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Escherichia coli SecY and SecE proteins appear insufficient to constitute the SecA receptor

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Abstract

In order to test whether SecY and SecE proteins constitute the SecA receptor inside out membrane vesicles where prepared from strains producing greatly different levels of these two proteins, and their SecA binding activity was quantitated. Substantial overproduction of SecE or SecY and SecE proteins resulted in no increase or only 50% increase, respectively, in the number of high affinity SecA binding sites. These results suggest that SecY and SecE proteins appear insufficient to constitute the primary SecA receptor. The existence of a cycle of SecA association with the inner membrane and its modulation by particular integral membrane proteins is discussed.

Key words: SecA receptor; SecY protein; SecE protein; Protein secretion; *Escherichia coli*

1. Introduction

Genetic studies of protein export in *Escherichia coli* have led to the identification of six genes, *secA*, *secB*, *secD*, *secE*, *secF*, and *secY*, required for protein targeting to and translocation across the inner membrane [1]. In vitro protein translocation systems have been developed to begin to assign functions to the individual components of the translocation machinery [2]. SecA protein has been shown to be a membrane-associated ATPase that is essential for functional binding and translocation of preproteins across IMV [3]. SecA has been proposed to interact with the signal peptide and mature portions of the preprotein [4,5], SecB [6], inner-membrane anionic phospholipids [5,7], and SecY or SecY/SecE protein [6,8]. Interaction of an export-competent preprotein with SecA bound to IMV bearing functional SecY protein stimulates SecA's translocation ATPase activity, which has been shown to be essential for both in vitro and in vivo protein translocation [9,10]. It has been suggested that this activity promotes successive cycles of of preprotein binding and insertion into the inner membrane [3,11]. Of the four integral membrane Sec components, only SecY and SecE have been shown to be required for reconstitution of in vitro protein translocation activity, while SecD and SecF were non-functional in this assay [12–14]. SecD may promote release of the translocated protein chain from the inner membrane, since addition of SecD antibodies to *E. coli* spheroplasts inhibited this process [15].

SecA association with the inner membrane appears to be complex and dynamic. SecA was shown to exist in both peripheral and integral states of association with the inner membrane [7,16]. Initial binding of SecA to the inner membrane appears to be mediated by anionic phospholipids [5,7]. Subsequent interaction of SecA with SecY protein has been proposed based on the ability of high concentrations of SecA to suppress the protein translocation defect found normally for heat-inactivated *secY24(Ts)* IMV as well as the requirement of translocation ATPase for functional SecY protein [8,9]. Other studies led to the proposal that SecY or SecY/SecE protein serves as the primary SecA receptor, since SecY antibody reduced the affinity of SecA for IMV 3-fold (but not the number of binding sites) and SecA addition to IMV afforded a 2-fold protection of SecY to proteolysis by trypsin [6]. However, antibody inhibition studies of membrane proteins have been shown to give rise to questionable results [17,18]. Furthermore, recent studies demonstrated that high affinity binding of SecA to IMV required anionic phospholipids, which have been shown to promote SecA penetration into model membranes [19–21]. Thus, the proposed interaction of SecA with SecY or SecY/SecE protein may occur only after penetration of SecA into the inner membrane.

Since the identity of the SecA receptor remains uncertain, we decided to test directly whether SecY and SecE

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Abbreviations: IMV, inverted inner membrane vesicles; ISO-MV, inside-out membrane vesicles; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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proteins fulfill this function. We found that SecA-binding activity did not increase in proportion to the increased content of SecY and SecE proteins present in membranes derived from overproducing strains [22], despite an increase in translocation ATPase and proton antiport activity [23], suggesting strongly that these proteins appear insufficient to constitute the primary SecA receptor.

2. Materials and methods

2.1. Media and chemicals

LinA [24] and M63 minimal medium [25] contained 50 μg/ml ampicillin and/or 30 μg/ml kanamycin where needed. Antibiotics, amino acids, IPTG, and most other common chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). [^{125}I]Donkey anti-rabbit IgG (Fab fragment) was purchased from Amersham Corp. (Arlington Heights, IL). Nitrocellulose membrane for immunoblotting was obtained from Schleicher & Schuell (Keene, NH).

