslocated, signal sequence-bearing proteins in their cytoplasm under nonpermissive conditions. The extent of a secretion defect in cells can thus be directly assessed by examining the amount of precursor species versus mature species of a given secreted protein. The analysis of the Sec dependence of protein export is made complicated by the fact that different proteins are not equally sensitive to the export inhibition conferred by conditional sec mutations. For example, in a strain deleted for the secDF genes, ribose-binding protein and maltose-binding protein (MBP) are exported relatively poorly (4). In these same strains, OmpA is exported fairly well (the majority of protein synthesized in a short pulse is exported by 10 min), and DegP is exported with wild type kinetics. It is possible that OmpA and DegP have a greater affinity for the secretion apparatus than do ribose-binding protein or MBP.

The assessment of whether a protein is dependent upon the secretion apparatus for translocation is further confounded by the nature of the sec conditional mutations. These mutations, rather than causing the synthesis of a conditionally defective Sec protein, often result in lower levels of functional Sec protein and, in effect, reduce the quantity of functional secretion apparatus in the cell. This phenomenon has been thoroughly characterized for the secE cold-sensitive mutations (5). The translocation of proteins that have a higher affinity for this limited secretion apparatus would be less affected relative to those proteins that have a lower affinity. Recently, a more accurate assessment of the Sec dependence of proteins has been made possible by the development of strains in which individual Sec proteins can be conditionally depleted (4, 6). These strains contain either the secDF genes or the secE gene under control of the highly repressible araBAD promoter. Strains in which expression of the SecDF genes is repressed have a very severe and general secretion defect for signal sequence-containing proteins after depletion of the SecD and F proteins (4). In this study, we characterize the secretion defect of cells that have been depleted for the SecE protein.

Integral cytoplasmic membrane proteins often have large hydrophilic domains that must be translocated to the extracytoplasmic face of the membrane. However, most cytoplasmic membrane proteins are synthesized without deavable N-terminal signal sequences. Whether most of these polypeptides depend on the Sec machinery for translocation of hydrophilic domains through the hydrophobic membrane is unresolved. A few cytoplasmic membrane proteins have been tested for their dependence on the Sec machinery for membrane insertion, including M13 procot protein, leader peptidase (Lep), the methyl-accepting chemotaxis receptor protein Tsr, the ProW component of the ProU osmoregulatory system, and the Malf component of the maltose transport complex (1, 7–9). These analyses have suggested that the M13 procot, ProW, and MalF are Sec-independent, whereas Lep and Tsr are Sec-dependent.

Recently, an highly sensitive assay for the proper insertion of
the MalF protein into the cytoplasmic membrane and its subsequent assembly into the hetero-oligomeric maltose transport complex was described (10). This assay is based on the accessibility of the periplasmic domains of MalF (~3, 180, 30, and 30 residues, respectively; Ref. 11) to exogenous proteases. The sensitivity of this essay in combination with the new SecE depletion mutant led us to readdress the dependence of MalF on the Sec apparatus for the export of its periplasmic regions.

MATERIALS AND METHODS

Bacterial Strains—Strain CM124 was constructed from P5291 (secE19–111, pcloB80 zasl::(Tn10 Tc Str) plaP2 pruv, lacZ74, galE, galK, rpsL, relA::cat pBRU) by transformation with plasmid pCM22 (6), replacing plasmid pBRU (secE) to test the effects of depletion of SecE upon secretion of various periplasmic and outer membrane proteins. pCM22 has the secE gene expressed from the strongly repressible promoter of the araBAD operon.

The MalF protein in all experiments is expressed at physiological levels from the normal chromosomal copy of the gene in the malB locus at 92 min on the E. coli chromosome; therefore there are no artifacts from protein overproduction. Strains BT62, BT8, and BT10 are MalT derivatives of MC4100 (F−, ΔlacI169, thiA, relA, rpsL, araD139). BT8 and BT10 are Mal− and MalE−, respectively (described in Ref. 10). BT62 is MalF−malF+ and MalE−, respectively (described in Ref. 10). BT62 is MalF−malF+ and MalE−, respectively (described in Ref. 10).

