新規フォールディングプローブを用いた生細胞内におけるタンパク質膜透過の定量解析とその応用

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Contents

1. Abstract

Various proteins synthesized by ribosomes are imported into specific organelles. To elucidate the behavior of protein domains during import, we developed a folding probe, in which the capsid protease (CP) domain of the Semliki Forest virus was connected to EGFP. The probe was fused to appropriate N-terminal organelle-targeting signal sequences and expressed in cultured cells. When the entire CP-domain was present in the cytosol, it became folded and cleaved off the following EGFP-domain. Once cleaved, EGFP stability was not affected by upstream sequences. Based on EGFP localization, we estimated the extent of CP-domain folding in the cytosolic space. When fused to mitochondrial hydrophobic multispanning membrane protein ABCB10, more than half of the EGFP remained in the cytoplasm, while most of the CP-portion was in the mitochondrial fraction. When fused to the endoplasmic reticulum (ER) signal, the cleaved EGFP was observed only in the ER fraction, confirming that the CP-domain cannot fold on the cytoplasmic side during cotranslational ER translocation. Thus, import of the ABCB10 molecule was not as tightly coupled with chain elongation as ER translocation. Use of this probe to quantitatively examine stop-translocation at the ER translocon in living cells revealed that positively-charged residues on the translocating nascent chain stall at the ER translocon.

2. Introduction

Many proteins synthesized in the cytoplasm are imported into specific cell organelles. The organelle localizations of proteins are fundamental basis of cell architecture. In mammalian cells, targeting and translocation across endoplasmic reticulum (ER) membranes are tightly coupled to polypeptide chain elongation, referred to as a cotranslational process. When a signal sequence of the nascent chain emerges from the ribosome tunnel, it is recognized by a signal recognition particle (1), which arrests polypeptide chain elongation at the ribosome, and then the ribosome nascent chain complex is targeted to the ER protein-conducting channel, termed translocon (2). After targeting, the ribosome resumes polypeptide chain elongation and the chain emerging from the ribosome enters directly into the translocon channel. Therefore, there is no time for the elongating polypeptide chain to fold in the cytosol before translocation, except for tiny domains such as a zinc-finger sequence (3). In contrast to the cotranslational translocation in mammalian ER, a posttranslational translocation mechanism occurs in the ER of budding yeast. Posttranslational ER translocation depends on the subcomponents of the translocon, Sec62, Sec63, Sec71, and Sec72, but not on the signal recognition particle (4,5). The signal sequence apparently defines the targeting routes (4). Both modes are operative in *E coli* (6). We recently demonstrated that cotranslational translocation in the ER is tentatively arrested by positively-charged residues of the nascent chain and can resume uncoupled with polypeptide chain elongation (7,8). Such movement of the polypeptide after the provisional translocation arrest is recognized as posttranslational translocation. We developed a probe to examine the molecular mechanisms and modes of ER translocation in living cells.

It is generally considered that the majority of mitochondrial and peroxisomal

proteins are targeted to and imported into cell organelles after protein synthesis; that is, a posttranslational process (9,10). For example, a remarkable amount of precursor proteins destined to the mitochondrial matrix accumulate in the cytoplasm and are then imported into the mitochondria (11). In a cell-free experimental system, many precursor proteins can be imported into the mitochondria posttranslationally, whereas membrane proteins and secretory proteins cannot be inserted into the ER after their synthesis. Many mRNAs encoding mitochondrial precursor proteins are detected in free polysomes, but not in membrane-bound polysomes. The presequence of mitochondrial ABC transporter isoform B10 (ABCB10) can switch the targeting mode of membrane proteins from cotranslational ER-targeting to posttranslational mitochondrial-targeting (12). In contrast to data supporting the posttranslational mode of mitochondrial import, other findings suggest that some mitochondrial protein import occurs cotranslationally. For example, specific mRNAs are enriched on the mitochondria (13-16). Nascent chains stalled at the ribosome could occupy the mitochondrial translocator (17). Some reports support the possibility that import into the mitochondria occurs cotranslationally (18-20). Assessing the coupling between polypeptide chain elongation and import is thus the next critical step.

We utilized the capsid protein protease (CP) domain of the Semliki Forest virus to examine the modes of organelle targeting. The CP can cotranslationally fold upon exit from a ribosome tunnel or translocon channel (21). The CP cleaves off the peptide bond just after the domain, even when the C-terminus is still elongating at the peptidyl transferase center of the ribosome (21-23). Cleavage occurs in a *cis*-acting manner (24). If translocation of the CP-domain is tightly coupled to ribosomal elongation, the polypeptide chain has no time to fold on the cytoplasmic

side. If the CP-domain folds into its active conformation in the cytoplasm, it can be active in the cytoplasm.

Here we constructed a folding probe in which the CP-domain was connected to enhanced green fluorescent protein (EGFP), and then fused the probe downstream of organelle-targeted proteins. When the probe was fused to secretory protein and expressed in the cells, the CP-domain and cleaved EGFP were observed only in the organelle fraction. In contrast, when ER import was stopped by a hydrophobic segment, the EGFP-domain was observed only in the cytoplasm. In mitochondrial import, a remarkable amount of EGFP was observed in the cytoplasm, even when it was fused to ABCB10, which possesses multiple hydrophobic transmembrane (TM) segments. In peroxisomes, all of the cleaved EGFP was in the cytoplasm. This technique is useful for assessing the time period during which the CP-domain is in the cytosol during organelle import. Using the probe, we demonstrated in vivo that clusters of positively-charged amino acid residues of a nascent polypeptide actually arrest translocation across the ER translocon, even in the absence of a hydrophobic sequence.

3. Abbreviations

CP : capsid protease

DMEM : Dulbecco's modified Eagle's medium

DTT : dithiothreitol

EGFP : enhanced green fluorescent protein

EndoH : endoglycosidase H

ER : endoplasmic reticulum

FCS : fetal calf serum

GFP : green fluorescent protein

PBS : phosphate-buffered saline

PCR : polymerase chain reaction

RSA : rat serum albumin

SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFV : semliki forest virus

SP : signal peptide

SRP : signal recognition particle

TM : transmembrane

4. Materials and Methods

4.1. Materials

Anti-EGFP rabbit antiserum (25,26) and anti-H450 rabbit IgG fractions (27) were previously described. Anti-hsp60 monoclonal antibody was obtained from Biotechnologies Corp. (SPA807).

4.2. Construction of model proteins

In the following DNA manipulation procedures, the desired DNA fragments were obtained by chemical synthesis or polymerase chain reaction. The DNA fragments were designed to possess the appropriate restriction enzyme sites at both ends, which are described in parentheses. The DNA fragments were ligated into plasmid vectors digested with the indicated restriction enzymes. At each junction, six bases of the restriction enzyme site encoded two codons. The DNA fragment encoding CP was synthesized according to the nucleotide sequence of the protease domain (Met^{113}-Trp^{267}) of the Semliki Forest virus capsid protein (accession X78109). The DNA fragments encoding CP and (XbaI/BamHI) and EGFP (BamHI/ApaI) were subcloned to pRcCMV (XbaI/ApaI) to yield pCP-EGFP. Incorporation of the BamHI site allowed for the insertion of Gly-Ser between the CP and EGFP domains. The protease-inactive mutant of CP was constructed by generating a point mutation (Ser219Ile) according to the previous report (28). The DNA fragment encoding the N140 sequence of ABCB10 (HindIII/XbaI) was also included into pCP-EGFP (HindIII/XbaI) to yield pN140-CP-EGFP. A DNA fragment encoding a 180-residue sequence of rat serum albumin (RSA, XbaI/XbaI) was ligated to the XbaI site of pCP-EGFP to yield pER-CP-EGFP. For ER-TM-CP-EGFP, a DNA fragment encoding the 20-residue Leu-Ala repeating sequence (NheI/Aor51HI) was inserted