2.2 Bacterial strains and plasmids

E. coli W3110 M25 (pMAN510, pMAN809) an *ompT* strain containing plasmids with *secE* and *secY*, respectively, under the control of the *tac* promoter was kindly provided by Dr. S. Mizushima (University of Tokyo) [22,26]. MC4100 (F$^-$ *ΔlacU169 araD136 relA rpsL thi*) [27] containing pMAN809 or pMAN510 and pUS12, containing the *lacI* gene [28], was used for overproduction of SecE or SecY, respectively. BL21(λDE3) [29] containing either pT7secA [30] or pT7secA2 [31] was used for preparation of [^{3}H]SecA protein.

2 3. Preparation of [^{3}H]SecA protein and urea-treated ISO-MV

Purified [^{3}H]SecA protein was prepared either according to Cabelli [32] or with modification of the protein purification procedure as described by Mitchell and Oliver [33]. For preparation of membranes strains were grown in LinA medium containing appropriate antibiotics and IPTG was added at mid-logarithmic phase growth for a period of 2 h for induction of SecY or SecE. ISO-MV were prepared using the procedure of Matsushita et al. [34] with the following modifications. Cells were harvested by sedimentation, washed once in 50 mM Tris-HCl, pH 7.5, and resedimented. Cells were resuspended in 50 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$ at 6 ml/g wet weight of cells, and DNase I was added to 10 μg/ml. The cell suspension was passed through the French pressure cell twice at 5,000 psi. Unbroken cells were removed by sedimentation at 10,000 × *g* for 10 min, and a membrane pellet was obtained after sedimentation at 120,000 × *g* for 2 h. Membranes were resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10% glycerol, and 250 mM sucrose and stored at −80°C until use. In order to prepare urea-treated ISO-MV for SecA removal and inactivation [35], the membrane pellet was resuspended in 5 M urea, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10% glycerol, and 250 mM sucrose and held on ice for 30 min, followed by resedimentation, washing and final resuspension in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10% glycerol, and 250 mM sucrose.

2.4. Binding assays

Each 100 μl binding reaction contained varying amounts of [^{3}H]SecA protein and 30 μg protein urea-treated ISO-MV or 100 mg protein ISO-MV in 50 mM Tris-OAc, pH 7.6, 5 mM MgOAc, 1 mM spermidine, 8 mM putricine, 0.5 mg/ml BSA. Reactions were incubated on ice for 15 min, overlayed onto 100 μl 0.2 M sucrose, 50 mM Tris-OAc, pH 7.6, 5 mM MgOAc, 1 mM spermidine, 8 mM putricine, 0.5 mg/ml BSA and sedimented at 95,000 rpm for 30 min in a Sorvall RP100-AT2 rotor. Membrane pellets were resuspended in 2% SDS, 125 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 15% glycerol, 0.005% Bromophenol blue and the amount of [^{3}H]SecA was determined by liquid scintillation counting.

2.5. SDS-PAGE and Western blotting

SDS-PAGE utilized the discontinous system of Laemmli [36]. Western blot analysis of SecY and SecE employed a mixture of anti-SecY peptide and anti-SecE peptide antisera as the primary antibody and 0.5 μCi/ml [^{125}I]donkey anti-rabbit IgG (Fab fragment) as the secondary antibody as described previously [7].

