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Reconstitution of protein translocation from detergent-solubilized *Escherichia coli* inverted vesicles: PrlA protein-deficient vesicles efficiently translocate precursor proteins

(cholate/dialysis/cell-free translation)

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ABSTRACT **Proteoliposomes were reconstituted by detergent dialysis of a sodium cholate extract of inverted vesicles derived from *Escherichia coli* plasma membrane. The translocation of precursor proteins into reconstituted vesicles occurred at high efficiency and was SecB dependent. The protein composition of the reconstituted vesicles differed markedly from that of native vesicles. Immunoblot analysis of the sodium cholate extract and of the reconstituted vesicles indicated that PrlA (SecY) protein remained largely unsolubilized under the described conditions and was virtually absent from the reconstituted vesicles, suggesting that PrlA may not be required for *in vitro* translocation.**

A variety of mutant selection schemes have been devised to identify the proteins that mediate, either directly or indirectly, protein export in *Escherichia coli*. Through genetic analysis five genes, *secA* (1, 2), *secB* (3, 4), *secD* (5), *secE* (6, 7), and *prlA* (*secY*) (8–11) have been identified as essential for protein translocation. The products of the genes *secA* and *secB* are soluble proteins of molecular mass 102 kDa (12, 13) and 16 kDa (14–16), respectively. Purified SecA has been shown to restore translocation activity to vesicles derived from a *secA* amber mutant and thus appears required for protein export (12). Purified SecB has been observed to bind to the signal sequence of precursor proteins (17), although other reports have suggested that SecB binds to the mature portion (18, 19).

SecD, SecE, and PrlA (SecY) are integral membrane proteins of as yet unknown function. *prlA* was originally identified as a suppressor of signal sequence mutations of pre-LamB (8, 9) and, therefore, was suggested to interact directly with the signal sequence (8, 9, 20, 21). Moreover, data from cell-free translocation systems (22–24) strongly suggest a role for PrlA in protein export. Recently, signal sequence mutation suppressors were shown to be tightly linked to the *secE* gene, suggesting direct interaction between SecE and signal sequences (25). No explicit function has been ascribed to the *secD* product.

A biochemical description of the protein-mediated events occurring at and within the membrane during translocation requires solubilization and reconstitution of translocation-competent vesicles. Using procedures similar to those we had developed for reconstitution of translocation-competent vesicles from cholate-solubilized canine rough microsomes (26), we report here reconstitution of translocation-competent vesicles from cholate-solubilized *E. coli* inverted vesicles (INV).

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MATERIALS AND METHODS

Preparation of INV. INV were prepared from *E. coli* strain MRE600 according to Schnaitman (27). To remove peripheral proteins, INV were diluted with an equal volume of high-salt buffer (0.25 M sucrose/50 mM triethanolamine acetate (pH 7.5)/2 M KOAc/1 mM dithiothreitol) and incubated on ice for 1 hr. Washed vesicles were collected by centrifugation through a cushion consisting of 0.5 M sucrose/50 mM triethanolamine acetate (pH 7.5)/1 M KOAc/1 mM dithiothreitol for 2 hr at 225,000 × g_{av}, and resuspended into the original volume of membrane buffer [0.25 M sucrose/50 mM triethanolamine acetate (pH 7.5)/1 mM dithiothreitol]; then the wash was repeated.

Heterologous Cell-Free Translation/Translocation System. mRNA (500 ng) coding for pre-MBP* [a mutant form of the maltose-binding protein precursor with alterations in its carboxyl terminus, as described (28)] was translated in 25 μl of a wheat germ cell-free translation system as described (29). After completion of translation, a postribosomal supernatant was prepared by centrifuging the translation mixture for 30 min at 30 psi (1 psi = 6.9 kPa) (122,000 × *g*) in the Beckman Airfuge (A 100/18 rotor). The translocation reaction mixture (30 μl) contained 23 μl of the postribosomal supernatant, 5 mM ATP, 8 mM creatine phosphate, creatine phosphokinase at 40 μg/ml, 5 μl of buffer (10 mM triethanolamine acetate (pH 7.5)/60 mM KOAc/14 mM $Mg(OAc)_2$/1 mM dithiothreitol) containing 50 ng of SecB (0.1 μM) and either 1 μl of high-salt-buffer-washed INV (15 A_{280} units/ml) containing 5 nmol of phospholipid phosphorus or reconstituted vesicles containing 5–10 nmol of phospholipid phosphorus. Translocation reactions were performed for 60 min at 25°C. Membrane sedimentation, protease protection, and $NaDodSO_4$/PAGE analysis of samples were as described (24). Radioactivity was measured by direct analysis of the gels with a radioanalytic imaging system (Automated Microbiology Systems, San Diego), and translocation efficiency was calculated as described (28).

