Energy use by biological protein transport pathways

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The targeting of proteins into and across biological membranes to their correct cellular locations is mediated by a variety of transport pathways. These systems must couple the thermodynamically unfavorable processes of substrate translocation and integration with the expenditure of metabolic energy, using the free energy of ATP and GTP hydrolysis and/or a transmembrane protonmotive force. Several recent advances in our knowledge of the structure and function of these transport systems have provided insights into the mechanisms of energy transduction, force generation and energy use by different protein transport pathways.

The targeting of soluble and membrane-bound proteins to their correct subcellular location is essential for cell biogenesis. Because proteins are charged, sterically bulky heteropolymers, in the absence of a transport system their translocation across and insertion into the low dielectric barrier of biological membranes is thermodynamically unfavorable. Protein complexes – termed translocases – couple substrate protein transport and integration with the expenditure of metabolic energy. Nearly all translocases use the free energy of nucleoside triphosphate (NTP)-binding and hydrolysis: ATPases mediate energy transduction (the conversion of electrochemical energy into mechanical FORCE) and GTPases mediate signal transduction (acting as molecular binary switches). In addition, many translocases residing within energy-conserving membranes use transmembrane ion gradients to drive substrate transport.

A confluence of genetic, biochemical and structural research has led to the identification of the components that constitute the various translocation pathways, as well as the energy-converting steps involved. Protein translocation follows certain general principles [1]. Precursor proteins contain targeting sequences, typically in the N terminus, and are usually maintained in an unfolded transport-competent conformation, either by cytosolic chaperones (post-translational mode) or by halting the synthesis of nascent chains on the ribosome until targeting is complete (co-translational mode). Targeting factors and the associated receptors impart specificity to a targeting route, and transport across or into the membrane is typically mediated by a proteinaceous transmembrane hydrophilic channel. Based on evolutionary considerations, Schatz and Dobberstein [1] broadly divided protein translocation pathways into export systems in which the protein is directed to an extracytosolic compartment, and import systems whereby the protein is typically targeted post-translationally to a compartment equivalent to or evolutionarily derived from the cytosol. Schematic representations of these translocases highlighting the energy-transducing steps [both NTP hydrolysis- and/or protonmotive force (PMF)-dependent] involved in protein transport are shown in Figs 1 and 2, and provide a structural basis for the following discussion. For detailed descriptions of the individual pathways, the reader is directed to several excellent recent reviews [2–10].

Glossary

Brownian motion: the random motion of an entity in a fluid owing to ambient thermal energy, k_BT.
Brownian ratchet: an anisotropic device that biases random, non-equilibrium fluctuations in an isothermal medium to produce force and motion. A protein-transport motor based on this system requires a chemical reaction (e.g. binding of Hsp70 to the oscillating substrate) on one side of the membrane to trap the substrate and prevent retrograde movement, thereby imparting directionality.
***Cis*- and *trans*-acting ATP motor proteins:** the terms *cis* and *trans* are used here to describe the location of the translocation motor with respect to the starting and finishing side of the membrane, respectively, across which the protein transport reaction occurs.
Force: an action that causes an object with mass to accelerate, typically expressed in units of Newtons (N).
Gibbs free energy: a potential energy function that indicates the maximum amount of energy available to perform work. A process in which free energy is lost (exergonic, $\Delta G < 0$) will proceed spontaneously: a process in which free energy increases (endergonic, $\Delta G > 0$) is thermodynamically unfavorable and requires energetic input. Free energy is typically expressed in units of energy per mol (kJ mol^{-1}).
Kinetic solvent isotope effects: the effects on the rate constants of two reactions that differ only in the isotopic composition of the otherwise identical chemical reactants.
Positive inside rule: positively charged amino acids of membrane proteins most frequently reside on loops exposed to cytoplasm. This rule is frequently used in predictions of membrane protein topology.
Proteoliposomes: lipid vesicles containing purified membrane proteins that enable the characterization of the protein(s) without interference from other membrane components.
Proton well (or proton trap): is a device whereby the membrane electric potential (or pH gradient) is converted into an equivalently energetic pH gradient (or electric potential) within the membrane. This allows for the fact that, while thermodynamically equivalent, there is no mechanistic reason that the two potentials should contribute equivalently to, or yield the same kinetics of, a protonmotive force-dependent reaction.

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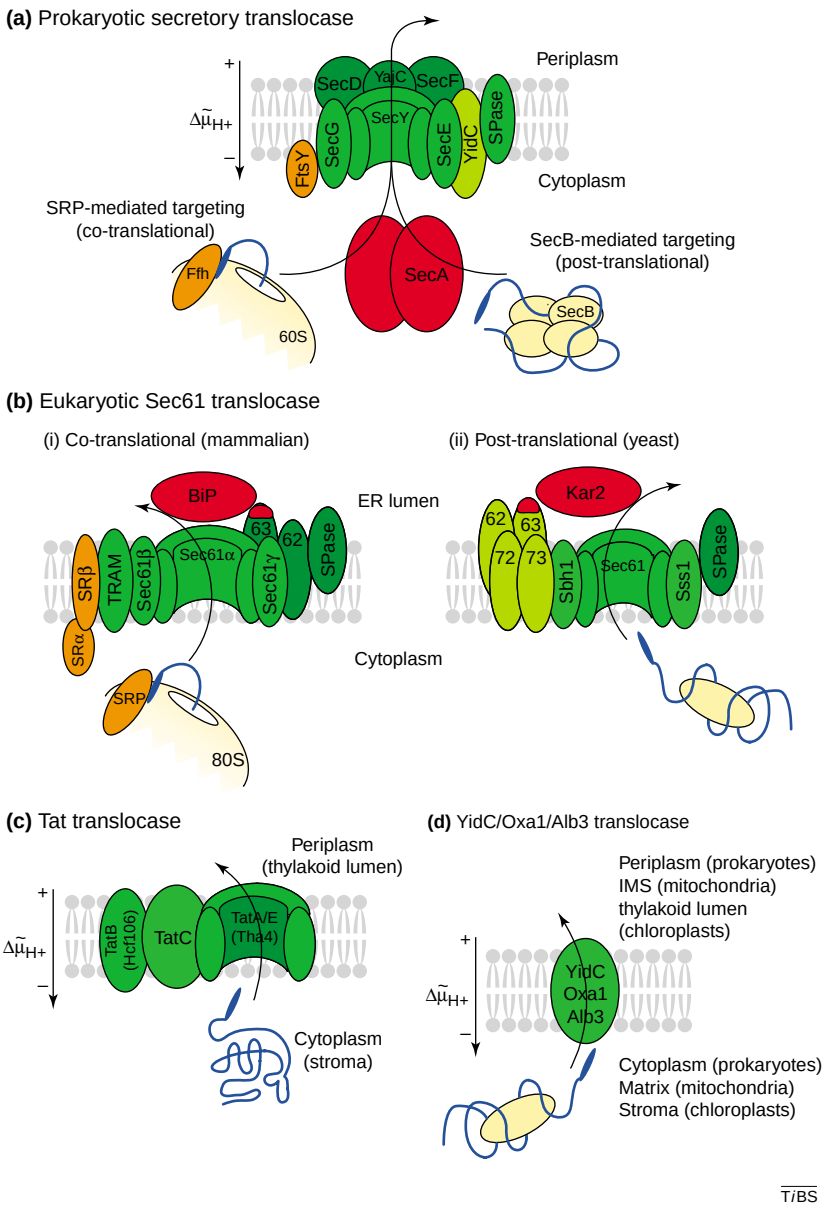