3. Results

In order to determine whether SecY and SecE constitute the SecA receptor we utilized three strains: MC4100 (pMAN809), MC4100 (pMAN510, pUS12), or W3110 M25 (pMAN510, pMAN809) were used for overproduction of SecE, SecY, or SecY and SecE proteins, respectively, using the regulated *tac* promoter [22]. Preparation of IMV from these strains revealed substantial cleavage and degradation of SecY protein, consistent with previous observations by other investigators [37]. However, use of the protocol developed by Matsushita et al. [34] yielded ISO-MV preparations where SecY protein was largely intact as shown in Fig. 1. Furthermore, the SecY and SecE content of ISO-MV prepared from W3110 M25 (pMAN510, pMAN809) was increased greatly after growth in the presence of IPTG, consistent with the prior estimate of a 50-fold and 80-fold overproduction of SecY and SecE proteins, respectively, under these conditions [14]. The SecE content of ISO-MV prepared from MC4100 (pMAN809) was increased greatly also after growth in the presence of IPTG, while the SecY content of ISO-MV prepared from MC4100 (pMAN510, pUS12) showed little or no increase after growth in the presence of IPTG, consistent with the requirement for SecE overproduction to achieve overproduction of SecY protein [22]. We performed binding assays of [^{3}H]SecA on these ISO-MV preparations and the results are given in Fig. 2. [^{3}H]SecA binding activity increased no more than 2-fold in ISO-MV preparations of W3110 M25 (pMAN510, pMAN809) that differed greatly in their SecY and SecE content (i.e. with or without IPTG induction). ISO-MV preparations of MC4100 (pMAN809) that differed greatly in their SecE content showed very similar [^{3}H]SecA binding activity. Identical conclusions were reached whether the high affinity or low affinity binding region of the curves was examined. Since our binding studies were performed with urea-treated membranes similar to previous studies [6], we considered the possibility that urea-treatment may damage the SecA receptor. Therefore, we repeated the binding studies using the same ISO-MV preparations prior to urea-treatment. The results are shown in Fig. 3. [^{3}H]SecA binding activity was decreased substantially in this case compared to the comparable urea-treated ISO-MV preparations, consistent with the idea that endogenous membrane associated SecA protein [7] or some other membrane associated component removed by urea depressed [^{3}H]SecA binding activity. However, little difference in [^{3}H]SecA binding activity was seen for ISO-MV preparations that differed greatly in either SecE or SecY and

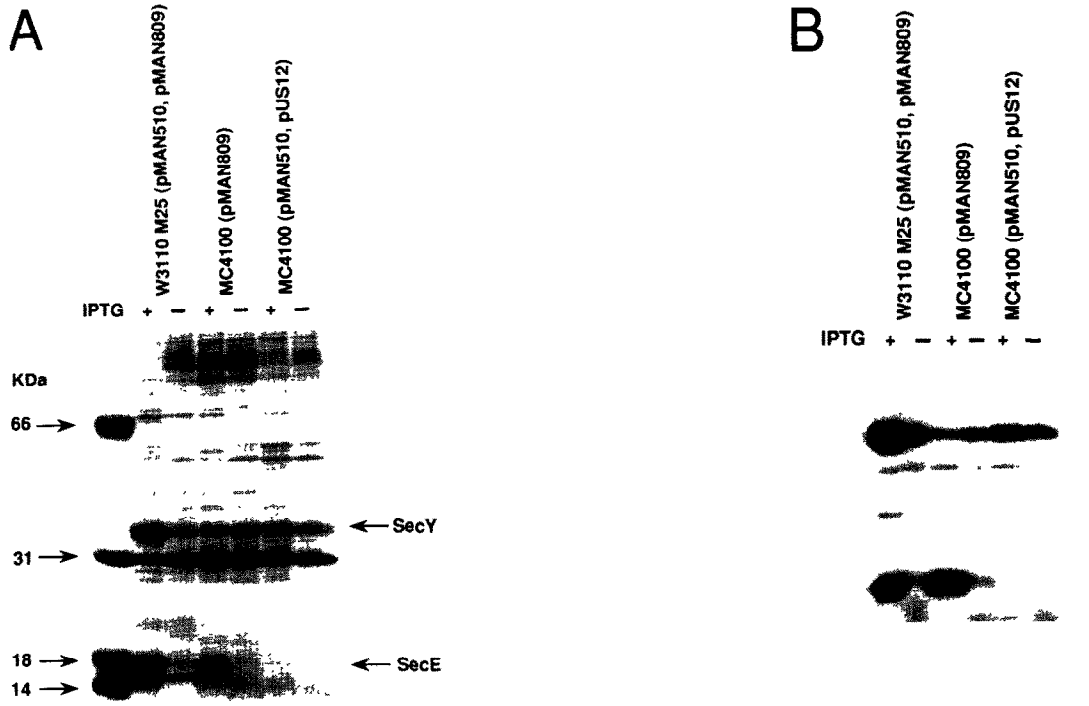

Fig. 1. SecY and SecE protein content of ISO-MV. Urea-treated ISO-MV were prepared from the strains indicated with or without IPTG induction as described in section 2. (A) 50 μg protein of each ISO-MV was analyzed by SDS-PAGE and staining with Coomassie brilliant blue R or (B) 30 μg protein of each ISO-MV was analyzed by Western blotting. Molecular weight standards of 66 kDa, 31 kDa, 18 kDa, and 14 kDa and the positions of SecY and SecE proteins are indicated.

