LETTERS

Signal peptides are allosteric activators of the protein translocase

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Extra-cytoplasmic polypeptides are usually synthesized as 'preproteins' carrying amino-terminal, 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 show that signal peptides have a new role beyond targeting: they are essential allosteric activators of the translocase. On 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; second, 'trapping' that engages non-native preprotein mature domains docked with high affinity on the secretion apparatus; and third, 'secretion' during which trapped mature domains undergo several 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. Because 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], 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), see refs 9–11). In contrast, this binding is reduced sevenfold once the mature region is carboxy-terminally 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 (Supplementary Fig. 2). 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 and Supplementary Fig. 3b and c). This implies similar recognition of mature domains by SecYEG-bound and cytoplasmic SecA, although the latter interaction is ~tenfold weaker. Because a synthetic proPhoA signal peptide binds to soluble SecA with fivefold less affinity than PhoA (Fig. 1a), the mature domain is the primary binding determinant.

Periplasmic PhoA folds into its native, enzymatically active structure after forming intramolecular disulphides[13]. In the reducing, cytoplasm-like, environment used above, proPhoA exists in a 'non-native' state, has no phosphatase activity (Supplementary Fig. 1c) and is translocation-competent (Supplementary Fig. 1a and b, lane 3). Oxidized, 'native' proPhoA is an active phosphatase, like PhoA[10,13] (Supplementary Fig. 1c), but is translocation-incompetent (Supplementary Fig. 1a, lane 4 and b, lane 2). 'Native' proPhoA cannot associate with either soluble or SecYEG-bound SecA (Supplementary Fig. 3a and d) 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; Supplementary Fig. 4)[14]. Translocating preproteins lower the ATPase activation energy (E_a) markedly (Fig. 2a; compare lane 3 to 1 and 2; Supplementary Fig. 4c) and stimulate multiple ATP turnovers by six- to ninefold ('translocation ATPase'; Fig. 2b, lane 1; Supplementary Fig. 4a)[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 (Supplementary Fig. 4d). 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, because neither signal peptide nor PhoA alone stimulated translocation ATPase (Fig. 2b, lanes 5 and 2), several 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* (protein localization) mutants in *sec* genes. We reasoned that these might bypass triggering because they allow some secretion of preproteins with defective or missing signal peptides *in vivo*[9–11,16–18]. Indeed, the PrlA4 (refs 10, 16 and 19) and PrlA3 (ref. 16) (not shown) mutant translocases are constitutively triggered in the absence of any preprotein (Fig. 2a, compare lane 10

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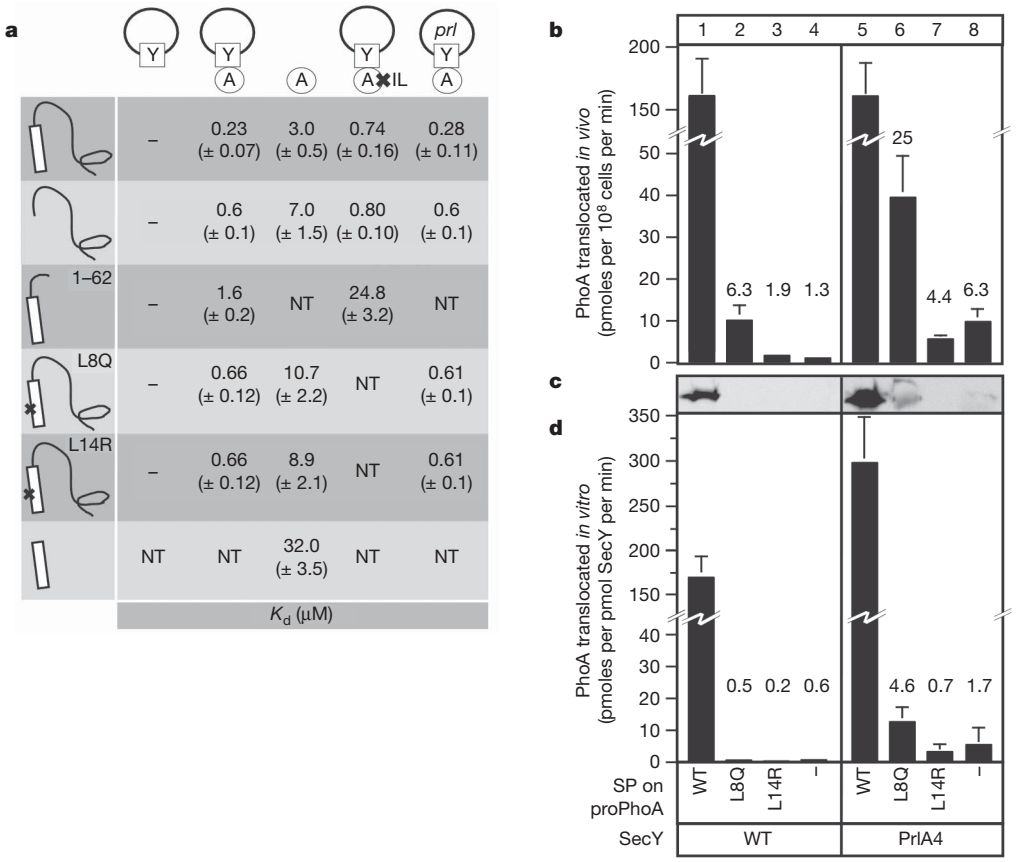