Solubilization of High-Salt-Buffer-Washed INV. High-salt-buffer-washed INV were resuspended at a final protein concentration of 3–4 mg/ml in 0.4 M sucrose/1 M KOAc/20 mM triethanolamine acetate (pH 7.5)/1.5 mM $Mg(OAc)_2$/1 mM EDTA. Sodium cholate [10% (wt/vol) in H_2O] or 10% (wt/vol) Nikkol was added to a final concentration of 0.75% or 1%, respectively. The mixture was incubated on ice for 60 min and subsequently centrifuged in the Beckman TLA-100.2

Abbreviations: INV, inverted vesicles derived from the *Escherichia coli* plasma membranes; rINV, reconstituted vesicles derived from cholate-solubilized extract of INV; pre-MBP*, precursor of mutant maltose-binding protein; nrINV and crINV, INV derived from a Nikkol or cholate extract, respectively.

rotor at 356,000 × *g* for 30 min. The supernatant was collected and stored on ice.

Reconstitution of Vesicles. The cholate extract was dialyzed at 15–22°C against 500–1000 vol of dialysis buffer [0.25 M sucrose/20 mM triethanolamine acetate (pH 7.5)/0.4 M KOAc/1.5 mM $Mg(OAc)_2$/1 mM EDTA] for 7–8 hr. Dialysis was performed with a Spectra-Por 1 dialysis membrane (Spectrum Medical Industries). After dialysis, the reconstituted vesicles (rINV) were collected by centrifugation in the Beckman TLA-100.2 rotor at 356,000 × *g* for 15 min. The Nikkol extract was incubated with an equal volume of SM-2 beads (Bio-Rad) that had previously been equilibrated in the dialysis buffer. Before addition of Nikkol extract, the buffer-equilibrated beads were packed in a disposable Poly-Prep column (Bio-Rad) and centrifuged at 2000 × *g* for 2 min to remove excess buffer. The detergent-binding reaction was performed in the Poly-Prep column for 1 hr on ice, and the flow-through was collected by centrifugation at 2000 × *g* for 2 min. The flow-through was maintained at room temperature for an additional 3–4 hr, and the vesicles were collected by centrifugation for 15 min at 356,000 × *g* in the Beckman TLA-100.2 rotor.

Mock-treated INV were prepared as follows: High-salt-buffer-washed INV were incubated in buffer without cholate, dialyzed, and the vesicles were collected by centrifugation as described above.

Immunoblot Analysis. Immunoblotting with rabbit antisera directed against a synthetic peptide representing the C-terminal sequence of PrlA was done as described (24).

Phospholipid Analysis. Phospholipids were quantitated by organic phosphorous analysis, as described in Ames and Dubin (30). Percent yield was calculated with the mock-treated INV as 100% control.

Electron Microscopy. High-salt-buffer-washed INV and reconstituted vesicles were fixed in suspension in 2.0% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2) for 30 min on ice. After centrifugation in the Beckman Airfuge rotor A100/18 for 30 min at 30 psi, pellets were washed in cacodylate buffer and treated with osmium for 1 hr on ice. Samples were block-stained with uranyl acetate, dehydrated, and embedded in Epon/Araldite. Sections were poststained with uranyl acetate and lead citrate.

RESULTS

INV of the *E. coli* plasma membrane were solubilized with sodium cholate/high-salt buffer, the mixture was centrifuged to sediment components of S value >24S, and the supernatant fractions were dialyzed to remove detergent and thereby allow reassembly of membrane vesicles. The translocation activity of rINV was subsequently assayed in a posttranslational translocation assay. mRNA coding for pre-MBP* was translated in a wheat germ cell-free translation system (28); a postribosomal supernatant was then prepared, supplemented with SecB, and subsequently incubated with INV or rINV. After incubation, the assay mixture was either centrifuged, to yield a supernatant fraction containing untranslocated chains and a pellet fraction containing membrane-bound and translocated chains, or treated with proteinase K to assay for translocated chains.