Fig. 1. Export-type protein transport pathways. (a) The prokaryotic secretory translocase consists of the heterotrimeric SecYEG core complex (green) associated with the heterotrimeric SecDFYajC complex (dark green), YidC (light green), the receptor FtsY (orange) and the peripherally associated ATPase motor protein SecA (red) [2]. Co- and post-translational targeting routes converge at the translocon. (b) The eukaryotic Sec61 translocase of the mammalian endoplasmic reticulum consists of the heterotrimeric Sec61 (α, β, and γ) and TRAM core (light green), the SRα/β signal-recognition particle (SRP) receptors (orange), and the lumenal ATPase BiP (red) [4]. The cognate translocase in yeast consists of homologous core (Sec61, Sbh1 and Sss1) and lumenal ATPase (Kar2) components, as well as the tetrameric Sec62–63 complex required for post-translational translocation [4]. (c) The bacterial Tat translocase consists of TatA/E (dark green), Tat B and TatC (green) – homologous to chloroplast Tat pathway components Tha4, Hcf106, and cpTatC, respectively [5]. (d) The YidC, Oxa1 and Alb3 membrane-insertion pathways of bacteria, mitochondria and chloroplasts, respectively, which mediate N-terminal tail export and polytopic membrane insertion, both post-translationally and co-translationally (not shown) [6]. All panels: core translocon components, green; ribosome and chaperones, yellow; ATPases and J-domains, red; GTPases, orange; precursor substrate, blue.

The role of ATP in protein translocation

Cycles of ATP binding and hydrolysis provide the majority of the driving force for protein transport for nearly all of the characterized pathways. Whether ATP-driven machinery is on the *cis* (pushing force) or the *trans* (pulling force) side of the membrane has fundamental implications for the type of energy-transducing mechanism used. The *CIS*-ACTING SecA and *TRANS*-ACTING Hsp70 proteins are the best-characterized energy-converting enzymes in protein transport machineries. As molecular motors, these proteins convert electrochemical energy (noncovalent ATP binding and hydrolysis) into mechanical energy through coupled conformational changes. Just as linear molecular motors (e.g. helicases and myosin) move along tracks (e.g. RNA, DNA or actin), these protein transport motors might be considered to move processively along the 'track' of an unfolded polypeptide substrate.

Cis-acting ATPase motor proteins

The SecA ATPase motor protein drives stepwise substrate transport by undergoing cycles of nucleotide-dependent conformational changes coupled to reversible binding to the SecYEG translocon [2,11,12] (Fig. 1a). SecA is a 102-kDa homodimeric protein whose structural elements

