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Signal peptides are allosteric activators of the protein translocase

Giorgos Gouridis[1,2], **Spyridoula Karamanou**[1], **Ioannis Gelis**[3], **Charalampos G. Kalodimos**[3], and **Anastassios Economou**[1,2,*]

[1] Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology-Hellas, Iraklio, Crete, Greece

[2] Department of Biology, University of Crete, Iraklio, Crete, Greece

[3] Chemistry & Chemical Biology, Biomedical Engineering, Rutgers University, 599 Taylor Rd, Piscataway, NJ 08854, USA

Abstract

Extra-cytoplasmic polypeptides are usually synthesized as “preproteins” carrying aminoterminal, cleavable signal peptides[1] and secreted across membranes by translocases. The main bacterial translocase comprises the SecYEG protein-conducting channel and the peripheral ATPase motor SecA[2,3]. Most proteins destined for the periplasm and beyond are exported post-translationally by SecA[2,3]. Preprotein targeting to SecA is thought to involve signal peptides[4] and chaperones like SecB[5,6]. Here we reveal that signal peptides have a novel role beyond targeting: they are essential allosteric activators of the translocase. Upon docking on their binding groove on SecA, signal peptides act *in trans* to drive three successive states: first, “triggering” that drives the translocase to a lower activation energy state; then “trapping” that engages non-native preprotein mature domains docked with high affinity on the secretion apparatus and, finally, “secretion” during which trapped mature domains undergo multiple turnovers of translocation in segments[7]. A significant contribution by mature domains renders signal peptides less critical in bacterial secretory protein targeting than currently assumed. Rather, it is their function as allosteric activators of the translocase that renders signal peptides essential for protein secretion. A role for signal peptides and targeting sequences as allosteric activators may be universal in protein translocases.

We sought to dissect the individual contributions of signal peptides and mature domains to membrane targeting and to post-targeting translocation steps. Since SecB is not universal or essential[6,8], we used the SecB-independent[9,10] substrate proPhoA (periplasmic alkaline phosphatase).

The affinity of proPhoA for inverted inner membrane vesicles (IMVs) containing SecYEG either alone or complexed with SecA was determined (Fig. 1a). ProPhoA associates with high affinity (0.23 μM) to SecYEG-bound SecA but not to SecYEG alone. Like proOmpA[5],

*Correspondence and Requests for materials should be addressed to: Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology-Hellas, PO Box 1385, Iraklio, Crete, Greece. aeconomo@imbb.forth.gr, Telephone: +302810-391166, Fax: +302810-391950.

Author Contributions G.G. cloned genes, performed *in vivo* and *in vitro* secretion experiments, phosphatase assays, membrane binding studies, Arrhenius conversions and developed the *in trans* reconstitution assay. G.G. and S.K. purified proteins, performed ATPase experiments, analyzed data, provided experimental ideas and contributed in writing the paper. S.K. developed thermal-dependence ATPase assay, contributed in assay development, performed preliminary ITC experiments and edited the paper. I.G. purified proteins, performed and analyzed ITC experiments. C.G.K. designed, guided and analyzed ITC experiments, contributed in experimental ideas and controls and in writing and editing the paper. A.E. conceived, designed and guided experiments, analyzed data and wrote the paper. All authors read and commented on the paper.

proPhoA requires SecA as an essential receptor. ProPhoA association to SecYEG-bound SecA is only marginally reduced if the signal peptide is impaired [proPhoA(L8Q); proPhoA(L14R) [9,10,11]]. In contrast, this binding is sevenfold reduced once the mature region is carboxyterminally truncated [proPhoA(1–62)]. Therefore, the mature domain moiety contributes substantially to ProPhoA translocase binding. This association was quantified for the first time: PhoA associated with SecYEG-bound SecA almost as strongly as proPhoA (0.6 μM), demonstrating that mature domains contain prominent targeting determinants. A large excess of signal peptide added *in trans*, does not out-compete PhoA binding (Fig. S2). Thus, signal peptide[4] and mature domain[12] binding sites on SecA must be distinct.

Soluble SecA also binds proPhoA and its derivatives tightly, with ~1:1 stoichiometry (Fig. 1a; Fig. S3b and c). This implies similar recognition of mature domains by SecYEG-bound and cytoplasmic SecA, although the latter interaction is ~10fold weaker. Since a synthetic proPhoA signal peptide binds to soluble SecA with fivefold less affinity than PhoA does (Fig. 1a), the mature domain is the primary binding determinant.

