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ER import of small human presecretory proteins: components and mechanisms

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Protein transport into the mammalian endoplasmic reticulum (ER) used to be seen as strictly cotranslational, that is temporarily and mechanistically coupled to protein synthesis. In the course of the last decades, however, several classes of precursors of soluble and membrane proteins were found to be post-translationally imported into the ER, without any involvement of the ribosome. The first such class to be identified were the small presecretory proteins; tail-anchored membrane proteins followed next. In both classes, the inherent address tag is released from the translating ribosome before the initiation of ER import, as part of the fully synthesized precursor. In small presecretory proteins, the information for ER targeting and -translocation *via* the polypeptide-conducting Sec61-channel is encoded by a classical N-terminal signal peptide, which is released from the ribsosome before targeting due to the small size of the full-length precursor. Here, we discuss the current state of research on targeting and translocation of small presecretory proteins into the mammalian ER. In closing, we present a unifying hypothesis for ER protein translocation in terms of an energy diagram for Sec61-channel gating.

Keywords: endoplasmic reticulum; post-translational protein import; Sec61 complex; signal recognition particle; small presecretory proteins

Transport into the endoplasmic reticulum (ER) is the first step in the biogenesis of roughly 10 000 different proteins which makes about one-third of the proteome in mammals [1–3]. These are soluble and membrane-embedded proteins which mainly reside in the organelles of the endo- and exocytic pathways as well as the plasma membrane and the extracellular space. Thus, transport into the ER either implies the complex insertion of membrane proteins into the ER membrane or the import of soluble proteins into the ER lumen. Information for both, ER transport and ER targeting is encoded by specific signals or zip codes in the respective precursor polypeptides and, subsequently, decoded by various transport components present in the cytosol, the ER membrane, and the ER lumen (in more detail reviewed in [4,5]). In the case of various types of membrane proteins – except for tail-anchored (TA) ones – the initial insertion into the polypeptide-conducting channel is followed by integration into the ER membrane. In the case of soluble proteins, it is followed by completion of translocation into the ER lumen. The information for ER targeting and initial membrane insertion is encoded within the precursor either by an N-terminal signal peptide, which is typically cleaved-off from the precursor upon ER entry, or by a more or less N-terminally located transmembrane

Abbreviations
ER, endoplasmic reticulum; GET, guided entry of tail-anchored proteins; SND, SRP-independent; SR, SRP-receptor; SRP, signal recognition particle; TA, tail-anchored; TMD, transmembrane domain; TRAP, translocon-associated protein; TRC, transmembrane recognition complex.

helix, which serves as a signal peptide but remains part of the mature protein. N-terminal signal peptides have a tripartite structure with a positively charged N-region, a central H-region, and a slightly polar C-region [6–9]. With the exception of ER-resident proteins, for all proteins passing quality control, their transport into the ER is followed by vesicular transport to the functional intra- or extracellular location [4,5].

The first transport components, which were identified and characterized at the molecular level, were the cytosolic signal recognition particle (SRP) and its ER-membrane resident receptor, the heterodimeric SRP-receptor (SR) (Fig. 1, Table 1) [10–20]. Today these components represent one of several ER-targeting pathways for precursor polypeptides, which in this case operates cotranslationally [4,5]. Notably, however,

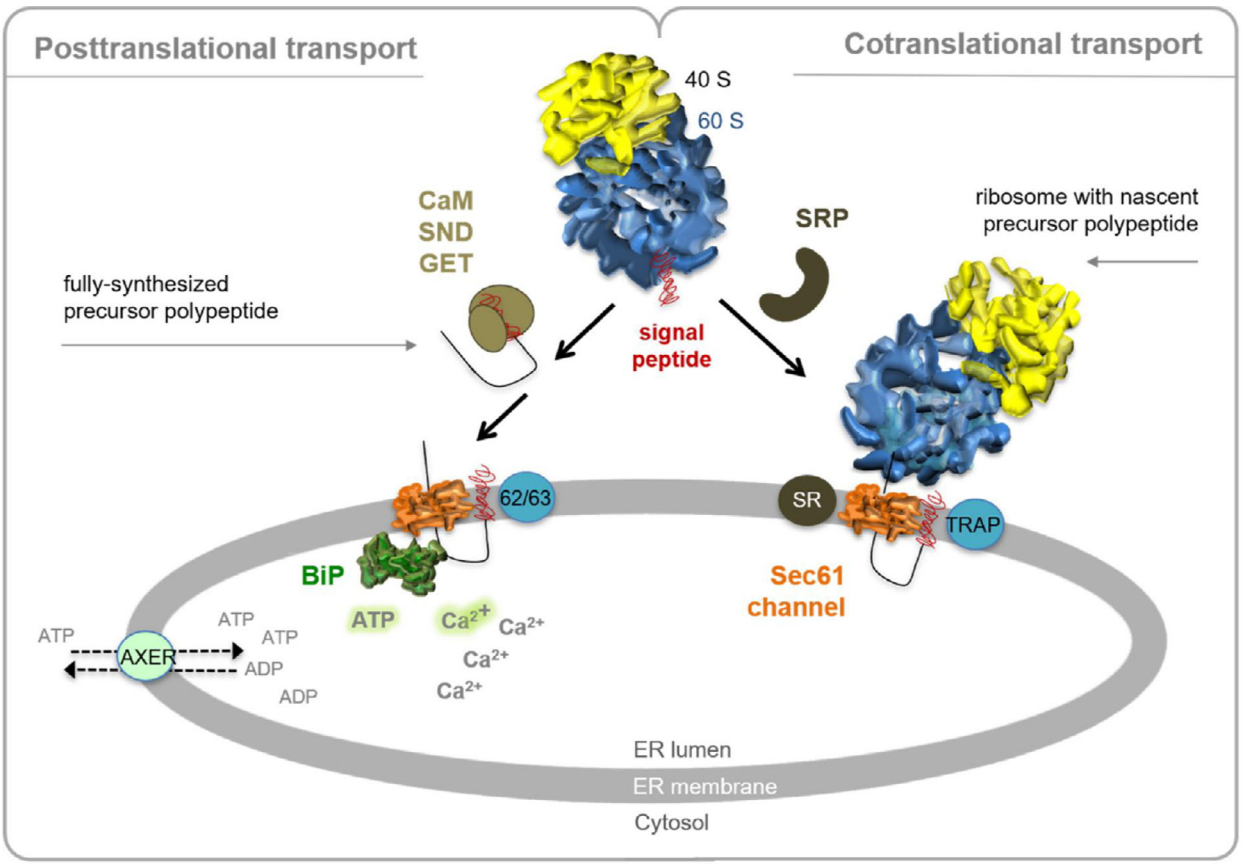

Fig. 1. Schematic view of co- and post-translational protein transport into the mammalian ER. Transport of newly synthesized proteins into the ER can be envisaged like going to a new exclusive club in a big city. First, you have to find the right place (the targeting reaction). Then, you have to actually get through the entrance door (the translocation reaction). See text for details. In protein transport, ER targeting and either translocation or membrane integration involve signals within the precursor polypeptide, such as the N-terminal signal peptide of small presecretory and other soluble proteins or the first transmembrane helix of membrane proteins. Depending on whether the two stages are coupled to protein synthesis at the ribosome or not, two modes of transport are distinguished. In cotranslational transport, SRP together with the membrane-embedded SR target nascent precursor polypeptides to the polypeptide-conducting channel in the ER membrane, the Sec61 complex. In post-translational transport, three pathways can target fully synthesized precursor polypeptides to the Sec61 channel, the SND- or the GET-pathway, or CaM (the respective receptors are not shown, see text and Table 1 for details). Opening of the Sec61-channel is facilitated by the signal peptide plus one of several allosteric effectors of the Sec61 complex, the ribosome plus the ER-membrane resident TRAP-complex in cotranslational transport and the ER-membrane resident Sec62/Sec63-complex plus the ER-lumenal BiP in co- and post-translational transport. BiP is a Hsp70-type molecular chaperone [99,100], which is recruited to the Sec61-channel by the Hsp40-type co-chaperone Sec63 [89,90]. BiP is also involved in protein folding and assembly in the ER and depends on calcium ions (Ca^{2+}) and the hydrolysis of ATP for its activity. AXER, systematically termed SLC35B1, is an ATP/ADP exchanger in the ER membrane and, therefore, indirectly involved in BiP-functionality, including BiP-dependent protein transport into the ER [76]. For the sake of simplicity, we omitted cytosolic Hsc70 and its co-chaperones and nucleotide exchange factors since they do not directly contribute to ER targeting and we omitted translocating chain-associated membrane protein 1 (TRAM1) and its paralogs TRAM1L1 and TRAM2 because there is no indication that they play a role in ER import of small precursor polypeptides so far.