Figure 1 | Translocase binding and export of proPhoA and its derivatives. **a**, Equilibrium dissociation constants (K_d) of proPhoA and variants for the translocase. SecA(I304A/L306A) (marked 'IL') or PrlA4/SecYEG were used (n = 3–7). A, SecA; Y, SecY; **x**, Mutant derivative; −, no detectable binding; NT, not tested. **b**–**d**, *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 percentage of translocated material compared to that of the wild-type proPhoA (100%) is indicated above each bar (n = 9). In all the experiments the mean (or average value) of the independent experiments (n) is indicated. Error bars (Figs 1b, d, 2b, 3b) represent the standard deviation (or the square root of the variance) of all the values of the independent experiments.

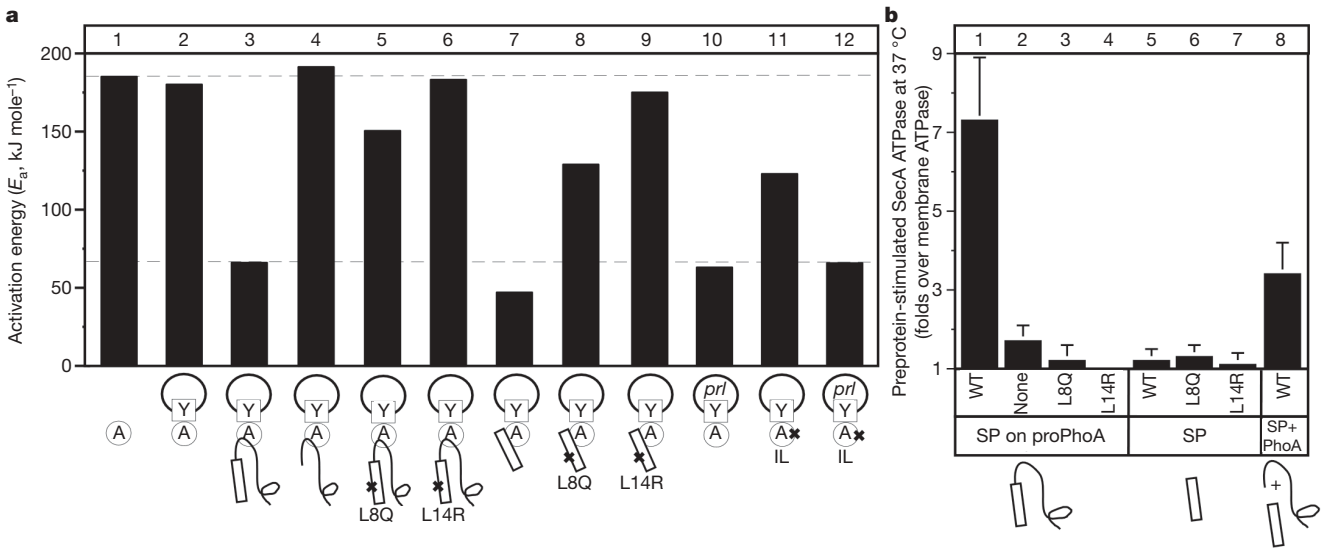

Figure 2 | Activation energy and stimulation of SecA ATPase under different regimes. **a**, The activation energy (E_a; kJ mole^{-1}) of the wild-type translocase and variants was determined in the presence of various preprotein derivatives and in the presence of synthetic signal peptides, as indicated. SecA or SecA(I304A/L306A) and wild type or PrlA4/SecYEG were used (n = 4–15). **x**, Mutant derivative. Mutated residues are indicated in capitals. **b**, The K_{cat} values (pmoles Pi per pmol SecA protomer per min) of the translocation ATPase activity of SecA at 37 °C divided by those of the corresponding membrane ATPase activity (Supplementary Fig. 3a) represent the folds of stimulation achieved by the various preprotein segments as indicated (n = 4–15).

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 mutant secreted more wild-type proPhoA *in vitro* than the wild-type translocase (Fig. 1c and d; compare lane 5 to 1)[19], indicating that 'triggering' is a rate-limiting step for secretion. PrlA4 (refs 10, 16 and 19) associated with PhoA, proPhoA(L8Q) and