Periplasmic PhoA folds into its native, enzymatically active structure after forming intramolecular disulfides[13]. In the reducing, cytoplasm-like, environment used above, proPhoA exists in a “non-native” state, has no phosphatase activity (Fig. S1c) and is translocation-competent (Fig. S1a and b, lanes 3). Oxidized, “native” proPhoA is an active phosphatase, like PhoA[10,13] (Fig. S1c), but is translocation-incompetent (Fig. S1a, lane 4 and b, lane 2). “Native” proPhoA cannot associate with either soluble or SecYEG-bound SecA (Fig. S3d and a) although it carries a functional signal peptide. Thus, mature domain targeting signals required for docking at the membrane are only presented on “non-native” preproteins.

We next turned to post-targeting events. Is docking of a mature domain to the translocase sufficient to ensure secretion across the membrane? In contrast to proPhoA that was proficiently secreted *in vivo* or *in vitro* (Fig. 1b–d, lane 1)[9,10], secretion of PhoA (lane 4), proPhoA(L14R) (lane 3) or proPhoA(L8Q) (lane 2) was marginal. Clearly, functional signal peptides are essential for translocation of docked mature domains.

To identify what is the essential role of signal peptides in translocation we examined their effect on ATP hydrolysis by the translocase (Fig. 2; Fig. S4)[14]. Translocating preproteins lower the ATPase activation energy (E_a) dramatically (Fig. 2a; compare lane 3 to 1 and 2; Fig. S4c) and stimulate multiple ATP turnovers by 6–9 fold (“translocation ATPase”; Fig. 2b, lane 1; Fig. S4a)[14,15]. Wild-type proPhoA synthetic peptide alone fully retains the ability to lower E_a to a similar extent (Fig. 2a, lane 7) and in a saturable manner (Fig. S4d). In contrast, its L8Q or L14R derivatives (Fig. 2a, lanes 8 and 9), mature PhoA, proPhoA(L8Q) and proPhoA(L14R) (lanes 4–6) all fail to lower E_a. Therefore, functional signal peptides are necessary and sufficient to lower the activation energy state of the translocation ATPase, an effect we term “triggering”. However, since neither signal peptide nor PhoA alone stimulated translocation ATPase (Fig. 2b, lanes 5 and 2), multiple ATP turnovers[15] require ongoing “secretion” of mature domains (Fig. 2b, lane 1)[7,14,15]. These two-steps, “triggering” and “secretion”, are ordered and can be experimentally dissected.

To uncouple them we used *prl* (*pr*otein *l*ocalization) mutants in *sec* genes. We reasoned that these might by-pass triggering since they allow some secretion of preproteins with defective or missing signal peptides *in vivo*[9,10,11,16,17,18]. Indeed, the PrlA4[10,16,19] and PrlA3[16] (not shown) translocases are constitutively triggered in the absence of any preprotein (Fig. 2a, compare lane 10 to 2). Presumably, *prl* mutations mimic signal peptide-induced triggering by stabilizing the same low energy conformational state of the translocase.

Prl mutants enabled us to examine the requirements of post-triggering “secretion” reactions. PrlA4 secreted more wild type proPhoA *in vitro* than the wild type translocase (Fig. 1c and d;

compare lane 5 to 1)[19], suggesting that "triggering" is a rate-limiting step for secretion. PrlA4[10,16,19] associated with PhoA, proPhoA(L8Q) and proPhoA(L14R) indistinguishably from the wild type translocase (Fig. 1a) but secreted them 4–10 times more either *in vivo* or *in vitro* (Fig. 1b–d, compare lanes 2–4 to lanes 6–8). Nevertheless, secretion of mutant substrates, by either wild type or PrlA4, is still severely compromised compared to that of wild type proPhoA (Fig. 1b–d; compare lanes 6–8 to lane 5)[9]. Clearly, *prl* mutations only by-pass "triggering". For efficient protein "secretion", even the PrlA4 translocase requires functional signal peptides.

These data argue strongly that "secretion" requires the physical presence of signal peptides. To test this directly we used *in vitro* reconstitution. Wild type synthetic peptide added *in trans* stimulated PhoA translocation into wild type SecYEG IMVs (Fig. 3a, compare lane 7 to lane 5) to levels comparable to those seen with proPhoA (lane 3) and translocation ATPase (Fig. 2b, lane 8). Similar results were obtained with the PrlA4 translocase (Fig. S5). Signal peptides that are defective for proPhoA secretion (Fig. 1b–d, lanes 2 and 3) are either severely (L8Q; lane 9) or completely (L14R; Fig. 3a, lane 10) compromised in driving PhoA secretion *in trans*. Signal peptide-stimulated PhoA translocation requires physiological temperature (compare lane 8 to 7), ATP (compare lane 7 to 6) and the "non-native" state of PhoA (Fig. S6b), depends on signal peptide concentration (Fig. S6a), proceeds with similar kinetics to those of proPhoA (Fig. S6b) and is not affected by the order of ligand addition (Fig. S5). Clearly, signal peptides are essential after triggering to drive mature domain secretion. For this role, their covalent linkage to mature chains is unnecessary.