SRP and SR can also act post-translationally, as has been observed for TA membrane proteins [19]. In cotranslational transport, SRP binds to transmembrane helices or to equivalently hydrophobic N-terminal signal peptides as they emerge from the ribosomal tunnel exit, that is during translation. As a result, translation is slowed down until the ribosome-SRP-nascent chain complex interacts with SR at the ER surface in a GTP-regulated process. Next, both the translating ribosome and the nascent precuror polypeptide chain are handed-over to the so-called protein-translocon in the ER membrane and translation is allowed to pick up speed. In the course of this hand-over, the signal peptide – with the help of the ribosome – initiates insertion of the nascent precursor polypeptide into the ER membrane, or – more precisely – into the ER-membrane resident and heterotrimeric Sec61 complex, which forms an aqueous polypeptide-conducting channel in its fully open state (Table 1) [21–32]. Ideally, signal peptides thus facilitate not only ER targeting but also full Sec61-channel opening. *A priori*, signal peptides or their equivalents can insert into the Sec61-channel in a head-on ($N_{ER\text{-}lumen}$-$C_{cytosol}$) or in a loop ($N_{cytosol}$-$C_{ER\text{-}lumen}$) configuration. In any case, signal peptides start sampling the cytosolic funnel of the Sec61-channel pore, that is start their dwell time in the Sec61-channel pore as brilliantly visualized for cotranslational transport by Zhang and Miller [30] (https://www.cell.com/cms/10.1016/j.celrep.2012.08.039/attachment/cd0b8007-ca63-44e5-afa1-454133428f79/mmc2.mp4). According to these simulations, sampling or dwell time is influenced by deleterious charges, hydrophobicity, mature protein domain length, and translation speed, which is dependent on pause sites, rare codons or hairpins in the mRNA and arrest peptides or polyproline motifs in the polypeptide [33–37]. Nascent membrane proteins leave the Sec61-channel with their transmembrane domain(s) (TMD) moving laterally into the phospholipid bilayer *via* the so-called lateral gate, while the nascent precursors of soluble proteins leave it axially into the ER lumen. In either case, N-terminal signal peptides – if present – are cleaved-off from the nascent polypeptide chain by the ER-membrane resident signal peptidase, which has its active site(s) in the ER lumen (Table 1) [38,39].

Since N-terminal signal peptides for transport into the ER typically comprise 25–30 amino acid residues and further 40–45 amino acid residues of a nascent polypeptide chain are burried in the ribosomal tunnel at any given time of elongation, a nascent precursor polypeptide with a minimum of 65–75 amino acid residues is required for ER transport to operate cotranslationally. This is the reason why precursors with less than these roughly 70 amino acid residues in overall length were expected to behave differently [40–49].

Small presecretory proteins

Since the 1970s, small secretory proteins and even peptide hormones are known [50,51]. Typically, however, they are synthesized as large precursor proteins with more than 70 amino acid residues in overall length, often with additional pro-peptides, which are cleaved from the pro-protein by converting enzymes late in the secretory pathway (Table S1). Classical examples are preproinsulin or prepro-opiomelanocortin, which give rise to insulin and the bio-active peptides corticotropin and melanotropin gamma, respectively. These precursors of more than 100 amino acid residues involve typical cotranslational ER targeting (*via* SRP and SR) and ER import. However, preproinsulin appears to also have the capacity for post-translational ER import, despite its 110 amino acid residues (see below).

With the beginning of DNA cloning and sequencing in the 1980s, the first small presecretory proteins with less than 70 amino acid residues in overall length were identified in insects and amphibia [52]. As expected, they were found to have the ability for post-translational transport into mammalian rough microsomes (i.e., vesicles derived from the rough ER, here, of canine pancreas), using rabbit reticulocyte lysate as cell-free system for *in vitro* translation and transport [40–49]. Examples are honeybee prepromelittin, *Xenopus laevis* prepropeptide GLa (originally termed PYLa), and *Hyalophora cecropia* preprocecropin A and have sizes between 60 and 70 amino acid residues (Table S2). These studies established that small presecretory protein import can indeed occur in a both ribosome- and SRP/SR-independent fashion (termed ribonucleoparticle-independent transport) and that these characteristics are related to the small size plus intrinsic features of the precursors, which allow them to stay transport competent in the cytosol at least with help from molecular chaperones (Fig. 1). Consistently, the import reaction was shown to involve the hydrolysis of ATP. This ATP-requirement was originally attributed to the cytosolic Hsp70-type chaperone Hsc70, which together with an Hsp40-type co-chaperone helps the precursors to stay transport competent in the cytosol. Later, the ER-lumenal Hsp70-type chaperone BiP plus its nucleotide exchange factor Grp170 were found to additionally facilitate translocation at two different stages. In initial Sec61-channel insertion, BiP binds to the channel and mediates its opening, while in subsequent completion of translocation, BiP binds to the incoming polypeptide and

Table 1. Protein transport components/*complexes* in HeLa cells. Alternative names of components/subunits are given in parentheses. We note that a more comprehensive list of transport components is given in [5]. For clarity, we omitted cytosolic Hsc70 and its co-chaperones and nucleotide exchange factors since they do not directly contribute to ER targeting and we omitted TRAM1 and its paralogs TRAM1L1 and TRAM2 because there is no indication that they play a role in ER import of small precursor polypeptides so far. Furthermore, we note that oligosaccharyltransferase exists as two paralogs, comprising Stt3a or Stt3b. Abundance is given in nM [109]; 1 nM corresponds to roughly 1000 molecules per cell. C, cytosol; CVID, Common Variable Immune Deficiency; ERL, ER lumen; ERM, ER membrane; TKD, Tubulo-Interstitial Kidney Disease; ?, Uncharacterized.

Component/subunit	Abundance	Location	Linked diseases
SRP[c]		C	
SRP72	355		Aplasia, Myelodysplasia
SRP68	197		
SRP54	228		
SRP19	33		
SRP14	4295		
SRP9	3436		
7SL RNA			
SRP receptor		ERM	
SRα (docking protein)	249		
SRβ	173		
hSnd1	?		
Snd receptor			
hSnd2 (TMEM208)	81	ERM	
hSnd3	?		
Bag6 complex[c]		C	
Get4 (TRC35)	171		
Ubl4A (Get5)	177		
Bag6 (Bat3)	133		
SGTA	549	C	
Get3 (TRC40, Asna1)	381	C	
TA receptor		ERM	
CAMLG (CAML, Get2)	5		
Get1 (WRB,CHD5)	4		Congenital Heart Disease
Calmodulin	9428	C	
Sec61 complex[c]		ERM	
Sec61α1	139		Diabetes[b], CVID, TKD
Sec61β	456		Polycystic Liver Disease (PLD)
Sec61γ	400		Glioblastoma
Sec62[c]	26	ERM	Prostate Cancer, Lung Cancer
Sec63	168	ERM	Polycystic Liver Disease (PLD)
BiP (Grp78, HSPA5)	8253	ERL	Hemolytic Uremic Syndrome (HUS)
Grp170 (HYOU1)	923	ERL	
Sil1 (BAP)	149	ERL	Marinesco-Sjögren-Syndrome (MSS)
TRAP complex[c]		ERM	
TRAPα (SSR1)	568		
TRAPβ (SSR2)			
TRAPγ (SSR3)	1701		Congenital Disorder of Glycosylation (CDG)
TRAPδ (SSR4)	3212		Congenital Disorder of Glycosylation (CDG)
Oligosaccharyltransferase[c]		ERM	
RibophorinI (Rpn1)	1956		
RibophorinII (Rpn2)	527		
OST48	273		Congenital Disorder of Glycosylation (CDG)
OST4			
TMEM258			
DAD1	464		
Stt3A[a]	430		Congenital Disorder of Glycosylation (CDG)
Stt3B[a]	150		Congenital Disorder of Glycosylation (CDG)
Kcp2			

Table 1. (Continued).

