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MINI-REVIEW

Protein Translocation *In Vitro*: Biochemical Characterization of Genetically Defined Translocation Components

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Abstract

Recent years have seen the convergence of both genetic and biochemical approaches in the study of protein translocation in *E. coli*. The powerful combination of these approaches is exemplified in the use of an *in vitro* protein synthesis–protein translocaltion system to analyze the role of genetically defined components of the protein translocation machinery. We describe in this review recent results focusing on the function of the *secA*, *secB*, and *secY* gene products and the demonstration of their requirement for *in vitro* protein translocation. The SecA protein was recently shown to possess ATPase activity and was proposed to be a component of the translocation ATPase. We present a speculative working model whereby the translocator complex is composed of the integral membrane proteins SecY, SecD, SecE, and SecF, forming an aqueous channel in the cytoplasmic membrane, and the tightly associated peripheral membrane protein SecA functioning as the catalytic subunit of the translocator or "protein-ATPase."

Key Words: Protein secretion; *in vitro* protein translocation; *E. coli* membrane vesicle; energetic requirement; ATP hydrolysis; SecA, SecB, and SecY proteins; protein translocator.

Introduction

Great insights into the process of protein sorting in eukaryotes were first provided as a consequence of the development of a reliable *in vitro* protein

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synthesis–protein translocation system. In fact, the development of the signal sequence hypothesis was dependent upon such a system (Blobel and Dobberstein, 1975). The *in vitro* system facilitated the identification of the signal recognition particle (SRP) (Walter and Blobel, 1981) and the docking protein (Meyer *et al.*, 1982).

Biochemical studies, including the use of *in vitro* systems, in *Escherichia coli* have also greatly enhanced our understanding of the protein translocation process in which metabolic energy is required (see reviews in Wu and Tai, 1986; Randall *et al.*, 1987; Tai, 1990). However, the power of the genetic system first provided important information in identifying protein export factors (Silhavy *et al.*, 1983; Oliver, 1985; Beckwith and Ferro-Novick, 1986). The signal sequence is an essential requirement for protein translocation (Silhavy *et al.*, 1983), yet the export of proteins also requires additional protein components. These protein components together form the translocation machinery. The isolation of mutations that pleiotropically affect protein export identifies genes that encode components of the translocation machinery, and mutations within secretory proteins identify aspects of the signal sequence. The genetic approach was very successful and identified many potential candidates for export machinery components and structural features of the signal sequence. The isolation of suppressors to mutations within a secretory protein was to identify protein components that may interact with the secretory protein, and suppressors of pleiotropic export defects were to identify possible interactions between translocation machinery components as well as identify new components (Brinkman *et al.*, 1984; Shiba *et al.*, 1984; Oliver, 1985; Shiba *et al.*, 1986). However, although the isolation of suppressors of specific signal sequence defects has proven fruitful (Silhavy, this volume), suppressors of pleiotropic export defects often were found to reside in genes encoding components of the protein synthesis machinery (Oliver and Liss, 1985; Shiba *et al.*, 1986). In fact, mutations or antibiotics that slowed the rate of protein synthesis in the cells were able to suppress the conditional pleiotropic export defects (Lee and Beckwith, 1986). Consequently, suppression analysis for general export defects became exceedingly difficult.

In vitro protein translocation systems are well suited to studying a problem which requires the separation of protein synthesis from protein translocation. Indeed, such an *in vitro* system has been established for protein translocation across *E. coli* membrane vesicles in which post-translational translocation is almost as efficient as co-translational translocation, unlike the endoplasmic reticulum system (reviewed in Tai, 1986). In this review we will discuss how the recent convergence of both genetic and biochemical approaches, which had otherwise remained surprisingly separate has provided us with a more confident list of the protein components that make up

the translocation machinery, and also with insights as to the possible interactions between some of these components. Our focus will be primarily on the employment of *in vitro* protein translocation systems for the biochemical characterization of the genetically defined translocation machinery components.

In Vitro Protein Translocation Systems

The *in vitro* protein translocation system is basically comprised of a soluble protein fraction (S30) capable of synthesizing protein when programmed with mRNA, and a membrane fraction of inverted cytoplasmic membrane vesicles (Rhoads *et al.*, 1984; Muller and Blobel, 1984a). In some cases, purified precursor molecules are used (Crooke and Wickner, 1987; Yamane *et al.*, 1988; Tian *et al.*, 1989). In these systems proteins are considered translocated if processing of the signal sequence coincides with protection from proteolytic digestion, and in some systems (Rhoads *et al.*, 1984), by cosedimentation of the processed protein with the membrane vesicles. The protection of the processed protein from protease digestion and cosedimentation with the membrane vesicles are taken as indications that the protein is translocated into the lumen of the vesicle, although it is possible that some of the protein is merely embedded in the membrane. Protein translocation can be examined cotranslationally by synthesizing protein in the presence of membrane vesicles, or posttranslationally by blocking synthesis prior to the addition of membrane vesicles (Chen *et al.*, 1985).