Translation of pre-MBP* mRNA in the wheat germ system yields two products (28), a translocation-competent form (p) representing full-length pre-MBP* and a translocation-incompetent form (i) resulting from initiation at Met-18 or -19 of the 26-amino acid signal sequence (Fig. 1*A*, lane 1). When native INV were present in the translocation assay ≈90% of the precursor was translocated, as evident from cosedimentation with INV (lane 5) and resistance to proteinase K digestion (lane 6). About half of the translocated precursor was cleaved by signal peptidase, yielding the mature form (m) (lanes 5 and 6).

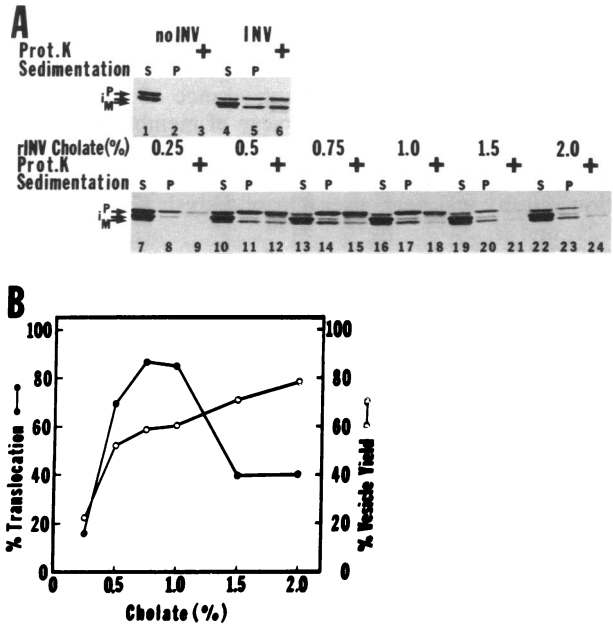

FIG. 1. Cholate dose dependence for recovery of translocation-competent vesicles. One-hundred-microliter aliquots of cholate extract of INV prepared at different cholate concentrations were dialyzed for 8 hr at room temperature against 1000 vol of dialysis buffer. rINV were collected by centrifugation, resuspended in 10 μl of membrane buffer, and assayed for translocation activity (*A* and *B*) and vesicle yield by organic phosphorus analysis (*B*) as described. Quantitation of the results shown in *A* is shown in *B*. Supernatant fraction (S) and pellet fraction (P) after centrifugation of the translocation mixture. p, i, and m (by arrows), precursor, intermediate form resulting from translation initiation at Met-18 or -19, and mature form of pre-MBP*, respectively. Prot. K, proteinase K.

Recovery of translocation activity in the reconstituted vesicles was dependent on the concentration of cholate used to solubilize INV. Maximal translocation activity was obtained when INV had been solubilized with 0.75–1% cholate (Fig. 1*A*, lanes 13–18). However, unlike native INV (lane 5), these rINV yielded more vesicle-bound precursor than mature form (lanes 14 and 17), and whereas most vesicle-bound precursor was translocated—i.e., resistant to proteinase K, less than half of the vesicle-bound mature form was resistant to proteinase K (lanes 15 and 18). The proteinase K-sensitive mature chains may represent translocation intermediates in which the signal sequence is cleaved by the luminal signal peptidase and the C-terminal portion is exposed on the vesicle exterior and, therefore, sensitive to degradation by exogenous protease. Interestingly, the mature form can also be generated in a "soluble"—i.e., not vesicle-bound form. A small amount of this soluble, processed precursor is generated even by native INV (see faint band migrating slightly faster than intermediate form in lane 4). Increasing quantities of soluble mature form were produced by rINV derived from INV solubilized at increased cholate concentrations (compare lanes 7, 10, 13, 16, 19, and 22). Possibly this soluble mature form is generated by vesicles containing externally exposed signal peptidase. Alternatively, the soluble mature form could represent a translocation intermediate that gained access to the luminally disposed signal peptidase but was not further translocated and subsequently released into the soluble fraction. Evidence for such an "aborted" translocation has recently been reported for canine rough microsomes (31).

The yield of vesicles, assayed as lipid phosphorous, increased as a function of the detergent concentration during solubilization (Fig. 1*B*). The relative yield of vesicles did not, however, correlate with translocation activity. We have titrated the amounts of INV and rINV and found that maximal

translocation activity required about twice as much phospholipid phosphorus for rINV as for INV (data not shown).