Component/subunit	Abundance	Location	Linked diseases
DC2			
TUSC3			Congenital Disorder of Glycosylation (CDG)
MagT1	33		
Signal peptidase		ERM	
SPC12	2733		
SPC18[a]			
SPC21[a]			
SPC22/23	334		
SPC25	94		

[a]Catalytically active subunit. [b]In mice. [c]Ribosome associated.

mediates unidirectional translocation by acting as a molecular ratchet. The latter was also observed to improve efficiency of the classical SRP/SR-dependent transport, which was demonstrated by the use of proteoliposomes, comprising the full complement of microsomal membrane proteins plus either BiP or avidin in combination with biotinylated nascent bovine preprolactin chains [49]. Notably, avidin did not work as a ratchet for nonbiotinylated precursors, while BiP did.

However, the general feeling in the field remained that post-translational transport of signal peptide bearing soluble precursors proteins is a rare if not artificial mechanism, which can be used by only a couple of small exotic precursor polypeptides. Recently, this view has started to change because of the simultaneous discovery – in four laboratories – of small and some not so small human precursor polypeptides (Tables S1 and S2), which can be post-translationally and ribosome-independently transported into the mammalian ER (β-defensin 133, C-C motif chemokine 2, preresistin, preproinsulin, preproapelin, prestatherin) [53–58]. Moreover, post-translational transport into the ER of intact human cells was demonstrated for one of the exotic precursor polypeptides, preprocecropin A [57]. Subsequently, the combination of siRNA-mediated gene silencing and protein transport into the ER of semi-intact human cells in rabbit reticulocyte lysate showed that transport of preprocecropin A occurs independently of the SRP/SR-targeting system and involves the ER membrane proteins Sec62 and Sec63 (a Hsp40-type co-chaperone of BiP), as well as the ER-lumenal Hsp70-type chaperone BiP [56].

Small presecretory proteins in mammals

With the advancement of sequencing projects and bioinformatic tools in the early years of the 21st century, the first systematic compilations of small proteins and small presecretory proteins in mice and humans were made based on collections of cDNAs (FANTOM consortium and Swiss-Prot), as they show an advantage over the search for proteins in the complete genome [59]. Using CRITICA as novel identification tool for coding regions, it turned out that 3701 mouse proteins are shorter than 100 amino acids, which traditionally limited the size of small proteins. In addition, only 232 of these proteins matched database entries at the time and 495 of them lacked similarity to any known proteins in UniRef90. Furthermore, 91 of 1240 newly annotated small ORFs were predicted to code for signal peptides, 117 of the corresponding proteins were grouped into 38 families with two or more members, and 844 transcripts of the small ORFs were found to mainly code for hormones or antimicrobial peptides, the latter being reminiscent of the originally described amphibian and insect small presecretory proteins. Furthermore, most of the small ORFs were observed to be expressed in a highly tissue-specific fashion, that is in neuronal tissue, haemopoietic cells and tissues, and embryonic cells and tissues. Later, ribosome profiling demonstrated active translation of hundreds of regions coding for proteins with less than 70 amino acids including the signal peptide [60–63] However, the early compilations were the starting point for several labs to seriously look into the biogenesis of small human presecretory proteins (Table S2) [53–58,64] and raise the question why it may have made sense in the course of evolution to allow development of both, small and large presecretory proteins (see below).

Targeting of small human presecretory proteins to the human ER

Originally, several small human precursor polypeptides of varying sizes between 60 and 110 amino acids were

observed to translocate post-translationally and ribosome-independently into the human ER (see above; Tables S1 and S2). For a subset of them, i.a. prestatherin and preproapelin with 62 and 77 amino acid residues, ER targeting was subsequently reported to occur independently of SRP and SR, and to involve cytosolic-guided entry of TA proteins (GET)3 (originally termed transmembrane recognition complex (TRC)40 or Asna1), calmodulin (CaM) or Sec62 in the ER membrane (Figs 1 and 2, Table 1) [53,55,57,64]. From these studies, the concept emerged that Sec62 and the GET-system, comprising GET3 in the cytosol in cooperation with its heterodimeric receptor in the ER membrane (GET1/GET2), may act as alternative signal peptide recognition proteins in post-translational ER targeting. In addition, it also became clear from these studies that the presumed 100 amino acid residues content of small presecretory proteins is not that strict [53] and that even the current small model presecretory proteins are quite different with respect to which targeting pathway they can use most efficiently [64]. Apparently, even the small precursors preproapelin and prestatherin can use the SRP/SR-system in both its co- and post-translational mode of action. Although smaller in size, prestatherin actually prefers SRα over Sec62-mediated targeting, which may be due to a C-terminally located peptide motif in the mature region of prestatherin, which is reminiscent of the translation-arrest peptide of XBP1. In contrast, preproapelin does the opposite, which may be related to the comparatively low hydrophobicity of its signal peptide (free energy of membrane insertion, delta G^{pred}: -0.19 kcal·mol^{-1}, versus -0.91 kcal·mol^{-1} of prestatherin) (Fig. 2). Taken together with the observation that C-terminal extension of preproapelin or prestatherin by the cytosolic protein dihydrofolate reductase (187 amino acid residues) leads to Sec62-independence, the data reiterated the notion that small precursor polypeptides use the SRP/SR-system for ER targeting in mammalian cells less effectively, simply because they are more likely to be released from ribosomes before SRP can efficiently interact [44,53,64]. Mammalian Sec62, however, does not only act as a signal peptide receptor, but also plays a role in Sec61-channel gating (priming and/or full opening of the channel, see below).

Furthermore, Ca^{2+}-CaM, which is known to have an affinity for TMDs, was described as an additional cytosolic signal peptide binding protein in post-translational ER targeting. Notably, it was proposed to productively cooperate with an IQ-motif in the cytosolic N terminus of Sec61α, for example in the case of targeting of β-defensin 133 and β-defensin 2 [57]. Interestingly, Ca^{2+}-CaM can also bind to tail-anchors but by doing so inhibits their membrane insertion [65]. In addition, yet another SRP-independent (SND) ER-targeting pathway was discovered in yeast, the SND-pathway, which was shown to involve an ER-membrane protein with a human ortholog, hSnd2 [66–68]. In yeast, Snd2 together with Snd3 forms another heterodimeric receptor in the ER membrane, in this case for the cytosolic precursor- and ribosome-binding protein Snd1 [66]. Notably, however, mammalian orthologs of the yeast Snd1 and Snd3 components have not been identified. So far, only ER targeting of one of the small presecretory proteins (prestatherin) showed an hSnd2 involvement, which was detected only in the simultaneous absence of the GET-system [64].

Precursor variant	Length (aa)	Sec62	Sec63	BiP	CAM741	Scheme
Preproapelin	77	++	++	++	++	+++ RRK-OPG2
Preproapelin-DHFR	266	–	+	++	++	+++ RRK-GT-DHFR
Preprolactin-proapelin	85	++	–	+++	+++	-OPG2
*Pr*eproapelin-AAA	77	++	++	–	–	AAA-OPG2
Prestatherin	62	++	++	–	–	-MM-OPG2
Prestatherin-DHFR	251	–	+	–	–	-GT-DHFR
Preprolactin	229	–	–	–	–	

Fig. 2. Model proteins for post-translational transport into the mammalian ER. Presecretory proteins, which are discussed in the text in detail, and their characteristics (i.e., features, translocation dependencies, CAM741 sensitivity). OPG2 refers to a C-terminal oligopeptide-tag, which is derived from opsin and contains two sites for N-glycosylation [55].