SecE content. These results suggest that damage of SecY or SecE by urea-treatment is not a likely explanation for the failure to observe an increase in SecA binding activity in this case. This interpretation is consistent with the maintenance of in vitro protein translocation activity of membranes after treatment with urea [16,35,38].

The data in Figs. 2 and 3 were used to calculate K_d values of SecA protein for different ISO-MV preparations as well as to determine the number of high affinity binding sites present in each case. The results are presented in Table 1. In general, the K_d values obtained were 5–9-fold higher than the previous estimate of 40 nM, but the number of high affinity binding sites was similar to the value of 140 pmol/mg membrane protein obtained previously [6]. The reason for the discrepancy in the former measurement is unclear, but may relate to differences in the strains employed, membrane preparation methodology, or SecA binding assays. Urea-treated ISO-MV preparations with greatly increased SecY and SecE content showed only a 50% increase in high affinity binding sites and a 50% decrease in K_d value compared to urea-treated ISO-MV from the uninduced strain. These same ISO-MV preparations prior to urea-treatment had similar K_d and binding site values despite their difference in SecY and SecE content. However, they displayed only 10% of the number of high affinity binding sites found for their urea-treated counterparts. Urea-treated ISO-MV preparations with greatly increased SecE content showed similar K_d and binding site values compared to ISO-MV preparations with normal SecE content (e.g. compare MC4100 (pMAN809) with MC4100 (pMAN510, pUS12)), indicating that overproduction of SecE alone did not lead to an increase in membrane binding activity of SecA protein either.

4. Discussion

Since previous investigations either did not address the question of whether SecA's interaction with SecY or SecE was primary or were based on marginal effects, we investigated this question directly by comparing SecA binding activity of ISO-MV preparations differing greatly in their content of these integral membrane proteins. ISO-MV preparations that differed greatly in their SecE content showed similar SecA binding activity, while ISO-MV preparations with greatly increased SecY and SecE content showed only a 50% increase in high affinity SecA binding sites compared to the control and an actual decrease in SecA affinity. Kawasaki et al. [23] showed that overproduction of SecY and SecE proteins in this strain led to a 5-fold increase in SecA-dependent translo-

