Is Ffh required for export of secretory proteins?

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Abstract In *Escherichia coli*, protein export from the cytoplasm may occur via the signal recognition particle (SRP)-dependent pathway or the Sec-dependent pathway. Membrane proteins utilize the SRP-dependent route, whereas many secretory proteins use the cytoplasmic Sec machinery. To examine the possibility that signal peptide hydrophobicity governs which targeting route is utilized, we used a series of PhoA signal sequence mutants which vary only by incremental hydrophobicity changes. We show that depletion of SRP, but not trigger factor, affects all the mutants examined. These results suggest secretory proteins with a variety of signal sequences, as well as membrane proteins, require SRP for export.

Key words: phoA; Protein export; Signal recognition particle; Secretory protein; Trigger factor; *Escherichia coli*

1. Introduction

Most exported proteins are translocated across the inner membrane of bacteria through the SecYEG complex or translocon [1]. In *Escherichia coli*, two pathways have been identified for targeting proteins to this translocon. The signal recognition particle (SRP)-dependent pathway involves binding of SRP to the nascent chain and its subsequent release upon docking of the SRP with FtsY near translocation sites. The SecA/SecB-dependent pathway involves binding of the preprotein to SecB and its subsequent transfer to SecA, the ATPase associated with the translocon.

There is conflicting evidence as to whether the SRP-dependent pathway is limited to the export of inner membrane proteins or is also involved in the transport of secretory proteins from the cytoplasm. The insertion of the inner membrane protein leader peptidase (Lep) and a Lep mutant, Lep-inv, were strongly affected by depletion of either Ffh (the bacterial homolog of the 54 kDa subunit of mammalian SRP) or 4.5S RNA, another component of bacterial SRP [2]. A separate study showed that depletion of Ffh resulted in an export defect of several periplasmic proteins such as alkaline phosphatase (PhoA), β-lactamase, and ribose-binding protein, as well as the outer membrane proteins, LamB and OmpF [3]. This evidence suggests that bacterial SRP is involved in the export of both secretory and membrane proteins. However, in Ffh depleted cells with the concomitant overexpression of exported proteins, only the overexpression of polytopic membrane proteins, but not secretory proteins, led to a loss of cell viability [4]. This genetic screening strategy suggested that only membrane proteins serve as SRP substrates. Furthermore, in vitro studies with purified components revealed that membrane proteins required SRP components, but not SecA/SecB, for membrane integration, whereas secretory proteins required SecA/SecB, but not SRP components, for translocation [5]. These latter studies suggest that most *E. coli* secretory proteins can utilize the SRP-independent pathway with high efficiency.

Trigger factor is a ribosome-associated chaperone which may play a role in directing proteins into the Sec-dependent export pathway and excluding nascent chains from association with SRP [6]. In vivo, trigger factor has been found to be non-essential. Lethality results only when trigger factor depletion occurs in combination with depletion of DnaK, the bacterial Hsp70 homolog [7,8].

In general, membrane proteins (type I or type II) contain signal anchor sequences which are more hydrophobic than those of secretory proteins. The substrate specificity of SRP for membrane proteins may reflect the higher affinity of SRP for relatively hydrophobic signal sequences. Consistent with this idea, in vitro crosslinking studies have shown that more hydrophobic signal sequences are better crosslinked to Ffh [9,10]. Furthermore, crosslinking to trigger factor was unaffected by hydrophobicity [9]. These studies utilized a series of signal peptides which varied in the ratio of alanine and leucine residues in the hydrophobic core, thereby varying the overall hydrophobicity in a systematic way.

In the present study, we have used equivalent signal sequences, which are hydrophobicity variants of the wild-type alkaline phosphatase [11]. The advantage of this approach is twofold. First, we can directly correlate the in vitro crosslinking data with the in vivo effect of SRP or trigger factor depletion on transport of a secretory protein. Secondly, involvement of Ffh in the export of secretory proteins with a variety of signal sequences can be represented with this simple model system. Surprisingly, our results show that depletion of SRP has a profound effect on the export of all the mutant proteins regardless of the hydrophobicity in the signal sequence. On the

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Abbreviations: Ffh, fifty-four homolog; MOPS, morpholinepropanesulfonic acid

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other hand, depletion of trigger factor has no effect on export of the same series of proteins. These results are consistent with the idea that the SRP-dependent pathway is involved in the export of not only membrane proteins, but secretory proteins as well.