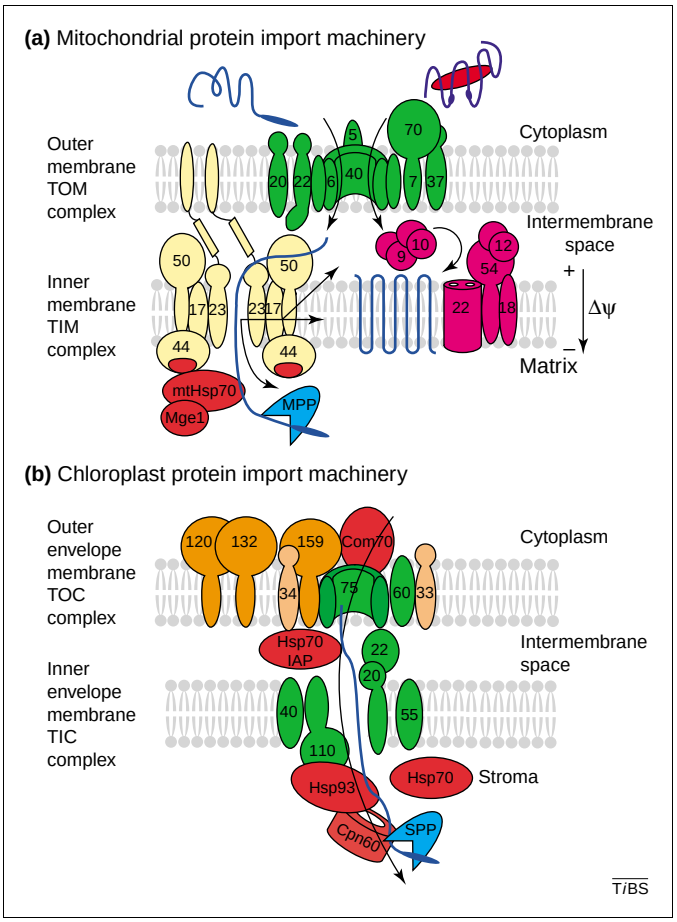

Fig. 2. Import-type protein transport pathways. (a) The mitochondrial import machinery. The TOM (translocase of the outer membrane) complex (green) is composed of the general insertion pore (the Tom40 channel, Tom22, 5, 6 and 7) and associated receptors [7]. Two functionally distinct TIM (translocase of the inner membrane) complexes mediate transport across or into the inner membrane. The TIM23 complex (presequence translocase; yellow) mediates the membrane insertion of monotopic proteins (stop-transfer pathway) in an inner membrane $\Delta\psi$-dependent manner, and the translocation of proteins into the matrix in successive steps requiring the $\Delta\psi$ and matrix-mtHsp70 ATPase activity. Cleavage of the presequence to generate the mature protein requires the soluble mitochondrial processing peptidase (MPP; light blue). The TIM22 complex (carrier translocase, pink) uses the $\Delta\psi$ to mediate the insertion of polytopic proteins [8]. ATPases, co-chaperones and Hsp70-binding regions are shown in red. (b) The chloroplast import machinery. The TOC (translocase of the outer chloroplast membrane) complex consists of a trimeric core (the Toc75 channel and Toc34/Toc159 receptors) [9] and associated ATPases Com70 and IAP [10]. The TIC (translocase of the inner chloroplast membrane) complex is associated with stromal chaperones (Hsp93, Cpn60 or Hsp70; red) [10]. Precursors initially bind to the TOC complex in a reversible and energy-independent step; early import intermediates form in a committed step of contact with TIC components in the presence of low concentrations of GTP and ATP in the intermembrane space and cytoplasm. Finally, stromal ATPases (red) drive translocation across both membranes. Cleavage of the signal sequence requires the soluble stromal processing peptidase (SPP; light blue). Non-NTP consuming components of the TOC and TIC components are shown in green; GTPases are shown in orange. Protein import across the outer and inner membranes can occur simultaneously by translocon coupling at contact sites (TOM–TIM and TOC–TIC in mitochondria and chloroplasts, respectively).

and domain interactions have been studied by a host of biochemical and biophysical techniques [13–16], and further elucidated by the recently determined crystal structures of cytoplasmic SecA from *Bacillus subtilis* [17] and *Mycobacterium tuberculosis* [18]. SecA consists of an N-terminal motor domain that is homologous to ATP-dependent RNA DEAD (Asp-Glu-Ala-Asp) superfamily II helicases [14,17–19], and a C domain with regulatory elements and ligand-binding regions [13] (Fig. 3a).

The ATPase catalytic cycle of SecA (Fig. 3b) is controlled by ligand interactions and interdomain communication. Binding of ADP to the nucleotide-binding site at the NBF-I–NBF-II junction stabilizes the compact conformation of cytoplasmic SecA [16] (Fig. 3bi). Futile ATP hydrolysis is prevented by the regulatory intramolecular regulator of ATP hydrolysis (IRA)-1 switch interacting with the N domain via the IRA2 region [13,14]. Association of SecA with SecYEG through its N or C domain [20] (Fig. 2bii) causes partial C-terminal unfolding of SecA. This relieves IRA1-mediated ATPase suppression [13], thereby stimulating ATPase activity [21] and enhancing signal peptide binding [15]. SecA binding might also nucleate the oligomerization of SecYEG [2]. The IRA2 region enhances ATPase activity by stimulating ADP release, which is the rate-limiting step in the SecA cycle [14]. ATP binding stabilizes a more unfolded conformation of SecA [17], driving the membrane insertion step (Fig. 3biii) in which portions of the N and C regions of SecA might extend into the periplasmic space [22]. SecA insertion drives the translocation of ~25 aminoacyl residues of the substrate polypeptide through the membrane [12]. The membrane-inserted conformation of SecA is stabilized by the SecDFYajC complex [23] and signal peptide binding [15], and is accompanied by the topological inversion of SecG [24]. Following ATP hydrolysis (Fig. 3biv), SecA assumes the compact ADP-bound conformation, causing substrate dissociation from SecA [11] and driving SecA de-insertion from SecYEG [2] in a manner promoted by the PMF [25]. The polypeptide substrate remains associated with SecYEG after SecA release [11], yet is free to diffuse within the channel. SecA might then undergo multiple catalytic turnovers to processively translocate the entire length of the substrate protein. Alternatively, after a significant amount of polypeptide has been inserted, translocation can be completed using the PMF through an unknown mechanism [11]. It is possible that SecA catalyzes processive protein transport in a mechanism similar to that used in its RNA helicase activity [19].

Other known, yet less well-characterized, *cis*-acting ATPases participating in protein transport are involved in the import-type machineries of chloroplasts (Fig. 2b) and peroxisomes (not shown). For instance, Com70, the Hsp70 homolog on the cytosolic face of the chloroplast outer membrane (Fig. 2b), interacts with precursors in an ATP-dependent manner, and might be involved in substrate unfolding or in providing a pushing force in the early stages of import [10].

Trans-acting ATPase motor proteins

Trans-side use of ATP drives organellar-protein import in mitochondria (Fig. 2a) and chloroplasts (Fig. 2b), and post-translational translocation across the endoplasmic reticulum (ER) membrane (Fig. 1b). The energy-converting ATPase in most of these systems is a member of the Hsp70 family of chaperones (BiP and Kar2 in the mammalian and yeast ER lumen, respectively, and mtHsp70 in the mitochondrial matrix), although much evidence points to an Hsp100 in this role in chloroplasts [10]. Hsp70 chaperones have a highly conserved N-terminal nucleotide-binding domain (NBD) of 44 kDa, followed by a