Due to the strength of the secretion defect observed in a malE derivative of BT62 (13), we reasoned that if MalF depends on SecE for its insertion into the cytoplasmic membrane, the three larger periplasmic domains of MalF are sensitive to cleavage by exogenously added proteases (such as trypsin) in spheroplasts of strains lacking the MalK or MalG proteins (10). We reasoned that if MalF depends on SecE for its insertion into the cytoplasmic membrane, then depletion of

![Fig. 1. Growth of SecE depletion strains.](image)

**RESULTS**

**SecE Depletion Causes a General Inhibition of Protein Export**—To examine the effects of depletion of SecE upon secretion, we utilized a strain deleted of its chromosomal copy of secE but containing a plasmid present at low copy number with secE under control of the strongly repressible promoter of the araBAD operon (6). This strain, CM124, is absolutely dependent upon the addition of arabinose to the growth medium for prolonged viability. After dilution of a CM124 culture from medium with medium with glucose (depletion conditions), cell growth continues for several hours at a rate indistinguishable from the rate of a culture in arabinose (Fig. 1). After 5.5–6.5 h in glucose, the growth rate of the strain decreases.

CM124 cultures were assayed for the extent of their secretion defect during the course of SecE depletion. At several time points after dilution into medium with glucose or arabinose, this secretion defect was visualized by pulse labeling cells and examining the ratio of precursor to mature form of several secreted proteins using immunoprecipitation and SDS-PAGE (Fig. 2). Cells expressing SecE (grown in arabinose) exhibit no accumulation of the precursor forms of the periplasmic proteins DegP and MBP or the outer membrane protein OmpA. In contrast, cells depleted of SecE exhibit a marked defect in secretion of these proteins after 3.5 h (a time at which the two cultures are doubling at the same rate), which becomes nearly complete at late time points. Precursor forms of exported proteins such as DegP, which had previously been difficult to observe under restrictive conditions for the conditional Sec mutants, were readily observed in the SecE-depleted cells. In addition, OmpA translocation during the course of a 5-min chase was substantially less efficient than that observed using Sec conditional mutants (13).

**Effect of SecE Depletion on the Membrane Incorporation of MalF**—Due to the strength of the secretion defect observed in SecE-deficient conditions, we asked whether we could detect a dependence of MalF on SecE for its insertion into the cytoplasmic membrane. The three larger periplasmic domains of MalF are sensitive to cleavage by exogenously added proteases (such as trypsin) in spheroplasts of strains lacking the MalK or MalG proteins (10). We reasoned that if MalF depends on SecE for its insertion into the cytoplasmic membrane, then depletion of
Sec Dependence of Membrane Protein Insertion

| 3.5 hr. | 5.5 hr. | 7.5 hr. |
|---------|---------|---------|
| +Ara    | +Glu    | +Ara    |
| +Glu    | +Ara    | +Glu    |
| 0 1 2 5 | 0 1 2 5 | 0 1 2 5 |

Fig. 2. Depletion of SecE results in a severe secretion defect. Strain CM124 was grown as described in Fig. 1. Pulse-chase labeling was performed at 3.5, 5.5, and 7.5 h. The translocation of MBP, DegP, and OmpA was assayed by visualization of immunoprecipitated precursor (p) and mature (m) forms of these proteins at the pulse (0 time point) and 1-, 2-, and 5-min chase time points. The precursor form of DegP appears as a diffuse doublet rather than a discreet band on these gels.

SecE might result in the appearance of a trypsin-resistant MalF species. The protease-resistant MalF protein would remain in the cytoplasm and not have its periplasmic domains accessible to cleavage by externally added proteases.