The discovery of posttranslational translocation indicated that arrest of translation prior to the secretory protein interaction with the membrane was not necessary, as had been postulated for protein translocation in eukaryotes (reviewed in Tai, 1986). It also clearly showed that the source of energy for the translocation process is not derived from translation. Thus, the *in vitro* system allows one to examine the energetic requirements of protein translocation; in particular, it allows one to separate and characterize the role of protonmotive force (PMF) and ATP (Chen and Tai, 1985). Both co- and posttranslational translocation appear to have the same requirements *in vitro* (Chen and Tai, 1987).

The Energetic Requirements of Protein Translocation

The energetic requirements of protein translocation have been reviewed recently (Tai, 1990). Many studies have demonstrated the importance of the protonmotive force in protein translocation both *in vivo* and *in vitro*.

Dissipation of the electrochemical gradient of cells by protonophores results in the accumulation of the precursor forms of several secretory proteins, including phage M13 procoat, outer membrane protein A (OmpA), alkaline phosphatase, and β-lactamase. It is difficult, however, to determine the precise effect of the protonophores. Although the dissipation of the proton electrochemical gradient clearly impairs protein translocation, it is not clear whether PMF acts directly as an energy source or acts indirectly by affecting the topology or the conformation of membrane protein or lipids.

PMF is not essential for protein translocation *in vitro*, although it was demonstrated that dissipation of the PMF across inverted membrane vesicles greatly inhibits the translocation of OmpA and alkaline phosphatase precursors and the translocation is stimulated by PMF at suboptimal concentrations of ATP (Chen and Tai, 1985; Chen and Tai, 1986a). Mizushima and coworkers reported that both ATP and PMF were required for *in vitro* protein translocation (Yamane *et al.*, 1987, 1988), but more recently they showed that it is not an absolute requirement for the translocation of pro-OmpA and a pro-OmpF-Lpp hybrid into inverted membrane vesicles: sufficient SecA eliminates the PMF requirement (Yamada *et al.*, 1989a, b). Geller *et al.* (1986) also reported that both PMF and ATP were required for translocation of OmpA, but have since confirmed the facilitatory role of PMF (Geller and Green, 1989). Additionally, the insertion of M13 procoat or proliprotein into liposomes or membranes also does not require PMF *in vitro* (Ohno-Iwashita and Wickner, 1983; Tian *et al.*, 1989).

These *in vitro* results are supported by the observation that *E. coli* can be conditioned to grow normally in the presence of the protonophore CCCP, and the growth rate and the rate of the translocation of proteins is normal (Kinoshita *et al.*, 1984; M. Miller, personal communication). Moreover, although PMF is also required for the translocation of proteins into mitochondria, the polarity is the reverse of the bacterial cytoplasmic membrane. And, PMF is not required for translocation of proteins into the endoplasmic reticulum or chloroplast (see Chen and Tai, 1985; Eilers and Schatz, 1988; Verner and Schatz, 1988).

It is clear that ATP is an absolute requirement for protein translocation. Membranes isolated from cells unable to generate a PMF due to lack of the H^+-ATPase are active in protein translocation in the presence of ATP (Chen and Tai, 1985; Chen and Tai, 1986a). The translocation activity of membranes can be inhibited by irradiation in the presence of photoreactive azido-ATP, and the inhibition is blocked if ATP is present, indicating the existence of at least an ATP-binding membrane protein component(s) involved in protein translocation (Chen and Tai, 1986b; Chen adn Tai, 1987). Studies with ATP analogs indicate that both the adenosine and phosphate moieties, and ATP hydrolysis are required for protein translocation (Chen and Tai,

1986b). The ATP analog 2′,3′-cAMP inhibits protein translocation but not protein synthesis during cotranslational translocation, and this inhibition of translocation is relieved by the addition of ATP, suggesting that ATP is also required for cotranslational translocation (Chen and Tai, 1987).

The essentiality of ATP in the translocation has also been demonstrated for the translocation of proteins into the endoplasmic reticulum, mitochondria, and chloroplasts (see Verner and Schatz, 1988).

Biochemical Characterization of Genetically Defined Components

Fractionation of the *in vitro* system facilitates definition of the components of the translocation machinery and their interactions. To examine the effects of mutation on the functions of the gene products in the *in vitro* system, either the soluble protein fraction or the membrane fraction of the *in vitro* system can be isolated from the mutant in question, or the role of a genetically defined component by immunochemically removing it from the soluble fraction can be examined. We have recently used both approaches to successfully demonstrate the requirement for SecA, SecB, and SecY in the *in vitro* system.

