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Pleiotropic Effects of Membrane Cholesterol upon Translocation of Protein across the Endoplasmic Reticulum Membrane

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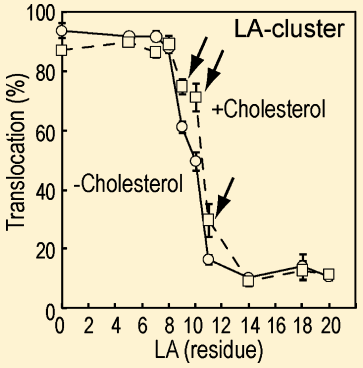
ABSTRACT: Various proteins are translocated through and inserted into the endoplasmic reticulum membrane via translocon channels. The hydrophobic segments of signal sequences initiate translocation, and those on translocating polypeptides interrupt translocation to be inserted into the membrane. Positive charges suppress translocation to regulate the orientation of the signal sequences. Here, we investigated the effect of membrane cholesterol on the translocational behavior of nascent chains in a cell-free system. We found that the three distinct translocation processes were sensitive to membrane cholesterol. Cholesterol inhibited the initiation of translocation by the signal sequence, and the extent of inhibition depended on the signal sequence. Even when initiation was not inhibited, cholesterol impeded the movement of the positively charged residues of the translocating polypeptide chain. In surprising contrast, cholesterol enhanced the translocation of hydrophobic sequences through the translocon. On the basis of these findings, we propose that membrane cholesterol greatly affects partitioning of hydrophobic segments into the membrane and impedes the movement of positive charges.



Several diverse secretory and membrane proteins are translocated across and inserted into the membrane of the endoplasmic reticulum (ER).[1] Nascent chains elongating from ribosomes are targeted to the translocation channel together with the synthesizing ribosomes. Just after the signal sequence emerges from the ribosome, it is recognized by the signal recognition particle and the signal recognition particle−ribosome−nascent chain complex is targeted to the ER.[1−3] This is the default pathway of hydrophobic transmembrane (TM) segments when there is no regulatory sequence on the upstream polypeptide chain.[4,5] The signal sequence is then released from the particle by its receptor and transferred to the translocon.[6−8] The hydrophobic segment penetrates the translocon to initiate translocation. In eukaryotes, the main part of the translocon comprises a Sec61 complex.[8] X-ray structures of the archaeal and bacterial SecY complex, a homologue of the Sec61 complex, suggest that the translocation pore is surrounded by 10 TM helices of the SecY molecule,[9,10] providing an aqueous environment through which a variety of hydrophilic polypeptides can be translocated. The pore appears to open laterally to allow hydrophobic TM segments of the translocating polypeptide chain to exit into the membrane lipid environment.[8]

Signal sequence functions are mainly determined by their hydrophobic segment, and their orientations are modulated by the flanking positively charged residues.[11−13] Signal sequences are categorized into two types based on the membrane orientation. The type I signal anchor (SA-I) causes translocation of the upstream portion, forming an N(lumen)−C(cytosol) orientation, while the type II signal anchor (SA-II) and the signal peptide cause translocation of the downstream portion, forming a C(lumen)−N(cytosol) orientation. In both cases, insertion of the hydrophobic segment of the signal sequence into the translocon is a common key process for initiation of translocation. The positive charges are retained on the cytoplasmic side of the membrane, and the less positive side is eventually positioned in the lumen. The signal sequences not only trigger membrane targeting but also provide a significant translocation motive force.[14] In the case of SA-I, N-terminal translocation is synchronized with partitioning of the hydrophobic segment into the lipid environment.[15] The motive force depends on the amino acid sequence of the hydrophobic TM segment. Hydrophobic interaction via partitioning initiates translocation. Translocation of an elongating polypeptide chain can be pushed by protein synthesis at the ribosome.

After the initiation step, movement of the polypeptide chain through the translocon is restricted by the hydrophobic sequence and positive charges. These residues are termed translocation-resistor sequences. Hydrophobic transmembrane sequences of the polypeptide chain stop the movement and insert into the lipid bilayer. Hydrophobicity is the primary determinant of stop translocation.[16,17] Simple hydrophobic partitioning between the aqueous and hydrophobic environment causes membrane insertion.[16−19] In some cases, a marginally hydrophobic sequence stops translocation depending on the downstream positive charges.[20] Such cooperation between the hydrophobic segment and positive charges occurs even when they are separated by more than 60 amino acid residues: a positively charged residue cluster causes stop translocation of a marginally hydrophobic segment that is located more than 60 residues upstream.[20,21] The positive charges arrest translocation even in the absence of a

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hydrophobic segment, indicating that the positive charges are translocation regulators that are independent of the hydrophobic sequences.[22]

Regardless of the importance of a hydrophobic interaction between the TM helix and the lipid bilayer in membrane protein topogenesis, there are few studies of the effect of membrane lipid components on protein translocation. Cholesterol supplementation of rough microsomal membrane (RM) vesicles inhibits targeting of a leader peptidase to the translocon, and the overall integration of leader peptidase B (LepB) protein is inhibited.[23] In *Escherichia coli*, the phosphatidylethanolamine content significantly affects the orientation of transporter proteins such as lactose permease, phenylalanine permease, and γ-aminobutyric acid permease.[24−27]

Here, we performed a detailed study of the effect of cholesterol on translocon function. Cholesterol greatly inhibited translocation of the C-terminal domain of LepB, as reported by Nilsson et al.[23] It also inhibited initiation of translocation by SA-I. In clear contrast, it had little inhibitory effect on the initiation of translocation by a signal peptide of rat serum albumin (RSA). The cholesterol resistance of the RSA signal peptide allowed us to examine the effects of cholesterol on the subsequent processes. Cholesterol greatly inhibited the movement of positive charges, whereas it enhanced the movement of hydrophobic segments toward the lumen. Cholesterol affects various translocon functions, initiation of translocation by the signal sequence, movement of the positive charges, and movement of marginally hydrophobic sequences.