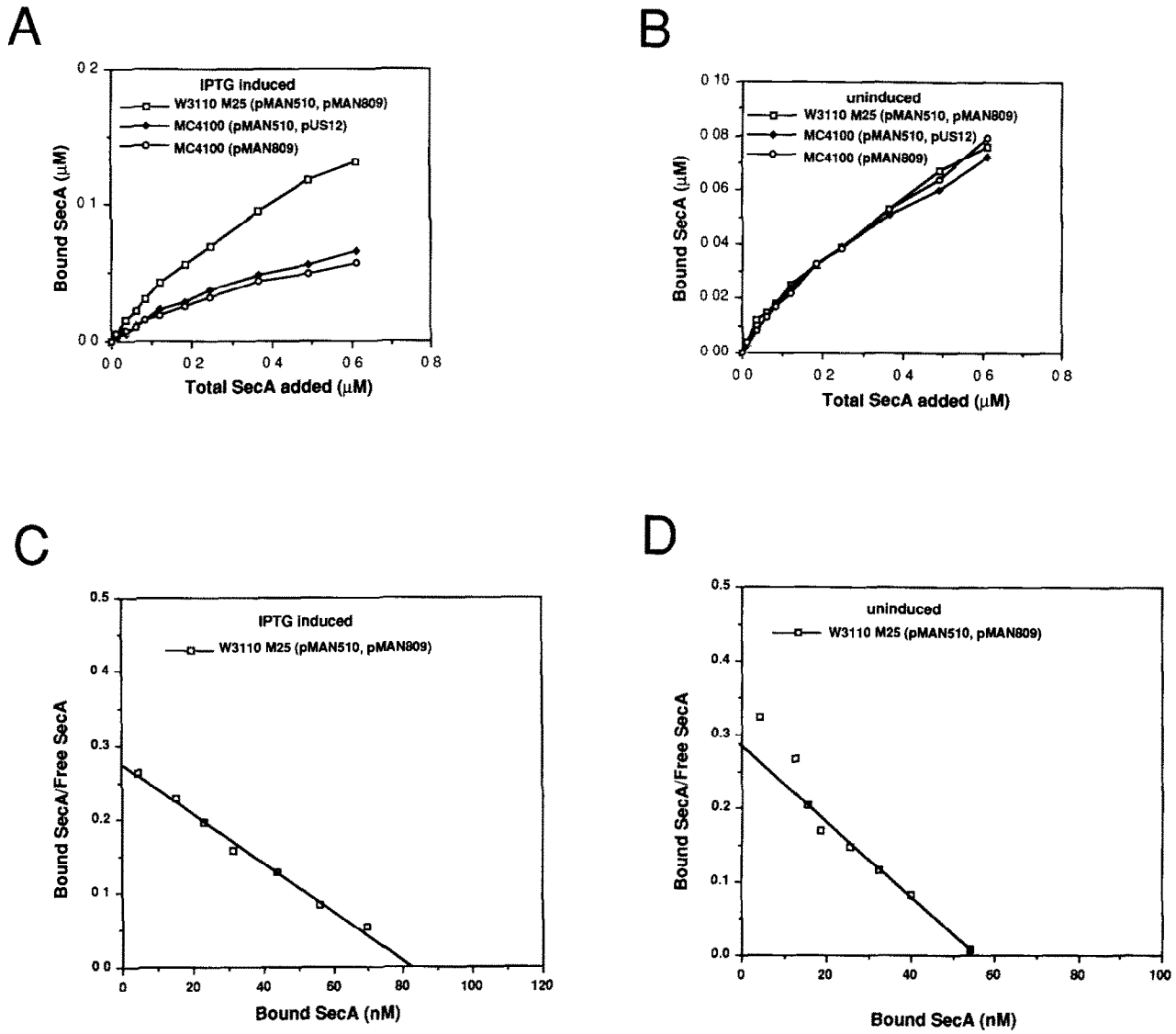

Fig. 2. SecA binding activity of urea-treated ISO-MV differing in SecY and SecE content. Binding assays of [^{3}H]SecA protein with urea-treated ISO-MV prepared from the strains indicated grown with or without IPTG induction were performed as described in section 2. (A,B) Both direct plots and (C,D) Scatchard plots of the data are shown. The data for W3110 M25 (pMAN510, pMAN809) are representative of three experiments, while that for MC4100 (pMAN510, pUS12) and MC4100 (pMAN809) are representative of two experiments.

cation ATPase activity as well as an increase in preprotein-dependent proton antiport activity, both of which have been shown previously to be measures of SecY or SecY/SecE protein activity [9,39]. These results, combined with a report that protein translocation has been reconstituted from the overproduced SecY and SecE proteins in this strain [13], argues strongly that the overproduced proteins were active in this case. Taken to-

Table 1
Summary of Scatchard analysis of SecA binding activity of ISO-MV differing in SecY and SecE content

ISO-MV type	IPTG	Urea-treatment	K_d (nM)	Binding sites (pmol/mg membrane protein)
W3100 M25 (pMAN510, pMAN809)	+	+	300	274
W3100 M25 (pMAN510, pMAN809)	–	+	200	187
W3100 M25 (pMAN510, pMAN809)	+	–	283	28
W3100 M25 (pMAN510, pMAN809)	–	–	263	20
MC4100 (pMAN510, pUS12)	+	+	352	192
MC4100 (pMAN809)	+	+	350	116