SecA

The *secA* gene encodes a 102-kDa soluble protein which is also found peripherally bound to the cytoplasmic membrane (Oliver and Beckwith, 1982; Schmidt *et al.*, 1988). SecA is essential for cell growth, and mutations in *secA* can result in pleiotropic loss of protein translocation (Oliver and Beckwith, 1981) or in the suppression of signal sequence defects (*prID* alleles) (Bankaitis and Bassford, 1985; Fikes and Bassford, 1989; Stader *et al.*, 1989). In addition the synthesis of SecA is regulated by the activity of the translocation machinery (Oliver and Beckwith, 1982). SecA synthesis increases when the cells accumulate precursor proteins as a consequence of a mutation in another component of the translocation machinery (Rollo and Oliver, 1988) or the overexpression of an export-defective hybrid protein (Oliver and Beckwith, 1982; Gardel *et al.*, 1987). Schmidt and Oliver (1989) have shown that SecA inhibits translation of its own mRNA.

The essential requirement of SecA for *in vitro* protein translocation was recently demonstrated (Cabelli *et al.*, 1988). Membranes with greatly reduced levels of SecA due to a conditional *secA* amber mutation are inactive for protein translocation only if the soluble component of the *in vitro* system was also greatly reduced in its level of SecA, due either to a conditional amber mutation or immunochemical depletion of the soluble fraction (Cabelli *et al.*,

1988). It is also possible to reconstitute the translocation activity of SecA-depleted membranes by preincubating the membranes with SecA (Cabelli *et al.*, 1988). These reconstituted membranes are active even if assayed in the absence of soluble SecA, suggesting that SecA activity functions on the membrane.

Cytoplasmic membranes that have been washed with urea lose their translocation activity and can be restored by the addition of purified SecA to the *in vitro* mixtures (Cunningham *et al.*, 1989; Kawasaki *et al.*, 1989), or by preincubating the membranes with SecA (L. Chen and P. Tai, in preparation), indicating that the loss of translocation activity is associated with a possible loss of SecA. However, quantitation of the urea-washed membranes shows that significant amounts of SecA remain on the membranes even after extensive urea washes. The membrane-associated SecA appears to exist in two forms. One form is sensitive to proteinase K digestion, whereas another is resistant to proteinase K digestion (L. Chen and P. Tai, in preparation). The tight binding of SecA to the membrane may be due to its incorporation into a protein complex in the membrane, or its incorporation into the membrane.

The activity of the urea-washed membranes can be fully restored by the addition of purified SecA, whereas the activity of proteinase K-treated membranes is only partially restored (L. Chen and P. Tai, in preparation). The inability of proteinase K-treated membranes to be fully restored may be due to digestion of another component of the translocation machinery; SecY has been shown to be susceptible to protease digestion (Akiyama and Ito, 1987).

It has been reported that the SecA protein is a component of the protein translocation ATPase and has two to three ATP binding sites (Lill *et al.*, 1989). The purified SecA protein and urea-treated membranes have equally low ATPase activity when assayed alone, but the addition of SecA to the membranes treated with urea or azido-ATP results in increased ATPase activity. When purified OmpA precursor is added to the reactions, ATPase activity increases up to 100-fold (Lill *et al.*, 1989). Interestingly, the stimulation by OmpA precursor requires that the precursor be competent for translocation. Neither the signal sequence alone nor the mature OmpA can stimulate SecA-associated ATPase activity (Cunningham and Wickner, 1989). These results suggest that the ATPase activity is associated with the process of protein translocation.

SecY

The *secY* gene (also known as *prlA* gene) encodes a 49-kDa integral membrane protein (Cerretti *et al.*, 1983; Akiyama and Ito, 1985) with ten transmembrane domains (Akiyama and Ito, 1987). Mutations in *secY*, an

essential gene, have been isolated which result in a pleiotropic defect in protein export (Shiba *et al.*, 1984; Riggs *et al.*, 1988). Other mutations, the *prlA* alleles, were isolated as suppressors of signal sequence defects (Emr *et al.*, 1981). Considering these two mutant phenotypes, and its location in the cytoplasmic membrane, SecY is a good candidate for a central component of the translocation machinery.

In cells bearing the temperature-sensitive *secY24* mutation (Shiba *et al.*, 1984), the precursor forms of secretory protein begin to accumulate when cells are grown at the nonpermissive temperature (42°C). In experiments to test if *secY24* affects protein translocation *in vitro*, membranes prepared from these cells grown at 42°C were found to be defective in protein translocation (Bacallao *et al.*, 1986; Fandl and Tai, 1987). This effect can be relieved by the introduction into the cell of a plasmid bearing the wildtype *secY* gene, suggesting that the defect of the membranes is due to the SecY24 protein (Bacalleo *et al.*, 1986). However, the membranes from $SecY^+$ cells grown at 42°C also have reduced activity, although they are 10-fold more active than SecY24 membranes from cells grown at 42°C (Fandl and Tai, 1987). As precursors accumulate in *secY24* cells at 42°C, the membranes may become inactive *in vitro* as a consequence of the membrane-associated translocation machinery becoming "jammed"; jamming may be directly due to the *secY24* mutation. This possibility has long been raised regarding studies of the genetic defects *in vivo*. However, the temperature-sensitive *secY24* translocation defect was demonstrated *in vitro*, in the absence of protein synthesis. Membrane vesicles prepared from *secY24* cells grown at the permissive temperature (30°C) were shown to be active, but could be inactivated by incubation at 40°C, whereas $SecY^+$ membranes were unaffected. This observation demonstrates that the defect of the *secY24* membranes is directly due to the *secY24* mutation and not indirectly due to the growth defect of *secY24* cells at 42°C (Fig. 1; Fandl and Tai, 1987). These studies mark the first instance of the convergence of biochemical and genetic approaches in providing important information in protein translocation.