Translocation of small human presecretory proteins into the human ER

The Sec61 complex

Post-translational import of small human presecretory proteins into the human ER was shown to involve the Sec61 complex (comprising Sec61α1, Sec61β, and Sec61γ), as evaluated by the combination of siRNA-mediated *SEC61A1*-gene silencing and protein transport into the ER of semi-intact HeLa cells. While Sec61 represents the polypeptide-conducting channel of the classical SRP-dependent and cotranslational transport pathway [64], it is not involved in membrane insertion of TA membrane proteins (for a timely review on the biogenesis of TA proteins see Borgese and Schwappach, [69]). The opening of the Sec61-channel during early steps of translocation can be envisaged in analogy to a ligand-gated ion channel. Here, the ligand is represented by the nascent or fully synthesized presecretory protein with its signal peptide. However, gating by the ligand alone is not sufficient, allosteric channel effectors have to support it. Channel opening occurs in two stages, a priming step, which involves the ribosome as allosteric channel-effector in cotranslational transport [32], and possibly, Sec62 with or without Sec63 in post-translational transport (Figs 2 and 3). In yeast, however, there is the so-called SEC-complex, a permanent assembly of a heterotrimeric Sec61 complex plus the heterotetrameric Sec62/Sec63/Sec71/Sec72-complex that is dedicated to post-translational protein import [70,71]. In contrast, the mammalian complex of Sec61, Sec62, and Sec63 appears to be assembled on demand rather than permanently, which was observed for cotranslational ER import of the precursors of the prion protein and the ER-lumenal protein ERj3 [72]. This may be related to the fact that the mammalian ER, in contrast to the yeast ER, also serves as the main intracellular calcium (Ca^{2+}) reservoir and thus, would not tolerate an even partially open Sec61-channel [58,73]. We further note, that the human genome also codes for Sec61α2, which does not seem to be present in HeLa cells to any significant extent, nor was it over-produced under conditions of *SEC61A1*-gene silencing by means of a compensatory mechanism. Databases actually indicate that expression of *SEC61A2* is limited to the tissues of

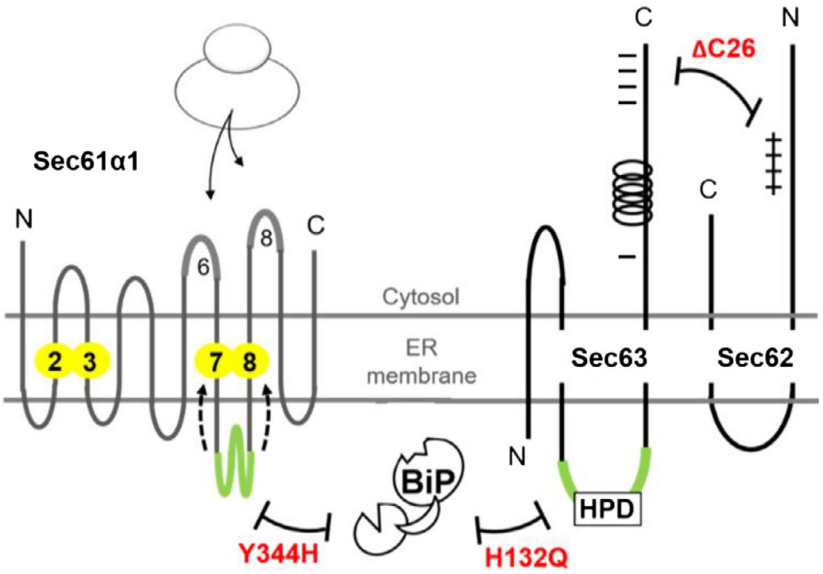

Fig. 3. Components of Sec61-channel gating. The scheme depicts the α-subunit of the heterotrimeric Sec61-channel with its three allosteric channel effectors (Sec62, Sec63, and BiP), which are involved in initial insertion of preproapelin into the Sec61-channel. In the Sec61 α-subunit transmembrane helices 2, 3, 7, and 8 forming the lateral gate (in yellow) and cytosolic loops 6 and 8 involved in ribosome binding are highlighted, as is ER-lumenal loop 7 (in green) containing the interaction site for BiP's substrate-binding domain; inactivation of the interaction by the Sec61α Y344H mutation inhibits initial insertion of preproapelin into the Sec61-channel [58,64,79]. In addition, the membrane topologies of Sec62 and Sec63 are depicted together with the interaction sites between Sec63's J-domain with BiP's nucleotide-binding domain and between a positively charged cluster within the N-terminal domain of Sec62 and a negatively charged cluster at the C-terminal end of Sec63 [64,89,90]. Notably, deletion of the negatively charged cluster at the C-terminal end of Sec63, which prevents the Sec62/Sec63-interaction, and the H132Q mutation in the crucial HPD-motif in Sec63's J-domain both inhibit initial insertion of preproapelin into the Sec61-channel.

brain and testis, though ranging at low estimated levels. Aside from that, the precursors of the prion protein and the ER-lumenal protein ERj3 involve Sec62, Sec63, and BiP in cotranslational import [54,58,74,75] (Schorr, S., personal communication). For productive precursor insertion into the Sec61-channel (priming) and subsequent opening of its aqueous pore, a high hydrophobicity/low delta G^{pred} value for the H-region of the signal peptide is conducive [8]. Apparently, H-region hydrophobicity is decoded by the so-called hydrophobic patch in the Sec61α transmembrane helices 2 and 7, which line the lateral gate