between Ser^{168} and Ser^{169} of RSA, as described previously (29). For ABCB10-fusions, DNA fragments (XbaI/XbaI) encoding various length sequences of ABCB10 were inserted into the XbaI site of pCP-EGFP to generate various fusions that were named according to their ABCB10-domain length (Fig. 4). For pPTS2-CP-EGFP, the DNA fragment encoding the 59-residue N-terminal segment of rat thiolase (HindIII/XbaI) was ligated between the HindIII and XbaI sites of pCP-EGFP. For EGFP-PTS1, the DNA sequence encoding Ser-Lys-Leu was generated at the EGFP C-terminus. For constructions with various clusters of Leu-Ala, Lys, and Arg sequences, the corresponding oligonucleotides were synthesized, phosphorylated, annealed, and inserted between the NheI and Aor51HI sites of the RSA sequence.

For EGFP glycosylation mutants, potential glycosylation sites were created at various sites on the molecular surface; Phe^{131}-Lys-Glu, Gly^{135}-Asn-Ile, His^{140}-Lys-Leu, Gly^{175}-Ser-Val, Leu^{195}-Leu-Pro, and Asn^{199}-His-Tyr were changed to Asn-Ser-Thr by the quick-change method. The most efficiently glycosylated mutant (Phe^{131}-Lys-Glu to Asn-Ser-Thr) was used.

For a CP expression plasmid, a DNA fragment encoding CP (BamHI/BamHI) was subcloned into the BamHI site of pET28b (Novagen), in which the CP-domain was fused to an N-terminal His_6-tag.

4.3. Preparation of anti-CP antibodies

E. coli strain BL21(DE3) was transformed with the CP expression plasmid in which CP was inserted into pET28b. The His_6-tagged CP protein was expressed, extracted, and recovered in the supernatant fraction after cell-disruption by sonication. The CP protein was bound to Ni-Sepharose resin (GE Healthcare) and

eluted with 200 mM imidazole. The eluate was essentially pure His_6-CP protein. Because the imidazole elution buffer caused CP aggregation during freezing, the elution buffer was exchanged with buffer [20 mM Tris (pH7.5), 200 mM NaCl, and 0.5 mM DTT] using a PD10 desalting column (GE Healthcare). The purified protein was used for rabbit immunization at Hokkaido System Science Co.

4.4 Expression in the cells and cell fractionations

HeLa and COS7 cells were maintained in Dulbecco's Modified Eagle Medium plus 10% fetal bovine serum under 5% CO_2 atmosphere. The expression plasmids were transiently transfected into the cells using FuGENE6 reagent (Promega) according to the manufacturer's instructions. To avoid the effect of overexpression, the plasmid DNA was titrated and the minimum amount used. The total amount of the DNA was compensated by vacant plasmid DNA (pRcCMV). Cells were cultured for 24 h after transfection.

To analyze the location of the EGFP-domain, we developed a simple procedure (peeling method), by which cytoplasmic proteins were quantitatively extracted from the cells. Cells in the plastic well were washed once with 1 ml PBS and then with the peeling buffer [20 mM HEPES (pH7.5), 250 mM sucrose, 2.5 mM $Mg(OAc)_2$, and 2.5 mM EGTA (pH7.5)]. The cells were then perforated by scraping them in the presence of 1 ml peeling buffer using a Cell-Lifter (Corning). Equal volumes of KCl-peeling buffer [20 mM HEPES (pH7.5), 250 mM sucrose, 2.5 mM Mg(OAc)2, 2.5 mM EGTA (pH7.5), and 50 mM KCl] were added to the scraped cells, and then the cells were suspended by extensive pipetting and incubated 10 min on ice. The mixture was centrifuged at 21,500g (15,000 rpm) for 10 min. All the membrane organelles were precipitated and the cytoplasm remained in the

supernatant. Proteins in the supernatant were precipitated in 6% trichloroacetic acid and the precipitates were rinsed with cold acetone. The protein precipitates and organelles in the precipitate were solubilized in SDS-PAGE sample buffer. The EGFP, CP-containing portion, and marker proteins for the cytoplasm (H-450) and mitochondrial matrix (hsp60) were probed with the appropriate antibodies. Endoglycosidase H (New England BioLabs) treatment was performed as described under SDS-denaturing conditions.

Immunoblotting analysis was performed essentially as described previously (30). Proteins separated by SDS-PAGE were transferred to a nitro-cellulose filter with a semi-dry transfer apparatus according to standard procedures. The filter was blocked with blocking buffer [2% (w/v) skim-milk, 0.2 % (v/v) Tween20, 150 mM NaCl, and 20 mM K-phosphate buffer (pH 7.4)] for 20 min. The filter was incubated with antibodies in the blocking buffer. The non-ionic detergent, Tween20, was critical for preventing over-blocking by the skim milk. The detection limitation of our system was 1 ng EGFP per lane. After the antibody reactions, the filters were washed with washing buffer [20 mM Na-phosphate buffer (pH 7.4), 150 mM NaCl, and 0.2% Tween 20]. Antibodies on the filter were visualized with ECL-reagent (GE Healthcare) and LAS4000mini (GE Healthcare).

5. Results

5.1 Auto-cleavage by the CP-domain in HeLa cells

The folding probe comprised the CP and EGFP domains (Fig. 1A). To target the probe to organelles, either the 140-residue N-terminal sequence (N140) of the mitochondrial ABCB10 or the RSA with the ER targeting signal was fused to the N-terminus of the probe. If organelle import is tightly coupled with polypeptide chain elongation, the CP-domain cannot fold before translocation across the organelle membrane. If the import is posttranslational, the CP-domain can fold before import and cleaves the EGFP domain on the cytoplasmic side.

The free CP-EGFP probe was transiently expressed in cultured HeLa cells, and the CP and EGFP domains were detected by immunoblotting (Fig. 1B). No uncleaved high molecular weight product was observed (Fig. 1B, lanes 1 and 7). When a Ser residue of the active site was mutated to Ile (CPm), only the uncleaved form was observed (Fig. 1B, lanes 4 and 10). Even when the CP-EGFP fusion was overexpressed in *E. coli* cells, the product was completely cleaved (data not shown). The junction between CP and EGFP was Gly-Ser (corresponding to the ligation site of BamHI), which is suitable for cleavage. Other hinge sequences of Ser-Arg or $(Gly)_5$ were also compatible, whereas Pro-Arg was rarely cleaved (data not shown).

For mitochondria and ER targeting fusions, both constructs were efficiently cleaved, leaving the EGFP (Fig. 1B, lanes 2 and 3) and upstream portions, including CP (Fig. 1B, lanes 8 and 9). Because the 105-residue presequence for mitochondrial import in the N140 sequence was cleaved by the processing peptidase in the mitochondrial matrix (31,32), the CP-portion polypeptide was 35 residues larger than free CP (Fig. 1B, lane 8). With the CPm, no cleaved EGFP was observed (Fig. 1B, lanes 5 and 6), and larger polypeptides were instead observed with anti-EGFP

antibodies. Larger polypeptides were also observed with anti-CP antibodies (Fig. 1B, lanes 11 and 12, open squares). The Mito-CPm-EGFP fusion was largely degraded (Fig. 1B, open triangles); the main band of 25 kDa was likely a degraded fragment including the EGFP domain and a small portion of the CP sequence. Hence the fragment strongly reacted with anti-EGFP antibody, whereas it reacted only weakly with the anti-CP antibody. The polypeptide of CP connected with EGFP seems to react poorly with anti-CP antibodies for some unknown reason.