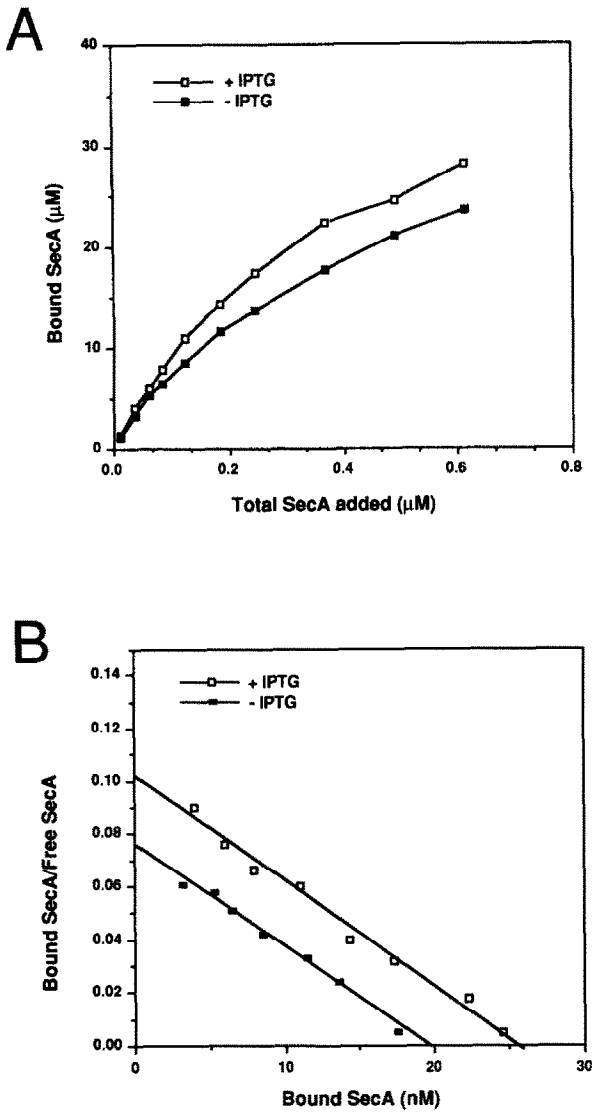

Fig. 3. SecA binding activity of ISO-MV differing in SecY and SecE content. Binding assays of [^{3}H]SecA protein with ISO-MV prepared from W3110 M25 (pMAN510, pMAN809) grown with or without IPTG induction were performed as described in section 2. (A) Both direct plots and (B) Scatchard plots of the data are shown.

gether, these data suggest strongly that SecY and SecE proteins appear insufficient to constitute the SecA primary receptor.

There have been several recent reports indicating the existence of at least three additional integral membrane proteins that may play roles in protein secretion. A protein termed band 1 was found in a complex with SecY/SecE protein, where it appears to promote stabilization of this complex, since no increase in the amount of SecY/SecE complex was detected during overproduction of these latter proteins [37]. Our data are in agreement with this result (Kim and Oliver, unpublished results), although it is difficult to rationalize the requirement of elevated SecE levels for SecY overproduction unless these two proteins form a complex in vivo albeit a labile one. The failure of overproduced SecY and SecE proteins to stably oligomerize into a SecA receptor could explain our negative results, although the overproduced proteins have been shown to be active by a number of criteria [13,23]. Reconstitution of another integral membrane protein, p12, into proteoliposomes containing SecY and SecE has been reported to cause more than a 20-fold increase in Sec-dependent in vitro protein translocation activity [40]. Finally, a third integral membrane protein, Ydr, was isolated as a multicopy suppressor of a dominant-negative *secY* mutant, and it displayed synthetic lethality with the *secY24(Ts)* allele as well as allowing SecY overproduction [41]. Whether any of these newly identified integral membrane proteins play primary or secondary roles in SecA association with the inner membrane will require a direct study to address this point. We have found recently that SecA localization is altered dramatically in a strain that carries a plasmid borne copy of the *secD secF* operon [42], since only the integral membrane form of SecA protein (resistant to extraction by sodium carbonate) was detected in this case (Y. Kim, T. Rajapandi, and D. Oliver, manuscript in preparation). This result is consistent with the recent report that the integral membrane form of SecA protein was fully active in promoting in vitro protein translocation [16]. Analysis of SecA binding activity of urea-treated ISO-MV from this strain showed an increased affinity but decreased number of high affinity sites (153 nM and 67 pmol/mg membrane protein, respectively). These data suggest that SecA binding, penetration, and release from the inner membrane is complex and will require a thorough analysis to reveal the biochemical interactions involved. Given the large number of integral membrane proteins that have been proposed to play roles in protein secretion, a careful analysis of this problem will be required to unambiguously identify the SecA receptor and to elucidate which interactions with SecA are primary versus secondary.

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