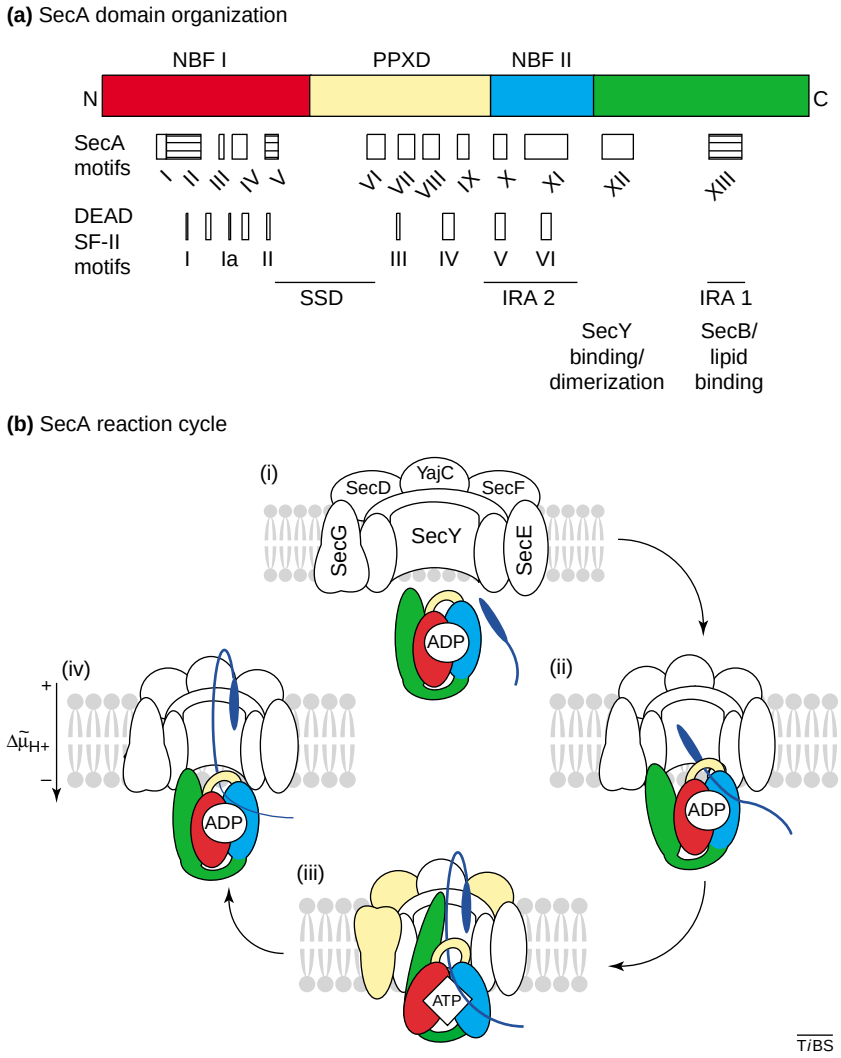

Fig. 3. The SecA ATPase cycle. (a) The domain structure of SecA. The N-terminal DEAD (Asp-Glu-Ala-Asp) helicase motor domain contains two nucleotide-binding folds – NBF I (red) and NBF II (blue) – surrounding a pre-protein crosslinking domain (PPXD; yellow) in tandem with a regulatory C-terminal domain (green). Conserved SecA regions include Walker Box sequences A and B (motifs II and V, respectively) within the DEAD motor, the signal-sequence-binding domain (SSD), and the regulatory regions in the N-terminal [IRA2 (intramolecular regulator of ATP hydrolysis 2), motifs X and XI] and C-terminal (IRA1, motif XIII) regions. Conserved motifs (I–VI) homologous to DEAD helicases of superfamily II are indicated. (b) The SecA reaction cycle. (i) The thermodynamically stable soluble SecA–ADP (closed conformation) is prevented from ATPase activity by IRA1 interaction with the N domain via the IRA2 subdomain. (ii) SecA interacts with SecYEG in a step gated by an endothermal transition which partially relieves IRA1-mediated suppression, thereby enhancing ATPase activity and signal-sequence binding. (iii) ATP binding stabilizes the domain-dissociated conformation, driving the insertion of N- and C-terminal regions into the SecYEG channel, and concurrently moving polypeptide substrate (20–30 aminoacyl residues) across the membrane. Translocon proteins specifically involved in this step are colored yellow: SecG displays an inverted topology, and the membrane-inserted SecA is stabilized by SecD and SecF. (iv) SecA de-insertion is coupled to ATP hydrolysis and stimulated by the protonmotive force.

C-terminal region that includes a 25-kDa substrate-binding domain (SBD) in tandem with a more variable 10-kDa region of unknown function [26] (Fig. 4a).

The structural determination of the ATPase [27] and substrate-binding regions [28] of Hsp70s has complemented a wealth of biochemical work in elucidating the Hsp70 catalytic cycle. Among many other functions, Hsp70 chaperones promote productive folding of polypeptides by undergoing iterative cycles of substrate binding and release: Hsp70–ATP has low affinity for substrate; ATP hydrolysis transmits a conformational change to the substrate-binding domain (the closing of a lid subdomain over the binding cavity), resulting in a stable Hsp70–ADP–substrate ternary complex with high substrate-binding affinity; nucleotide exchange of ATP for ADP resets the cycle [26]. This catalytic cycle is modulated by co-chaperones: DnaJ-type proteins that stimulate the low (rate-limiting) intrinsic rate of Hsp70 ATP hydrolysis through their signature J-domains, and nucleotide-exchange factors that accelerate the exchange of ATP for ADP. J-domain-bearing translocon subunits recruit cognate Hsp70s to the *trans* side of the membrane, concentrating the motor proteins at the point of substrate exit from the translocon. In the ER translocon (Fig. 1b) this role is played by Sec63 [29], and in mitochondria, Tim44 contains a J-related segment at the TIM23 complex (Fig. 2a) for delivery of mtHsp70 to the channel [7]. Relevant nucleotide-exchange factors include Mge1 in mitochondria and perhaps the recently discovered BAP in the ER [30].

Two distinct, but not mutually exclusive, models have been proposed for the mechanism by which Hsp70 proteins