The time course for recovery of translocation activity is shown in Fig. 2 *A* and *B*. The appearance of translocation activity correlated with the recovery of membrane vesicles (Fig. 2*B*). The reconstitution process was essentially complete after 4 hr of dialysis, and only slightly higher translocation activity was seen after 8 hr of dialysis (Fig. 2*A*, lanes 16–21, and *B*).

The process of reassembly of translocation-competent vesicles was relatively insensitive to temperature over the range 7.5°C–25°C (Fig. 3*B*). When dialysis was performed at 0°C, processing of precursor to mature form and membrane association of precursor were seen, but the resistance of both precursor and mature form to exogenous protease was reduced (Fig. 3*A*, lanes 7–9). Dialysis of the detergent extract at 37°C resulted in a marked loss of translocation activity (Fig. 3, lanes 19–21). The vesicle yield was, however, unaffected by the temperature at which dialysis was performed (Fig. 3*B*). The loss of function observed during dialysis at relatively low or high temperatures may represent the cold sensitivity or thermolability of different components or an as yet undefined aspect of the reconstitution process.

As shown (17) for native INV, translocation depends on SecB (see also Fig. 4, compare lanes 4–6 of both panels). Likewise, SecB is also required for translocation across rINV (Fig. 4, compare lanes 10–13 of both panels).

Vesicles reconstituted after solubilization of INV in the nonionic detergent Nikkol (similar in physical properties to Triton X-100) (nrINV) did not exhibit translocation activity (Fig. 4, lanes 7–9). Interestingly, these vesicles generated substantial quantities of soluble mature form, in a SecB-independent fashion (Fig. 4, compare lanes 7 of both panels). The possible origin of soluble mature form has been discussed above.

nrINV and cholate-derived rINV (crINV), although markedly different in terms of functional characteristics, were morphologically similar. Electron micrographs indicate that the reconstituted vesicles (Fig. 5 *B* and *C*) are significantly

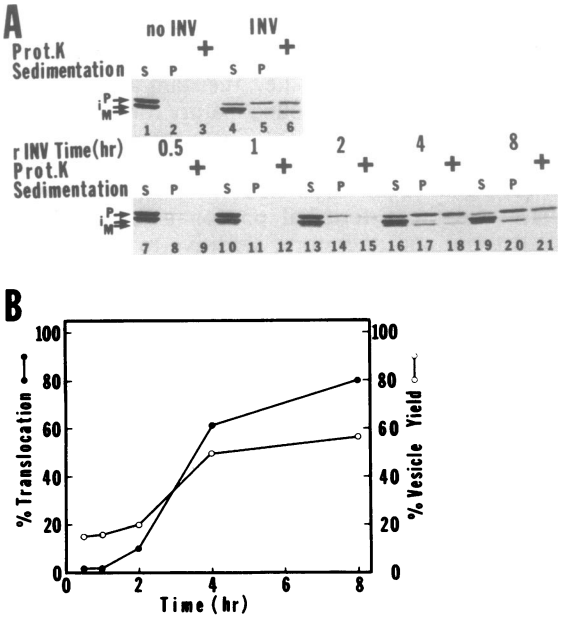

FIG. 2. Time course of vesicle reconstitution. A cholate extract of INV was prepared, as described, and dialyzed at room temperature against 1000 vol of dialysis buffer. At indicated times, aliquots were removed, and vesicles were collected by centrifugation and assayed for translocation activity (*A*) and vesicle yield (*B*). For details, see Fig. 1 and text.

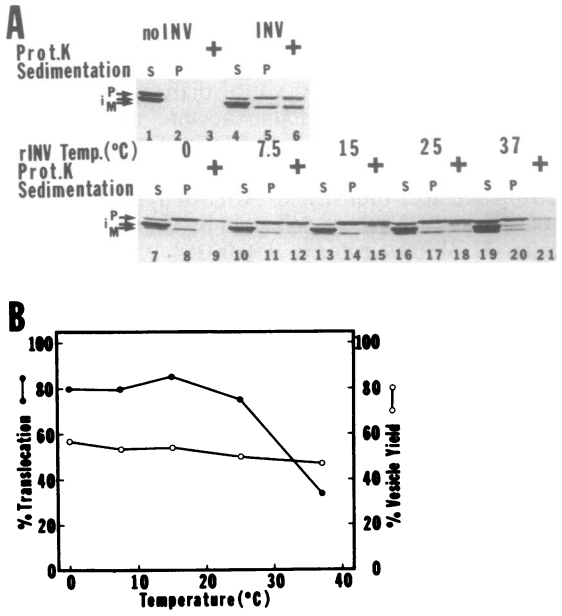

FIG. 3. Temperature dependence of vesicle reconstitution. Aliquots of a cholate extract of INV prepared as described were dialyzed for 8 hr against 1000 vol of dialysis buffer at the indicated temperatures. Membranes were collected by centrifugation and assayed for translocation activity (*A*) and vesicle yield (*B*). For details, see Fig. 1 and text.