The growth defect of cells that overproduce secretion-defective hybrid proteins was postulated to be the consequence of the jamming of some component of the translocation machinery. By way of an elegant genetic test, Bieker and Silhavy (1989) demonstrated that the lethal phenotype of cells that overproduce a secretion-defective LamB-LacZ hybrid protein is due to the inhibition of SecY/PrlA function. Moreover, translocation of LamB into membrane vesicles is inhibited by antibodies specific for SecY (Watanabe and Blobel, 1989a). Functional SecY is also required for the activity of the translocation ATPase (Lill *et al.*, 1989).

Together, these results clearly show that the *secY/prlA* gene product is required for protein translocation. The insertion of the major prolipoprotein

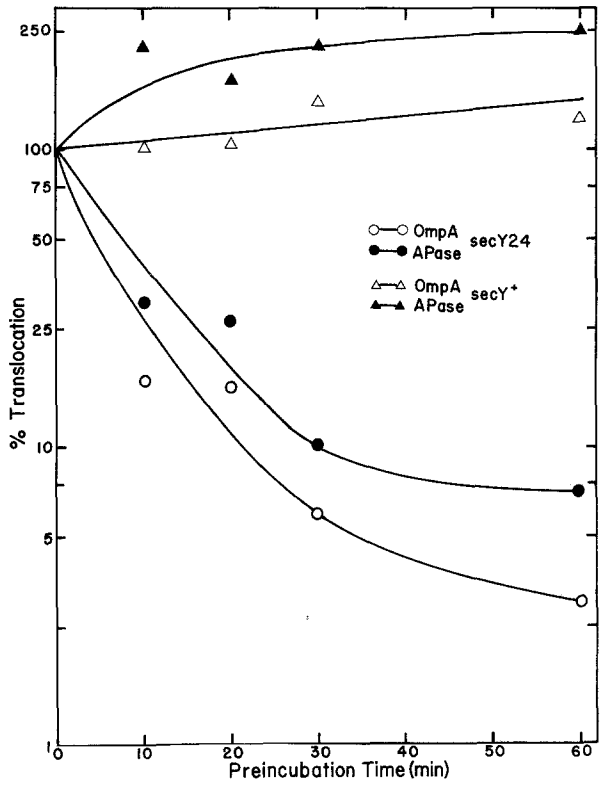

Fig. 1. *In vitro* heat inactivation of SecY24 membrane protein translocation activity. Membranes from IQ85 (SecY24) and from IQ86 (SecY$^+$) cells grown at 32°C were incubated at 40°C for the times indicated and were then assayed for the translocation of alkaline phosphatase (APase) and OmpA protein (Fandl and Tai, 1987).

(pLpp) into membrane vesicles occurs spontaneously even in the absence of functional SecY protein (Tian *et al.*, 1989). However, translocation and processing of pLpp is blocked in SecY24 membranes, suggesting that the functional involvement of SecY in the translocation process occurs after the initial interaction of the precursor with the membrane.

The *secY24* mutation results in the substitution of an aspartic acid for glycine-240, predicted to be exposed on the cytoplasmic face of the membrane between transmembrane domains 6 and 7 (Akiyama and Ito, 1987). One explanation for the defect associated with the mutation is that it interferes with the interaction between SecY and some other soluble components of the translocation apparatus. Accordingly, an increase in the relative concentration of the soluble factor might compensate for the loss of affinity. Indeed, the addition of soluble protein, beyond what is normally

found in the *in vitro* system, suppresses the translocation defect of the inactive SecY24 membranes (Fandl and Tai, 1987). It was subsequently shown that the SecY24-suppressing activity in the soluble protein fraction was primarily due to the *secA* gene product (Fandl *et al.*, 1988), and will be discussed further below. Implicit in this result is the fact that *secY24* is not an irreversible defect, although returning heat-inactivated SecY24 membranes to 30°C fails to restore membrane activity (Fandl and Tai, 1987). Membranes prepared from a strain that has a conditional amber mutation in *secY* contain less than 3% of wild-type levels of SecY and are inactive in protein translocation but can be partially restored by the addition of soluble SecA (J. P. Lian and P. C. Tai, unpublished). Whether the additional SecA compensates for the SecY24 defect or merely bypasses SecY24 by enhancing or creating another pathway that is independent of SecY is not known and awaits the construction of membranes which completely lack SecY.