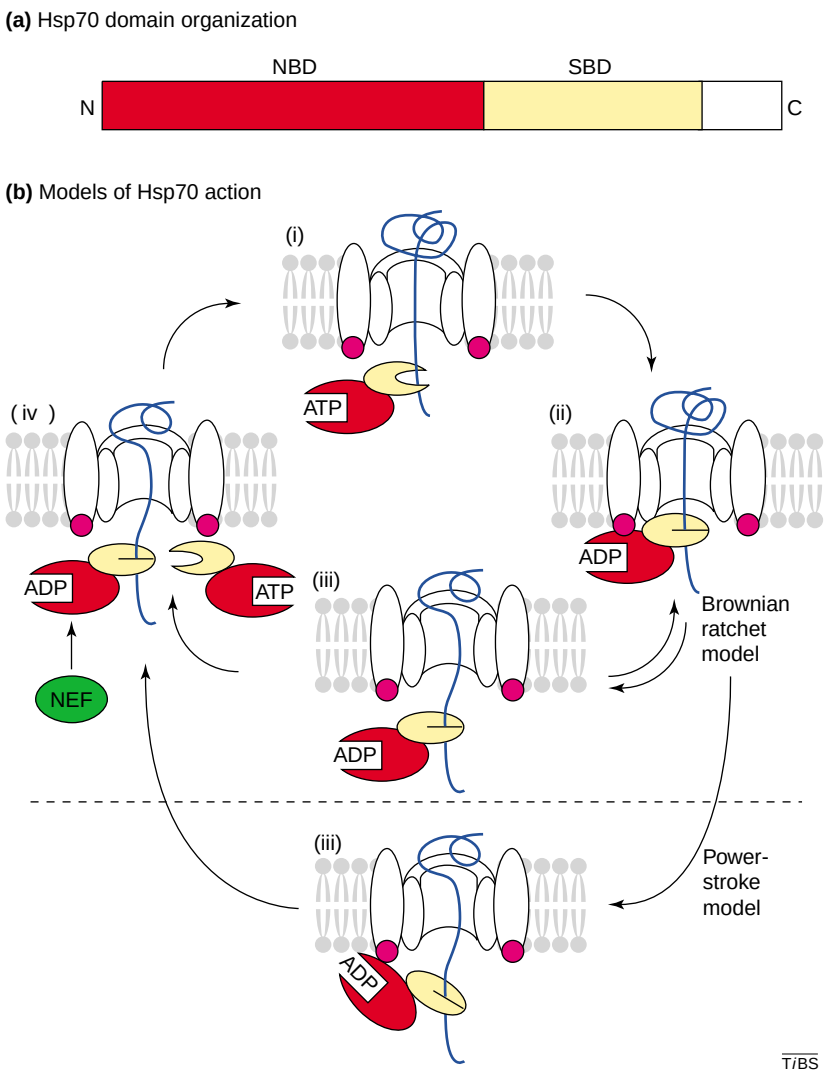

Fig. 4. The Hsp70 ATPase cycle. (a) The domain structure of Hsp70. The nucleotide-binding domain (NBD) and substrate-binding domain (SBD) are shown in red and yellow, respectively. (b) Models of Hsp70 action. The Brownian ratchet model: (i) an unfolded segment of substrate (blue) spans the channel, exposing a chaperone-binding site, and Hsp70–ATP is recruited to the J-domain site (pink). (ii) J-activated ATP hydrolysis by Hsp70 leads to a high-affinity interaction with the substrate. (iii) Thermal breathing and local unfolding of the polypeptide substrate allow for Brownian oscillations, in which segments of substrate diffuse freely within the channel. (iv) Once a sufficient stretch of substrate has diffused toward the *trans* compartment to expose a new Hsp70-binding site, a second Hsp70–ATP is recruited to the translocon and the cycle is repeated. Exchange of ATP for ADP is facilitated by nucleotide exchange factors (NEF). The power stroke model: (i) and (ii) an unfolded segment of substrate is bound to Hsp70 by J-activated ATP hydrolysis as above. (iii) Following ATP hydrolysis, Hsp70 undergoes a second conformational change, generating a pulling force on the bound substrate. Although it has not been shown experimentally, such a power stroke could be associated with the release of energy stored in an elastic element associated with P_i release, similar to the myosin power stroke. (iv) A single Hsp70 could remain bound to the translocon and undergo repeated cycles, or remain bound to the substrate, allowing additional Hsp70–ATP molecule recruitment to the translocon.

drive protein translocation (Fig. 4b). In the 'BROWNIAN RATCHET' cycle, an unfolded segment of substrate diffuses spontaneously through the translocon channel by BROWNIAN MOTION, and *trans*-side Hsp70 binding biases the thermal fluctuation, thereby causing vectorial translocation. In the power stroke cycle, Hsp70 generates a pulling force on the substrate perpendicular to the plane of the membrane by anchoring onto the cognate J-site for leverage and undergoing a conformational change after ATP hydrolysis to actively drive vectorial translocation. The mechanism by which folded domains of the precursor protein are unfolded by the transport machinery differs fundamentally between these two models: an Hsp70-driven power stroke could actively pull on a segment of the substrate to partially collapse the cooperatively folded mature protein, whereas a passive ratchet would rely on thermal breathing of the substrate, with spontaneous unfolding events rectified by Hsp70 binding. Experimental support for the requirement of a power stroke step in mitochondrial import has come from the observation that import rates exceed rates of spontaneous substrate unfolding [31], that substrate–channel interactions lead to low diffusion rates [32] and that mtHsp70 must interact with Tim44 – the membrane fulcrum in the power stroke – through its ATPase domain to import folded substrates [33,34]. Support for the sufficiency of passive ratcheting to drive transport comes from several observations of free substrate diffusion through the channels of mitochondria [35] and the ER [36], that the substrate-binding domains of mtHsp70 and BiP interact with Tim44 [37] and Sec63 [38], respectively, and that antibody–substrate interactions can drive transport [36]. Spontaneous forward movement

during mitochondrial import was shown using substrates with stretches of polypeptide lacking Hsp70-binding sites between the presequence and a folded passenger protein [39]. Efficient import of these substrates suggested that spontaneous local unfolding of the mature protein is necessary, and that a molecular ratcheting mechanism is sufficient for import. Additional support of the ratchet model comes from recent evidence showing that release of substrate-bound mtHsp70 from Tim44 precedes ATP hydrolysis [40]. It is possible that Hsp70-driven transport uses a combination of a targeted ratcheting mechanism (whereby the motor Hsp70 protein is directed to the translocon by Tim44 or Sec63 interactions) and an active power stroke for tightly folded substrate domains.