larger and of more heterogeneous size distribution than native INV (Fig. 5*A*). The protein profiles of nrINV and crINV are, however, quite different. As shown in Fig. 6*A*, essentially all proteins present in INV were solubilized in the Nikkol/high-salt buffer and recovered with the vesicles after Nikkol depletion by SM-2 beads. In contrast, only a subset of the proteins present in INV were solubilized in the cholate/high-salt buffer and recovered during detergent dialysis. The differences in protein composition of the two vesicle preparations are more clearly evident in immunoblot analysis of PrlA content. As depicted in Fig. 6*B*, PrlA was completely solubilized by Nikkol/high-salt buffer and efficiently recovered in rINV. PrlA was poorly solubilized by the cholate/high-salt buffer, sedimenting almost entirely in the insoluble fraction. rINV prepared from the cholate supernatant fraction were, therefore, largely depleted of PrlA, with densitometric analysis of the autoradiogram depicted in Fig. 6*B*, indicating recovery of <1% of PrlA in the reconstituted vesicles (data not shown).

DISCUSSION

We report that proteoliposomes prepared by detergent dialysis of sodium cholate extracts of INV mimic native vesicles

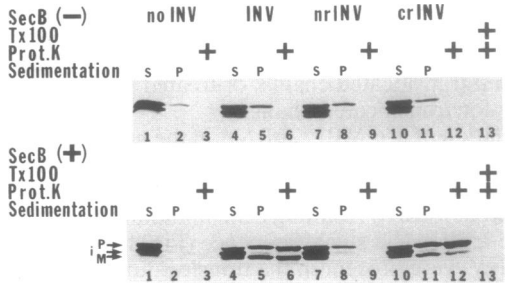

FIG. 4. SecB dependence of pre-MBP* translocation across different vesicle preparations. Translocation was assayed without (lanes 1–3) or with INV (lanes 4–6), nrINV (lanes 7–9), or crINV (lanes 10–12). For details, see Fig. 1 and text.

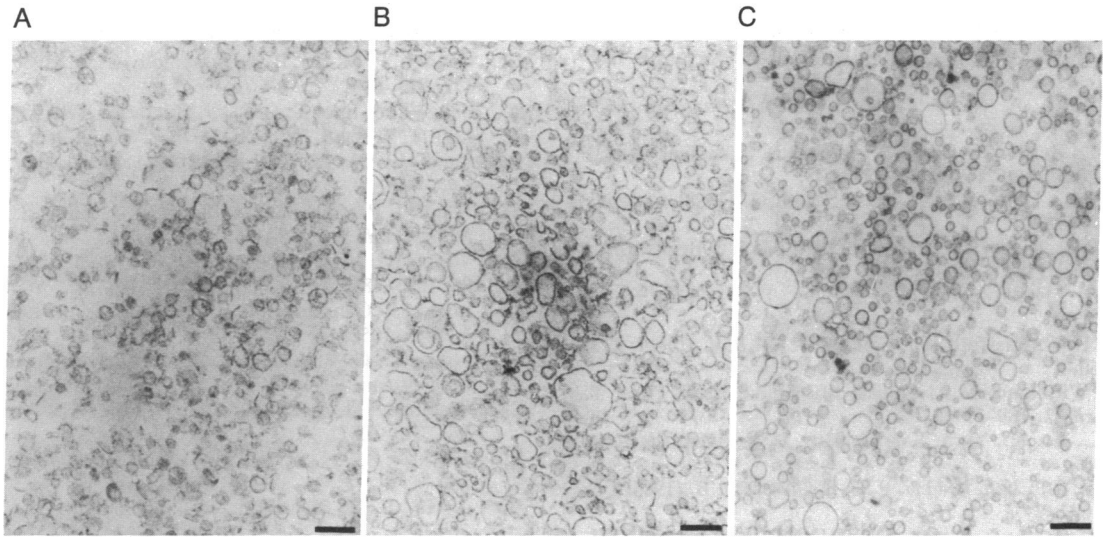

FIG. 5. Electron micrographs of INV, nrINV, and crINV. Aliquots of INV, nrINV, and crINV were fixed in solution and processed for electron microscopy as described. (*A*) INV. (*B*) nrINV. (*C*) crINV. (Bar = 0.2 μm.)

in *in vitro* translocation assays. Translocation of pre-MBP* occurred at very high efficiency and depended upon SecB. Like native vesicles, reconstituted vesicles also translocate wild-type pre-LamB, but not a translocation-defective pre-LamB mutant containing a charged residue in the hydrophobic core of the signal sequence (M.W. and G.B., unpublished observations).