2. Materials and methods

2.1. Strains and plasmids

E. coli AW1043[Δ*lac galU galK* Δ(*leu-ara*)*phoA* E15 *proC*::Tn5] was used for the generation and replication of mutant forms of alkaline phosphatase. *E. coli* WAM121(MC4100, *araD*$^+$; *ffh*::*kan*1; *attB*::R6-Kori, ParaBAD$^-$*ffh*$^+$, gift from G.J. Phillips) was used to construct WAM823. *E. coli* WAM823 (WAM121 Δ*phoA*), and *E. coli* BLR(DE3) (Novagen, Madison, WI, USA) were used for transport analyses with SRP. C600 and BB4802 (C600 Δtig::kan) (gifts from B. Bukau) were used for the trigger factor studies. The mutant alkaline phosphatase plasmids have been described previously [11,12].

2.2. Deletion of the chromosomal phoA gene from WAM121

A *Xho*I site was created in the DNA corresponding to amino acid position 283 of WT-Nhe in the *phoA* gene by oligonucleotide-directed mutagenesis [13]. This plasmid was digested with *Sal*I and *Xho*I and self-ligated, resulting in WT(Δ*Sal*I/*Xho*I) which is missing the first two thirds of *phoA* and does not have an appropriate open reading frame of *phoA*. WT(Δ*Sal*I/*Xho*I) was cut with *Hin*dIII and *Bam*HI to generate a fragment containing the upstream sequence of *phoA* (288 bp), partially deleted *phoA*, and the downstream sequence of *phoA* (1444 bp). The fragment was ligated to the corresponding sites of pMAK700. The resultant plasmid, pMAKΔPhoA, was introduced into WAM121 to replace the chromosomal copy of the *phoA* gene according to Hamilton et al. [14]. A *phoA* knockout mutant of WAM121 was named WAM823.

2.3. Depletion of Ffh

Strain WAM823 carrying plasmids encoding alkaline phosphatase and β-lactamase was grown overnight in 5 ml of M9 medium [15] supplemented with thiamine hydrochloride (2 μg/ml) and arabinose (0.2%), and containing 250 μg/ml ampicillin and 50 μg/ml kanamycin. The cells (0.5 ml) were washed twice in the same medium (1 ml each) lacking a carbon source and finally resuspended in the same medium (0.5 ml). Cells (0.15 ml) were used to inoculate 10 ml of the same medium containing either arabinose or 0.2% glucose and further cultured at 37°C until filamentation was detected (about 7 h) [3]. Cells were then washed twice with morpholinepropanesulfonic acid (MOPS) (without phosphate) and resuspended in the same medium supplemented with amino acids (20 μg/ml, minus methionine) in the presence of either arabinose or glucose. Cells were incubated at 37°C for 15 min to induce the expression of alkaline phosphatase. Cells were radiolabeled with [^{35}S]methionine for 40 s and chased with unlabeled methionine (4 mg/ml final concentration) for 30 s. Half of the samples were immunoprecipitated with PhoA antiserum and the other half with β-lactamase antiserum [16].

2.4. Trigger factor studies

Strains C600 and BB4802, containing the *phoA* mutant plasmids in which alkaline phosphatase is under the control of the *lac* promoter, were grown in Luria-Bertani medium to mid-logarithmic phase. Cells were harvested, washed with MOPS medium without amino acids, but containing 40 mM phosphate, to repress chromosomal alkaline phosphatase expression and resuspended in the same medium supplemented with amino acids (20 μg/ml, minus methionine) and 0.4 mM isopropyl-1-thio-β-D-galactoside to induce plasmid alkaline phosphatase expression. Cells were incubated at 37°C for 15 min prior to radiolabeling and alkaline phosphatase was immunoprecipitated as described above.

2.5. Electrophoresis and quantitation of protein bands

Immunoprecipitated proteins were separated by electrophoresis on 7.5–15% sodium dodecyl sulfate-polyacrylamide electrophoresis gels [17]. The pattern was visualized by autoradiography as described in Kendall and Kaiser [18] and protein was quantified with a phosphorimager (Bio-Rad).