Chloroplast protein import uses multiple *trans*-acting ATPase chaperones (Fig. 2b) with an NTP affinity that decreases as the substrate protein progresses through the envelope transport machinery [10]. The Hsp70 of the intermembrane space (IAP) might bind the precursor during transport across the outer membrane, thereby maintaining the import competence of the substrate and preventing backsliding. Translocation across the envelope membranes requires internal ATP only [41], and the main import-associated chaperone, unlike the Hsp70s of mitochondria, could be the Hsp100 homolog Hsp93 [10]. Hsp93 might be recruited to the import channel by association with Tic110 in a manner similar to the mtHsp70–Tim44 association, but given the extensive differences between the two ATPases, the transport mechanism might be different. Although their precise roles are unknown, other stromal chaperones thought to be involved in import include Cpn60, a member of the Hsp60 chaperonin family involved in substrate folding, and multiple Hsp70s in the stroma with associated co-chaperones [10]. Given the similarity of Tic40 to the Hsp70 nucleotide exchange factor Hip, it is possible that this subunit plays a role similar to Mge1 in mitochondria [7].

The role of GTP in protein transport

GTPase proteins act as binary molecular switches, with the ability to change affinities for other macromolecules in temporal succession with cycles of GTP binding and irreversible hydrolysis, thereby imparting unidirectionality and binding specificity to biological processes. The prototypical GTPase cycle drives transitions among three conformations: the GTP-bound (active) state, and the GDP-bound and empty (inactive) states [42]. Regulatory proteins involved in this cycle include GTPase-activating proteins (GAPs), which enhance rates of irreversible GTP hydrolysis, and guanine-nucleotide-exchange factors (GEFs), which catalyze the exchange of bound GDP for GTP [43]. Molecular switch models that impart steady-state proofreading typically involve branched reaction pathways of intersecting GTPase cycles [44]. In the case of protein transport, such mechanisms ensure substrate–receptor specificity.

Signal recognition particle (SRP)-mediated targeting of ribosome-nascent chain complexes (RNCs) in eukaryotes involves three GTPases: SRP54 of the targeting factor SRP, and SRα and SRβ of the SRP receptor (Fig. 1b). The cognate prokaryotic pathway involves Ffh and FtsY (homologs to SRP54 and SRα, respectively) [45] (Fig. 1a). SRP54 and SRα belong to a subfamily of Ras-like GTPases with unique structural features, and SRβ is a member of Arf GTPases [45]. Targeting involves the intersecting GTPase cycles of the SRP and the SRP receptor (Fig. 5). Prior to interaction, SRP54 and SRα might assume the GTP-bound state owing to GEF activity of the ribosome and translocon, respectively. Upon interaction, SRP54 and SRα could act as reciprocal GAPs, thereby stimulating GTP hydrolysis. In this reaction series, targeting fidelity is imparted through a kinetic proofreading mechanism by GTP hydrolysis. Alternatively, SRP-mediated targeting could follow an unusual 'concerted-switch' mechanism in which both SRP54 and SRα can assume stable empty or GTP-bound states, but GTP-binding affinity for both

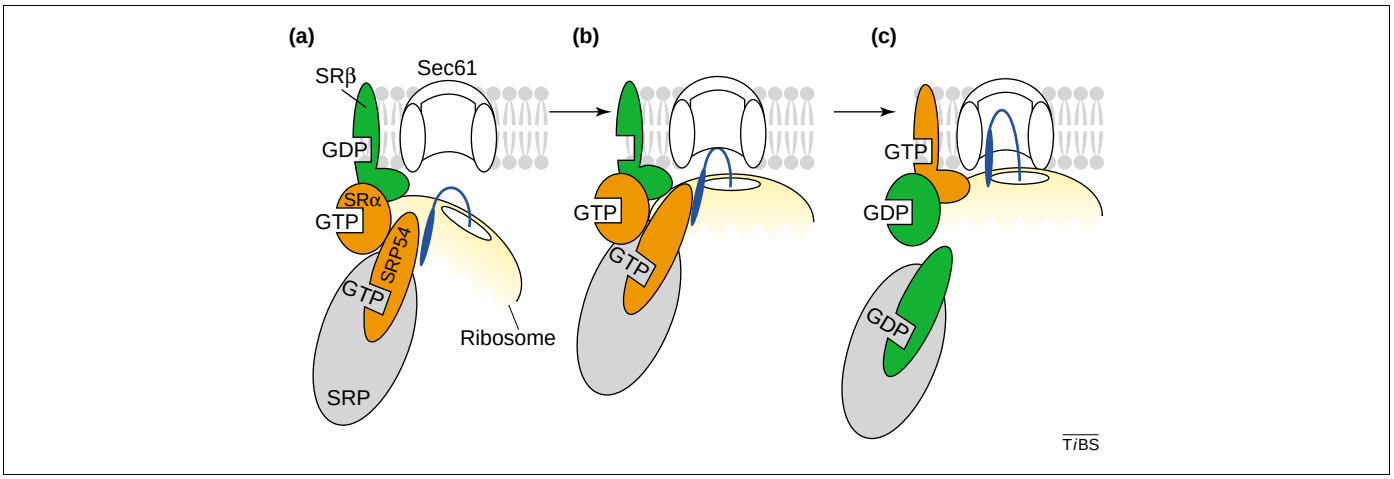

Fig. 5. The GTPase cycle of signal recognition particle (SRP)-mediated targeting. Nucleotide-free and GDP-bound (green) and GTP-bound (orange) forms of GTPases are shown; SRP is gray, the ribosome is yellow and substrate is blue. (i) Binding of SRP to the signal peptide causes temporary translation arrest, and targeting of the ribosome nascent-chain complex (RNC) to the endoplasmic reticulum membrane occurs by a specific interaction between SRP and the SRP receptor. SRP54 and SRα might assume a GTP-bound state before interaction, or a nucleotide-free ('primed') state. (ii) Homotypic interaction between SRP54 and SRα via their NG domains leads to stable GTP binding, and interaction between the ribosome and SRβ stabilizes the empty form of SRβ. (iii) Reciprocal GTPase stimulation between SRP54 and SRα promotes GTP hydrolysis, resulting in the dissociation of SRP and SRP receptor. GTP binding to SRβ results in the translocon-dependent release of the signal sequence from SRP, and the nascent chain is transferred to the Sec61 translocon to begin co-translational translocation.