proPhoA(L14R) indistinguishably from the wild-type translocase (Fig. 1a), but secreted these preproteins 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 translocase, 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 bypass 'triggering'. For efficient protein 'secretion', even the PrlA4 translocase requires functional signal peptides.

These data show 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 (Supplementary Fig. 5). Signal peptides that are defective for proPhoA secretion (Fig. 1b–d, lanes 2 and 3) are either severely (L8Q; Fig. 3a, lane 9) or completely (L14R; Fig. 3a, lane 10) compromised in driving PhoA secretion *in trans*. Signal-peptide-stimulated PhoA translocation requires physiological temperature (Fig. 3a, compare lane 8 to 7), ATP (Fig. 3a, compare lane 7 to 6) and the 'non-native' state of PhoA (Supplementary Fig. 6b), depends on signal peptide concentration (Supplementary Fig. 6a), proceeds with similar kinetics to those of proPhoA (Supplementary Fig. 6b) and is not affected by the order of ligand addition (Supplementary Fig. 5). 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 (Fig. 3b, compare lane 2 to 1). However, 2 min pre-incubation of bound [^{35}S]-PhoA with signal peptide before chase completely prevented exchange with unlabelled PhoA (Fig. 3b, 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-hydrolysable 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 (Fig. 3b, lanes 11–12). Trapped PhoA represents an early translocation intermediate because, first, trapping is reversed readily after brief chilling (lane 9) and second, all PhoA that is trapped at 2 min 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 shown 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 (Supplementary Fig. 7) 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), whereas addition of the proPhoA signal peptide *in trans*