5.2 Convenient assay of EGFP in the cytosol

To assess whether cleaved EGFP was in the cytoplasm or in the organelle, we developed a simple procedure (termed the "peeling method"), by which cytoplasmic proteins were easily separated from cell organelles (Fig. 2). In the procedure, the plasma membranes of HeLa cells were perforated by scraping the cells cultured on a plastic dish in peeling buffer. Then, the cells suspensions were centrifuged to separate the supernatant of the cytoplasm and the precipitates of organelles (Fig. 2B). As a marker of cytoplasmic soluble protein, we probed H450, which is also called cystathionine synthase (27). Mitochondrial hsp60 was used as a mitochondrial marker. Both were quantitatively recovered in the supernatant and precipitate fractions, respectively (Fig. 2B, lower panel). In the case of the free CP-EGFP, both EGFP and CP were largely recovered in the supernatant fraction (Fig. 2B, lanes 1 and 7). Thus, cytoplasmic EGFP could be separated from the cell organelles using the peeling method.

5.3 CP-domain can be active in the cytosol during mitochondrial import

In the case of Mito-CP-EGFP, more than half of the cleaved EGFP was recovered in

the supernatant, whereas the CP-portion was largely recovered in the precipitate (Fig. 2B, lanes 3 and 10). When the mitochondria-targeting N140 sequence was fused directly to EGFP (Mito-EGFP), the EGFP portion was completely recovered in the precipitate (Fig. 2B, lane 6). During mitochondrial import mediated by the N140 sequence, more than 50% of the CP-domain folded and cleaved the following EGFP in the cytoplasm.

5.4 CP distinguishes cotranslational import into the ER and posttranslational import into the mitochondria and peroxisomes

To examine the coupling between the synthesis and import of full-length ABCB10, CP-EGFP was fused to the C-terminus of the ABCB10 (Fig. 3). When the fusion was transiently expressed in HeLa cells for 24 h, more than half of the cleaved EGFP was observed in the supernatant, while the preceding ABCB10-CP portion was fully recovered in the precipitate (Fig. 3B, lanes 1 and 12). It was not determined whether the folded CP-domain was fully imported or retained outside the mitochondria. Even in the case of such a highly hydrophobic multispanning membrane protein, the C-terminal portion was retained in the cytoplasm for a considerable amount of time during mitochondrial import (Fig. 3C).

As a case of cotranslational import, we examined ER-CP-EGFP, where CP-EGFP was fused to RSA of 175 residues. Both the EGFP and upstream RSA-CP portion were recovered in the precipitate (Fig. 3B, lanes 4 and 14). The CP-domain did not fold during cotranslational translocation across the ER membrane. To retain the CP-EGFP on the cytoplasmic side of the ER membrane, a hydrophobic TM segment (comprising 10 Leu and 10 Ala residues, termed the 10L segment) was inserted between RSA and CP (ER-TM-CP-EGFP). The TM-segment was expected to stop

translocation through the translocon as a stop-transfer sequence and to anchor the product on the membrane (33,34). When it was expressed in HeLa cells and the cells were separated into supernatant and precipitate fractions by the peeling method, the cleaved EGFP was recovered in the supernatant and the CP-portion was recovered in the precipitate (Fig. 3B, lanes 5 and 16). The CP-domain folded and cleaved the EGFP-domain on the cytoplasmic side of the ER membrane (Fig. 3C). The CP-EGFP probe should thus be useful for examining the membrane topology of a specific segment of a nascent chain on the ER membrane. Furthermore, we expect that the CP-EGFP will be useful for analyzing the provisional retention of a specific polypeptide domain in the cytoplasm during membrane translocation on the ER.

When the probe was targeted to the peroxisome by the N-terminal peroxisomal targeting signal (so-called PTS2) of human thiolase (35), EGFP was only observed in the supernatant, whereas the CP-portion was largely recovered in the precipitate (Fig. 3B, lanes 7 and 18). As a control for EGFP in the peroxisome matrix, EGFP was C-terminally fused with PTS1 (SKL sequence) (36). This was largely observed in the precipitate (Fig. 3B, lane 10). Some peroxisomes might be disrupted during the separation procedure. The CP-domain also folds prior to import into the peroxisomes by PTS2.

The CP-domain can be active in the cytoplasm, in the mitochondrial matrix, on the cytoplasmic surface of the ER membrane, and in the ER lumen. Recovery of EGFP in the supernatant is a clear indication of folding of the CP before import. Because the CP-domain might be maintained in an unfolded form, even in cases of posttranslational import by various cytoplasmic chaperones, recovery of EGFP in the cell organelles does not directly indicate that the process is cotranslational. On the other hand, recovery of significant amounts of the EGFP domain in the

supernatant is a clear indication of folding of the CP-domain in the nascent chain before import. Thus, the C-terminal portion of full-length ABCB10 can be imported posttranslationally.

5.5 Import of mitochondrial multispanning membrane protein ABCB10 is not coupled to synthesis

Import of some portions of the long ABCB10 sequence, including several TM segments, might be coupled with ribosomal elongation. To examine this possibility, the CP-EGFP probe was fused at various positions in ABCB10 (Fig. 4A). In all cases, more than 50% of the EGFP was recovered in the supernatant, whereas the CP-containing portions were largely found in the precipitate. Despite the hydrophobic nature of the ABCB10 molecule, every portion was retained on the cytoplasmic side for a substantial amount of time, which allowed the CP-domain to fold during import. In other words, at least half of the molecule was imported posttranslationally. The mitochondrial import of the ABCB10 molecule was not so stringently coupled to protein synthesis as the ER-translocation system. It should also be noted that the CP-containing portions became unstable as the fusion position shifted downstream, while the cleaved EGFP could be similarly detected regardless of the sequence fused to the CP-domain (Fig. 4B).

5.6 Quantitative analysis of translocation arrest at ER translocon

The CP-domain folds only after exiting the translocon tunnel when cotranslationally targeted to the ER (23); the CP-domain does not fold on the cytoplasmic side of the ER membrane during cotranslational translocation. We expected that if movement of the polypeptide chain through the translocon was

stopped upstream of the CP-domain, the CP would fold on the cytoplasmic side of the membrane and cleave the downstream portion. To more conveniently detect the translocation, a glycosylation site was created in the EGFP-domain. To achieve efficient glycosylation, we created glycosylation sites at various points in the EGFP. The most efficiently glycosylated construct was used. The fusion protein was transiently expressed in cultured COS cells in the presence of a proteasome inhibitor (MG132). The glycosylated EGFP fragment was detected (Fig. 5B, lane 1). In the case of CPm, the larger uncleaved product was detected (Fig. 5B, lane 5). With anti-CP antibodies, the RSA-CP portion was detected, which was also glycosylated (Fig. 5B, lanes 9 and 10). The cleaved EGFP and the CP-RSA portion were converted to smaller ones by EndoH treatment, indicating that both of them were translocated into the lumen and glycosylated.