SecA is a soluble protein but is also found tightly associated with the membrane (Oliver and Beckwith, 1982; Schmidt *et al.*, 1988; L. Chen and P. Tai, in preparation). Is the SecA suppression of the SecY24 defect a soluble activity, or an activity associated with the membrane-bound SecA? The isolated SecY24 membranes incubated with soluble SecA can be restored to near wild-type translocation levels, indicating that the SecY24-suppressing activity of SecA occurs on the membranes, and suggests an interaction between SecA and SecY (Fandl *et al.*, 1988). The possible interaction between SecA and SecY has also been suggested by genetic analysis: manipulation of the SecA levels *in vivo* influence the extent of *prlA4* suppression of maltose-binding protein signal sequence defects (Oliver and Liss, 1985).

The functional SecY protein has not been purified, hindering more detailed studies of its function.

SecB

The *secB* gene encodes a 16-kDa soluble protein which is not essential for cell growth (Kumamoto and Beckwith, 1983). The primary role of SecB appears to be modulation of the folding kinetics and translocation activity of some precursor proteins (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989; Watanabe and Blobel, 1989c; Bassford, this volume; Kumamoto, this volume).

The *secB* gene product also has *secY24*-suppressing activity *in vitro* (Kumamoto *et al.*, 1989). Purified SecB, however, stimulates the translocation activity of SecY24 membranes to about 30% the level achieved with purified SecA. SecB does not exert its activity on the membranes, as indicated by the observation that preincubating SecB with SecY24 membranes does not restore membrane activity (Kumamoto *et al.*, 1989). It is possible that the

suppression activity is a consequence of the antifolding activity of SecB or an as yet to be defined activity (see below). SecB was also shown to stimulate the translocation activity of a heat-inactivated *in vitro* system (Weng *et al.*, 1988), although SecB itself is heat stable under the conditions tested (Kumamoto *et al.*, 1989).

Cytoplasmic Factors and Competence of Precursors

Although the overall process of protein translocation in bacteria and in eukaryotic cells has been assumed to be similar, a structural equivalent of the eukaryotic signal recognition particle, SRP (Walter and Blobel, 1981), has not been found in bacteria (Lee *et al.*, 1985). Using various *in vitro* translocation assays, Muller and Blobel (1984b) identified an "export factor" which was later found to consist of SecB (Watanabe and Blobel, 1989c), and Crooke and Wickner (1987) reported a "trigger factor" that stabilizes the translocation-competent form of OmpA precursors (Crooke *et al.*, 1988a). By employing *in vivo* and *in vitro* analysis, SecB has been found to possess antifolding and targeting activity and allow precursors to be translocated (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Weiss *et al.*, 1988; Kumamoto, 1989). Both trigger factor (Crooke *et al.*, 1988b) and SecB (Watanabe and Blobel, 1989d) have been suggested to function as bacterial SRP, and the eukaryotic SRP can marginally substitute trigger factor for antifolding of OmpA precursors (Crooke *et al.*, 1988b). This notion needs to be further sustained. In fact, whether SecB interacts with signal sequence (Watanabe *et al.*, 1988; Watanabe and Blobel, 1989d) or with the mature region (Collier *et al.*, 1988; Gannon *et al.*, 1989) of the precursor molecules is still controversial.

The assay of trigger factor involved the study of its ability to prevent the loss of proOmpA competency and it is not required for the translocation of urea-diluted precursors (Crooke and Wickner, 1987; Crooke *et al.*, 1988a). Using a different approach, Weng *et al.* (1988) found that soluble cytoplasmic factors *stimulate* the translocation of partially purified proOmpA proteins, which may be in a partially folded conformation that differs from the completed unfolded form of urea-diluted molecules or from the trigger factor-or SecB-complexes. These cytoplasmic translocation factors (CTFs) can be fractionated into three active fractions (Weng *et al.*, 1988). One probably is SecB (Kumamoto *et al.*, 1989). The activities of the other two CTF fractions are roughly additive. The major activity is about 8S and 120 KD and the other is 4S and 60 KD. These CTFs have been further purified to near homogeneity and are distinct entities, each composed of two nonidentical polypeptide chains, and have not been identified with SecA, trigger factor, or the heat-shock proteins GroEL or DnaK (L. Chen and P. Tai, unpublished).

The discovery of the multiple distinct proteins each being able to stimulate the translocation suggests that these factors are redundant. It is quite likely that different factors can perform the same function; this redundancy may make it difficult to demonstrate the physiological role of each factor *in vivo* and may explain why no other additional mutants have been detected to affect protein secretion, despite exhaustive genetic searches (Schatz *et al.*, 1989).

Recent work (Lecker *et al.*, 1989) suggests that SecB, trigger factor, and GroEL (Bochkareva *et al.*, 1988) may function as chaperones (Hendrix, 1979; Ellis, 1987; Hemmingsen *et al.*, 1988) to stabilize a translocation-competent structure (Randall and Hardy, 1986). These chaperones are presumed to function to prevent the folding of the precursors into a structure which is incompatible with the protein entering the translocation pathway. Heat-shock proteins have been reported to be involved in yeast protein translocation (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). Whether this is the physiological role *in vivo* remains to be determined.