EXPERIMENTAL PROCEDURES

Chemicals and Plasmids. Cholesterol, methyl-β-cyclodextrin (MCD), biotin, and cycloheximide (CHX) were purchased from Sigma-Aldrich. Streptavidin (SAv) was purchased from Wako pure chemicals. Proteinase K (ProK) was purchased from Merck Inc. Plasmids harboring model proteins were previously described.[20,28] Dog pancreas RM[29] and rabbit reticulocyte lysate[30] were prepared as previously described. LepB was previously described.[23]

Supplementation of Microsomes with Cholesterol. To supplement the microsomes with cholesterol, a complex of cholesterol and MCD was prepared as described previously[23] with a slight modification. Cholesterol (1 mg) was dissolved in 500 μL of chloroform. The solution was dried in a glass test tube under a nitrogen gas stream to make a thin film of cholesterol. One milliliter of an MCD solution [containing 26 mg of MCD, 0.25 M sucrose, and 20 mM HEPES-KOH (pH 7.5)] was added to the cholesterol film. The molar ratio of cholesterol to MCD was 1:8. The cholesterol was extensively dissolved by sonication in a bath sonicator and vortexed until the turbid solution became clear. The cholesterol−MCD complex was frozen until it was used. Microsome vesicles were incubated with the indicated amount of the cholesterol−MCD complex (see figures) at 30 °C for 10 min. The RM supplemented with cholesterol was stocked in the freezer. The translation mixture also included the same amounts of the cholesterol−MCD complex.

Measurement of the Cholesterol Content of the RM. After translation, the RM was sedimented by ultracentrifugation at 100000*g* for 10 min. Cholesterol was extracted with a chloroform/water mixture [1:1 (v/v)]. The organic phase was collected and evaporated to dryness. The extracted cholesterol was dissolved in chloroform, applied to a high-performance thin-layer chromatography plate, and separated with a hexane/diethyl ether/acetic acid mixture [130:30:2 (v/v/v)]. We visulalized the choleterol spots by staining the plates with cupric acetate [3% (w/v) and 8% (w/v) H_3PO_4] and heating the plates for 15 min at 150 °C to develop the color. The chromatogram was recorded with a CanoScan 9950F image scanner (Canon) and quantified with ImageJ version 1.45s (National Institutes of Health). RMs corresponding to 46 μg of total RM protein were assayed for cholesterol determination. The cholesterol level in samples treated with or without 0.1 μg/μL cholesterol was found to be 2.6-fold in the treated sample [mean of three experiments (data not shown)].

In Vitro Transcription and Translation. In vitro protein synthesis and translocation experiments were performed essentially as previously described.[31] Briefly, mRNAs were synthesized by in vitro transcription using T7 RNA polymerase. Template plasmids were linearized with AflII for SytII derivatives, XhoI for RSA derivatives, and BamHI for LepB. The mRNA for RSA models and LepB included the termination codon, but the mRNA for SytII-related models did not contain an in-frame termination codon and ended at Arg^{200}.

The translation reaction solution consisted of 32% reticulocyte lysate and 15.5 kBq/mL EXPRESS protein-labeling mix (Perkin-Elmer). The mRNAs were translated in the absence or presence of the RM at 30 °C for 20−60 min. ^{35}S-labeled proteins were detected with Bioimage Analyzer (Fuji, BAS1800). The percentage of diglycosylation was calculated using the following formula: (diglycosylated form) × 100/[(nonglycosylated form) + (monoglycosylated form) + (diglycosylated form)].

For the translocation chase experiments, translations with or without the cholesterol−MCD complex (final concentrations of 0.1 μg/μL cholesterol and 2.6 μg/μL MCD) were performed for 20 min, terminated with 2 mM CHX, and then chased for the indicated time periods. An aliquot was treated with ProK (final concentration of 0.2 mg/mL) for 60 min at 0 °C, and the reaction was terminated via addition of 15% trichloroacetic acid. For posttranslational addition of cholesterol, the translation reactions were performed in the presence of SAv (1 mg/mL) for 60 min and terminated with 2 mM CHX. The cholesterol−MCD complex (final concentrations of 0.1 μg/μL cholesterol and 2.6 μg/μL MCD) was then added, and the solution was incubated for 10 min at 30 °C to equilibrate the RM with cholesterol. After incubation, 2 mM biotin was added to induce translocation of the N-terminal portion of the model proteins and aliquots were sampled at the indicated times.

SytII derivatives were subjected to 15% trichloroacetic acid precipitation before sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The protein precipitates were washed with acetone, dissolved in sample buffer containing RNaseA to remove the tRNA moiety (1 mg/mL), and incubated for 10 min at 30 °C and for 5 min at 95 °C.

Calculation of the Translocation Percentage for RSA Models. The fully translocated molecule was expected to be diglycosylated and resistant to ProK. A significant amount of the monoglycosylated form was ProK-resistant, indicating that some of the monoglycosylated form was fully translocated but still remained monoglycosylated. To calculate the percentage of translocation, we quantified the diglycosylation percentage (p) within ProK-resistant polypeptides. The translocation percentages were calculated using the following formula: [diglycosylated form] × p^{-1}/([monoglycosylated form] + [diglycosylated form]).