When the hydrophobic TM segment (10L) was included upstream of the CP-domain, the polypeptide chain was expected to stop statically at the membrane and the downstream chain to be retained on the cytoplasmic side (34,37). As expected, the cleaved EGFP was not glycosylated. The CP-domain was active on the cytoplasmic side of the ER membrane. In the ER translocation system, the CP-domain did not fold in the cytosol and became active in the lumen only after translocation. Once cleaved off, the stability of the glycosylatable EGFP (EGFPg) domain would not be affected by the upstream sequences, although the upstream portion was artificially changed. The CP-EGFPg domains would thus be useful probes of the translocation of various artificially engineered sequences in the cells and would detect uncoupling of translocation induced by marginally hydrophobic segments or cluster of positively charged residues.

5.7 Quantitation of translocation arrest by the hydrophobic segments

Onishi et al. demonstrated that a marginally hydrophobic segment of the nascent chain transiently arrests the translocation and uncouples the movement from the elongation using the in vitro experimental system (38). To examine the translocation arrest by hydrophobic segments, we systematically changed the contents of the hydrophobic 10L-segment (Fig. 5C); the number of Leu residues was systematically reduced and the central Ala was changed to Cys for other experiments. The constructs were expressed and the EGFP was probed by immunoblotting. Unglycosylated EGFP increased depending on the number of leucines. Use of this system allowed us to conveniently quantify the translocation arrest of marginally hydrophobic segment at the ER translocon in living cells.

5.8 Positive-charge cluster arrests translocation in living cells, even in the absence of a hydrophobic segment

Positively charged residues of the nascent chain transiently arrest the translocation and uncouple the movement from the polypeptide chain elongation (7,8). We then examined the effect of the number of positive charges on translocation efficiency in the cells. Positive-charge clusters were inserted in the middle of the model instead of the hydrophobic segment (Fig. 6A). When expressed in COS7 cells, the cluster suppressed the translocation of EGFPg depending on the number of positive charges (Fig. 6). Although the positive-charge cluster on the nascent chain might inhibit polypeptide chain elongation and cause degradation of the ribosome (17,39,40), we observed a comparable amount of EGFP. Positive-charge clusters containing more than 10 lysine residues could be read through up to the CP and EGFP domains. These data provide in vivo support that the cluster of positive

charges induces translocation arrest even in the absence of a hydrophobic segment.

5.9 Cooperation between the hydrophobic segment and its downstream positive charges

Using the folding probe CP-EGFPg, we examined the cooperation between a positive charge-cluster and a marginally hydrophobic segment (LA8; Fig. 7). The LA8 segment and the 6K-cluster had a weak and small translocation arrest effect (Fig. 7B, lanes 2 and 3). When the 6K-cluster was positioned after the LA8-segment, the translocation arrest was greatly enhanced (Fig. 7B, lane 4). On the other hand, when the 6K was positioned before the LA8-segment, the translocation was not affected, but rather enhanced (Fig. 7B, lane 5). It was clearly demonstrated in vivo that the positively-charged residues effectively enhanced the translocation arrest of the hydrophobic segment only when they followed a hydrophobic segment.

6. Discussion

Here we described the development and application of a unique probe (CP-EGFP) to examine the folding of a protein domain during organelle import. Experiments using this probe demonstrated that import of a highly hydrophobic multispanning membrane protein (ABCB10) into mitochondria is not so tightly coupled with ribosomal elongation. The CP-domain can be active in the cytosol before import. On the other hand, cotranslational translocation into the ER does not allow the CP-domain to fold before translocation. These findings are consistent with the notion that ER targeting and translocation of proteins are tightly coupled to polypeptide chain elongation by the bound ribosome. The probe can also be used to monitor the stalling of cotranslational ER translocation. We estimated the threshold of the hydrophobic segment for the stop-translocation at the ER translocon in living cells. Further, we provided in vivo evidence that the positive-charge cluster of the nascent chain arrests translocation through the ER translocon and that the positive-charge cluster cooperates with a marginally hydrophobic segment to stop translocation.

The CP-domain can become active in the cytosol, the ER lumen, and the mitochondrial matrix, and on the cytoplasmic surface of the ER membrane. If the CP-domain folds in the cytosol, it generates an apparent EGFP footprint. Using the peeling method to separate cytosolic soluble proteins from cell membrane organelles, free CP and EGFP were largely recovered in the cytosol. When the CP-EGFP probe was fused to the ER targeting sequence, both EGFP and CP were located in the organelles, indicating that the CP does not fold during ER translocation. With the N-terminal mitochondrial presequence, the CP-domain can fold more than 50% of the way before import into the mitochondria. With the N-terminal

peroxisome-targeting signal (PTS2), the CP-domain was almost completely cleaved from the EGFP-domain in the cytosol, and largely located in the organelle. Although the upstream sequence was artificially changed, the cleaved EGFP was similarly stable. We could thus assess the translocation behavior of systematically designed sequences.

In vivo analysis of the stop-translocation at the ER translocon with the CP-EGFP probe estimated the hydrophobicity required for 50% stalling of ER translocation (Fig. 5). The required number of leucine residues for the stop-translocation was essentially the same as that determined by pulse-labeling experiments in HeLa cells, using model proteins, including systematically constructed model proteins (41). The developed probe allows for quantitative estimation of the effect of the nascent chain sequence on translocation behavior.

The CP-probe provided in vivo evidence indicating that the cluster of positively-charged residues stalls at the ER translocon. Using a cell-free system, we demonstrated that 3 to 4 lysine residues slow down the movement of the nascent chain by several 10s of seconds, which then moves forward (7,8,42). Here we obtained in vivo evidence that 8 to 9 lysine residues are required to allow enough time for the CP-domain to elongate, fold, and exert activity on the cytoplasmic surface of the ER membrane.

The folding probe would distinguish the translocation mode. In budding yeast, both cotranslational and posttranslational modes are operative even in the ER. The modes have been distinguished based on the requirements of the signal recognition particle (4). The CP-EGFP would be a critical probe for distinguishing the targeting processes in yeast. On the other hand, a cotranslational mode of mitochondrial import has been suggested. Various mRNAs are enriched on the mitochondrial

surface (13,14). Site-specific ribosome profiling demonstrated that mRNAs encoding the mitochondrial inner membrane and matrix are enriched on the mitochondrial surface with their nascent chain (16). Further, the nascent chain bound to ribosomes can stack at the ribosome translocator and suppress translocator function (17). These previous findings strongly suggest that import of the nascent chain into the mitochondria occurs cotranslationally. On the other hand, the present findings suggested that the import of even such a hydrophobic protein as ABCB10 was not as tightly coupled to polypeptide chain elongation as the ER. The CP-EGFP probe would clarify the details of coupling between the ribosome and various organelle translocators.

The dihydrofolate reductase domain has been used as a probe to monitor protein domain-folding before organelle import. When fused to the N-terminal mitochondrial presequence, it folds and can bind to a substrate analog inhibitor (methotrexate). The binding stabilizes the fold and inhibits import into the mitochondria (43) . When fused to the N-terminus of the signal-anchor sequence with N_{out}/C_{in} topology, the reductase domain can be translocated through the ER translocon (44,45). Translocation is also inhibited by methotrexate not only in a cell free system but also in cultured cells (44). In both organelle membranes, the protein domains fold before translocation and should be unfolded for translocation through the translocators of the mitochondrial (43) and ER membranes (44,45). Observations with the reductase domain could, however, be greatly affected by the stability of the fusion partner. In contrast, once the CP-domain folded and cleaved the EGFP-domain, the EGFP-probe remained stable despite the variety of preceding sequences. Glycosylation in the ER is easily detected and quantified, and thus this newly developed probe provides more sensitivity, convenience, and

versatility than the reductase domain.