Box 1. The protonmotive force

The electrochemical proton potential ($\Delta\tilde{\mu}_{H+}$) comprises the thermodynamically equivalent electric potential ($\Delta\psi$) and H^+-concentration difference across the membrane (ΔpH) by the following relation (Eqn 1):

$$\Delta\tilde{\mu}_{H+} = F\Delta\psi + 2.3RT\Delta\text{pH} \quad \text{(Eqn 1)}$$

This term can be recast in electrical units as the protonmotive force (PMF; Eqn 2):

$$\text{PMF} = \frac{\Delta\tilde{\mu}_{H+}}{F} \quad \text{(Eqn 2)}$$

GTPases increases with successive targeting steps, and GTP hydrolysis is required only for dissociation of SRP54 and SRα [46]. GTPase activity of SRβ might mediate the translocon-dependent release of the signal sequence from SRP. Similarly, chloroplast homologs of SRP54 and FtsY are required for post-translational insertion of some thylakoid membrane proteins in a GTP- and Alb3-dependent manner.

GTPase-mediated substrate–receptor interactions are also involved in post-translational protein translocation. The homologous Toc159 and Toc34 GTPases of the chloroplast TOC (translocon at the outer chloroplast membrane) trimeric complex (Fig. 2b) interact with precursor proteins in temporal succession through GTPase cycles that might be under the control of receptor phosphorylation [9]. Toc159 assumes both soluble and membrane-bound forms and targets to Toc34 by GTP-dependent interaction through the cognate G-domains [47]. Therefore, it is possible that Toc159 acts as a mobile substrate receptor, and that the Toc159–Toc34 GTPase targeting cycle is functionally analogous to the SRP system. A recent report suggests that a pushing force is provided by the Toc159 and Toc34 GTPases via a membrane insertion or de-insertion step analogous to SecA activity [48].

The role of the protonmotive force in protein transport

Most protein transport systems that use the energy contained in transmembrane gradients (Box 1) do so to enhance ATPase- or GTPase-driven cycles, or to act sequentially with them. How transport systems transduce these gradients into a mechanical driving force remains unknown, and must depend on the exact nature of the energetic input. In mitochondria, energy storage across the mitochondrial inner membrane (Fig. 2a) is required for matrix-targeted protein transport (via the TIM23 complex) and membrane-protein integration (via the TIM23 and TIM22 complexes) [7,8]. For the former reaction it has been shown that only the inner membrane potential $\Delta\psi$, not the total PMF, contributes to the energetics of protein import [49]. Thus, it can be postulated that the $\Delta\psi$ electrophoretically pulls the positively charged presequence of matrix-targeted proteins through the TIM23 machinery before mtHsp70 interaction. Indeed, the $\Delta\psi$ generates a force sufficient to catalyze substrate unfolding [50]. TIM22-mediated insertion involves two voltage-dependent steps: $\Delta\psi$-dependent docking of precursor to the translocase and $\Delta\psi$-dependent gating transitions of a double-pore complex followed by membrane insertion [51].

By contrast, the Sec translocon in bacteria (Fig. 1a) is a true PMF-using apparatus, wherein the ΔpH and $\Delta\psi$ can contribute equivalently to the driving force [52]. As first pointed out by Mitchell [53], although the ΔpH and $\Delta\psi$ are thermodynamically equivalent, there is neither mechanistic reason to expect that reactions depending on both electric potential and acid–base chemistry should do so in such a way that the two should be additive thermodynamically, nor should they be expected to produce the same reaction kinetics. To explain this functional interchangeability, Mitchell proposed that chemiosmotic systems operate through a PROTON WELL (or conversely, a PROTON TRAP), wherein the membrane electrical (or pH) gradient is converted to a pH (or electrical) gradient within the membrane, usually at a point corresponding to the active site of the proton-coupled enzyme. Evidence has accumulated over the years to support the operation of a proton well in chemiosmotic ATP synthesis (cf. [54]). The observation that the rate of protein transport through the bacterial Sec pathway depends on the total PMF suggests that this translocation apparatus operates through a proton well or trap in a similar way. This point of view places certain restrictions on the possible mechanisms of energy coupling to protein translocation, and indicates that we should not, for instance, be looking for steps in which an electric potential operates in one energy-dependent step while acid–base chemistry operates on another.

Although we do not know the mechanism by which the PMF drives protein transport on the Sec pathway, we do know several points at which it enters the reaction scheme (Fig. 3b) to impart unidirectionality and enhanced transport rates. For instance, it can be used to complete transport once a sufficient length of substrate has been inserted through ATP-dependent SecA cycles [11]. The PMF also optimizes the SecA ATPase reaction cycle by stimulating SecA de-insertion from SecYEG and promoting substrate release [11,25] (Fig. 3biv), enhancing the rate-limiting exchange of ADP for ATP following SecA de-insertion [55] (Fig. 3bii) and facilitating the initiation step of transport [56]. Large KINETIC SOLVENT ISOTOPE EFFECTS [57] and substrate-induced ΔpH consumption in a reconstituted system [58] suggest that vectorial H^+ flux is involved in Sec-mediated transport. The PMF might also act directly on SecY because transport with PROTEOLIPOSOMES containing SecYEG only is PMF-enhanced, and because SecY mutants have been identified in which transport is independent of PMF [59]. Interestingly, the homologous Sec61 translocase of the eukaryotic ER, which does not maintain a PMF, does not have a subunit homologous to SecG.

The Tat pathway (Fig. 1c) is unusual in that it uses membrane energization as the sole energy source to transport folded proteins across membranes (but see [60]), with no requirement for NTP hydrolysis or soluble *cis*-acting factors [5]. It is not known whether this pathway, which is powered by the pH gradient in thylakoids, can use an electric field. This question has not been addressed in bacteria and is difficult to answer in

thylakoids owing to the almost complete parsing of the steady-state PMF in this membrane into the ΔpH term [61] (although this latter point has recently been questioned). Thus, it remains to be determined whether the Tat translocon is a PMF- or ΔpH-using device. Whatever the mode of energy coupling, the thylakoid Tat machinery uses the membrane energization only after an initial binding step of the substrate to specific Tat proteins is completed [62,63]. And, as observed for the Sec pathway, a significant solvent isotope effect on the kinetics of Tat transport points to the involvement of a proton-transfer step [64], and a transport-coupled ΔpH consumption [65] indicates that a vectorial counter-movement of H^+ drives translocation.