To assay for MalF insertion using the proteolysis assay, spheroplasts were derived from a SecE depletion strain BT62 (malF F"G K") grown in either glucose (depleted of SecE) or arabinose (SecE). We chose time points early in the SecE depletion process to maximize the likelihood that we were testing the direct effects of SecE depletion on MalF membrane insertion. In addition, the strength of the secretion defect for MBP was monitored in parallel as described for Fig. 2, so that a comparison of protein secretion versus cytoplasmic membrane protein insertion could be made. In cells that are either Sec+ or depleted of SecE for 2 h, the MalF protein was inserted into the cytoplasmic membrane in a trypsin-sensitive form, with its periplasmic domains accessible to protease (Table I, +Ara and +Glu, 2 h; +Ara samples shown in Fig. 3A, lanes 3–6). In contrast, at later time points of SecE depletion, a substantial proportion of MalF became insensitive to trypsin proteolysis (Table I, +Glu, 2.75 and 3.5 h; +Glu at 3.5 h shown in Fig. 3A, lanes 7–10). This MalF protein became trypsin-sensitive if the spheroplasts in these experiments were lysed prior to the addition of the protease (data not shown), indicating that this form of MalF was not intrinsically protease-resistant. We speculate that in secretion-compromised cells, MalF, that is insensitive to externally added protease is localized to one of two compartments. Because of the hydrophobic nature of the transmembrane domains of MalF, uninserted protein might aggregate in the cytoplasm. Alternatively, insertion of some or all of the transmembrane domains might occur, resulting in MalF that is apposed to or partially inserted into the cytoplasmic membrane. This latter species would have some or all of its periplasmic domains (that are normally sensitive to external protease) in the cytoplasm. To exclude the possibility that SecE-depleted cells were resistant to spheroplast formation, which would also result in protease-resistant MalF, steady state populations of MalF were shown to be sensitive to externally added protease by Western blot analysis (data not shown). This population of MalF molecules presumably would have been properly incorporated into the membrane during the Sec+ conditions of the depletion time course and persisted to late times in the experiment.

The amount of radio-labeled MalF detected in the different samples does not reflect significant variation in the recovery or the integrity of the spheroplasts in each case. A fraction of each of the solubilized cell extracts from the samples shown in Fig.
The effect of Sec inhibition on MBP and MalF localization. The abundance of precursor MBP and protease-resistant MalF is shown for after NaN₃ treatment or at different time points in the SecE depletion time course for BT62. The background level of protease-resistant MalF in cultures grown in arabinose was subtracted from the data shown in Table I.

*Previous work had suggested that MalF is not dependent on the SecA component of the secretion apparatus for its insertion into the cytoplasmic membrane (1). In these experiments, cells were treated with NaN₃ to inhibit SecA function (14), and then MalF insertion was assayed by a protease sensitivity assay similar to the one described here. Two important facts led us to re-examine the dependence of MalF insertion on SecA. First, as described in the previous section, MalF insertion was dependent on expression of SecE, one of the core components of the secretion apparatus. Because the basic mechanism of secretion is thought to involve the actions of SecE, SecY, and SecA, it seemed likely that SecA might also be involved in MalF insertion. Second, the experiments of McGovern and Beckwith (1) were performed in a Mal⁺ strain background. Normally, MalF expressed in a Mal⁺ strain acquires a protease-resistant form that reflects its inclusion into a stable complex with MalG and MalK; absence of either of these components of the complex results in protease-sensitive MalF (10). It is possible that in the Mal⁻ strains, MalF could acquire protease resistance by two different mechanisms, one in which MalF becomes complexed with MalG and MalK and one in which MalF is not inserted into the membrane due to a secretion defect. To address this latter possibility, both Mal⁻ and Mal⁺ strains were used to assess NaN₃ sensitivity of MalF insertion into the cytoplasmic membrane.