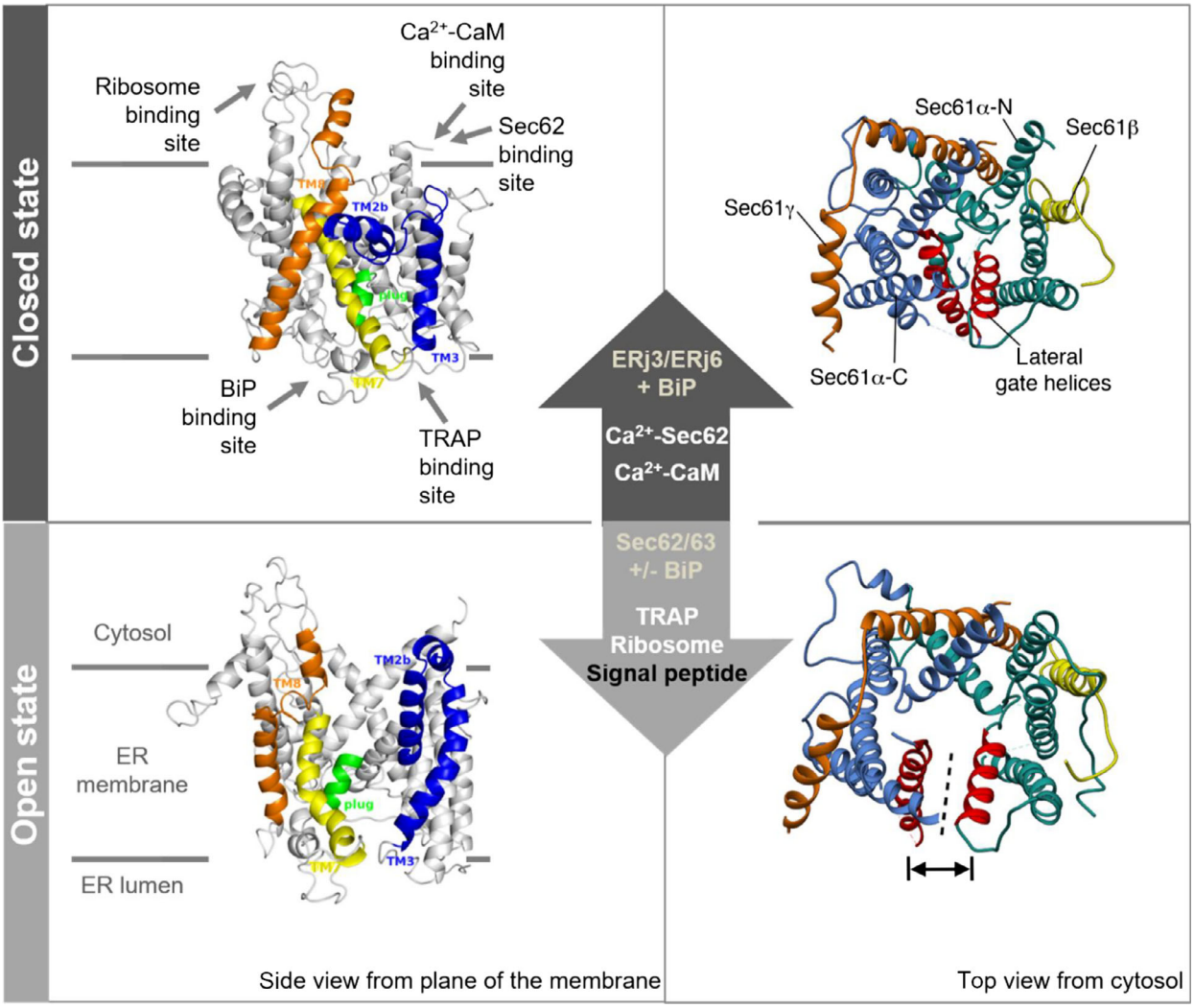

Fig. 4. Model for the dynamic equilibrium and gating of the human Sec61 complex. Structural model for the closed human Sec61 complex based on the cryo-EM structure of Sec61α from dog (*Canis familiaris*) (PDB code: 4cg7), which used information from the X-ray structure of archaean SecY (PDB code: 1rhz.) Transmembrane helices forming the front of the lateral gate and the plug are indicated in color. The binding sites of the allosteric factors ribosome, TRAP, Sec62, BiP, and Ca^{2+} -CaM are shown. Homology model for the open human Sec61 complex based of the cryo-EM structure of SecY from *Escherichia coli* (PDB code: 3j46). On the left, views from the plane of the membrane (lateral gate front) are shown. Atomic model for the laterally closed Sec61 complex (PDB code: 3j7q). Atomic model for the laterally opened Sec61-channel (PDB code: 3jc2). On the right, views from the cytosol are shown. N- and C-terminal halves of the Sec61 α-subunit are shown in green and blue, respectively, lateral gate helices 2 and 7 are shown in red, and cytosolic loops are not shown for clarity. The open state is induced by interaction with the ribosome plus a strong signal peptide or N-terminal transmembrane helix of a precursor polypeptide or a weak signal peptide or N-terminal transmembrane helix plus allosteric effectors, such as TRAP or Sec62/Sec63(+/−BiP). During protein translocation, the lateral gate is typically occupied by a signal peptide and the central aqueous pore by the polypeptide chain in transit. Notably, efficient closing of the Sec61-channel can also involve allosteric effectors, such as BiP with its ER-lumenal Hsp40-type co-chaperones ERj3 plus ERj6 or Ca^{2+}-bound Sec62 plus Ca^{2+}-CaM [58,95,101,102].

of the channel [32] (Fig. 4). Furthermore, high hydrophobicity of the signal peptide favors its partitioning *via* the lateral gate into the phospholipid bilayer. This can be expected to contribute to full channel opening by a free energy gain, in analogy to the hydrophobic effect in protein folding. Typically, the signal peptide-orientation in the Sec61-channel follows the 'positive-inside rule'. Thus, positively charged residues in the N-region of the signal peptide – which are absent in preproapelin – support its loop insertion, while positively charged side chains downstream of the signal peptide – which are present in preproapelin – interfere with loop formation and favor head-on insertion [8,9,64].

The Sec62/Sec63 complex

It was observed by the combination of siRNA-mediated gene silencing and protein transport into the ER of semi-intact HeLa cells that import of small precursor polypeptides involves the ER membrane proteins Sec62 and Sec63 (preproapelin, prestatherin) plus the ER-lumenal Hsp70-type chaperone BiP (preproapelin). Specifically, BiP cooperates with the Hsp40-type co-chaperone Sec63 (Figs 3 and 4) [64]. The role of BiP in this early phase of preproapelin import was indirectly confirmed by ATP-depletion from the ER *via* depletion of the ER-membrane resident ATP/ADP exchanger AXER [76]. From these studies the concept emerged that Sec62, Sec63, and BiP are involved in productive initial insertion of some precursor polypeptides into the Sec61-channel (such as preproapelin). The same had originally been proposed for the three yeast orthologs, Sec62p, Sec63p, and Kar2p, but was dismissed later on – in our eyes prematurely [77,78]. According to the current model, small presecretory proteins are not able to trigger full opening of the Sec61-channel, because of an inefficiently gating signal peptide [64]. Channel opening has therefore, at this early stage of protein translocation, to be supported by Sec62 and Sec63 (prestatherin) or even by Sec62/Sec63-mediated binding of BiP to the ER-lumenal loop 7 of Sec61α (preproapelin). This view was supported by the observations that the murine diabetes linked mutation of tyrosine 344 to histidine within loop 7 destroys the BiP binding site and, when introduced into HeLa cells, prevents import of BiP-dependent precursor polypeptides, such as preproapelin [58,64,79] (Fig. 3).

Our observation – by chemical cross-linking – that small precursor polypeptides (such as preproapelin) accumulate within the Sec61-channel upon Sec62-, Sec63-, or BiP depletion suggested a Sec61-gating function for these three proteins [64]. Furthermore, the small presecretory protein prestatherin presented a remarkable phenotype, as it apparently involves Sec63 and Sec62 independently of BiP. Thus, at least in certain cases, Sec63 itself can contribute to Sec61-channel gating, that is without involving BiP, most likely *via* its direct interaction with the Sec61 complex. Therefore, the question arising is which features of preproapelin or prestatherin determine their dependence on Sec63 in Sec61-channel gating. Signal peptide swap variant pre_{ppl}–proapelin (with the bovine preprolactin signal peptide preceding proapelin) suggests that the signal peptide contributes to requiring Sec63, at least for Sec63/Sec62 and most likely intrinsic Sec63 function. Apparently, there are signal peptides efficient enough to trigger full opening of the ribosome-primed Sec61 channel, such as the bovine preprolactin signal peptide (Figs 2 and 3). We attribute this efficiency to the consecutive interactions of the H-region with the hydrophobic patch within the channel and the phospholipid bilayer [32]. In contrast, other signal peptides like the signal peptides of preproapelin and prestatherin require help from the auxiliary transport component Sec63. In addition to its intrinsic activity in protein translocation, Sec63 acts as Hsp40-type co-chaperone for ER-lumenal Hsp70-type chaperone BiP. The collaboration of Sec63 and BiP involves the characteristic HPD-motif within the ER-lumenal J-domain of Sec63 and the interacting surface of the ATPase domain of BiP (Fig. 3). Preproapelin, which depends on Sec63 plus BiP for productive insertion into the Sec61 complex and efficient Sec61-channel gating to the fully open state, was therefore sensitive to the SEC63H132Q and SEC61A1Y344H mutations (Fig. 3). In contrast, prestatherin was not BiP-dependent and not sensitive to the two mutations. Consequently, as has been detected by chemical cross-linking, Sec63 and BiP depletion resulted in an accumulation of preproapelin within the Sec61-channel, due to the lack of Sec62/Sec63, BiP/Sec63, and possibly intrinsic Sec63 action.

BiP

Although the signal peptide of preproapelin was identified as a factor contributing to Sec63 dependence, it appeared not to be associated with requiring BiP [64]. Instead, the mature region contributed to the inefficiency of preproapelin in Sec61-channel gating. The mature region of preproapelin contains a cluster of three positively charged amino acid side chains near the C terminus that weakens its gating property and causes the additional requirement for support by BiP.