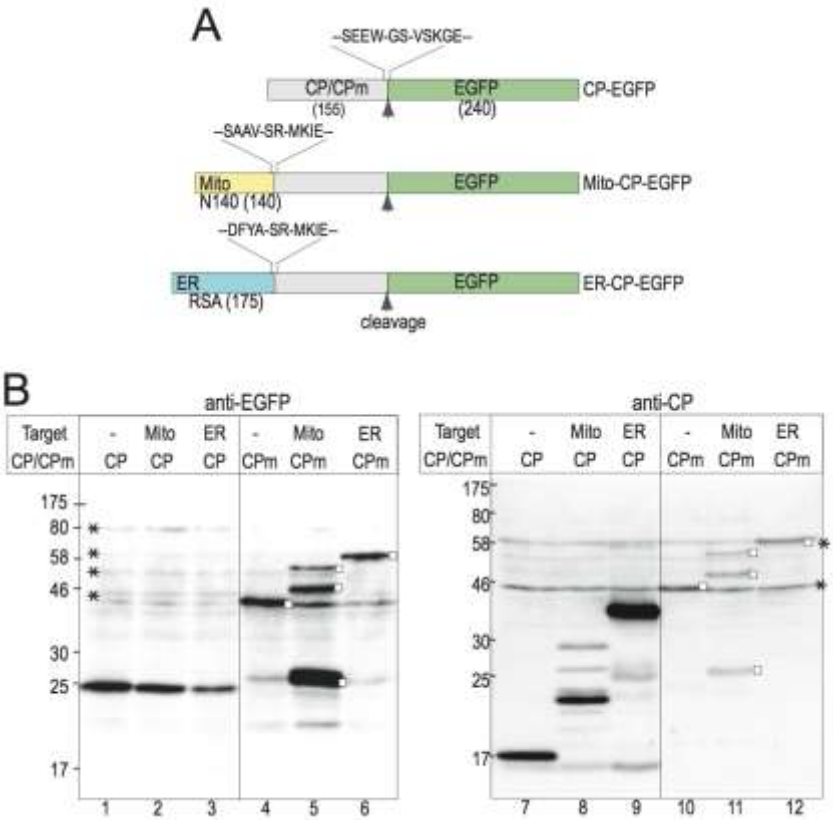

Figure 1 Auto-cleavage by the CP-domain

(A) CP-EGFP probe and fusion proteins. The CP-domain is connected to the N-terminus of EGFP (CP-EGFP). The N-terminal 140 residues of ABCB10 (N140), including the mitochondrial targeting pre-sequence, was fused to the probe (Mito-CP-EGFP). The 175-residue N-terminal portion of RSA was fused to CP-EGFP (ER-CP-EGFP). Residue numbers of the domains are indicated in the parentheses. Amino acid sequences of each junction are indicated. Arrowheads show auto-cleavage sites. A protease inactive point mutant of CP (CPm) was also used. (B) The fusion proteins were transiently expressed in HeLa cells and analyzed by immunoblotting using anti-EGFP or anti-CP antibodies. Major bands detected with the CPm-constructs (open squares) and degraded proteins (open triangles) are indicated. Nonspecific bands are indicated (asterisks).

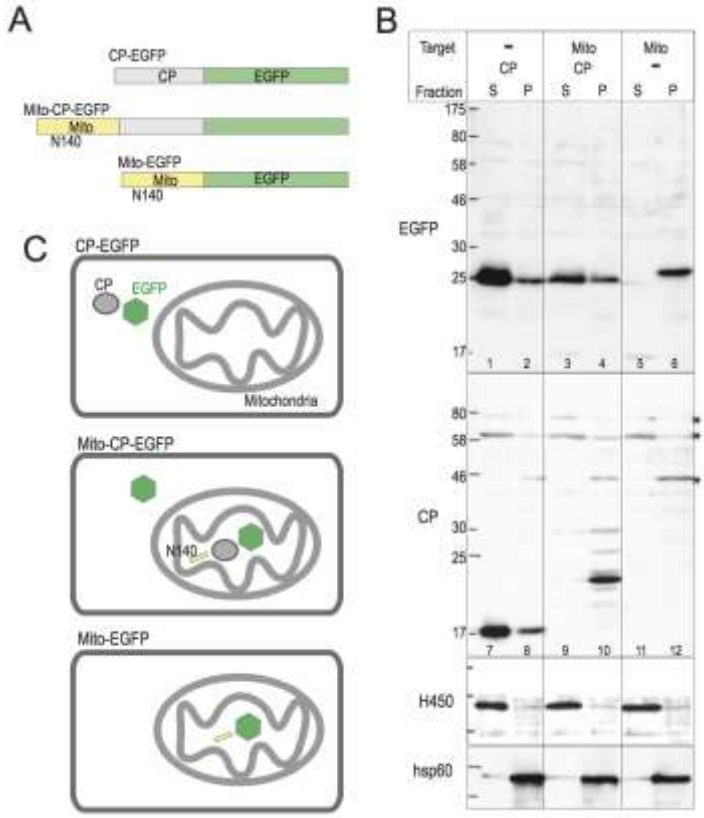

Figure 2. CP-domain folds before mitochondrial import

(A) Model proteins used. In the third model protein, the EGFP-domain was directly fused to N140. (B) The fusion proteins were transiently expressed in HeLa cells. Cytoplasmic fractions (S) were separated from membrane organelles (P) by the peeling method. The fractions were subjected to immunoblotting analysis. As controls for supernatant and precipitate, the H450 protein and mitochondrial hsp60 were probed, respectively. Nonspecific products are indicated (asterisks). (C) Summary of the localizations of the domains. In the absence of targeting signal (CP-EGFP), the CP-domain (gray oval) and EGFP-domain (green hexagon) were observed in the cytoplasm. In the presence of the mitochondrial targeting signal (Mito-CP-EGFP), the CP-domain was in the mitochondria, while large amounts of the cleaved EGFP domain were observed in the cytoplasm. In the absence of the CP-domain (Mito-EGFP), the EGFP domain located completely in the mitochondria.