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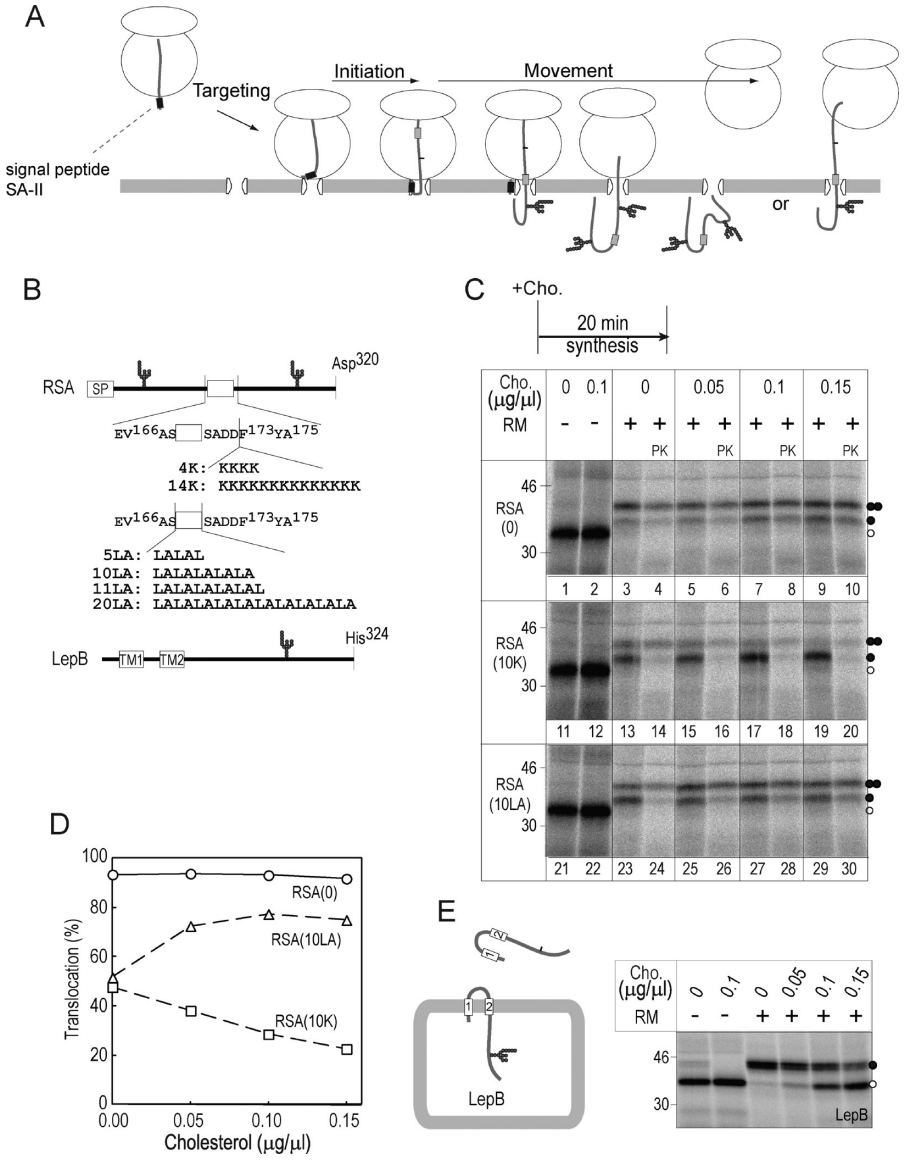

Figure 1. Cholesterol inhibits translocation of the 10K cluster and enhances translocation of the hydrophobic segment. (A) Stages of translocation of the nascent polypeptide chain induced by a signal peptide (black rectangle). During targeting, the nascent chain is targeted to the ER translocon with a ribosome. During initiation, the signal peptide initiates translocation of the downstream portion, forming the transmembrane orientation. During movement, the downstream polypeptide chain moves through the translocon. The movement is affected by the hydrophobic segment and/or positive charges on the chain (gray rectangles). The polypeptide chain in the lumen acquires sugar chains (forks). When the polypeptide chain is stalled at the translocon, only the upstream region is glycosylated. When the peptide is fully translocated, upstream and downstream portions acquire sugar chains in the lumen. (B) Model proteins used. Model protein comprising an RSA sequence containing the N-terminal signal peptide (SP), two glycosylation sites (forks), and a termination codon after Asp^{320} of RSA.[20] A cluster of lysine (K) or a Leu and Ala mixed sequence was inserted in the middle portion. Examples of the inserts are shown. *E. coli* leader peptidase B (LepB) possesses two transmembrane segments, and the C-terminal domain is translocated into the lumen. (C and D) Effect of cholesterol on translocation. The model proteins are translated in the absence (−) and presence (+) of the RM supplemented with the indicated amounts of the cholesterol−MCD complex. After translation, aliquots were treated with ProK (PK lanes). The nonglycosylated (○), monoglycosylated (●), and diglycosylated (●●) forms are denoted. Each glycosylated form before and after ProK treatment was quantified. Percentages of translocation were calculated. These data are listed in Table 1. The values are averages of two independent experiments. (E) Effect of cholesterol on the insertion of LepB. Translocation of the C-terminal domain was monitored on the basis of glycosylation (fork).