We suggest that, overall, the low gating efficiency of preproapelin results from the presence of two individual features, its signal peptide (Sec62/Sec63) and the cluster of positively charged amino acid residues within the mature region (BiP) (Figs 3 and 4). Notably, the clusters of positive amino acid residues within the mature region of preproapelin contain the dibasic cleavage site for furin and play a role in interaction of the mature hormone with its receptor. Thus, BiP compensates the deleterious effect of a cluster of charged residues within proapelin, which is required for the maturation and subsequent biological activity (Fig. 5). Interestingly, cotranslational ER import of the large precursors of the prion protein and the ER-lumenal protein ERj3 also involves Sec62, Sec63, and BiP, because of clusters of positive charges downstream of the signal peptides [54,58,74,75] (Schorr, S., personal communication). Although being deleterious for ER import, presence of those charges relates again to the biological activity of the mature protein. Here, however, their effect on ER import clearly depends on the properties of the preceding signal peptide and its capacity for compensation. Thus, the combination of both, an inefficiently gating signal peptide and downstream clusters of charges lead to a low gating efficiency of the prion protein and ERj3 precursors (Fig. 2). In contrast, low gating efficiency in the case of the small preproapelin is the result of individual features each one separately requiring specific factors for compensation. Therefore, the low gating capacity of the preproapelin signal peptide remains in the absence of charges and so does the requirement for Sec62/Sec63.

Based on these different observations on charged clusters in the mature regions of preproapelin and the precursors of prion protein and ERj3, we differentiate –depending on their distance to the signal peptide – between a 'cis-' and a 'trans-effect' on ER import, though all clusters of charges being part of the translocating polypeptide chain. In the case of preproapelin, the cluster of charges may act 'in trans' and impair precursor insertion into the Sec61-channel just like any charged peptide, endogenous or exogeneous, might do when in close proximity to the sampling signal peptide within the channel. In the case of the precursors of prion protein and ERj3, the cluster of charges has to be part of the mature polypeptide chain at a certain effective distance to the signal peptide to act 'in cis' and to impair insertion of the preceding signal peptide into the Sec61 channel according to the 'positive-inside rule'. We suggest that such a positive cluster may favor 'head-first' rather than 'loop' insertion of the signal peptide into the Sec61-channel, particularly in the case of precursors with a low number of positive charges in the N-region (such as in the case of prion protein and pre-ERj3). In this case, the required flip-turn of the signal peptide may pose a particularly high energetic barrier or activation energy for Sec61-channel opening.

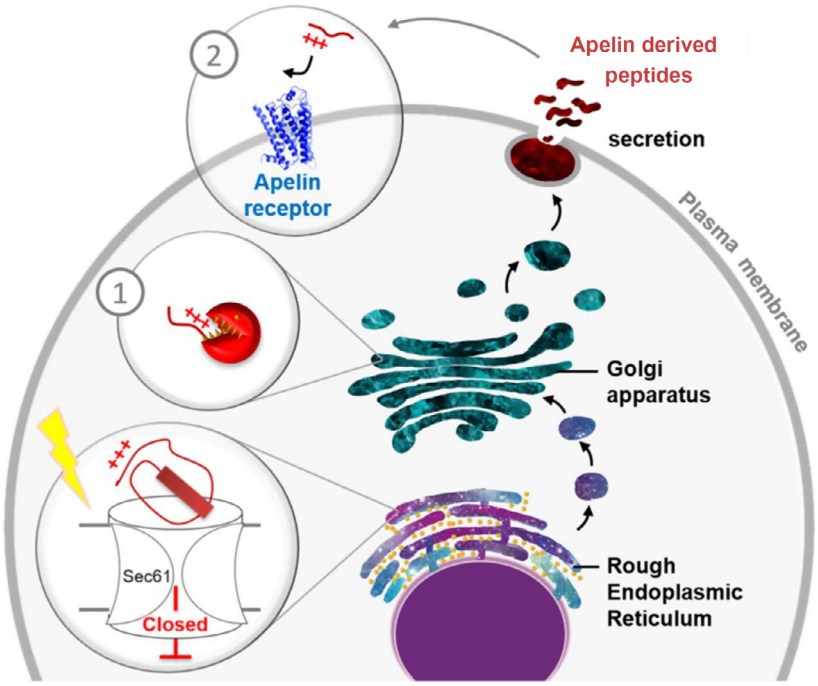

Fig. 5. Clustered charges in apelin play multiple roles during its biogenesis. ER import and thus, entry into the secretory pathway is the first step in biogenesis of the human hormone apelin. Presence of a cluster of charged amino acids in the mature region of preproapelin inhibits productive insertion into the Sec61 channel in the absence of BiP or in the presence of the inhibitor CAM741. Consequently, the channel remains closed and preproapelin accumulates at the cytosolic funnel of Sec61. Under normal conditions, however, the same charges are required in the Golgi apparatus (1) for processing of apelin into bioactive peptides of different length (red pacman: furin), termed maturation. Upon secretion, the clustered charges in the peptides contribute to the binding of apelin to its receptor in the plasma membrane (2).

Free energy diagram for Sec61-channel gating

On the basis of the findings described above, we favor discussing the effects of the allosteric Sec61-channel effectors in terms of free energy diagrams (Fig. 6). Accordingly, full Sec61-channel opening requires activation energy. The consecutive interactions of the H-region with the hydrophobic patch and the phospholipid bilayer lead to isosteric energy input and, therefore, lower the activation energy. When this is not sufficient, the translocon-associated protein (TRAP) – or Sec62/Sec63(+/–BiP)-interactions with the Sec61-channel have to provide additional – in this case allosteric – energy input, thereby accelerating the conformational changes of the channel or increasing the affinity for the transport substrate [64,80–85]. Alternatively or additionally, the allosteric effectors may affect the equilibrium between the closed and open channel conformations. Notably, the same cluster of positively charged residues within proapelin that determines BiP-dependence was found to be responsible for the sensitivity of preproapelin import towards cyclic heptadepsipeptide inhibitors of the Sec61-channel, such as CAM741 [64,86–88]. Replacement of this cluster by alanines relieves both BiP-dependence and CAM741-sensitivity (Fig. 6). Therefore, we favor the idea that the energetic barrier or activation energy for Sec61-channel opening is raised by cyclic heptadepsipeptide inhibitors, such as CAM741. Interestingly, cotranslational ER import of the ERj3 precursor protein is also CAM741-sensitive due to the cluster of positive charges within the mature region (Schorr, S., personal communication).

So far, there are no structural data on the mammalian Sec62/Sec63-complex. However, the recent structural analysis of the yeast heptameric SEC-complex elucidated extensive interactions between Sec63 and the Sec61 complex including contacts in their cytosolic, membrane and lumenal domains [70,71], which is perfectly in line with the above-discussed intrinsic Sec63 activity in ER import of prestatherin [64]. Notably, the yeast SEC-complex includes in addition to the heterotrimeric Sec61 complex and the heterodimeric Sec62/Sec63 complex the heterodimeric Sec71/Sec72-complex and is supposedly involved only in post-translational protein import into the ER. Of further note, the additional components, Sec71 and Sec72, are without known mammalian orthologs [89–91]. According to the yeast SEC-complex structure, the cytosolic Brl domain of Sec63 interacts with cytosolic loops 6 and 8 of Sec61α. In the membrane, Sec63 (transmembrane helix 3) contacts all three subunits of the Sec61 complex in the hinge region opposite of the lateral gate, including transmembrane helices 1 and 5 of Sec61α as well the tail-anchors of Sec61β and Sec61γ. In addition, the short lumenal N-terminus of Sec63 appears to intercalate on the lumenal side of the channel between the hinge loop (Sec61α loop 5) and Sec61γ. Thus, interactions of allosteric Sec61-channel effectors other than the ribosome with ER-lumenal loops of Sec61α appear to be a common principle for their action.