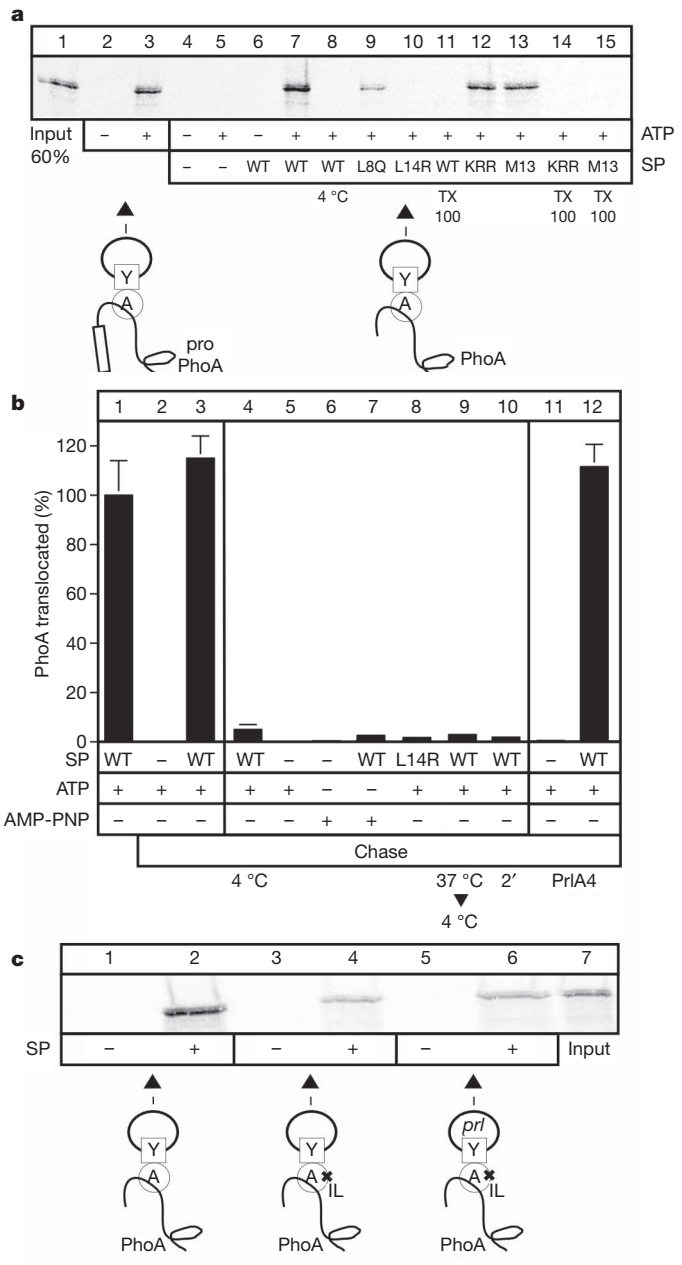

Figure 3 | Signal peptides added *in trans* promote PhoA translocation. **a**, [^{35}S]-PhoA translocation into wild-type SecYEG IMVs driven by proPhoA (WT, L8Q, L14R), M13 procoat or proLamB12 (KRR) signal peptides. Lanes 11, 14, 15 are identical to 7, 12, 13 except TX-100 was added before proteolysis. 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 9: after 2 min the reaction was chilled (4 °C) before translocation resumed. Lane 1, 100% ($n = 3$). AMP-PNP, Adenylyl-imidodiphosphate. **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); $n = 3$.

drives their secretion (Fig. 4b) as efficiently as their own signal peptides (Fig. 4c).

Preprotein signal peptides and mature domains have several 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[5,6,8,22]. An additional targeting route, universal in bacteria, was shown here (Fig. 4d, targeting). This involves direct recognition of 'non-native' mature domains by cytoplasmic or SecYEG-bound SecA. SecA is ubiquitous in Bacteria and