RESULTS

Cholesterol Inhibits the Movement of Positive Charges but Does Not Inhibit the Initiation of Translocation by the RSA Signal Peptide. The translocation of secretory proteins comprises three distinct steps: targeting to the translocon of the nascent chain together with the ribosome, initiation of translocation, and subsequent chain movement (Figure 1A). To examine the effect of membrane cholesterol on these processes, we supplemented RM vesicles with cholesterol and quantitatively examined the translocation of systematically designed model proteins in a cell-free translation system. The models comprised 320 residues of RSA as a backbone, which included the N-terminal signal peptide, various insertions in the middle portion, and glycosylation sites upstream and downstream of the inserted sequences (Figure 1B). The inserted

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Table 1. Band Intensities and Percentages of Translocation in Figure 1C[a]

		no treatment			proteinase K treatment			
		band intensity			band intensity			
model	[cholesterol] (μg/μL)	monoglycosylated	diglycosylated	% diglycosylated	monoglycosylated	diglycosylated	% diglycosylated	% translocation
RSA(0)	0.00	1690	4187	71	880	2845	76	93
	0.05	1441	2826	66	950	2295	71	94
	0.10	2345	3461	60	1662	2966	64	93
	0.15	2983	3621	55	2021	3014	60	92
RSA(10K)	0.00	2462	1415	36	342	1120	77	48
	0.05	2672	1000	27	280	702	71	38
	0.10	3401	822	19	288	614	68	29
	0.15	3041	513	14	201	356	64	23
RSA(10LA)	0.00	2134	1324	38	356	1023	74	52
	0.05	1588	1745	52	520	1356	72	72
	0.10	1685	1795	52	802	1610	67	77
	0.15	1740	1652	49	858	1599	65	75

[a]Values are averages of two independent experiments.

sequences comprised positively charged or hydrophobic amino acid residues. The model proteins were named according to the numbers of inserted amino acids; e.g., RSA(10K) contains a cluster of 10 lysine residues (10K cluster), and RSA(10LA) contains an insertion of a 10-residue Leu and Ala mixed sequence (Figure 1B). The models were expressed in the cell-free transcription and translation system in the presence of the RM. To supplement the RM vesicles with cholesterol, we utilized MCD as a carrier as previously described.[32] The translocation behavior of the model proteins was monitored on the basis of the glycosylation status (Figure 1A). If translocation was stalled at the inserted sequence, only the N-terminal glycosylation site was glycosylated and the product was monoglycosylated. If the polypeptide was fully translocated, both of the glycosylation sites were glycosylated. In some cases, the glycosylation of the second glycosylation site was affected by cholesterol. In these cases, the inhibition was taken into account to estimate the percentage of translocation (Figure 1C and Table 1).

Prior to examination of the RSA models, we confirmed the inhibitory effect of cholesterol on the membrane insertion of *E. coli* LepB (Figure 1E). The LepB model protein possesses two TM segments and a potential glycosylation site in the large C-terminal domain. When synthesized in the presence of RM, the product was efficiently glycosylated, indicating that the C-terminal domain was translocated into the lumen. When the RM was supplemented with cholesterol, translocation of the C-terminal domain was largely suppressed, as previously reported.[23]

An RSA model without insertions [RSA(0)] was translated in the presence of the normal RM for only 20 min. In the in vitro system used here, 12−15 min was required for translation of the full-length model protein.[22] The majority of the products were 4 kDa larger than the product synthesized in the absence of RM, indicating diglycosylation of the product (Figure 1C). When the translation products were treated with ProK, the diglycosylated form was resistant and the monoglycosylated form was largely digested, indicating that the diglycosylated and monoglycosylated forms represented the fully translocated polypeptide and translocation-interrupted polypeptide, respectively.

With cholesterol supplementation, the level of the monoglycosylated form of RSA(0) substantially increased in a concentration-dependent manner. Cholesterol supplementation also resulted in an increase in the level of the ProK-resistant monoglycosylated form, indicating that cholesterol inhibited glycosylation by the oligosaccharyl transferase in the lumen and some populations of fully translocated molecules remained monoglycosylated. Translocation efficiency (percent) was therefore calculated by taking into account the ProK-resistant monoglycosylated form (for details, see Experimental Procedures). Quantified data indicate that translocation of RSA(0) after a 20 min translation was not affected by cholesterol (Figure 1D). In all cases, the model proteins were monoglycosylated or diglycosylated, and nonglycosylated forms were not observed, indicating that at least an upstream portion has been in the lumen. In contrast to the case of LepB, cholesterol did not inhibit membrane targeting, initiation of translocation by the signal peptide of RSA, or chain movement of the RSA polypeptide.

To examine chain movement with the positive charges in the RSA context, the 10K cluster was inserted in the middle of the model peptide. When the peptide was translated in the presence of the RM for 20 min, the major product was 2 kDa larger than the nonglycosylated form, indicating that the majority of the product was stalled at the translocon and the product was monoglycosylated (Figure 1C). The level of the diglycosylated form was decreased in a cholesterol-dependent manner. The movement of RSA(10K) was inhibited by cholesterol. Again, glycosylation of the upstream portion was not inhibited at all. Even under these conditions, the nonglycosylated form was not observed. The first glycosylation site is an efficient substrate of oligosaccharyl transferase.

When examined with model proteins possessing various K clusters (Figure 2A), the percentage of translocation was dependent on the residue numbers, as recently reported.[22] Translocation of the 4K and 6K clusters was not affected by cholesterol as was the original RSA polypeptide chain, whereas the inhibitory effect of cholesterol was apparent with the 8K, 10K, and 12K models.

Cholesterol Enhances the Translocation of Marginally Hydrophobic Segments. We then examined the effect of cholesterol on the movement of hydrophobic sequences (Figure 1C,D). A 10-residue mixed LA sequence cluster was inserted into the middle portion of the model peptide. After the 20 min translation, the product was mainly monoglycosylated. No nonglycosylated forms were observed. In contrast to the inhibitory effect on the translocation of the K cluster, translocation of the 10LA segment was rather enhanced by