To identify post-triggering steps required for secretion, complexes of [^{35}S]-PhoA bound to SecA-SecYEG IMVs were isolated (Fig. 3b). Excess of unlabelled PhoA readily replaces bound [^{35}S]-PhoA and prevents signal peptide-driven translocation seen in the absence of chase (compare lane 2 to 1). However, two minute pre-incubation of bound [^{35}S]-PhoA with signal peptide prior to chase completely prevented exchange with unlabelled PhoA (compare lane 3 to 2). Presumably, signal-peptides cause mature domains to become physically "trapped" in the translocase. Trapping requires concomitant incubation with signal peptide and ATP at 37° C (Fig. 3b, lane 3). Low temperature (lane 4), ATP-alone (lane 5), non-hydrolyzable ATP (lanes 6 and 7) or a non-functional signal peptide (lane 8), all failed to drive the reaction. Identical results were obtained with the PrlA4 translocase (lanes 11–12). Trapped PhoA represents an early intermediate of the secretion state since, firstly, trapping is reversed readily after brief chilling (lane 9) and secondly, all of PhoA that is trapped at two minutes, is protease-accessible (lane 10) and therefore still largely exposed to the cytoplasm.

The signal peptide binding groove on SecA[4] is essential for all of the sub-reactions dissected here. Inactivation of the signal peptide binding cleft, reduces proPhoA affinity to that of PhoA (Fig. 1a) and severely compromises triggering (Fig. 2a, compare lane 11 to 3) as well as trapping (Fig. 3c, compare lane 4 to 2). Trapping remains defective (Fig. 3c, lane 6) even after use of a PrlA4 translocase to impose the triggered state artificially (Fig. 2a, lane 12).

The properties revealed here are not only valid for proPhoA but are likely to be universal. Two other signal peptides, from proLamB[4] and proM13coat[20], drive triggering (Fig. S7) and mature PhoA secretion (Fig. 3a, lanes 12 and 13). Four other mature domains bind to SecA with high affinity in the absence of signal peptides (Fig. 4a), while addition of the proPhoA signal peptide *in trans* drives their secretion (Fig. 4b) as efficiently as their own signal peptides (Fig. 4c).

Preprotein signal peptides and mature domains have multiple distinct roles in secretion (Fig. 4d)[15,20,21]. Signal peptides and, in some cases, SecB binding, stabilize "non-native" states and preproteins are then targeted to the translocase[22,5,6,8]. An additional targeting route, universal in bacteria, was revealed here (Fig. 4d, I). This involves direct recognition of "non-native"

mature domains by cytoplasmic or SecYEG-bound SecA. SecA is ubiquitous in the Bacteria and shuttles between cytoplasm and membrane[5]. Mature domain targeting signals could be degenerate sequences that become buried in “native” structures, reminiscent of chaperone recognition[23,24].

Mature domains are main contributors to the docking of several preproteins on SecA (Fig. 4a; Fig. 4d, II). This prominence was previously unsuspected. In some cases, signal peptides slightly enhance preprotein binding (Fig. 4a, see phosphatase, β-glucosidase, PPIase); in others they have no contribution (see amylase and sulphatase). Signal peptides with higher affinities[14,20] or those attached to short mature domains [e.g. proPhoA(1-62); Fig. 1a] could contribute more to preprotein docking. Mature domains (Fig. 1a)[15,25] and signal peptides[4] dock at non-overlapping SecA sites. These must be proximal since proPhoA, in which signal peptide and mature domain are covalently connected, binds 2–3 fold more tightly than PhoA (Fig. 1a). Being significantly larger, mature domains might facilitate positioning of signal peptides over their SecA binding cleft[4].

Tight signal peptide binding to SecA promotes triggering of the translocase holoenzyme (Fig. 4d, III) possibly by priming the protein-conducting channels[11,26,27] for opening. Next, it drives trapping (Fig. 4d, IV) of the first amino-terminal segment of mature PhoA in the translocase so that mature domains become irreversibly engaged in the channel. Trapping is tightly coupled to subsequent complete secretion (Fig. 4d, V) through multiple rounds of ATP hydrolysis and engagement of succeeding mature domain segments. Signal peptides could come off after trapping (Fig. 4d, V left) or may remain bound throughout secretion (right). Following triggering, signal peptides are expected to form additional intimate interactions with the SecYEG channel[18,28].

This cascade of events imposes multiple checkpoints that ensure efficient sorting of secretory proteins from cytoplasmic residents. Cytoplasmic proteins fold rapidly and will not be recognized by SecA. Without a signal peptide, the occasional illicit cytoplasmic binder can not trigger the translocase and as a result of this proofreading-like function it will be rejected[17,18].