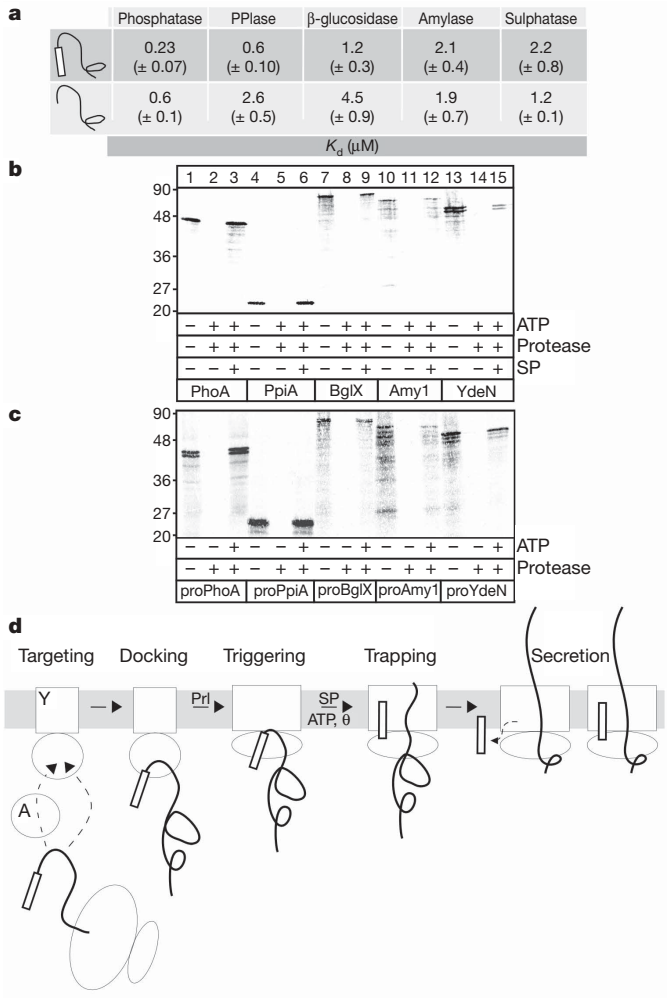

Figure 4 | Generality and working model of bacterial secretory protein translocation. a, Equilibrium dissociation constants (K_d) of precursor and mature forms of the indicated secretory *Escherichia coli* proteins for the translocase. **b**, **c**, *In vitro* translocation reactions containing [^{35}S]-labelled mature forms and synthetic proPhoA signal peptide (**b**) or [^{35}S]-labelled precursor forms of the indicated secretory proteins (**c**, as in Fig. 3a). **d**, Model of post-translational bacterial protein secretion (see text for details). A nascent secretory chain (thick line) carrying a signal peptide (rectangle) is shown to exit the ribosome. A, SecA; Y, SecY. Elongated shapes depict the triggered conformational state.

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, d, docking). This prominence was previously unsuspected. In some cases, signal peptides slightly enhance preprotein binding (Fig. 4a, see phosphatase, β-glucosidase, peptidyl-prolyl *cis-trans* isomerase (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 (for example, 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 (Supplementary Fig. 2). These must be proximal because proPhoA, in which signal peptide and mature domain are covalently connected, binds two- to threefold 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, triggering) possibly by priming the protein-conducting channels[11,26,27] for opening. Next, it drives trapping (Fig. 4d, trapping) 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, secretion) through several rounds of ATP hydrolysis and engagement of succeeding mature domain segments. Signal peptides could come off after trapping (Fig. 4d, secretion, left) or may remain bound throughout secretion (Fig. 4d, secretion, right). Following triggering, signal peptides are expected to form additional intimate interactions with the SecYEG channel[18,28].

This cascade of events imposes several 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 cannot trigger the translocase and as a result of this proofreading-like function it will be rejected[17,18].

METHODS SUMMARY

Strains and reagents. Bacterial strains expressing proPhoA and derivatives have been described previously[14,19]. SecA and urea-treated IMVs were prepared as described[29]. SecY amounts were quantified using western blots with anti-SecY immunostaining. All genetic constructs and antibodies are described in the Supplementary 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; 6 M urea; 10% glycerol v/v) and were stored in buffer D (50 mM Tris-HCl pH 8.0; 50 mM KCl; 6 M 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% dimethylsulphoxide at 4 °C.

Biochemical and biophysical assays. [^{35}S]-labelled 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 2 mM Tris (2-carboxyethyl) phosphine (TCEP) to maintain a non-native state), while SecA (1 mM) 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 $MgCl_2$) supplemented with 0.4 μM SecA; 0.5 mg ml^{-1} BSA; 1 mM ATP and 1.5 mM dithiothreitol (unless otherwise specified). 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 (Supplementary Fig. 4c).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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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, analysed 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 analysed ITC experiments. C.G.K. designed, guided and analysed ITC experiments, contributed in experimental ideas and controls and in writing and editing the paper. A.E. conceived, designed and guided experiments, analysed data and wrote the paper. All authors read and commented on the paper.