Components of the Translocator

Both SecA and SecY at times have been referred to as the "translocator," the protein component directly responsible for the physical movement of proteins across the cytoplasmic membrane. Since SecA may interact with SecY (Fandl *et al.*, 1988) and SecA possesses weak ATPase activity which is greatly stimulated by a functional SecY (Lill *et al.*, 1989), these two components may constitute the protein translocator, much analogous to the anion-pump ATPase (Hsu and Rosen, 1989) with SecA as the catalytic subunit. However, genetic studies (see below) have shown that three other membrane proteins may also be involved in protein translocation, and we define the translocator not as a single polypeptide, but rather, a discrete complex of tightly interacting proteins found in the cytoplasmic membrane, perhaps similar to the other multi-component ion or amino acid transport systems.

Today, the products of *secA/prlD*, *secD*, *secE/prlG*, *secF*, and *secY/prlA* genes are the best candidates for playing central roles in protein export in the membrane (Silhavy, this volume). The *secA* and *secY/prlA* genes were described above. The *secD* (Gardel *et al.*, 1987), *secE* (Riggs *et al.*, 1988; Schatz *et al.*, 1989; Stader *et al.*, 1989), and *secF* (J. Beckwith, personal communication) genes are also essential for cell growth and pleiotropically affect protein export but are not yet as well characterized. The proteins encoded by these genes share some common features. (i) These genes encode essential functions. (ii) Mutations in these genes can result in pleiotropic defects in protein translocation. (iii) Mutations in *secA/prlD* (Bankaitis and Bassford, 1985; Fikes and Bassford, 1989; Stader *et al.*, 1989), *secE/prlG*

(Stader *et al.*, 1989), and *secY/prlA* (Emr *et al.*, 1981) can suppress signal sequence defects. (iv) They all are integral membrane proteins or, in the case of SecA, tightly associated with the membrane. (v) *secA* (Schmidt *et al.*, 1988), *secE* (Schatz *et al.*, 1989), and *secY* (Cerretti *et al.*, 1983) are found within the operons encoding translation/transcription machinery components. (vi) Some alleles of all these genes have cold-sensitive phenotype (J. Beckwith, personal communication), consistent with the notion that conformational or assembly defects of subunits at low temperatures affect the formation of a functional complex, similar to the prevalence of cold-sensitive alleles in ribosome assembly mutants. In addition to these gene products, signal peptidase I, encoded by the *lep* gene, and the lipoprotein-specific signal peptidase II, encoded by the *Isp* gene (see Ray *et al.*, 1986) are membrane proteins essential for growth. Although protein translocation can occur *in vivo* and *in vitro* without the cleavage of the signal sequence (Lin *et al.*, 1978; Rhoads *et al.*, 1984; Yamane *et al.*, 1988), the signal peptidase is probably closely associated with the translocator.

Singer *et al.* (1987) first postulated the existence of a complex of homologous but nonidentical subunits within the cytoplasmic membrane that would provide a hydrophilic channel for the translocation of proteins. We envision the translocator as a complex of the integral membrane proteins SecD, SecE, SecF, and SecY, which together form the postulated aqueous channel in the cytoplasmic membrane. This membrane channel is associated with the peripheral membrane protein SecA, which possesses the catalytic activity of the translocator, thus forming a complex analogous to the F_0F_1 H^+-ATPase (Fig. 2). In essence, the translocator can be viewed as a protein-ATPase, where the aqueous channel is analogous to the F_0 membrane complex and SecA is analogous to the membrane-associated F_1-ATPase. The existence of a translocator complex predicts that such a complex could be purified.

A tight association of the proteins forming the translocator, which may be assembled in the presence of precursor molecules, is suggested by the observation that all signal sequence suppressors are not allele-specific, but rather are always active in the suppression of several different signal sequence mutations, albeit with different efficiencies. It has been postulated that this is the result of a mutation in one component of the translocator complex affecting the function of the entire complex, due to the tight interaction of the components, rather than one mutant component individually interacting with the signal sequence (Stader *et al.*, 1989).

A Model for Protein Translocation

Secretory proteins may be localized in the cytoplasmic membrane, the periplasmic space, or in the outer membrane. Consequently, the final