Methods summary

Strains and biochemicals

Bacterial strains expressing proPhoA and derivatives were described previously[14,19]. SecA and urea-treated IMVs were prepared as described[29]. SecY amounts were quantitated using western blots with α-SecY immunostaining. All genetic constructs and antibodies are described in the Supplementary Materials and Methods. Preproteins were purified by Ni^{+2} affinity chromatography under denaturing conditions in Buffer C (50 mM Tris-HCl pH 8.0; 50 mM KCl; 6M Urea; 10% glycerol v/v) and were stored in buffer D (50 mM Tris-HCl pH 8.0; 50 mM KCl; 6M Urea; 1 mM EDTA; 10% glycerol v/v). Alkaline phosphatase units were determined *in vivo* using *p*-nitrophenol phosphate (Sigma) as described [10,13] and were converted to secreted protein mass using a standard curve obtained by determining the units of increasing amounts of purified native PhoA. Signal peptides (chemically synthesized; Genscript) were stored at 15 mM in 100% DMSO at 4°C.

Biochemical and biophysical assays—[^{35}S]-labeled proPhoA and derivatives were prepared by *in vitro* transcription/translation (Promega) and bound to inverted inner membrane vesicles as described[29]. Binding of proPhoA and derivatives to SecA in solution was determined by Isothermal Titration Calorimetry (VP-ITC, Microcal) at 8°C as described[4]. ProPhoA and derivatives were kept in the ITC measuring cell (80 μM; 20 mM Tris-HCl pH 8.0, 20 mM KCl, supplemented with 2mM TCEP to maintain a non-native state), while SecA

(1mM) was added in 20 μl injection steps. Thermal ATPase assays were performed as described [14] in buffer B (50 mM Tris-HCl pH 8.0; 50 mM KCl; 5 mM MgCl2) supplemented with 0.4 μM SecA; 0.5 mg/ml BSA; 1 mM ATP and 1.5 mM DTT (unless specified otherwise). For membrane ATPase, IMVs (0.4 μM SecY) were added. For translocation ATPase, proPhoA or derivatives were further added at indicated amounts. Activation energies were derived from Arrhenius transformations (Fig. S4c).

Online Methods

Determination of equilibrium dissociation constants (K_D) of proPhoA variants for SecYEG bound SecA

Non-radiolabeled proPhoA variants were serially diluted in buffer E (50 mM Tris-HCl pH 8.0; 50 mM KCl; 6 M urea; 1 mM EDTA; 1 mM DTT; 5% glycerol v/v). 0.7 μl from each dilution was added to the final reaction (20 μl) to achieve the desired protein concentration. Non-radiolabeled proPhoA variants were added in a concentration range of 1–30.000 μM (depending on the K_D of each variant for the SecYEG-bound SecA). IMVs containing over-expressed SecYEG (2 mg/ml total membrane protein) were diluted 25 times in buffer B (50 mM Tris-HCl pH:8.0; 50 mM KCl, 5 mM MgCl2), and 5 μl of these were mixed with 4.3 μl of SecA (0.2 μg/μl). The SecA/SecYEG complex (9.3 μl) was incubated on ice for 10 min and then added in the reactions. 5 μl of the reaction mix (prepared by mixing 0.8 ml of BSA 100 mg/mL; 8 mL of 10X buffer B and distilled water up to 20 ml) and 5 μl of [^{35}S]-proPhoA or its variants dilution were also added to the reactions. Reactions were then incubated on ice for 20 min, overlaid on an equal volume of BSA/sucrose cushion (prepared by mixing 1.37 g sucrose with 5 ml of the reaction mix and made up to 20 ml with distilled water) and ultracentrifuged (320.000 × g; 30 min; 4°C). The membrane bound material in the pellet was isolated and resuspended in buffer B (50 mM Tris-HCl pH:8.0; 50 mM KCl, 5 mM MgCl2) and then immobilized by spotting the resuspended pellets on a nitrocellulose membrane using a vacuum manifold (Bio-Rad). Data were analyzed by non-linear regression fitting for one binding site using Prism (Graph Pad) as described[29]. IMVs were prepared from cells that over-express either a wild type *secY/secE/secG* operon[30] or the *prlA4(i.e. secY(I408N/F286Y)/secE/secG*[16] operon. SecA and SecA(I304A/L306A) (mutated in the signal peptide binding groove[4]), were prepared as described. After synthesis of [^{35}S]-proPhoA variants, buffer exchange was accomplished using G-50 resin equilibrated with buffer E (50 mM Tris-HCl pH 8.0; 50 mM KCl; 6 M urea; 1 mM EDTA; 1 mM DTT; 5% glycerol v/v) and an ~8 fold dilution was made in buffer B supplemented with 1 mM DTT before adding them in the reactions. Non-radiolabeled proPhoA variants were treated with 2 mM DTT for 8 h to maintain them in a “non-native”, translocation-competent state.