Undoubtedly, gating of the Sec61-channel to the closed state, that is efficient and fast closing of the channel, also requires activation energy (Figs 4 and 6). This is of particular importance for mammalian cells, where the ER serves as the main intracellular Ca^{2+} reservoir. In light of the energy diagram for Sec61-channel gating, it may not come as a surprise that BiP can also accelerate channel closure (in more detail reviewed in Ref [5]). Apparently, it supports the involved conformational change by interaction with the same ER-lumenal loop 7 of Sec61α, which is involved in channel opening [58].

What defines inefficiently gating signal peptides of small human presecretory proteins?

Among the low performing precursor proteins – small or large – we found two different types of signal peptides, those with low overall hydrophobicity in combination with high glycine- plus proline-content and those with low H-region hydrophobicity in combination with detrimental features within the mature part. In both cases, full Sec61-channel opening in cotranslational transport is supported by allosteric Sec61-channel effectors, the TRAP-complex or the Sec62/Sec63-complex with or without BiP [64,75,85]. Notably, lower signal peptide hydrophobicity has also been found to be decisive for Sec62p/Sec63p-involvement in post-translational ER import in yeast [92]. Based on the fact that all so far-analyzed small human presecretory proteins showed a requirement for Sec62 [53,64], we had a closer look at the signal peptides of small human presecretory proteins with respect to overall hydrophobicity, delta G^{pred}, glycine plus proline-content, N-region net charge, H-region hydrophobicity, and C-region polarity, as previously done for TRAP clients [85]. Strikingly, here, higher than average overall hydrophobicity and higher than average H-region hydrophobicity appear to define inefficiently gating signal peptides in the context of small precursor proteins, which is in sharp contrast to the signal peptides of precursor polypeptides in cotranslational and ribosome-dependent transport mentioned above (Fig. 7).

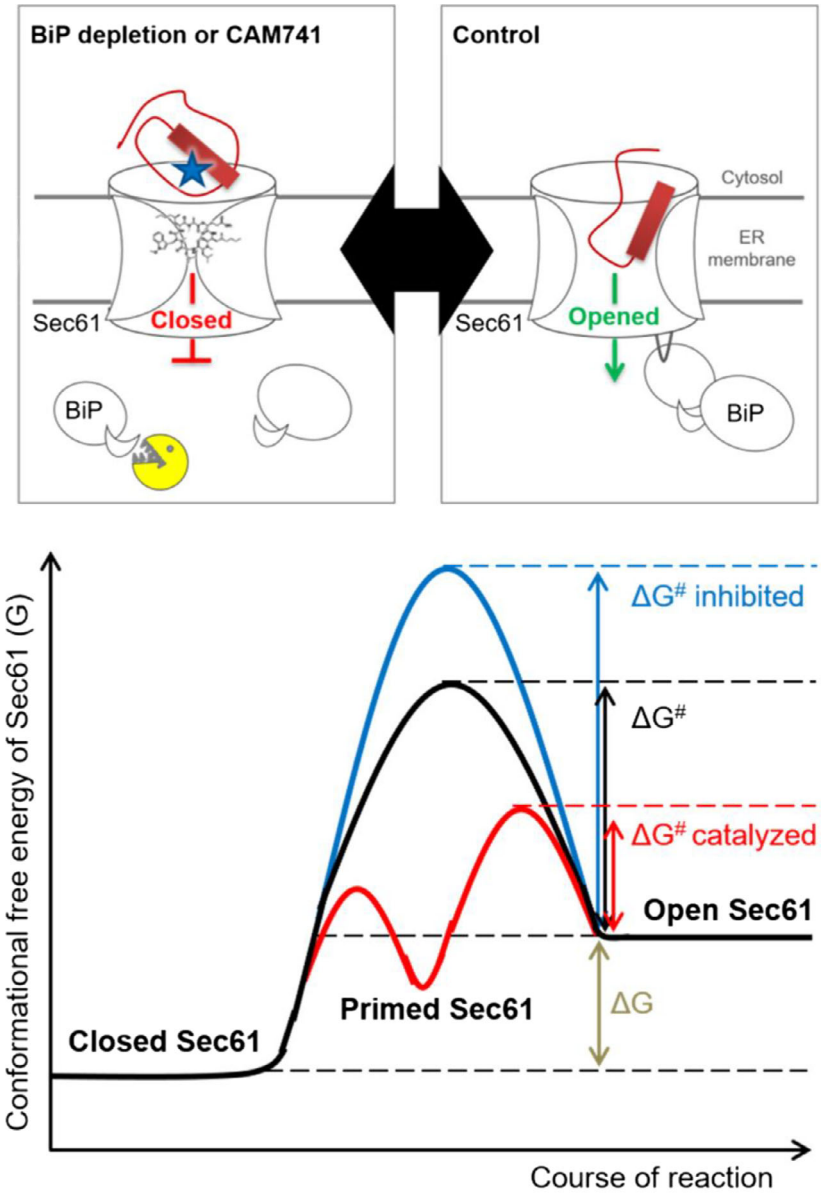

Fig. 6. Scheme and energy diagram for Sec61-channel gating. The cartoon illustrates that complete BiP-depletion by treatment of cells with subtilase cytotoxin SubAB (yellow pacman) [64,103] and Sec61 inhibition by CAM741 [64,86–88] both prevent productive insertion of preproapelin into the Sec61-channel, that is opening of the channel. This allows crosslinking of preproapelin to Sec61α (blue star). For illustrative purposes, we discuss the TRAP- or Sec62/Sec63+/- BiP-mediated Sec61-channel gating in analogy to an enzyme-catalyzed reaction. Accordingly, TRAP, Sec63, or BiP reduce the energetic barrier or activation energy for full channel opening, which can apparently be reinforced by Sec61-channel inhibitors, such as cyclic heptadepsipeptides (e.g., CAM741). Of note, binding of other inhibitors like [64,86–88] or certain eeyarestatins (e.g., ES1, ES24) [73] within the channel pore arrests the channel in a partially open state (termed 'foot in the door'), which maybe identical with the primed state and allows Ca^{2+}-efflux but is not compatible with full channel opening for protein translocation [73]. TRAP and BiP contribute to full channel opening by direct interaction with ER-lumenal loops 5 or 7 of Sec61α. Notably, *SEC61A1*-mutations can also increase the energy barrier for channel opening *per se* (V85D or V67G mutation) or indirectly, such as by interfering with BiP binding (Y344H mutation) [79,104,105]. Furthermore, *SEC61B*-, *SEC63*-, and *TRAP*-mutations can increase the energy barrier or prevent the action of the respective effector, which caused us to propose the term Sec61-channelopathies for these diseases [106–108]. Notably, all these effects are precursor specific because the N-terminal signal peptides are either efficient or inefficient in driving Sec61-channel opening and do or do not involve allosteric effectors, besides the ribosome. Typical for an enzyme-catalyzed reaction, BiP can also support efficient gating of the Sec61-channel to the closed state, that is the reverse reaction [58]. $G^{\#}$, activation energy.

Therefore, the question is how these apparently contradictory findings can be reconciled. We hypothesize that both higher and lower than average signal peptide hydrophobicity may extend the sampling or dwell time of the signal peptide in the Sec61 channel, simply because the interactions with the hydrophobic patch are either too strong, that is disfavoring reversibility, or not strong enough to trigger spontaneous opening of the lateral gate and accompanying full channel opening, which obviously remains to be experimentally tested. Therefore, allosteric effectors have to come into play (Fig. 4), in particular when aberrant hydrophobicity coincides with low-signal peptide helix propensity (as in the case of TRAP action) [85] or with deleterious features downstream of the signal peptide in the mature region (as in the case of Sec62 and Sec63 action) [64,74,75]. Interestingly, there appear to be some cotranslationally translocated precursors polypeptides, which can involve Sec61-channel gating by either the TRAP or the Sec62/Sec63-complex (Schorr, S., personal communication). Thus, there is a certain redundancy in ER protein import at the level of Sec61-channel gating, too. However, this does not seem to extend to the post-translational import of small presecretory proteins, which may be related to the fact that the signal peptides of the latter lack the tendency towards a high glycine- and proline-content that characterizes the signal peptides of TRAP-dependent precursors [85] (Fig. 7).