To confirm the results of McGovern and Beckwith (1), we tested the protease sensitivity of pulse-labeled MalF in the Mal⁻ strain BT8 with and without treatment with NaN₃. Without NaN₃ treatment, MalF initially inserts into the membrane in a trypsin-sensitive form and is rapidly converted into a trypsin-resistant conformation as a consequence of complex assembly with MalG and MalK (Table I). The initial trypsin-sensitive form is detectable as a portion (roughly half) of the population of labeled MalF molecules after a short pulse time point in this experiment; the MalGK-complexed, trypsin-resistant MalF form is already abundant at the pulse time point and becomes the predominant species after a chase (Table I). Consistent with previous results, cells treated with NaN₃ show no difference in the amount of trypsin-resistant MalF at the pulse time point compared with nontreated cells. In contrast, our added quantitation of the amount of trypsin-resistant MalF after a 10-min chase in the NaN₃-treated versus untreated samples showed a significant difference (35 versus 65%). The inability to distinguish between the two possible populations (described above) of trypsin-resistant MalF in these Mal⁻ cells makes interpretation of the data difficult. However, the lower level of protease-resistant MalF at the end of the chase in NaN₃-treated Mal⁻ cells suggests that the ability of MalF to assemble into the MalFGK complex in the membrane was compromised in the treated cultures.

The ambiguities created by using Mal⁻ cells were alleviated by an analysis of the protease sensitivity of MalF in the similar conditions.
malG strains BT62 and BT10. (For comparison with the SecE depletion data, the data from NaN₃-treated BT62 cells are shown in Figs. 3 and 4 and in Table I. The quantitation of the BT10 data is given in Table II. NaN₃-treated control samples of the ΔmalF strain KM1089 are shown in Fig. 3.) In the cultures not treated with NaN₃, the majority of MalF synthesized in a pulse is inserted into the cytoplasmic membrane in a trypsin-sensitive form, consistent with previous results (10). This population of MalF remains trypsin-sensitive after chase, because it is unable to form a native complex. In NaN₃-treated cultures, 30–40% of the MalF synthesized during a pulse-labeling is resistant to exogenous trypsin. Because none of this resistant species attributable to MalFGK complex formation, it represents noninserted MalF.

As was the case in the SecE depletion experiments, this uninserted population of MalF in NaN₃-treated cells was susceptible to endogenous proteases, as evidenced by its gradual disappearance during the course of a 10-min chase (Table II). The stability of MalF over the course of a 10-min chase in NaN₃-treated cultures ranges from 48–68%, compared with a slight increase in the amount of MalF detected in the chase compared with the pulse time points in untreated Sec- cells. The decrease in MalF stability in Sec-inhibited cells is observed in both Mal⁺ and the malG⁻ strains.

To show that the species of MalF that is sensitive to endogenous proteolysis in NaN₃-treated cells was actually the result of a block in SecA activity, we characterized the stability of the protein in the Mal⁻ strains BT7 and BT63. BT7 produces a wild-type SecA protein. BT63 produces a SecA mutant that is partially resistant to NaN₃ (from the azi-4 allele; Ref. 14). The trypsin sensitivity of MalF in pulse-labeled cultures of BT7 with and without NaN₃ treatment is analogous to that observed in BT8 under similar conditions (Table II). However, the sensitivity of MalF to trypsin and endogenous proteases in BT63 is relatively unchanged by the presence or the absence of NaN₃ treatment at both the pulse and the chase time points. The NaN₃ treatment of BT63 apparently did not impair either the membrane insertion of MalF or its subsequent assembly into the MalFGK complex. This result strongly suggests that the NaN₃ treatment of azi-4-sensitive cells affects the proper membrane insertion of MalF and the stability of MalF to proteolysis directly via the inhibition of SecA protein and not indirectly by a general disruption of the cell’s physiology.

These results indicate that the proper insertion of MalF requires the activity of SecA in addition to SecE. As with the SecE-depleted cells, the inhibition of MalF membrane insertion and MBP export caused by NaN₃ is diminished at the end of a 10-min chase after pulse-labeling. The inhibition of membrane insertion for MalF is not as strong with NaN₃ as with SecE depletion at the 3.5 h time point (Fig. 4 and Table I). However, the inhibition of MBP export is as strong with NaN₃ as with SecE depletion in BT62 at 3.5 h.

### DISCUSSION

The involvement of the Sec proteins in the export of signal sequence-containing proteins such as OmpA and MBP is well documented. However, a general mechanism for the insertion of proteins into the cytoplasmic membrane, including translocation of their hydrophilic domains across this membrane, has not been established. This issue has been addressed in only a few cases, and the examined proteins fell into two classes: 1) Sec-independent, e.g., M13 procoat, ProW, and MalF, and 2) Sec-dependent, e.g., Lep, SecY, and Tsr (1, 7–9, 15). In contrast to earlier work, we show here that MalF insertion into the cytoplasmic membrane requires both SecE and SecA.