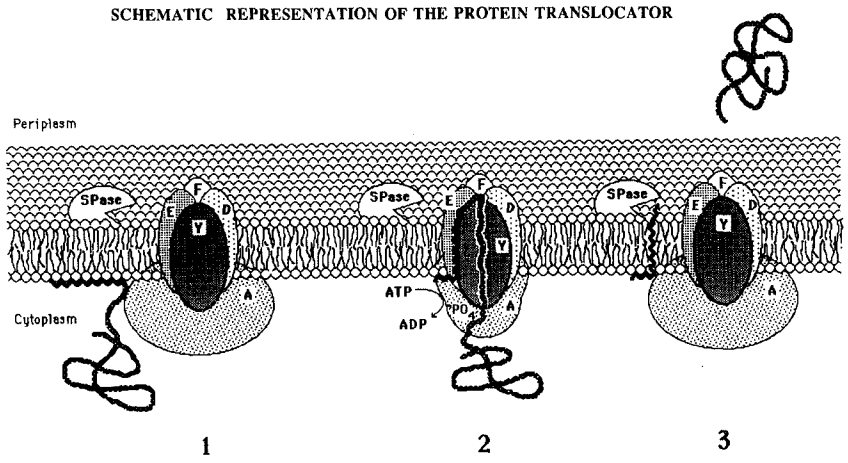

Fig. 2. A speculative model of protein translocator. The putative translocator consists of a membrane complex that is formed by SecY, SecD, SecE, and SecF (represented by Y, D, E, F, respectively) and SecA(A) which is tightly associated with the menbrane. 1. Initial interaction between the secretory protein and the cytoplasmic membranes occurs spontaneously near SecA, directed by signal sequence (solid black zig-zag line). 2. The signal sequence of the inserted secretory protein (speckled line) associates with the translocator, and the cyclic hydrolysis of ATP mediated by SecA causes the conformational change of the translocator and results in the transport of the secretory peptides through the aqueous translocation channel formed. 3. Translocation of the secretory protein is associated with cleavage of the signal sequence, dissociation of the signal sequence from the translocator, and return of the translocator to the resting state.

localization of the exported protein influences, to some degree, the possibly acceptable tertiary conformations. Evolution, we believe, precludes the existence of a common structure within the mature portion of exported proteins that serves as a signal for translocation. No unifying structure among the proteins that are exported in *E. coli* other than that of the signal sequence has been identified (Gierasch, this volume).

Secretory proteins are most likely entered rapidly into the translocation machinery shortly after onset of their synthesis, although protein translocation occurs both cotranslationally and posttranslationally in *E. coli*. The structure and charge distribution of the signal sequence is such that it will spontaneously insert into membranes or liposomes and assume an orientation with the N-terminus in the cytoplasm and the C-terminus of the signal in the periplasmic space (Briggs and Gierasch, 1986). In such an orientation it will of necessity pull the N-terminal part of the mature protein through the membrane as well, and initiate the translocation process. In addition, *E. coli* has but one membrane to which it must target proteins, unlike a eukaryotic cell which contains several membranous organelles. Therefore, the need for

a translocation factor that guides a secretory protein to the membrane is not apparent, and we postulate that the nascent precursor spontaneously inserts, by electrostatic binding and hydrophobic interaction, into the cytoplasmic membrane without benefit of a targeting factor (Tian *et al.*, 1989). However, this does not preclude the involvement of cytoplasmic factors and chaperones in maintaining translocation competence.

Given the fluid nature of the membrane, one can envision the membrane-bound precursor proteins diffusing into the translocator. Purified competent pOmpA can bind to urea-washed membrane vesicles without the addition of SecA, but are unable to be translocated even if SecA is added subsequent to the binding (Cunningham *et al.*, 1989). Similarly, purified Lpp precursor that has spontaneously inserted into the membrane in the absence of SecA is also unable to be translocated when SecA is then introduced into the *in vitro* system (N. Yu and P. Tai, unpublished). These results suggest that the insertion takes place at or near the SecA site of the translocator or in the presence of functional translocator. However, if the precursor folds into a conformation not competent for translocation while inserted in the membrane in the absence of SecA, and SecA added subsequent to the folding is unable to unfold or refold the precursor, then these results may also be simply the consequence of a loss of translocation competence. Purified pOmpA and pLpp are known to rapidly assume a translocation-incompetent form in the absence of translocation factors (Crooke and Wickner, 1987; N. Yu and P. Tai, unpublished).

Secretory proteins that spontaneously insert into the membrane are then introduced into the translocator which, with the hydrolysis of ATP, translocates the secretory protein across the membrane with concomitant cleavage of the signal sequence. There is no question that the thermodynamic requirements of passing a hydrophilic protein through a hydrophobic membrane requires input of energy. Since it has become apparent that the precursor is required to be in a relaxed or unfolded state, the driving force for translocation is probably not derived from a conformational change in the precursor, *per se*, upon interaction with the membrane or translocator. Rather, the energy more likely derives from the activity of the translocator. Although we define the translocator as a complex rather than a single component of the translocation machinery, it is possible that one component possesses the catalytic activity which induces the translocator, as a whole, to function.

We postulate that SecA is the catalytic component of the translocator, much as F_1 is the active subunit of the F_1F_0 H^+-ATPase. As described above, SecA is part of the membrane-associated translocation ATPase (Lill *et al.*, 1989). The hydrolysis of ATP mediated by SecA may result in a conformational cycling of SecA and, as a consequence, the translocator. Conformational changes occurring in SecA are suggested by the observation that SecA

exists as a soluble protein as well as tightly associated with the membrane, and the membrane-associated SecA can be found in two distinct forms. Each of these forms of SecA may have a unique conformation. Even small conformational changes in SecA may result in relatively large conformational changes in the translocator as a whole (Singer *et al.*, 1987). The conformational change in the translocator, specifically the aqueous channel, may provide the direct driving force for the translocation of polypeptide or polypeptide domain (Fig. 2, Step 2). Further support for a catalytic role for SecA is provided by the observation that SecA is able to relieve every form of *in vitro* protein translocation defect thus far tested. Membranes defective due to the lack of SecA, or inactivated by azido-ATP, urea washing, or protease treatment, or the *secY24* mutation or deprivation of SecY are effectively restored to a significant level of wild-type activity by the addition of soluble SecA.