Determination of equilibrium dissociation constants (K_D) of proPhoA variants and chemically synthesized proPhoA signal peptide for free SecA

K_D determination of proPhoA variants and chemically synthesized proPhoA signal peptide for free SecA was determined by ITC as described[4] and explained in the legend to Fig. S3.

In vivo translocation of proPhoA and its derivatives

The wild type *secY/secE/secG* operon[30] or the *prlA4 (i.e. secY(I408N/F286Y)/secE/secG*[16] operon were cloned in **pET610** plasmid which is under the control of a *trc* promoter[30]. Genes encoding proPhoA and its derivatives were cloned in the compatible **pBAD33** plasmid, under the control of the arabinose promoter[11]. The two plasmids were co-transformed in JM109 cells. Cells were grown at 37°C until OD_{595}= 0.2, SecYEG synthesis was induced by addition of 0.2 mM IPTG, while synthesis of proPhoA and derivatives was based on the read-through of the arabinose promoter[11] in the absence of arabinose. 20 min after IPTG induction, cells were pelleted by centrifugation (3.000 rpm, 4 min) and resuspended in 1 M Tris pH:8.0 followed

by addition of p-nitrophenyl phosphate (15 mM). The reaction was incubated at 37 °C for an appropriate time until a strong yellow color was observed and then stopped with a 10 % (v/v) of a solution obtained by mixing 1 volume 0.5 M EDTA pH 8.0 and 4 volumes 2.5 M K_2HPO_4). After addition of the non-ionic detergent TX-100 (1% v/v) and centrifugation (15.000 rpm, 4 min) in order to remove cell debris, the absorbance of p-nitrophenol was determined at 420 nm. Units of alkaline phosphatase were calculated as described[10] and converted to mass of secreted protein by using a standard curve with purified PhoA. Secretion of the proPhoA derivatives mediated by chromosomal SecYEG was measured under the same conditions using an empty **pET610** plasmid and these values were subtracted.

In vitro translocation of proPhoA and its derivatives

Reactions were performed in 100 μl buffer B; 0.5 mg/ml BSA, 2.5 mM ATP, 1 mM DTT by addition of SecA (0.4 μM), wild type or PrlA4-SecYEG IMVs (1.0 μM SecY) and proPhoA or its derivatives (8.5 μM). Reactions were incubated at 37°C for 12 min and translocation into the lumen of the IMVs was terminated by addition of proteinase K addition (1 mg/ml; 20 min; 4°C). Proteins were precipitated with trichloroacetic acid (TCA; 15% w/v), analyzed by SDS-PAGE (13% acrylamide) and immuno-stained with α-PhoA antibody. In all cases quantitation is carried out compared to the stain of a fraction of the protease-untreated [^{35}S]-PhoA material.

Calculation of activation energies (Ea)

The activation energies (*E*a) of the translocase were derived from Arrhenius plots using measured K_{cat} values (pmoles Pi/pmol SecA protomer/min) of basal, membrane and translocation ATPase activities of SecA, as a function of temperature[14] (as described in Fig. S4c). The Y axis in the Arrhenius plots represents the natural logarithm of the K_{cat} values [ln (K_{cat})] and the X axis the inversed temperature values (1/T) expressed in Kelvin. The activation energies (E_a) of the translocase under different regimes was calculated (in kJ/mole) using the slopes of the linear parts of the curves. SecA or SecA(I304A/L306A) mutant were used at 0.4 μM; wild type or PrlA4/SecYEG at 0.4 μM SecY; proPhoA derivatives at 8.5 μM; synthetic signal peptides at 15 μM.

In vitro reconstitution of [^{35}S]-PhoA translocation into wild type SecYEG IMVs by in trans addition of synthetic signal peptides

Reactions were performed in 100 μl buffer B; 0.5 mg/ml BSA, 2.5 mM ATP, 1 mM DTT by addition of SecA (0.4 μM), SecYEG IMVs (1.0 μM SecY), freshly prepared [^{35}S]-PhoA or [^{35}S]-proPhoA (~300 fmoles) and synthetic signal peptides (50 μM). Reactions were incubated at 37°C for 12 min and translocation into the lumen of the IMVs was terminated by proteinase K addition (1 mg/ml; 20 min; 4°C). Proteins were precipitated with trichloroacetic acid (TCA; 15% w/v) and analyzed by SDS-PAGE (13% acrylamide). Molecular weights (kDa) were derived from five marker proteins (from top to bottom: β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, lysozyme). The gel was incubated with 1 M sodium salicylate (1 h) and then visualized by phosphorimaging. Signal peptides (chemically synthesized; Genscript) were stored at 15 mM in 100% DMSO at 4°C, diluted at 1 mM in 10 mM Tris-HCl pH 7.0 before being added to the reactions. In all cases quantitation is carried out compared to the stain of a fraction of the protease-untreated [^{35}S]-PhoA material taken as 100%. In Fig. 4c. samples in lanes 8 and 9, 11 and 12, 14 and 15 were loaded with three times more material than that of lanes 7, 10 and 13 respectively and were quantified as follows: in b, lane 3, 180(±20) %; lane 6, 61(±10) %; lane 9, 16(±8) %; lane 12, 20(±6) %; lane 15, 4(±2) %. In c, lane 3, 165(±35) %; lane 6, 110(±12) %; lane 9, 22(±4) %; lane 12, 17(±8) %; lane 15, 13(±4) %.