Systems to study the Sec dependence of cytoplasmic membrane protein insertion have been plagued by two major problems. First, a general assay (such as signal sequence cleavage for fully secreted proteins) for cytoplasmic membrane protein insertion does not exist. To compensate for this, several studies have used an indirect assay for Sec dependence that relies on a comparison of the activity or localization of an enzymatic/polyepitope tag in fusion protein constructs in Sec⁻ or Sec⁻ conditions (8, 9). Although the results from these experiments are suggestive, the notion that the marker tag of the fusion protein does not affect the normal process of membrane insertion for the target protein is largely untested. A few specific proteins can be directly tested for Sec involvement in membrane insertion. For example, the studies on the membrane insertion of Lep assay its Sec dependence for insertion by the protease sensitivity of a large periplasmic domain in Sec⁻ and Sec⁻ conditions (8, 9). The inhibition of export of this domain results in the accumulation of protease-resistant Lep. Unfortunately, relatively few integral cytoplasmic membrane proteins can be analyzed by this method because of their general resistance to exogenous proteases in extracytoplasmic domains (e.g., Tsr; Ref. 16). MalF rapidly acquires resistance to exogenous proteases upon its export to the cytoplasmic membrane when assembled into the oligomeric MalFGK complex. However, in spheroplasted MalF⁻ cells, properly localized MalF is sensitive to added proteases (10). This finding allows for a sensitive MalF insertion assay in which one can attribute protease-resistant MalF in MalF⁻ cells unambiguously to noninserted MalF.

The second major pitfall encountered in previous studies was the use of relatively weak conditional sec mutations. As has been shown for secE mutations, many conditional sec mutations likely cause a decrease in the amount of functional secretion apparatus in the cell (5, 13). It seemed possible to us that if cytoplasmic membrane proteins in general had a greater

| Strainᵃ | Treatment | Pulse | Chase | Pulse | Chase |
|---------|-----------|-------|-------|-------|-------|
| BT10 (secA⁻, malEFG⁻) | NaN₃ | 13 | 13 | 110 | NDᵇ | NDᵇ |
| BT10 | + NaN₃ | 40 | 24 | 68 | ND | ND |
| BT8 (secA⁺, Mal⁻) | no NaN₃ | 46 | 65 | 110 | ND | ND |
| BT8 | + NaN₃ | 46 | 35 | 64 | ND | ND |
| BT7 (secA⁺, Mal⁻) | no NaN₃ | 38 | 86 | 110 | 0 | 0 |
| BT7 | + NaN₃ | 55 | 60 | 64 | 85 | 81 |
| BT63 (azi-4, Mal⁻) | No NaN₃ | 36 | 76 | 110 | 0 | 0 |
| BT63 | + NaN₃ | 26 | 71 | 99 | 34 | 31 |

ᵃ The strain names are given with the relevant genetic markers.
ᵇ ND, not determined.
affinity for the secretion apparatus than fully secreted proteins, then their membrane insertion and export of hydrophilic domains would be relatively less affected by the limited amount of secretion apparatus than that of fully secreted proteins. In fact, various members within the class of fully secreted proteins in E. coli, such as DegP, are only slightly affected by partial secretion defects in the cell; others, such as MBP, are extremely sensitive to even slight Sec defects. To address this issue, we sought to utilize a stronger conditional allele of the secE gene. One such conditional SecE mutant, in which the secE gene is expressed from the araBAD promoter, has been described in previous studies (6). Because SecE is an essential component of the secretion apparatus, strains containing the para-secE construct are absolutely dependent on arabinose for prolonged growth (Fig. 1).