The stoichiometry of ATP hydrolysis during a translocation cycle is unknown. If ATP hydrolysis serves to induce a simple opening of the aqueous channel, then the hydrolysis of one ATP may be sufficient. However, if the hydrolysis of one ATP is required for the translocation of one polypeptide domain, the stoichiometry may vary with the particular secretory protein; multiple cyclic ATP-ase activity is probably required.

The exact function of SecA remains unclear. Although soluble SecA appears to be abundant in the cell, it is not clear if SecA cycles off the membrane during the translocation process. Evidence thus far suggests that it is the membrane-bound SecA that is important for the restoration of the activity of the translocation defect of SecA-depleted membranes, urea-washed membranes, and SecY24 membranes (Cabelli *et al.*, 1988; Fandl *et al.*, 1988).

Regulation of Translocation

What is the role of soluble SecA? SecA is the only component of the proposed translocator that is not an integral membrane protein and whose synthesis is known to be regulated (Schmidt and Oliver, 1989). Another role of SecA may be to mediate communication between cellular translocation requirement and formation of a functional translocator. When SecA is not actively involved in translocation, it may dissociate from the translocator on the membrane and act as an indicator of translocation requirement. The soluble SecA in turn directly moderates the translation of SecA mRNA (Schmidt and Oliver, 1989), thus regulating the concentration of the catalytic component of the translocator, and translocation activity. This implies that the integral membrane protein complex of the translocator, the aqueous

channel, is in excess at any given time, or the rate-limiting step in the process is the function provided by SecA.

We previously noted the existence of a soluble inhibitory activity (Fandl *et al.*, 1988). This factor, which we refer to as SecI (for secretion inhibitor), was identified as an inhibitor of SecA suppression of SecY24 membranes, suggesting that it may act to inhibit SecA function, and this inhibition was relieved by SecB, thus adding another dimension to the regulation of the translocation process (J. Fandl and P. Tai, unpublished). SecI also inhibits the activity of wild-type membranes, and binds very weakly to membranes (J. Fandl and P. Tai, unpublished). We have subsequently found that SecI is identical to trigger factor (TF) (Crooke *et al.*, 1988b). Soluble TF not complexed with precursor protein was reported to inhibit protein translocation *in vitro*, presumably by titrating TF-specific binding sites on the membrane, as additional membranes suppress the inhibition (Lill *et al.*, 1989).

A protein associated with ribosomes, of unknown function, was recently found to be identical to TF (J. Fandl and P. Tai, unpublished), and its synthesis is regulated by the growth rate of the cell (Subramanian *et al.*, 1976). Trigger factor is tightly associated with the ribosomes (Lill *et al.*, 1989) and as such may act as a signal for the activity of the protein synthesizing machinery. When the ribosomes are actively synthesizing proteins, then TF is associated with the ribosomes, but when the cell growth decreases and ribosomes dissociate, then the increase in the soluble free TF will inhibit the activity of SecA, thus releasing it from the translocator. The free SecA will in turn inhibit its own synthesis and the formation of additional translocator complexes. It was recently reported that SecB is more active than TF in stabilizing OmpA precursor competence (Lecker *et al.*, 1989) and we found that SecB relieves the inhibition of SecI on SecA. In the light of these observations, it is possible that the primary function of TF/SecI is the modulation of SecA activity of the protein-ATPase, perhaps analogous to the inhibition of F_0 by the ε protein (Sternweis and Smith, 1980).

Conclusions and Perspectives

The power of the combined genetic and biochemical approaches to the study of a fundamental problem in biology is illustrated well by recent advances in our understanding of the mechanism of protein translocation in *E. coli*. The *in vitro* protein translocation system is a powerful tool for the identification and characterization of translocation machinery components in its own right, but it has also proven to be extremely useful in the characterization of genetically defined components. We have used the *in vitro* system to establish the requirement of both the peripheral membrane protein

SecA and the integral membrane protein SecY for *in vitro* protein translocation, and together with the results of genetic analysis, provide strong evidence for central roles for these two proteins in protein export. Recent advances in the field have come, in part, from the ability to fractionate the *in vitro* system in order to isolate and characterize specific components of the system. This ability is best exemplified by studies on SecA. However, the majority of the components of the translocator are integral membrane proteins, and as such may prove more difficult to characterize. Clearly, the near goal for biochemical studies is the purification and characterization of all the translocator components with the aid of genetic manipulation and the reconstitution of translocation activity into liposomes.

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