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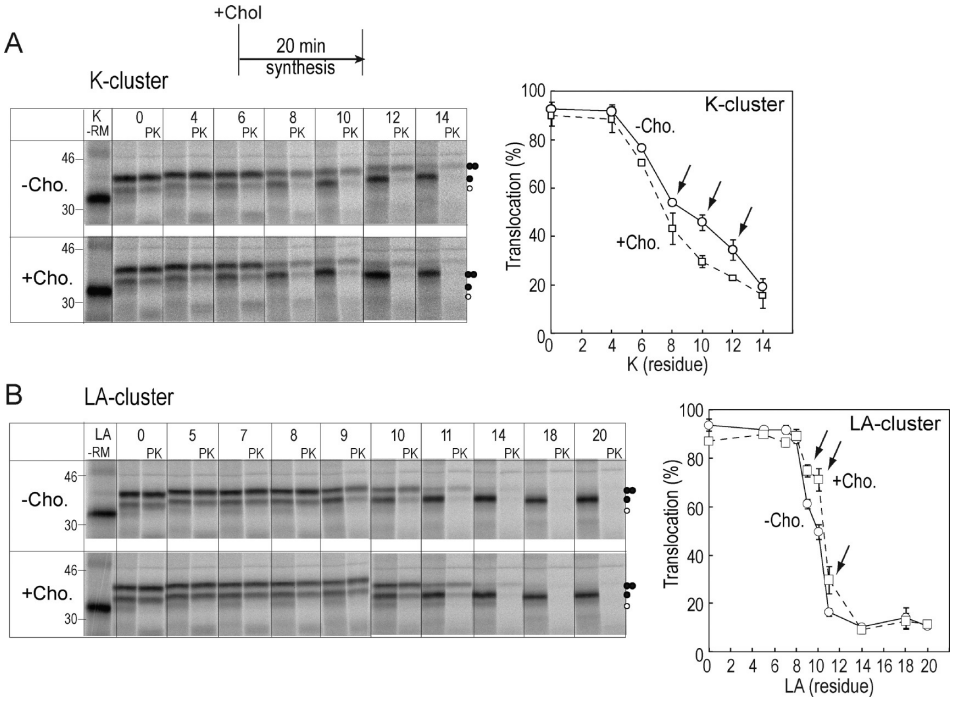

Figure 2. Effect of cholesterol on the movement of positive and hydrophobic residues. Model proteins with the K cluster (A) or LA cluster (B) of the indicated residue numbers are translated for 20 min. Where indicated, the RM was supplemented with cholesterol (final concentration of 0.1 μg/μL). Percentages of translocation were calculated. Values were calculated from three independent experiments, and the means are shown with the standard deviation. Points with a significant effect of cholesterol are denoted (arrows).

cholesterol. The enhancement was reproducibly observed with the 9LA, 10LA, and 11LA segments (Figure 2B).

Cholesterol Delays the Posttranslational Movement of the 10K Cluster. Positively charged clusters, such as the 10K cluster, of the translocating polypeptide chain tentatively stalled on the cytoplasmic side of the membrane and then moved forward (Figure 3A).[20,22] In this case, the polypeptide chain moved across the membrane in the absence of the pushing force of chain elongation. We examined the effect of cholesterol on the movement of the 10K cluster after the chain had been fully synthesized (Figure 3B). The 10K model was translated for 20 min in the presence of the RM; chain elongation was terminated with CHX, and then the translocation was chased for the indicated periods. After a 20 min translation, the majority of the nascent chain was still in the monoglycosylated form, which was sensitive to ProK. The accumulated monoglycosylated form was converted to the diglycosylated form with an increased chase time. The conversion to the diglycosylated form was apparently slowed by cholesterol supplementation. Cholesterol supplementation clearly inhibited the posttranslational movement of the 10K cluster.

Similar translocation chase experiments were performed with the RSA(10LA) model (Figure 3C). The diglycosylation levels obtained with the cholesterol-supplemented RM were higher than those obtained with the normal RM. In the absence of cholesterol, the percentage of translocation gradually increased during the chase. Half-times for the 10K cluster without cholesterol, the 10K cluster with cholesterol, the 10LA segment without cholesterol, and the 10LA segment with cholesterol were estimated to be 9, 19, 41, and 25 min, respectively. These data confirmed our conclusion that cholesterol supplementation enhanced translocation of the 10LA segment and inhibited the translocation of positive charges.

Cholesterol Affects Translocation of the Upstream Region of SA-I. SA-I induces translocation of the upstream polypeptide chain (Figure 4A). We next examined the effect of cholesterol on translocation. The models included a backbone of mouse synaptotagmin II (SytII), two potential glycosylation sites, a 38-residue spacer sequence, and a streptavidin binding peptide tag (SBP tag) (Figure 4B, SBP-38-SytII). The distance between glycosylation sites was 76 residues. In this context, the second glycosylation site is accessible to the glycosylation enzyme even when the N-terminus is fixed on the cytoplasmic side.[28] To examine the effect of a positive charge, a 4K cluster was included just after the SBP tag (SBP-4K-38-SytII). The mRNA encoding these models was truncated at Arg^{200} of SytII to construct a ribosome nascent chain complex, where the nascent chain is retained in the ribosome as a peptidyl tRNA and the N-terminal region was translocation-competent in this cell-free system.

When translated for 60 min in the presence of the normal RM, the SBP-38-SytII model was largely diglycosylated (Figure 4C). With cholesterol supplementation, significant amounts of the nonglycosylated form accumulated, whereas no monoglycosylated form was observed, indicating that cholesterol inhibited the initiation of translocation; movement after the initiation of translocation was not affected. Thus, the initiation of translocation became a rate-limiting step in the presence of cholesterol. This result contrasts with that of RSA models, in which the initiation of translocation was barely inhibited by cholesterol supplementation.

In the presence of the 4K cluster (SBP-4K-38-SytII), the main product was largely diglycosylated after the 60 min translation reaction. Upon cholesterol supplementation, both the monoglycosylated form and the nonglycosylated form accumulated. The nonglycosylated form mainly accumulated with a higher concentration of cholesterol (Figure 4C, lanes 9−12).