		↓
WT	MKQST **IALALLPLLF** TPVTKA	RTP
1L9A	MKQST **AAAAAAAALA** TPVTKA	RTP
3L7A	MKQST **LAAAAALALA** TPVTKA	RTP
4L6A	MKQST **LALAAAALAL** TPVTKA	RTP
5L5A	MKQST **LALALALALA** TPVTKA	RTP
7L3A	MKQST **LALLLLLALA** TPVTKA	RTP
9L1A	MKQST **LALLLLLLLL** TPVTKA	RTP

Fig. 1. The amino acid sequences of the signal peptides of alkaline phosphatase are given with the hydrophobic core region shown in bold face. The signal peptide cleavage site is marked by an arrow.

3. Results and discussion

In order to test the hypothesis that the SRP interacts only with preproteins with hydrophobic signal sequences, we used a series of *E. coli* alkaline phosphatase (PhoA) mutants with signal sequences (Fig. 1) that cover a wide range of hydrophobicities [11]. When PhoA proteins containing different signal sequences were expressed in a Ffh-depleted strain, the precursor form of PhoA accumulated in every case except for the preprotein with the 9L1A signal sequence (Fig. 2A). The extent to which precursor proteins accumulated in the absence of Ffh, relative to accumulation in the presence of Ffh, increased as the hydrophobicity of signal sequences decreased. Recent evidence [19] suggested that preproteins with more hydrophobic signal sequences would be more affected by depleting Ffh. Interestingly, these results suggest that the role of SRP is not restricted to the transport of precursor proteins with very hydrophobic signal sequences or membrane proteins, but it is also responsible for the transport of proteins that have relatively weak hydrophobic signal sequences.

The 9L1A signal sequence has been known to crosslink well to Ffh [10], suggesting that it may follow a SRP-dependent pathway. However, under the conditions used here, transport of PhoA with the 9L1A signal sequence did not appear sensitive to limiting amounts of Ffh. We considered the possibility that the insensitivity of PhoA with a 9L1A signal sequence to limiting amounts of Ffh is not because it follows a SRP-independent pathway, but because it interacts with SRP so well it can still effectively utilize the small amount of Ffh left in the Ffh-depleted cells. If this is true, high expression of PhoA carrying the very effective signal sequences should interfere with the transport of proteins carrying less effective signal sequences that use Ffh, resulting in accumulation of precursor proteins with these less effective signal sequences. To examine this, we analyzed the processing of β-lactamase, another SRP-dependent protein [3], in the same strains harboring PhoA containing the various signal sequences (Fig. 2B). When the amount of Ffh is not limiting (+arabinose), the precursor form of β-lactamase accumulated to only a small degree with coexpression of PhoA-9L1A or PhoA-7L3A. In contrast, when the amount of Ffh is limiting (−arabinose), the precursor form of β-lactamase showed significant accumulation with coexpression of 9L1A or 7L3A, suggesting not only that PhoA and β-lactamase are competing for a limited source of Ffh, but also that PhoA with 9L1A or 7L3A signal sequences are transported in a SRP-dependent manner. These results also point to a limitation of Ffh-depletion experiments; that is, complete depletion is rarely achievable and a residual