The secretion defect in cells that have been depleted for the SecE protein is much stronger than that observed in other conditional sec mutants. The magnitude of this defect is evidenced by two findings. First, CM124 cells depleted of SecE accumulate large amounts of precursor species of proteins such as DegP, which have been difficult to visualize previously (Fig. 2). Second, precursors of proteins, such as OmpA, that accumulate under SecE depletion conditions are very slowly converted to the mature species during the course of a chase. This is in contrast to the fate of precursor OmpA accumulated in many other sec conditional mutants where a large proportion of this precursor species eventually becomes exported (13). Conversely, there is no detectable secretion defect in the SecE depletion strains when grown under permissive conditions, in contrast to other secE conditional alleles (Fig. 2; Ref. 13). The ability to both elicit a strong secretion defect in SecE-depleted cells and to assay MalF insertion in a sensitive manner led us to readdress whether MalF has a Sec requirement for its insertion into the cytoplasmic membrane.

In the SecE depletion strain BT62 (malF::malG) grown in arabinose, MalF is inserted into the membrane and is sensitive to trypsin in spheroplasted cells (Fig. 3 and Table I). This trypsin sensitivity is indistinguishable from that observed in wild type secE+ malF::malG cells (Table I, Table II; also see 10). In cells depleted of SecE for 3.5 h, there is a dramatic increase in the amount of pulse-labeled MalF protein that is resistant to proteolysis (Fig. 4). These data strongly suggest that MalF requires the SecE component of the secretion apparatus for its proper insertion into the cytoplasmic membrane.

The dependence of MalF on SecE for its membrane insertion prompted us to re-examine the involvement of the SecA protein in this process. Like SecE-depleted cells, NaN3-treated BT62 cells also contain an elevated level of trypsin-resistant MalF immediately after the synthesis of the protein (Fig. 3 and Table I). This elevated level of trypsin-resistant MalF is also seen in the secE+ malF::malG strain BT10 when treated with NaNa3 (Table II). These results are consistent with an additional dependence on SecA for the membrane insertion of MalF.

Previously, McGovern and Beckwith (1) had examined the protease sensitivity of pulse-labeled MalF in cultures of a mal+ strain with and without treatment with NaNa3 (1). In both cases, they observed a population of protease-sensitive MalF (−50%) giving rise to similar peptide patterns after SDS-PAGE. To directly compare our results with theirs, we examined the protease sensitivity of MalF in the Mal− strain BT8 (Table II). We found that a similar amount of trypsin-resistant MalF is present at the pulse time point, with or without NaNa3 treatment, consistent with the observations of McGovern and Beckwith (1). We further characterized the amount of MalF and its trypsin sensitivity after a chase. The increase in the level of trypsin-resistant MalF during a chase in NaNa3-un-treated cells reflects the continued incorporation of MalF into the MalFGK complex. In contrast, there is a decrease in the amount of trypsin-resistant MalF at the end of a chase in the presence of NaNa3. Therefore, proteolysis experiments that only examined the state of MalF at the pulse time point in Mal− cells led to incorrect conclusions regarding the Sec dependence of MalF.

In both SecE-depleted and SecA-inhibited (NaN3-treated) cultures, an unstable population of MalF is revealed in the unproteolysed controls (see Tables I and II, MalF stability). This turnover of newly synthesized protein during the course of the chase is a dramatic increase in the sensitivity of MalF to endogenous proteases. We suggest that when MalF is not properly inserted into the membrane, it becomes a substrate for cellular proteases, which recognize it as a misfolded protein. In BT63, containing NaNa3-resistant SecA, NaNa3 treatment did not result in a loss in MalF stability to endogenous proteases (Table II).