The reconstituted vesicles displayed a reduced capacity for signal sequence cleavage and, therefore, yielded relatively more translocated unprocessed precursor than native INV. At present it is uncertain whether these vesicles are simply deficient in signal peptidase or whether the described reconstitution procedures do not allow functionally appropriate reassembly of signal peptidase into the lipid bilayer.

Comparisons of the protein composition of native and reconstituted membranes indicate that the rINV contained only a subset of the proteins present in native membranes. These differences appear to arise through differential solubilization by sodium cholate of integral membrane proteins and are particularly striking with respect to the distribution of PrlA (SecY). PrlA remained largely unsolubilized by sodium cholate in the presence of 1.0 M KOAc and, as determined by immunoblot analysis, was essentially absent from the rINV. These results suggest that PrlA is not required for *in vitro* protein translocation across the INV membrane. We cannot rule out, however, that the <1% of PrlA present in the rINV may be sufficient for protein export, particularly if PrlA serves a catalytic role in the translocation process.

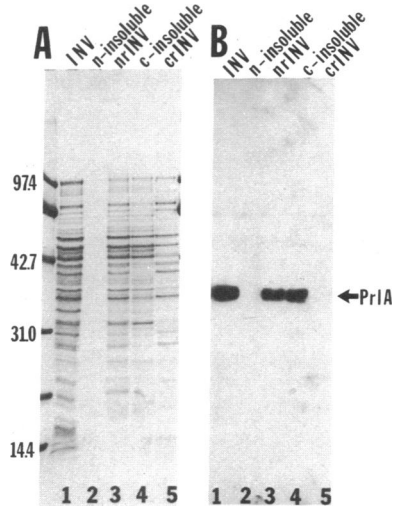

FIG. 6. PrlA is present in INV and nrINV but not in crINV. Fifty nanomoles of phospholipid phosphorus of INV (lane 1), nrINV (lane 3), and crINV (lane 5) and corresponding amounts of unsolubilized material [lane 2, Nikkol (n) solubilization; lane 4, cholate (c) solubilization] were processed for $NaDodSO_4$/PAGE. Polypeptides were either stained (*A*) or blotted onto nitrocellulose and probed with antisera directed against PrlA (*B*). Molecular weight markers are in kDa.

The observations reported here are somewhat difficult to reconcile with the extensive genetic and biochemical evidence indicating an essential function for PrlA in protein export. Because the *prlA* gene was identified as a suppressor of mutations in the signal sequence, PrlA has been suggested to function by direct interaction with the signal sequence (8, 9, 19, 20; see, however, ref. 32). A close association of PrlA with the translocation apparatus was also indicated by an inhibition of precursor binding and translocation by both IgG and Fab fragments directed against PrlA (24). Furthermore, accumulation of precursors of a number of periplasmic and outer membrane proteins was seen in a temperature-sensitive *secY* (*prlA*) strain grown at the nonpermissive temperature (10, 11). Vesicles prepared from the *secY* (*prlA*) mutant strain grown at permissive temperature were active but could be inactivated by preincubation of the vesicles at nonpermissive temperature (22). Inactivated vesicles could be reactivated when large amounts of SecA were added (23), suggesting an involvement of PrlA in protein export as well as an interaction between PrlA and SecA. An interaction of SecA and PrlA is also indicated by genetic data (33). A temperature-sensitive mutant of *secY* (*prlA*) displays enhanced synthesis of SecA (34) upon shifting to nonpermissive temperature. However, an increase in SecA synthesis has also been associated with mutations in *secD* or *secE* (34).

At present, conclusive evidence in support of a direct function for PrlA in protein export is lacking. As the data presented herein indicate that PrlA does not directly mediate, or regulate, protein export, it remains possible that the association of PrlA with protein export occurs in an indirect manner, perhaps through effects on membrane structure or membrane biogenesis.

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