Trapping of the polypeptide chain in the translocase holoenzyme

The translocase holoenzyme, assembled on SecYEG IMVs (1.0 μM SecY) by addition of 0.4 μM SecA in buffer B, was incubated on ice for 10 min with freshly prepared [^{35}S]-PhoA (~600 fmoles), overlaid on an equal volume of BSA/sucrose cushion (prepared as previously described) and ultracentrifuged (320.000×g; 30 min; 4°C). The SecYEG bound [^{35}S]-PhoA present in the pellet was isolated and resuspended in buffer B and then incubated for 2 min at 37°C with nucleotides (ATP, AMP-PNP; 1 mM) and/or synthetic proPhoA signal peptides (wt or L14R; 50 μM). Where previously omitted, reactions were supplemented with ATP (1.5 mM) and/or proPhoA signal peptide (50 μM) to initiate translocation into the lumen of IMVs by transfer at 37°C as previously described. At the same time all reactions were chased with excess of non-radiolabelled PhoA (1.5 μM). Translocation into the lumen of the IMVs was terminated by proteinase K addition (1 mg/ml; 20 min; 4°C). Proteins were precipitated with trichloroacetic acid (TCA; 15% w/v), analyzed by SDS-PAGE (13% acrylamide). The gel was incubated with sodium salicilate (1 h, 1 M) and then visualized by phosphorimaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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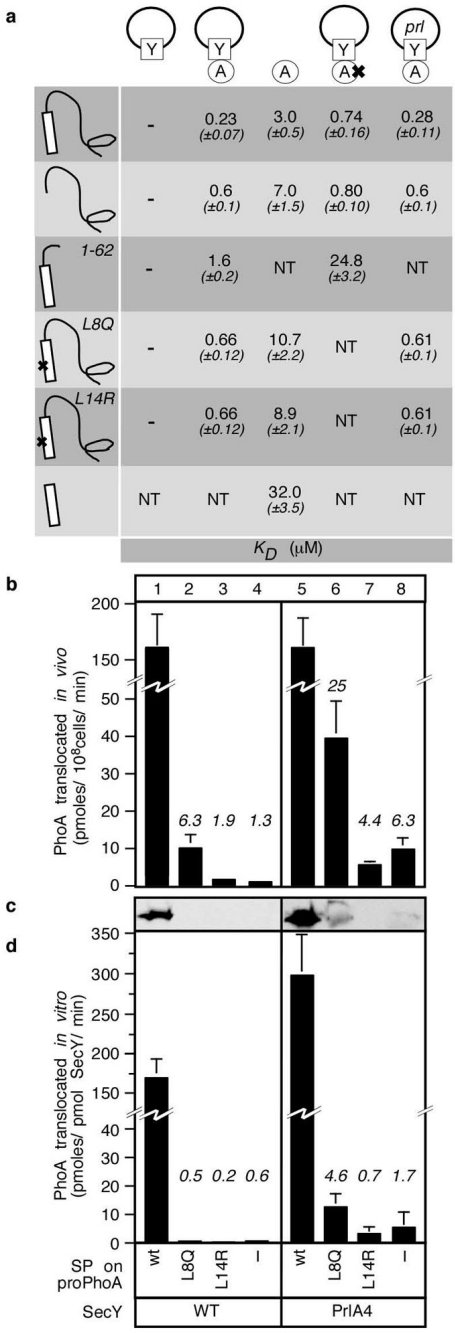

Fig. 1. Translocase binding and export of proPhoA and its derivatives

a. Equilibrium dissociation constants (*Kd*) of proPhoA and variants for the translocase. SecA (I304A/L306A) (marked "IL"), or PrlA4/SecYEG were used. **x**: mutant derivative. "–"u mnjh: no detectable binding, NT: not tested. (*n*= 3–7).

In vivo (**b**) or *in vitro* (**c** and **d**) translocation of proPhoA and derivatives by wild type or PrlA4/SecYEG translocase. In (b) phosphatase units were converted to protein mass. Proteins visualized by immunostaining (c) were quantified by phosphorimaging (d).

The percent of translocated material compared to that of the wild type proPhoA (100%) is indicated above each bar. (*n=9*).