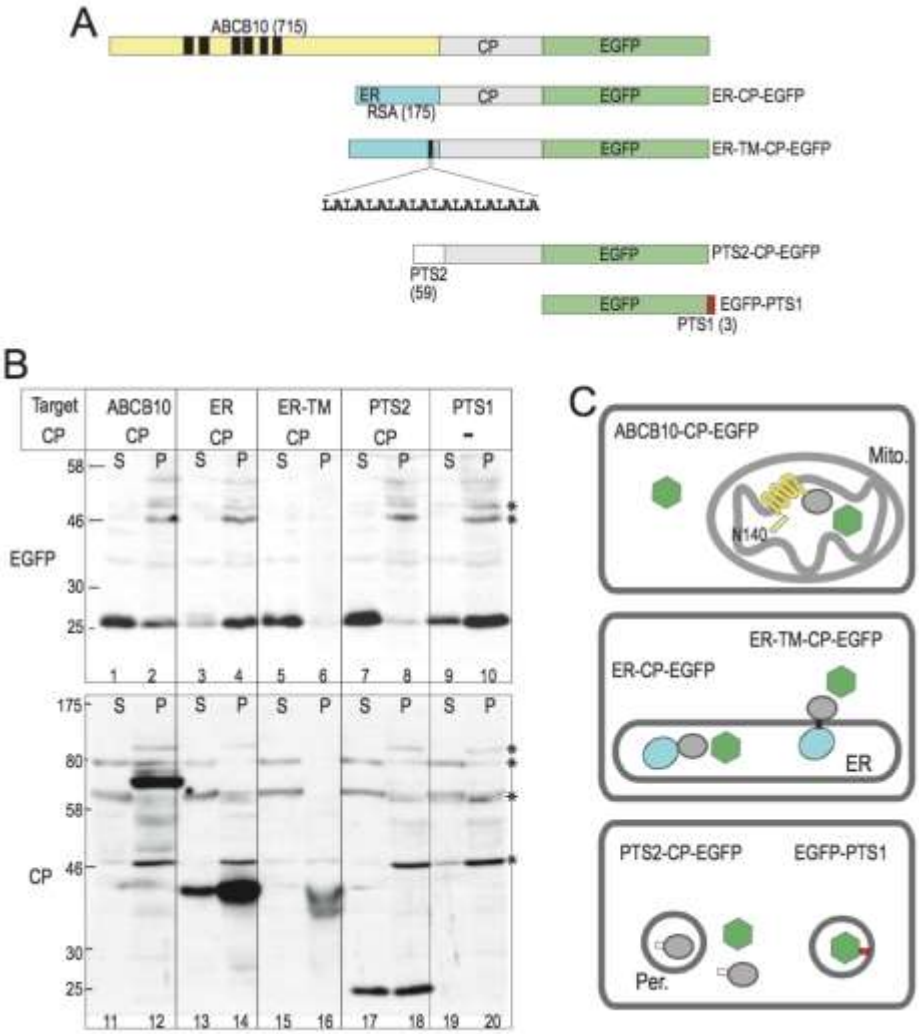

Figure 3 Import of mitochondrial multispanning membrane protein ABCB10 is not coupled to synthesis.

(A) Models used. Full-length ABCB10 was fused to the CP-EGFP probe. The postulated TM-segments are indicated (black rectangles). A hydrophobic TM-segment was included in ER-CP-EGFP (ER-TM-CP-EGFP). The 59-residue N-terminal portion of thiolase was fused to the probe (PTS2-CP-EGFP). As a control for peroxisomal EGFP, the PTS1 was directly fused to the C-terminus of EGFP (EGFP-PTS1). (B) The fusions were expressed in HeLa cells, separated into supernatant and precipitate fractions by the peeling method, and analyzed by

immunoblotting. Nonspecific bands are indicated (asterisks). (C) Summary of the localizations of the domains. A large amount of EGFP was cleaved in the cytoplasm, while almost all ABCB10-CP was observed in the cell organelles (ABCB10-CP-EGFP). The folded CP-domain might be retained outside the mitochondria. EGFP was translocated into the ER lumen when linked to the ER signal (ER-CP-EGFP). In contrast, when the TM-segment was included, the EGFP domain was observed in the cytoplasmic fraction (ER-TM-CP-EGFP). The N-terminal PTS2 targeted large amounts of the CP-portion into peroxisomes, while all of the EGFP was observed in the cytoplasm. PTS1 directed EGFP to the peroxisomes. EGFP (green hexagon), ABCB10 protein (yellow), RSA (blue oval), PTS2 (open box), PTS1 (red), and the CP-domain (gray oval) are indicated.

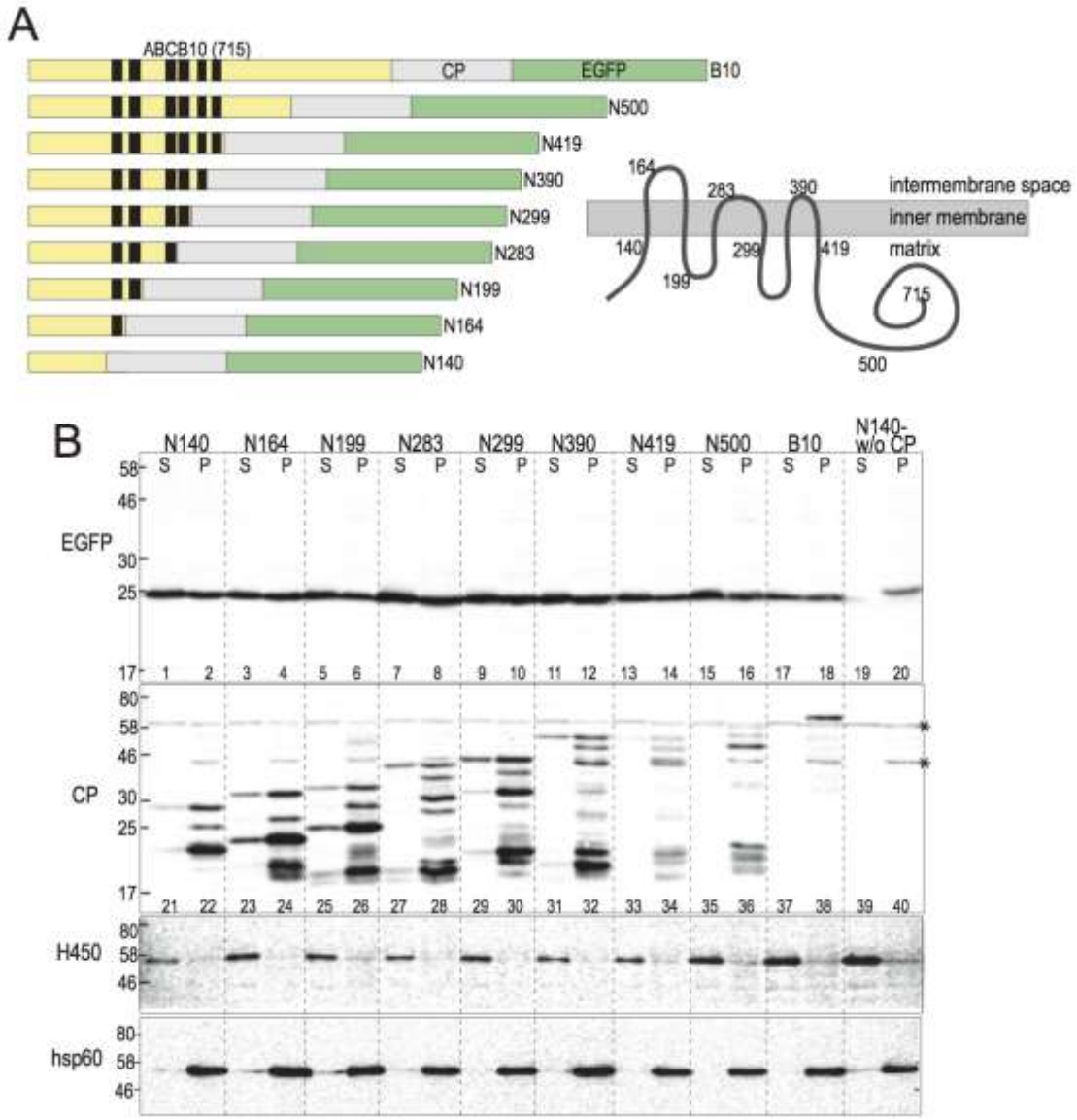

Figure 4 Every position of ABCB10 is retained in the cytoplasm before import

(A) The CP-EGFP probe was fused to various positions of ABCB10. The fusion site was located just after each TM-segment. The membrane topology is shown. Numbers indicate amino acid numbers at the fusion sites. (B) The constructs were expressed in HeLa cells. The supernatant and precipitate fractions were separated and probed by immunoblotting. Marker proteins of cytoplasm (H450) and mitochondria (hsp60) were probed. Nonspecific products are indicated (asterisks).