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METHODS

Determination of equilibrium dissociation constants (K_d) of proPhoA variants for SecYEG-bound SecA. Non-radiolabelled 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). An aliquot (0.7 μl) from each dilution was added to the final reaction (20 μl) to achieve the desired protein concentration. Non-radiolabelled 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 overexpressed SecYEG (2 mg ml^{-1} total membrane protein) were diluted 25 times in buffer B (50 mM Tris-HCl pH:8.0; 50 mM KCl, 5 mM $MgCl_2$), and 5 μl of these were mixed with 4.3 μl of SecA (0.2 μg μl^{-1}). The SecA–SecYEG complex (9.3 μl) was incubated on ice for 10 min and then added in the reactions. An aliquot (5 μl) of the reaction mix (prepared by mixing 0.8 ml of BSA 100 mg ml^{-1}; 8 ml of 10× 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 $MgCl_2$) and then immobilized by spotting the resuspended pellets on a nitrocellulose membrane using a vacuum manifold (Bio-Rad). Data were analysed by nonlinear regression fitting for one binding site using Prism (Graph Pad) as described[29]. IMVs were prepared from cells that overexpress either a wild-type *secY/secE/secG* operon[30] or the *prlA4* (that is, *secY(I408N/F286Y)/secE/secG*, see ref. 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 ~eightfold dilution was made in buffer B supplemented with 1 mM DTT before adding them in the reactions. Non-radiolabelled 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 Supplementary Fig. 3.

***In vivo* translocation of proPhoA and its derivatives.** The wild type *secY/secE/secG* operon[30] or the *prlA4* (that is, *secY(I408N/F286Y)/secE/secG*, see ref. 16) operon was 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 $D_{595} = 0.2$, SecYEG synthesis was induced by addition of 0.2 mM IPTG (isopropylthiogalactoside), while synthesis of proPhoA and derivatives was based on the read-through of the arabinose promoter[11] in the absence of arabinose. After IPTG induction (20 min), cells were pelleted by centrifugation (3,834 *g*; 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 colour was observed and then stopped with a 10% (v/v) of a solution obtained by mixing 1 volume of 0.5 M EDTA pH 8.0 and 4 volumes of 2.5 M K_2HPO_4. After addition of the non-ionic detergent TX-100 (1% v/v) and centrifugation (17,000 *g*; 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^{-1} 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 (1 mg ml^{-1}, 20 min, 4 °C). Proteins were precipitated with trichloroacetic acid (TCA; 15% w/v), analysed by SDS–PAGE (13% acrylamide) and immunostained with anti-PhoA antibody. In all cases quantification is carried out compared to the stain of a fraction of the protease-untreated [^{35}S]-PhoA material.

Calculation of activation energies (E_a). The activation energies (E_a) of the translocase were derived from Arrhenius plots using measured K_{cat} values (pmoles Pi per pmol SecA protomer per min) of basal, membrane and translocation ATPase activities of SecA, as a function of temperature[14] (as described in Supplementary Fig. 4c). The *y* axis in the Arrhenius plots represents the natural logarithm of the K_{cat} values and the *x* axis the inversed temperature values (1/T) expressed in Kelvin. The activation energies (E_a) of the translocase under different regimes were calculated (in kJ mole^{-1}) using the slopes of the linear parts of the curves. SecA or SecA(I304A/L306A) mutants 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^{-1} 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^{-1}; 20 min; 4 °C). Proteins were precipitated with trichloroacetic acid (TCA; 15% w/v) and analysed by SDS–PAGE (13% acrylamide). Molecular masses (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% dimethylsulphoxide at 4 °C, diluted at 1 mM in 10 mM Tris-HCl pH 7.0 before being added to the reactions. In all cases quantification 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 those 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 (adenylyl-imidodiphosphate); 1 mM) and/or synthetic proPhoA signal peptides (wild type 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^{-1}; 20 min; 4 °C). Proteins were precipitated with trichloroacetic acid (TCA; 15% w/v), analysed by SDS–PAGE (13% acrylamide). The gel was incubated with sodium salicilate (1 h, 1 M) and then visualized by phosphorimaging.

30. van der Does, C. *et al.* SecA is an intrinsic subunit of the *Escherichia coli* preprotein translocase and exposes its carboxyl terminus to the periplasm. *Mol. Microbiol.* **22**, 619–629 (1996).