In contrast, the mature region of small presecretory proteins might comprise deleterious clusters of positive charges, as they represent sites for their fragmentation into several biologically active peptides. The role of BiP in compensating their presence can thus be seen in the context of an inherent disability of the Sec61-channel to translocate protein regions with respective features (Fig. 5). We note that such deficiency might only be apparent when clusters of positive charges are implemented in the mature region (a) with additional structural features (intrinsically disordered domains,

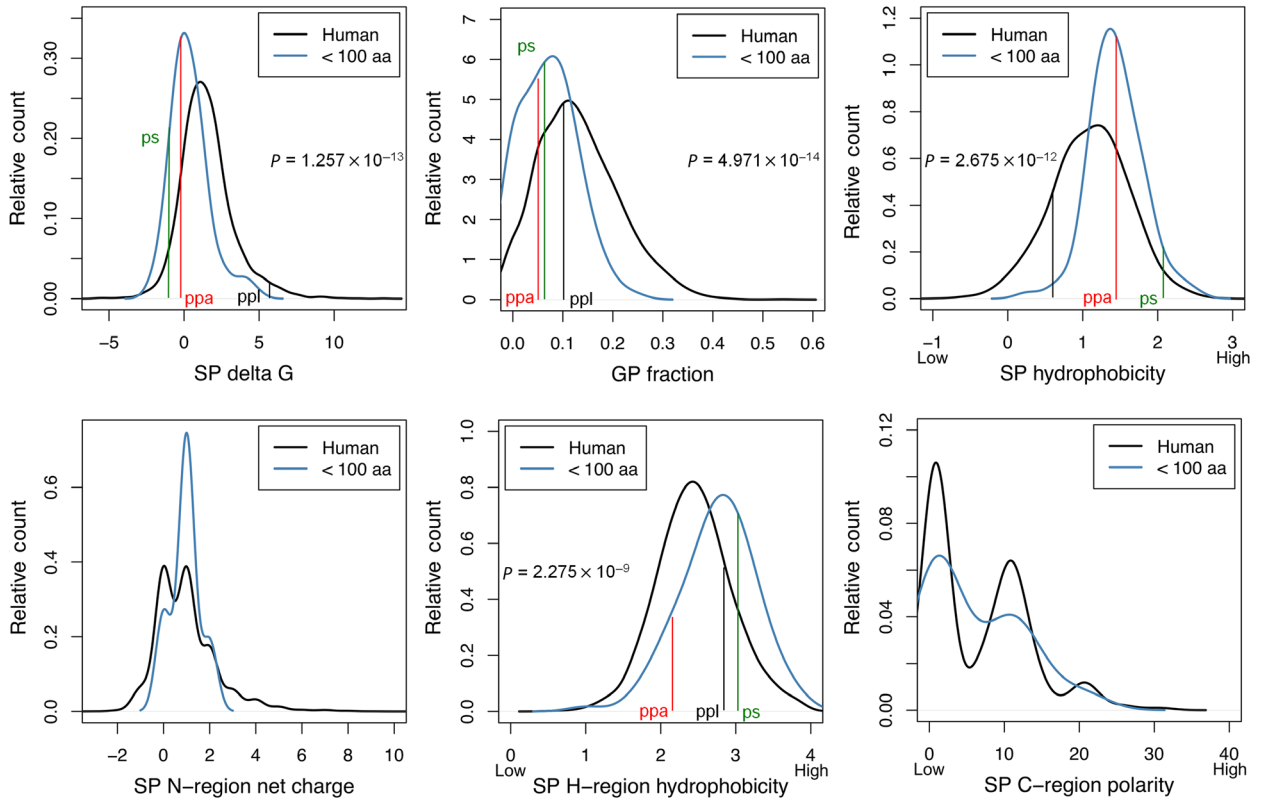

Fig. 7. Characteristics of signal peptides of small human presecretory proteins. Protein annotations of SP were extracted from UniProtKB entries using custom scripts. Using custom scripts, we computed the hydrophobicity score and glycine and proline (GP) content of signal peptides as described previously. Delta G^{pred} values were calculated with the delta G predictor (http://dgpred.cbr.su.se). Signal peptide segmentation prediction was carried out using the well-established prediction tool Phobius (http://phobius.sbc.su.se) to identify N-region, H-region, and C-region. Based on this, we calculated the total net charge of the N-region, the polarity of the C-region, and the hydrophobicity of the H-region as described previously [85]. Wilcoxon rank test $P < 0.1$ are indicated. ppa, human preproapelin; ppl, bovine preprolactin; ps, human prestatherin. The values are given in Tables S1 and S2.

see preproapelin and prion protein) or (b) at a specific location or distance to the signal peptide or (c) at a certain frequency and defined distances (reflecting the sizes of the bioactive peptides after maturation).

What is the point of having small presecretory proteins?

One can rephrase this question into the following ones, (a) what is the advantage of having small and large precursor polypeptides, (b) what is the advantage of having efficiently and inefficiently gating signal peptides, (c) what is the advantage of having precursor polypeptides, which do or do not depend on allosteric Sec61-channel effectors. We are convinced that the answer to all these questions was given in the course of evolution and is related to differential regulation of both ER targeting and import of precursor poylpeptides into the ER. When certain precursors, such as the ones larger than 100 amino acid residues (Table S1), have a preference for the SRP/SR-system, their ER import can be regulated independently from ER import of small presecretory proteins, which use alternative pathways. First, regulatory separation of the two translocation mechanisms, co- versus post-translational, can be simply achieved due to involvement of two different energy sources, GTP versus ATP. Thus, low cellular ATP-levels cause SRP-independent transport to stop, while allowing SRP-dependent transport to continue [43]. On the other hand, when certain precursor polypeptides, such as at least some of the small ones, depend on Ca^{2+}-CaM, their import – in contrast to the SRP/SR-dependent one – can be regulated in a Ca^{2+}-dependent fashion [57,65]. Furthermore, when certain precursor proteins depend on allosteric Sec61-channel effectors, such as the Sec62/Sec63-complex, their ER import can be regulated independently from the import of Sec62/Sec63-independent precursors. Additional involvement of the Hsp70-chaperone BiP adds another layer of regulation by the overall energy status of the cell or the specific ATP-level in the ER lumen. With the exception of the ATP involvement, the same can be said about the TRAP-complex. Notably, both the TRAP and the Sec62/Sec63-complex are subjected to phosphorylation and to Ca^{2+}-binding [93–95]. Therefore, it is tempting to speculate that these two modifications play an important and possibly even reciprocal regulatory role in ER protein import, an area, which has not been explored at all. To give just two potential examples: Calreticulin has a dual intracellular location, in the nucleus and the ER lumen, and depends on the TRAP-complex in its ER import [85,96]. $P58^{ipk}$, as Hsp40-type co-chaperone of BiP also termed ERj6, has been described to be a player in both cytosolic and ER-lumenal protein quality control in ER protein import and appears to involve Sec62 and Sec63 in its import [75,97,98]. We hypothesize that in both cases phosphorylation and/or Ca^{2+}-binding to the allosteric Sec61-channel effectors may favor one over the other possible intracellular location. These kinds of regulatory mechanisms may well be involved in the course of cell differentiation or specific cellular demands and certain conditions, such as stress.

Concluding remarks

As of today, several components and mechanisms for transport of precursor proteins into the human ER have been described in considerable detail. Two key characteristics are that there are overlapping substrate specificities or redundancies in both ER targeting as well as Sec61-channel gating. However, also many open questions remain: We cannot be sure that we already know all pathways for ER targeting of precursor polypeptides and all mechanisms of Sec61-channel gating, nor do we know anything about the contribution of even the known pathways and mechanisms in certain cell types. Finally, we hardly know anything about the mechanisms, which are at the disposal of cells to regulate all aspects of ER targeting and -translocation of precursor polypeptides.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Table S1. Large human presecretory proteins.
Table S2. Small human presecretory proteins.