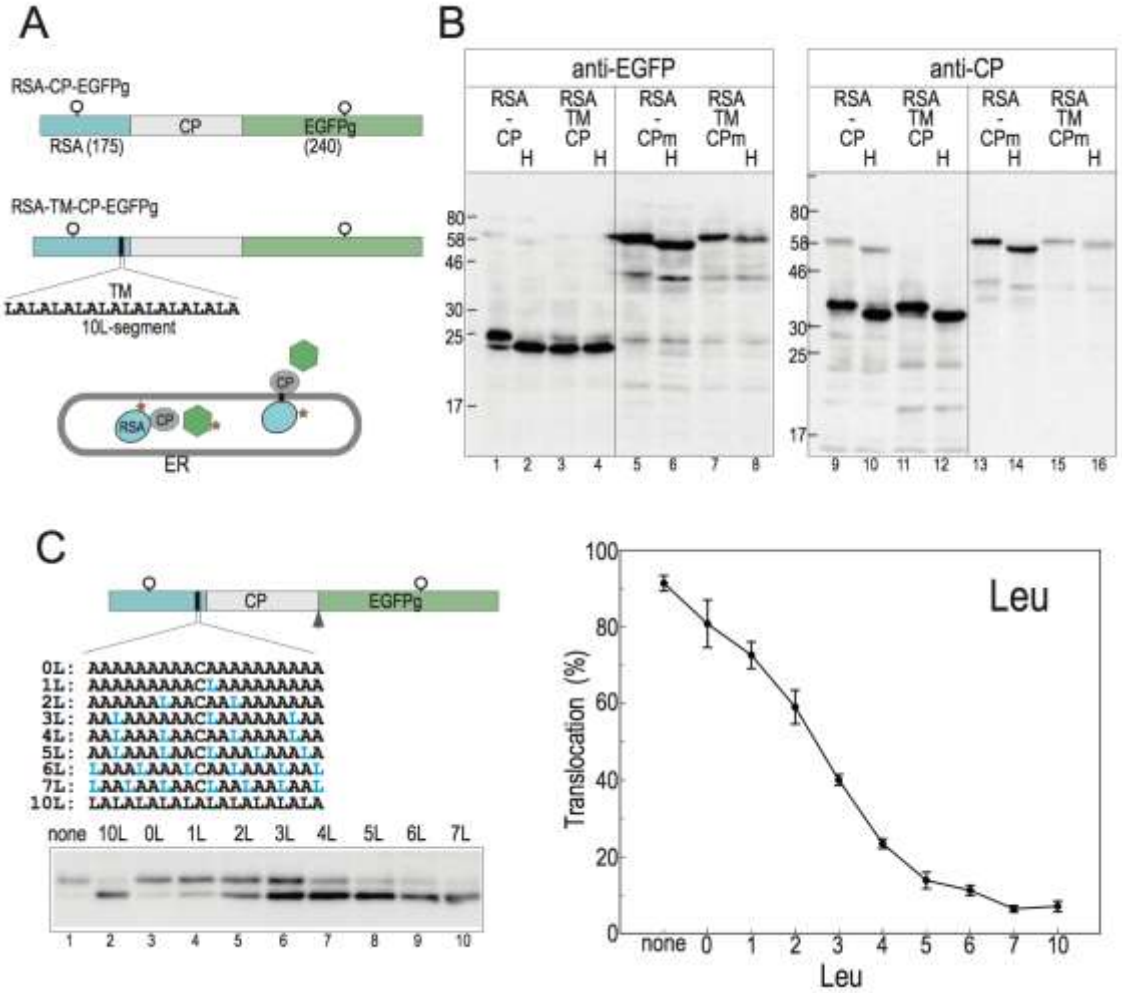

Figure 5 Hydrophobic segment induced stop-translocation at the ER translocon probed by CP-EGFPg

(A) A glycosylatable EGFP-domain (EGFPg) was used as a probe and a hydrophobic TM-segment (10L) was inserted before the CP-EGFPg. The TM-segment stopped at the translocon and allowed the CP-domain to fold on the cytoplasmic side of ER membrane. The potential glycosylation sites are indicated (circles). The ER translocated domains were glycosylated (stars) (B). The fusion constructs and their CP-inactive mutant versions (CPm) were expressed in COS cells and the expressed proteins were probed with anti-EGFP or anti-CP antibodies. Aliquots were treated with EndoH (H-lanes). (C) To examine the requirements of hydrophobic residues for

stop-translocation, Leu residues were serially changed to Ala. The constructs were named according to the numbers of Leu residues. After immunoblotting analysis, percent glycosylation was calculated. Mean and SD are presented.

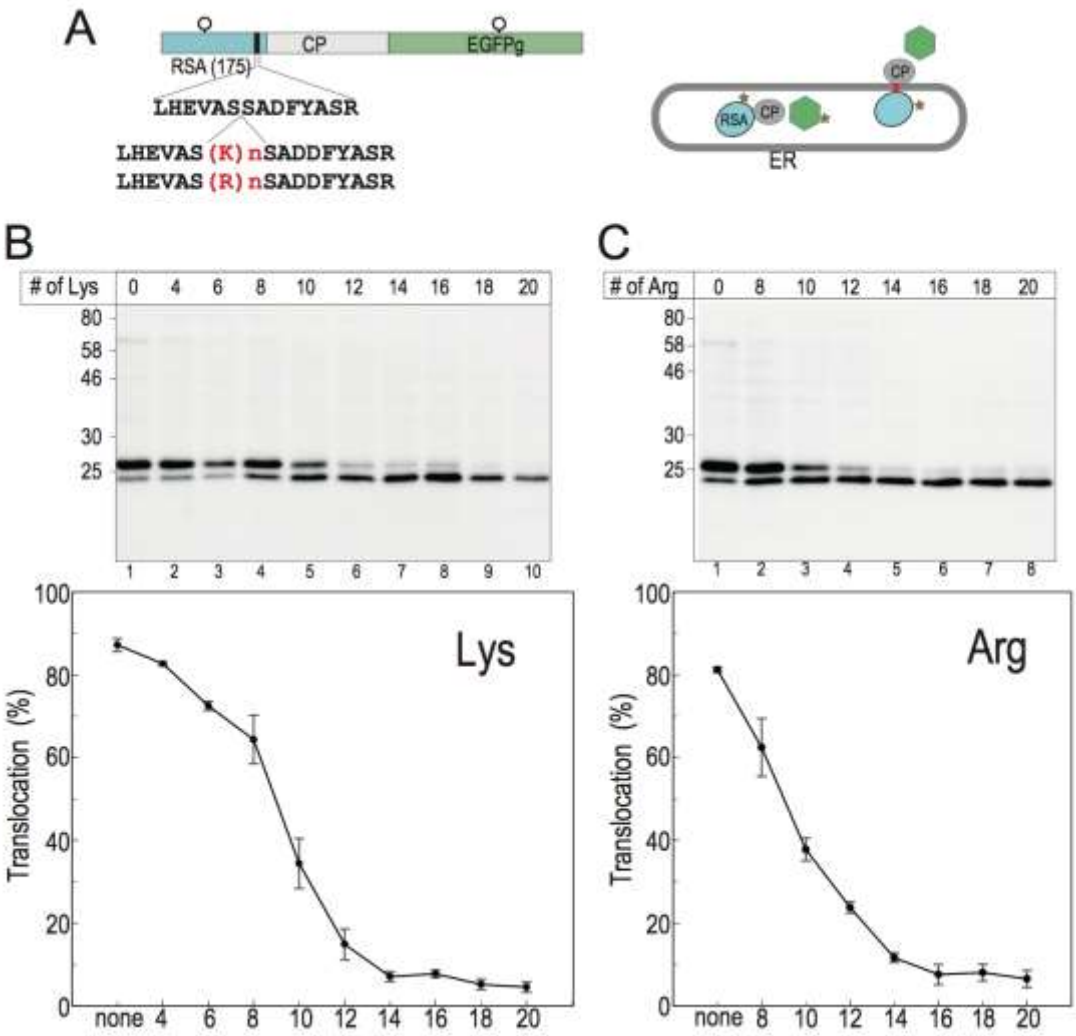

Figure 6 CP-EGFPg estimates translocation arrest at the ER translocon by positive charges in living cells