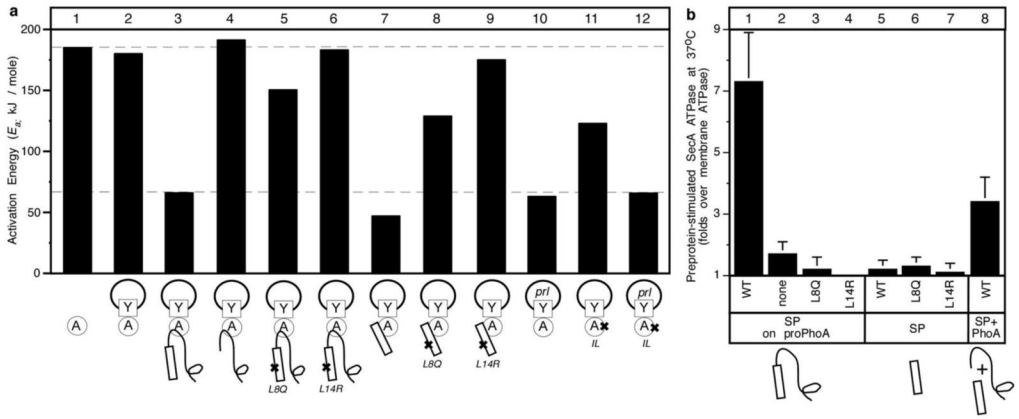

Fig. 2. Activation energy and stimulation of SecA ATPase under different regimes

a. The activation energy (*E*a; KJ/mole; Y axis) of the wild-type translocase and variants was determined in the presence of various preprotein derivatives and in the presence of synthetic signal peptides, as indicated. SecA or SecA(I304A/L306A) and wild type or PrlA4/SecYEG were used. **x**: mutant derivative. (*n=4–15*). Mutated residues are indicated in capitals.

b. The K_{cat} values (pmoles Pi/pmol SecA protomer/min) of the translocation ATPase activity of SecA at 37°C divided by those of the corresponding membrane ATPase activity (Fig. S3a) represents the folds of stimulation achieved by the various preprotein segments as indicated. (*n=4–15*).

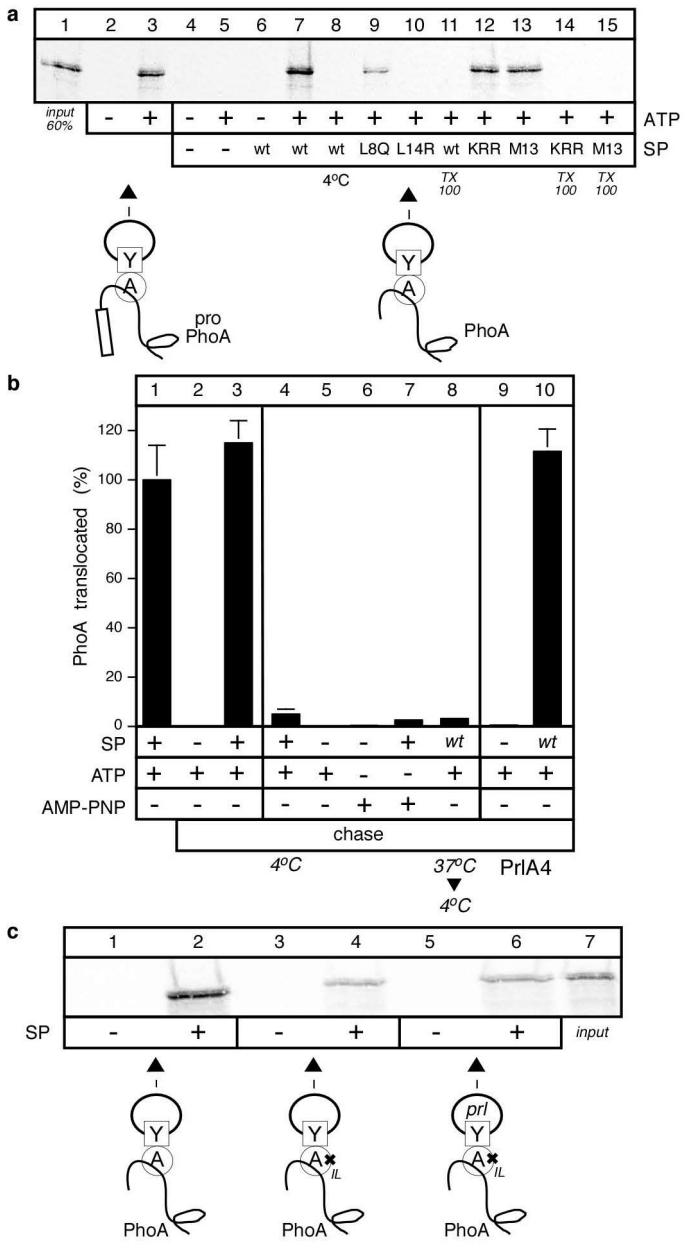

Fig. 3. Signal peptides added *in trans* promote PhoA translocation

a. [^{35}S]-PhoA translocation into wild type SecYEG IMVs driven by proPhoA (wt, L8Q, L14R), M13 procoat or proLamB12(KRR) signal peptides. Lanes 11, 14, 15 are identical to 7, 12, 13 except TX-100 addition prior to proteolysis. Lane 3 (100%): lane 7: 120 (±16) %; lane 9: 7 (±4) %; lane 12: 78 (±10) %; lane 13: 71 (±8) %. (*n =3*)