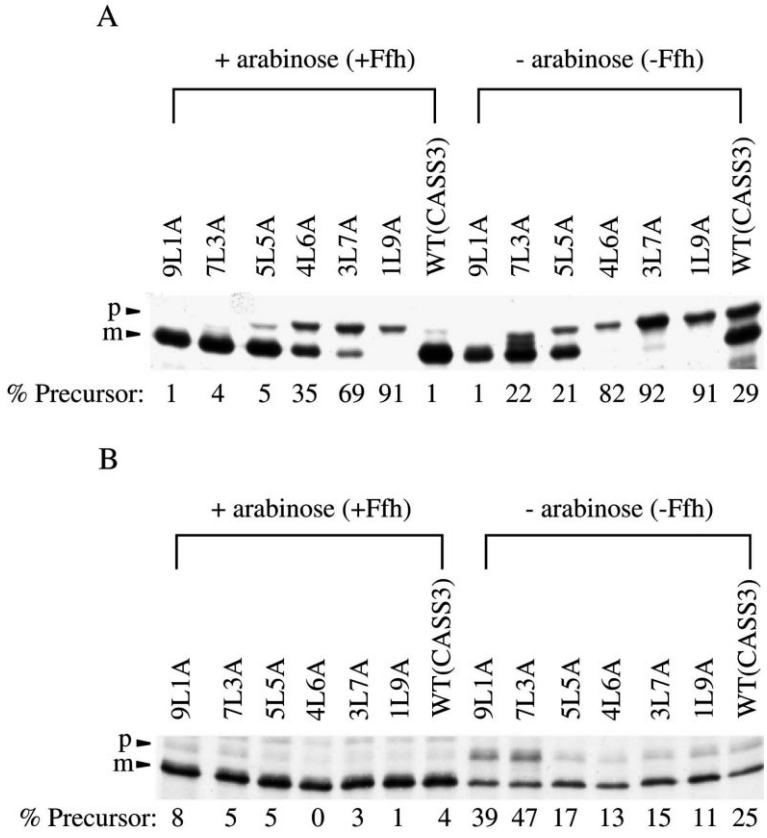

Fig. 2. Transport of PhoA with different signal sequences and wild-type β-lactamase in a Ffh-depleted strain, WAM823. Proteins were labeled with [^{35}S]methionine for 40 s and chased with excess cold methionine for 30 s and then immunoprecipitated. β-Lactamase is constitutively expressed from the plasmid carrying the PhoA mutants. Half of the samples were immunoprecipitated by anti-PhoA antiserum (A) and the other half by anti-β-lactamase antiserum (B). The positions of the precursor and mature forms of alkaline phosphatase are indicated by p and m, respectively. Percent precursor is indicated below each lane.

amount of Ffh can be effectively utilized by very efficient and hydrophobic signal sequences. Similarly, when SRP substrates are overproduced in a strain that is used to generate lysate, less Ffh is available for crosslinking to added nascent chains in vitro [9].

Perhaps if membrane proteins with highly hydrophobic signal anchors are overexpressed, these will interact most efficiently with the small amount of SRP remaining in Ffh depleteable cells and titrate it out more effectively than will secretory proteins with less hydrophobic signal peptides. Consequently, a loss in cell viability is likely, not because only membrane proteins use SRP, but rather because they have a higher affinity for SRP. Secretory proteins, which are necessary for cell viability, but which contain less hydrophobic signals, cannot compete and the cells die. This is consistent with the results from genetic selections for SRP substrates which employ the overproduction of exported proteins in Ffh-depleteable cells [4].

Trigger factor has been identified as a cytosolic component of the Sec-dependent (SRP-independent) pathway [6]. Transport of the PhoA mutant precursors was tested in the presence or absence of trigger factor. The extent of transport was nearly identical in the wild-type and trigger factor null strains for every precursor, regardless of the hydrophobicity of its signal sequence (Fig. 3). Thus, it appears that these PhoA precursors do not depend on trigger factor in the earliest steps of export, consistent with their dependence on SRP.

Recent findings imply that SRP-dependent inner membrane proteins can interact with SecA and SecY in the translocation site [10,20,21]. It is, therefore, possible that many proteins

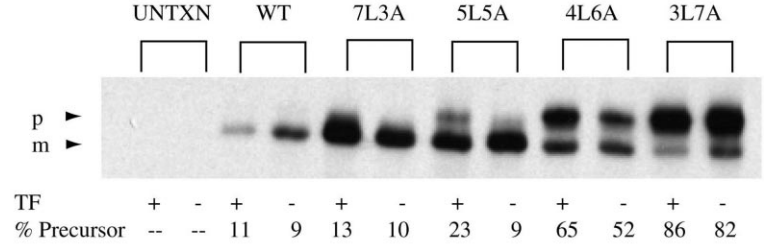

Fig. 3. Transport of PhoA with different signal sequences in a trigger factor null strain, BB4802. Proteins were radiolabeled as in the legend to Fig. 2, and alkaline phosphatase was immunoprecipitated. The positions of the precursor and mature forms are indicated by p and m, respectively. Percent precursor is indicated below each lane. UNTXN refers to untransformed cells.

move into the SecA/SecB pathway from SRP, and that these two pathways are not mutually exclusive. Consistent with this notion, PhoE utilizes a SecB-dependent pathway, yet the PhoE signal sequence has been found to crosslink with SRP upon readdition of wild-type lysate in a heterologous expression system [22] or when Ffh is added to physiological concentration in a homologous expression system [9]. In addition, some of the PhoA signal sequences, which are shown here to be sensitive to Ffh depletion, have also been shown to accumulate in the absence of SecB [12] and SecA [23] in vivo. Collectively, the results indicate that while signal peptide hydrophobicity may influence the affinity of the preprotein for these components, the substrate specificity of the SRP- and SecA/SecB-dependent pathways may overlap.

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