The PMF, or a component thereof, is required for the assembly of translocon subunits in diverse transport systems. The Δψ of the mitochondrial inner membrane promotes the formation of Tim23 [66] and Tim22 [67] voltage-gated and substrate-sensitive channels. Similarly, the thylakoid ΔpH is required for the assembly of the chloroplast Tat machinery [68]. The apparent PMF-dependent modulation of the SecYEG pore size [69] suggests a role of the PMF in component oligomerization of the Sec machinery.

Finally, the influence of transmembrane gradients on the topology of integrated membrane proteins is demonstrated by the 'POSITIVE INSIDE RULE' [70] in which charged residues flanking a hydrophobic region determine orientation. Transmembrane gradients are important over a range of membrane insertion pathways (e.g. TIM23-, TIM22- and YidC/Oxa1/Alb3-mediated insertion).

Force generation and the cost of protein transport

For efficient protein transport to occur, the substrate polypeptide must overcome opposing forces such as viscous drag and interactions between the substrate and channel. To date, there are no direct measurements on the magnitude of the forces generated by any protein transport system. However, it is possible to estimate the force generated during a catalytic turnover of an ATPase motor protein given the GIBBS FREE ENERGY of ATP hydrolysis (ΔG_{ATP}) and the distance of a putative power stroke (Box 2). A force similar in magnitude to that produced by myosin (5–10 pN) might be sufficient to transport unfolded substrates across the membrane. However, it has been observed that the immunoglobulin-like domains of titin, whose unfolding force measures many times this value, are imported efficiently into mitochondria when fused to a targeting sequence. This was taken as evidence that a single mtHsp70 power stroke would be insufficient to catalyze unfolding, indicating spontaneous local unfolding of the substrate during transport [39]. From another perspective, a comparison of the free energy available to the TIM23 complex to drive Δψ-catalyzed protein unfolding [50] with the activation energy required to unfold substrates (Box 2) indicates that the unfolding reaction is thermodynamically feasible if multiple charges exist on the substrate and/or if the transport machinery promotes an alternative unfolding pathway with a lower energetic barrier, as has been observed for the TIM23 complex [71].

A substrate-specific threshold ΔpH, ranging from one to two pH units, is required for cpTat-mediated transport [65]. Interestingly, a thermodynamic barrier of similar magnitude ($\Delta\psi \approx 100$ mV) is required for substrate-activated channel gating of the TIM23 [66] and TIM22 [67] complexes. The energetic cost of protein transport has been evaluated for the bacterial Sec and cpTat pathways. An unfolded polypeptide of 70-aminoacyl residues required an indeterminate, but small, energy expenditure for transport on the Sec pathway [72]. By contrast, proOmpA, a longer model substrate that can fold before translocation, requires the expenditure of between 1000 ATP per substrate in the presence of the synergistic PMF and 5000 ATP per substrate when the PMF has been dissipated [21,73]. Transport along the cpTat pathway also has a high energy cost, the consumption of 8×10^4 H^+ per substrate (the thermodynamic equivalent of 10^4 ATP) [65]. Such high energy costs in protein transport might be caused by significant slipping in the translocation machinery (compare the seven ATP per proOmpA calculated from the SecA translocation cycle step size [12] with the observed 1000–5000 ATP per proOmpA [21,73]), by processes such as substrate unfolding or, in the case of the Tat pathway, by the requirement to avoid significant ion leakage during the transport of a folded polypeptide. Determination of whether other protein transporters have similar high energetic demands awaits further experimentation.

Box 2. Force generation by protein translocases

Force generation by protein translocation ATPases

From the known free energy of ATP hydrolysis ($\Delta G_{ATP} \cong -60$ kJ mol^{-1} under cellular conditions), one can estimate the force (energy per molecule/distance) generated by a SecA or Hsp70 power stroke acting on an unfolded segment of substrate during a catalytic turnover. Due to the small conformational energy difference between the nucleotide-bound states of Hsp70 homologs [75], most of the energy from ATP hydrolysis can be used to drive transport. With power-stroke distances of 88 Å for SecA (assuming translocation of 25 unfolded residues per cycle) and 35 Å for Hsp70 (assuming a binding site every ten residues), these proteins could generate forces of 11 pN and 28 pN, respectively, which are forces comparable with the 5–10 pN produced by myosin [74].

The energetic requirement of substrate unfolding

The mitochondrial import machinery catalyzes the unfolding of the model substrate barnase by the Δψ (inner membrane potential) alone [50]. Here, the energetic input (assuming a Δψ of 150 mV, which translates into 14.4 kJ mol^{-1}, the maximum energy that can be gained moving a point charge along the gradient) is considerably lower than the activation energy of global unfolding for the model substrate barnase ($\Delta G^{\ddagger} \cong 80$ kJ mol^{-1}). This reaction might be thermodynamically feasible if multiple charges exist on the transported substrate or if the translocase lowers the energetic barrier of unfolding by promoting an alternate unfolding pathway, as has been demonstrated [71].

Concluding remarks

Several recent advances in our understanding of cellular protein transport systems have provided insights into the type of energetic input used by various translocases, as well as the steps in transport reactions that are energy coupled. However, the molecular mechanisms by which free energy is transduced into protein translocation and integration by these systems remain largely unknown,

presenting a challenge for future research. Progress towards this end will come on several fronts. *In vitro* assays using reconstituted transport systems comprising individual translocase components and mutants thereof will elucidate the mechanisms underlying the energy converting steps of individual translocon subunits. Further high-resolution structural information (e.g. motor proteins in different nucleotide-bound states) will help identify key conformational changes in the energy-transducing subreactions of protein transport. Finally, the application of single-molecule research to the study of protein transport might enable the direct measure of force generation, intermediate steps, kinetics and energy-coupling efficiency of various transport motor proteins interacting with polypeptide substrate. The combined techniques of optical trap technology and single fluorophore measurements have been applied to other motor protein systems (cf. [74]), and offer exciting possibilities for future work with protein transport machineries. By using a reductionist approach and novel experimental tools to understand the discrete energy transduction steps of protein transport, future research in this field will advance our knowledge of energy use by protein translocases and, from a broader perspective, provide insights into how protein transport is integrated with other endergonic cellular processes such as protein translation, folding and degradation.

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