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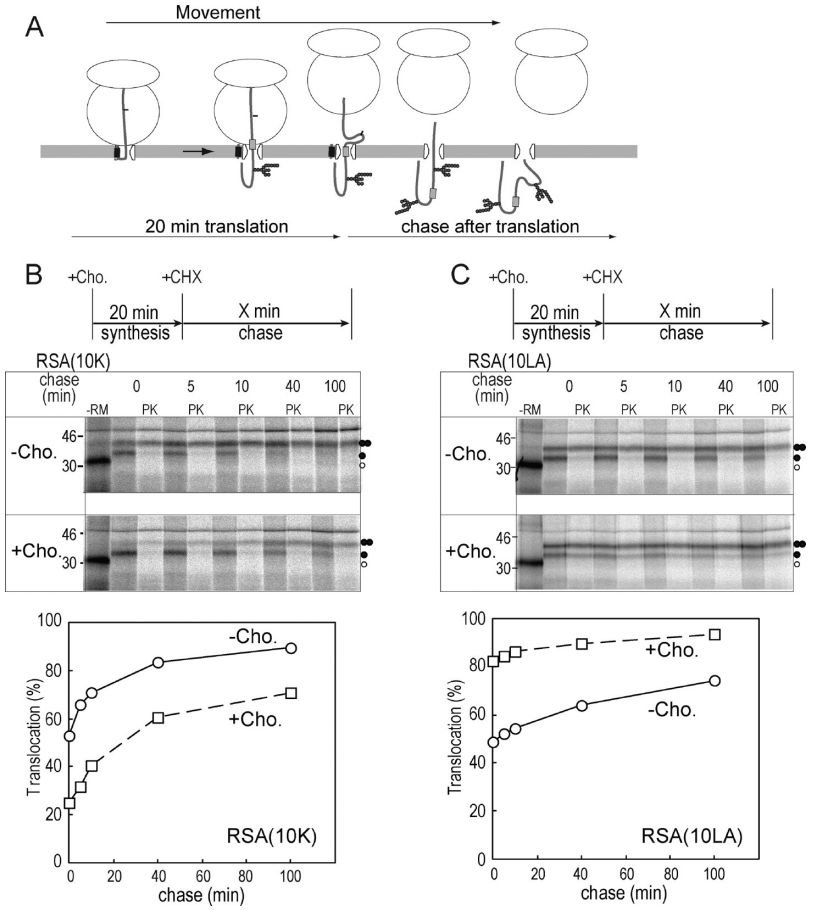

Figure 3. Effect of cholesterol on the posttranslational movement of the 10K cluster and 10LA segment. (A) A positively charged cluster (gray rectangles) of the translocating polypeptide chain stalls at the membrane and moves after translation. The stalled polypeptide is the monoglycosylated form, and the fully translocated form is diglycosylated. (B and C) Effect of cholesterol on the posttranslational movement of the 10K cluster and 10LA segment. The RSA(10K) and RSA(10LA) models were translated in the presence of RM for 20 min. Translation was terminated by CHX, and the translocation was chased for the indicated periods. The RM was supplemented with cholesterol (final concentration of 0.1 $\mu g/\mu L$). Aliquots were sampled at the indicated time points and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Aliquots of each sample were treated with ProK (PK). The percentage of translocation was calculated as described in Experimental Procedures. Values are averages of two independent experiments. We estimated the half-time of the translocation rate by fitting the equation $y = A - B \exp(-Cx)$, where y is the percentage of translocation, x is the time, and A, B, and C are fitting parameters. We found that half-times for the 10K cluster without cholesterol, the 10K cluster with cholesterol, the 10LA segment without cholesterol, and the 10LA segment with cholesterol are 9, 19, 41, and 25 min, respectively.

Movement of the 4K cluster became a rate-limiting step at low cholesterol concentrations, and the initiation of translocation became a rate-limiting step at higher cholesterol concentrations. Cholesterol supplementation, therefore, affected the SA-I-mediated initiation of translocation as well as movement of the 4K cluster.

We then chased the movement after a short translation (Figure 4D). The models were translated for 40 min; elongation was terminated with CHX, and translocation was chased. Cholesterol caused an accumulation of the nonglycosylated form of SBP-38-SytII. The nonglycosylated forms were converted into diglycosylated forms, indicating that cholesterol slowed the translocation initiation process. Again, no monoglycosylated forms were observed. On the other hand, the 4K cluster caused a significant accumulation of the monoglycosylated form after the 40 min translation (SBP-4K-38-SytII), even in the absence of cholesterol. Supplementation with cholesterol enhanced the accumulation of the monoglycosylated form. We confirmed that cholesterol supplementation slowed the movement of the 4K cluster. Collectively, both the initiation of translocation and chain movement induced by SA-I were inhibited by cholesterol supplementation. The movement of the 4K cluster was more sensitive to cholesterol than the initiation of translocation.

Cholesterol Inhibits the Initiation of Translocation after Targeting and Chain Movement. To uncouple the initiation of translocation and chain movement from the membrane targeting process, we utilized the SBP tag strategy. Because the SBP tag tightly binds to SAv, the SBP tag on the nascent polypeptide chain is trapped by SAv outside of the membrane after the ER targeting (Figure 5A). Biotin causes a release of the SBP tag from the SAv and induces movement of the N-terminal region. In the absence of the 38-residue spacer (SBP-SytII model), SAv arrested translocation at the initiation stage, where the hydrophobic segment of SA-I was retained in the aqueous environment of the translocon.[15] In the presence of the spacer (SBP-38-SytII and SBP-4K-38-SytII), translocation was arrested at the chain movement stage, where the N-terminus was trapped on the cytoplasmic side, the downstream portion was in