(A) A cluster of positively-charged residues was inserted before the CP-domain. According to the translocation arrest effect, the CP-domain would fold at the cytoplasmic surface and cleave off the EGFP domain on the cytoplasm side. (B) The constructs were expressed in COS cells for 24 h and the EGFPg-domain was probed with anti-EGFP antibodies. The glycosylated and unglycosylated forms of the EGFP-domain were quantitated and percent translocation was calculated. Mean and SD are provided.

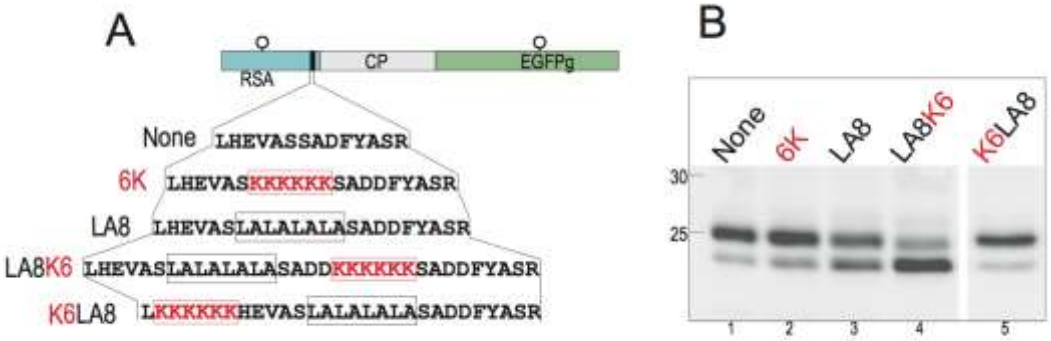

Figure 7 Cooperation between marginally hydrophobic segment and positive charges in living cells

(A) A positive-charge cluster (6K) and/or marginally hydrophobic segment (LA8) were inserted before the CP. (B) The model proteins were transiently expressed in COS7 cells and EGFP in the cells was probed by immunoblotting with anti-EGFP antibodies.

6. Discussion

Here we described the development and application of a unique probe (CP-EGFP) to examine the folding of a protein domain during organelle import. Experiments using this probe demonstrated that import of a highly hydrophobic multispanning membrane protein (ABCB10) into mitochondria is not so tightly coupled with ribosomal elongation. The CP-domain can be active in the cytosol before import. On the other hand, cotranslational translocation into the ER does not allow the CP-domain to fold before translocation. These findings are consistent with the notion that ER targeting and translocation of proteins are tightly coupled to polypeptide chain elongation by the bound ribosome. The probe can also be used to monitor the stalling of cotranslational ER translocation. We estimated the threshold of the hydrophobic segment for the stop-translocation at the ER translocon in living cells. Further, we provided in vivo evidence that the positive-charge cluster of the nascent chain arrests translocation through the ER translocon and that the positive-charge cluster cooperates with a marginally hydrophobic segment to stop translocation.

The CP-domain can become active in the cytosol, the ER lumen, and the mitochondrial matrix, and on the cytoplasmic surface of the ER membrane. If the CP-domain folds in the cytosol, it generates an apparent EGFP footprint. Using the peeling method to separate cytosolic soluble proteins from cell membrane organelles, free CP and EGFP were largely recovered in the cytosol. When the CP-EGFP probe was fused to the ER targeting sequence, both EGFP and CP were located in the organelles, indicating that the CP does not fold during ER translocation. With the N-terminal mitochondrial presequence, the CP-domain can fold more than 50% of the way before import into the mitochondria. With the N-terminal

peroxisome-targeting signal (PTS2), the CP-domain was almost completely cleaved from the EGFP-domain in the cytosol, and largely located in the organelle. Although the upstream sequence was artificially changed, the cleaved EGFP was similarly stable. We could thus assess the translocation behavior of systematically designed sequences.

In vivo analysis of the stop-translocation at the ER translocon with the CP-EGFP probe estimated the hydrophobicity required for 50% stalling of ER translocation (Fig. 5). The required number of leucine residues for the stop-translocation was essentially the same as that determined by pulse-labeling experiments in HeLa cells, using model proteins, including systematically constructed model proteins (41). The developed probe allows for quantitative estimation of the effect of the nascent chain sequence on translocation behavior.

The CP-probe provided in vivo evidence indicating that the cluster of positively-charged residues stalls at the ER translocon. Using a cell-free system, we demonstrated that 3 to 4 lysine residues slow down the movement of the nascent chain by several 10s of seconds, which then moves forward (7,8,42). Here we obtained in vivo evidence that 8 to 9 lysine residues are required to allow enough time for the CP-domain to elongate, fold, and exert activity on the cytoplasmic surface of the ER membrane.

The folding probe would distinguish the translocation mode. In budding yeast, both cotranslational and posttranslational modes are operative even in the ER. The modes have been distinguished based on the requirements of the signal recognition particle (4). The CP-EGFP would be a critical probe for distinguishing the targeting processes in yeast. On the other hand, a cotranslational mode of mitochondrial import has been suggested. Various mRNAs are enriched on the mitochondrial

surface (13,14). Site-specific ribosome profiling demonstrated that mRNAs encoding the mitochondrial inner membrane and matrix are enriched on the mitochondrial surface with their nascent chain (16). Further, the nascent chain bound to ribosomes can stack at the ribosome translocator and suppress translocator function (17). These previous findings strongly suggest that import of the nascent chain into the mitochondria occurs cotranslationally. On the other hand, the present findings suggested that the import of even such a hydrophobic protein as ABCB10 was not as tightly coupled to polypeptide chain elongation as the ER. The CP-EGFP probe would clarify the details of coupling between the ribosome and various organelle translocators.

The dihydrofolate reductase domain has been used as a probe to monitor protein domain-folding before organelle import. When fused to the N-terminal mitochondrial presequence, it folds and can bind to a substrate analog inhibitor (methotrexate). The binding stabilizes the fold and inhibits import into the mitochondria (43) . When fused to the N-terminus of the signal-anchor sequence with N_{out}/C_{in} topology, the reductase domain can be translocated through the ER translocon (44,45). Translocation is also inhibited by methotrexate not only in a cell free system but also in cultured cells (44). In both organelle membranes, the protein domains fold before translocation and should be unfolded for translocation through the translocators of the mitochondrial (43) and ER membranes (44,45). Observations with the reductase domain could, however, be greatly affected by the stability of the fusion partner. In contrast, once the CP-domain folded and cleaved the EGFP-domain, the EGFP-probe remained stable despite the variety of preceding sequences. Glycosylation in the ER is easily detected and quantified, and thus this newly developed probe provides more sensitivity, convenience, and

versatility than the reductase domain.

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