The amount of protease-resistant MalF caused by NaNa3 inhibition of SecA is smaller than that caused by SecE depletion of the cell (Fig. 4 and Table I). This difference in the inhibition of the membrane insertion of MalF caused by the different treatments is in contrast to a similar inhibition of MBP export due to NaNa3 treatment compared with SecE depletion at 3.5 h +Glu (Fig. 4). MalF also seems to be affected at later times than MBP in the SecE depletion time course. The greater defect in the membrane insertion of MalF caused by SecE depletion than by NaNa3 inhibition of SecA is consistent with two previous observations. First, Werner et al. (17) have observed in vitro that the cytoplasmic membrane mannitol carrier protein MtIA was dependent upon the SecY protein for proper membrane insertion. However, MtIA inserted into membranes normally in the presence of reduced levels of SecA. Second, Rusch et al. (18) have characterized the export of alkaline phosphatase precursors as a function of the relative hydrophobicity of their signal sequences. They found that signal sequences with very high hydrophobicity mediated precursor export that was not detectably inhibited by treatment of cells with NaNa3. Proteins localized by the Sec apparatus with very hydrophobic signal sequences or transmembrane domains (like MalF or MtIA) may have a higher affinity for the Sec machinery than more typical signal sequence-containing precursor proteins. Therefore, detecting a Sec dependence for this class of proteins may require a severe Sec defect stronger than most conditional Sec mutants at nonpermissive conditions.

This proposal is supported by work from two groups characterizing the protease export (SEC) pathway in Saccharomyces cerevisiae. Green et al. (19) and Stirling et al. (20) found that the insertion of proteins into the endoplasmic reticulum membrane is dependent on several proteins, including SEC61 (a homolog of the E. coli SecY protein). However, not all sec alleles can result in inhibition of membrane protein insertion. Apparently strongly defective mutants or double mutants are required in many instances to detect a SEC involvement in membrane protein insertion, whereas weaker mutants will inhibit the export of several soluble proteins with degradable signal sequences across the membrane.

We have determined that MalF depends on the Sec apparatus for proper membrane insertion using a direct protease assay coupled with two strategies for eliciting potent and specific secretion defects. In contrast, McGovern and Beckwith (1) concluded that MalF does not utilize the Sec apparatus for membrane localization by examining MalF and MalF fusion proteins in either SecDFc mutants or NaN3-treated cells. Our work does not address the dependence of MalF on SecDF for translocation of periplasmic domains. We did assay the export
of periplasmic domains of MalF-PhoA fusion proteins in NaN3-treated cells (data not shown). In results similar to those of McGovern and Beckwith (1), we also failed to detect any inhibition caused by NaN3 in the membrane assembly of the MalF fusion proteins. However, recent experiments of Sääf et al. (21) and Jander et al. (22) did demonstrate a Sec dependence for export of some periplasmic domains in other MalF fusion constructs. In the experiments of Sääf et al. (21), NaN3 treatment of cells expressing MalF-Lep fusions caused a weak defect in export of the large periplasmic domain of MalF, compared with the export of the precursor form of OmpA. The work of Jander et al. (22) demonstrated a role for both SecA and SecE in the export of biotinylated tags fused to different regions of Mal, but the degree of Sec involvement was not quantified.

Our study highlights the pitfalls and difficulties in determining the Sec involvement in integral membrane protein assembly. Our results suggest that great caution should be used in the interpretation of localization data from fusion protein studies, given that the MalF-PhoA fusions did not show a SecA dependence clearly evident from our analysis of the intact protein and from the analysis of some kinds of fusion proteins. The exclusive use of NaN3-inhibition of SecA as a secretion defect in this kind of experiment may be inadequate. We advise the additional use of strong Sec mutants (such as a SecE depletion strain) for the analysis of Sec dependence for protein localization studies.

Further studies are needed to determine whether membrane proteins without cleavable signal sequences generally rely on the Sec pathway for membrane assembly. Based on studies with an extensive collection of Lep fusion constructs, von Heijne and co-workers have proposed that the translocation of short (≤60 residues) extracytoplasmic domains is Sec-independent, whereas that of longer domains is generally Sec-dependent (reviewed in Ref. 23). The membrane insertion of MalF is now partially consistent with this model. However, because we quantified the amount of intact MalF after proteolysis, our results suggest that MalF may require the Sec machinery for translocation of each of its three periplasmic domains (with 180, 30, and 30 residues) that are susceptible to proteolytic cleavage, not just the largest. It may be that many, if not most, other membrane proteins will exploit the Sec machinery during the normal in vivo insertion and assembly process, regardless of the size of the hydrophilic domains to be translocated.

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