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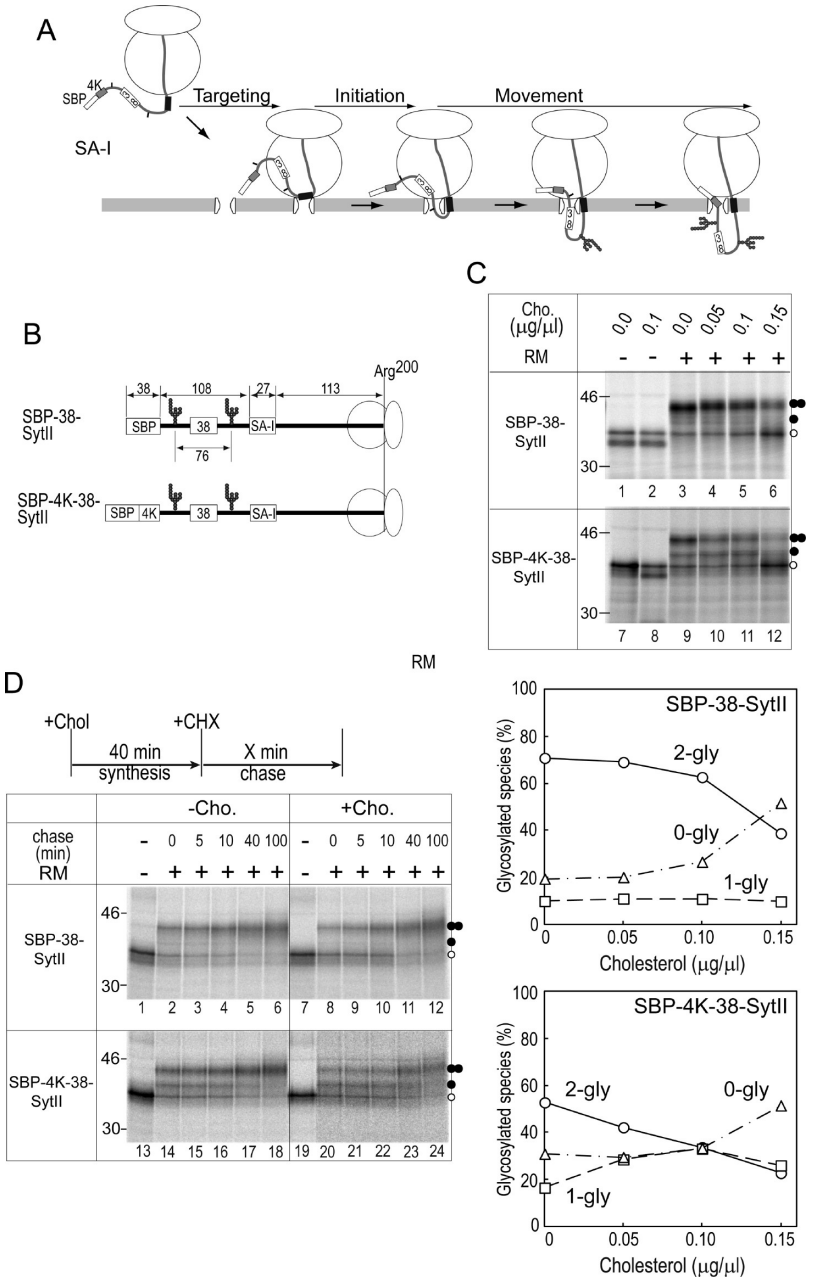

Figure 4. Effect of cholesterol on SA-I function. (A and B) SA-I (filled rectangles) mediates ER targeting and initiates translocation of the upstream portion. The model protein included a backbone of SytII, an N-terminal SBP tag, a 38-residue spacer, and two potential glycosylation sites (forks). The 4K cluster inserted after the SBP tag (gray rectangles) induced the weak arrest of translocation. The residue number of each portion is indicated. The polypeptides were truncated at Arg^{200}. When translocated in the lumen, the potential glycosylation sites are glycosylated (forks). (C) The model proteins were translated for 60 min in the absence (−) and presence (+) of the RM supplemented with the indicated amounts of cholesterol. Nonglycosylated (○), monoglycosylated (●), and diglycosylated (●●) forms are denoted. (D) Time dependence of the N-terminal translocation. The models were translated for 40 min, and the translocation was chased in the presence of CHX.

the lumen and glycosylated, and the hydrophobic segment was located in the membrane.

Model proteins were translated in the presence of SAv and the normal RM. After the translation was terminated by CHX, cholesterol was loaded into the RM, and then the translocation of the N-terminal portions was resumed by biotin (Figure 5). Membrane targeting normally occurs using this strategy, so the effect of cholesterol on the initiation of translocation and movement can be assessed.

When translated in the absence of SAv, the SBP-SytII model was almost completely diglycosylated. In the presence of SAv, it was mainly nonglycosylated (Figure 5B, lane 3), indicating that translocation was efficiently arrested. When translocation was resumed by biotin, the nonglycosylated form was converted to the diglycosylated form. The monoglycosylated form was not observed at all, indicating that translocation resumed and the chain was fully translocated. Cholesterol supplementation after membrane targeting clearly slowed conversion, indicating that the initiation of translocation by SA-I was affected by cholesterol after the targeting stage.