b. Trapping reaction. Translocase was incubated with [^{35}S]-PhoA and then with nucleotides and/or with signal peptides. Where previously omitted, ATP and/or signal peptide were added. Samples (except lane 1) were chased with non-radiolabelled PhoA at 37°C (except lane 4). Lane 8: after 2min the reaction was chilled (4°C) before translocation resumed. Lane 1 (100%). (*n =3*)

c. proPhoA signal peptide-driven [^{35}S]-PhoA translocation (as in "a"). Lane 2 (100%): lane 4, 5 (±1.1) %; lane 6, 12 (±2.4) %. (*n =3*)

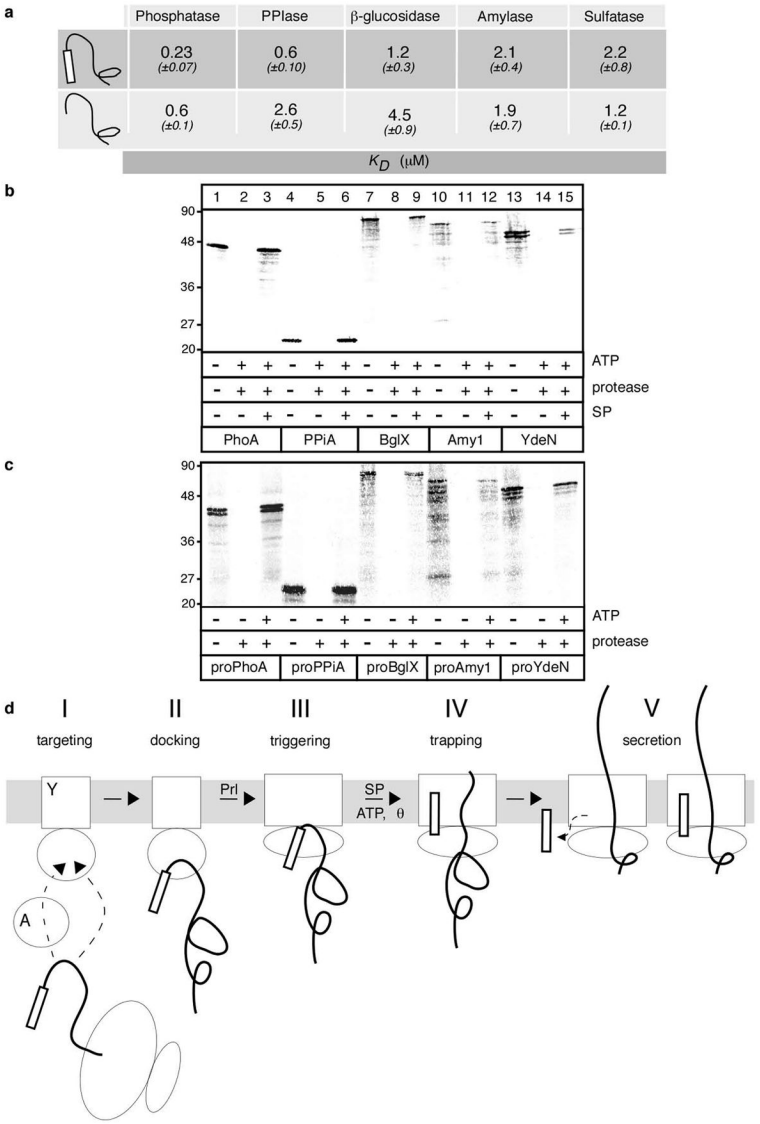

Fig. 4. Generality and working model of bacterial secretory protein translocation

a. Equilibrium dissociation constants (K_D) of precursor and mature forms of the indicated secretory *E. coli* proteins for the translocase.

b. and **c.** *In vitro* translocation reactions contain in b. [^{35}S]-labeled mature forms and synthetic proPhoA signal peptide whereas in c. [^{35}S]-labeled precursor forms of the indicated secretory proteins (as in Fig. 3a).

d. Model of post-translational bacterial protein secretion (see text for details). In “I” (bottom), a nascent secretory chain (thick line) carrying a signal peptide (rectangle) is shown to exit the ribosome. A, SecA; Y, SecY. Elongated shapes in “III–V” depict the triggered state.