The model with the 38-residue spacer (SBP-38-SytII) was also efficiently diglycosylated. Insertion of the 4K cluster caused a significant accumulation of the monoglycosylated form,

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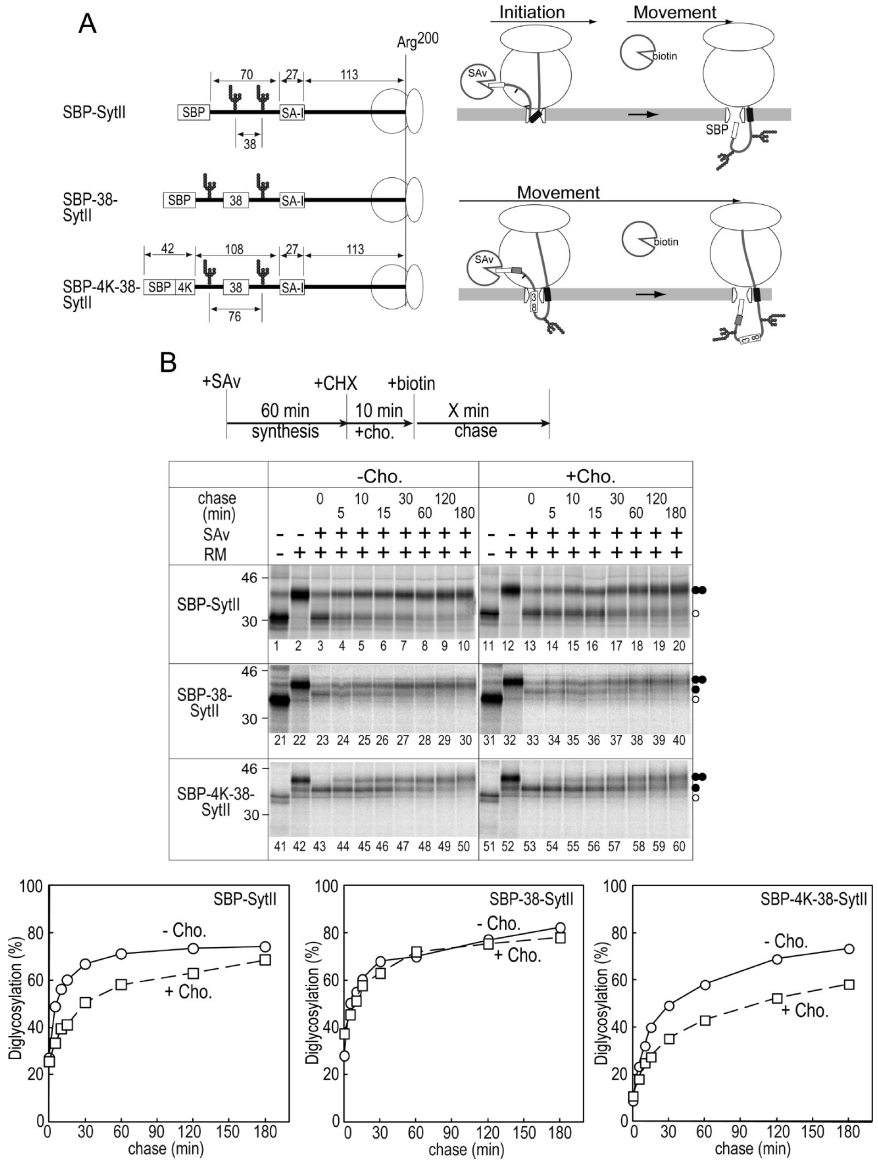

Figure 5. Cholesterol inhibits the initiation of translocation by SA-I and movement of the positive charge. (A) Model proteins and the arrest of translocation by SAv. The SA-I model protein without the 38-residue spacer was also examined (SBP-SytII). SAv binds the N-terminal SBP tag and arrests the translocation. Biotin releases the SAv from the SBP tag and resumes the translocation. SA-I (filled rectangles) and sugar chains (forks) are denoted. Using this system, cholesterol can be supplemented after the targeting stage. (B) Effect of cholesterol supplementation of the RM after the targeting. The model proteins were translated in the presence of the RM and SAv for 60 min; elongation was inhibited with CHX, and cholesterol was supplemented. Translocation was resumed by biotin to complete the translocation. After incubation for the indicated time, aliquots were sampled at the indicated time. The percentages of diglycosylated forms were calculated.

indicating that the positive charges slightly arrested the movement. When translated in the presence of SAv, the product was mainly monoglycosylated. The N-terminus was retained on the cytoplasmic side by the SAv, and the following portion was in the lumen[15] (Figure 5A). The polypeptide was in the chain movement stage,[14,28] during which the hydrophilic polypeptide passes through the aqueous pore of the translocon and corresponds to the movement stage of Figure 1A. Upon addition of biotin, the monoglycosylated form was converted to the diglycosylated forms during the chase. In the absence of the 4K cluster, diglycosylation was not affected by cholesterol, indicating that movement of the hydrophilic N-terminal polypeptide was not affected by cholesterol. On the other hand, in the presence of the 4K cluster (SBP-4K-38-SytII), cholesterol supplementation reduced the rate of diglycosylation. The 4K cluster inhibited polypeptide chain movement after the initiation of translocation, and the cholesterol did further inhibit the movement. Taken together, cholesterol supplementation of the RM inhibited the initiation of translocation driven by SA-I and the movement of the N-terminal positive charges.

DISCUSSION

Here we demonstrated that cholesterol affects various processes of protein translocation after ER targeting. Cholesterol inhibits the initiation of translocation, inhibits the movement of positive charges, and enhances the movement of marginally hydrophobic segments toward the lumen. The extent of the inhibition of initiation was dependent on the signal sequences. Insertion of LepB and SytII SA-I was largely inhibited, whereas initiation by the RSA signal peptide was not affected.

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Irrespective of cholesterol supplementation, newly synthesized RSA molecules were at least monoglycosylated, and the nonglycosylated form was not observed. The cholesterol resistance of the RSA signal peptide allowed us to further analyze the effects of cholesterol on the chain movement stages of translocation. Cholesterol clearly inhibited the movement of the positively charged cluster. In clear contrast, cholesterol enhanced the translocation of marginally hydrophobic segments. In other words, cholesterol inhibited the stop translocation of the marginally hydrophobic segments. Thus, cholesterol influences at least two independent processes of translocon function: movement of positively charged residues and the behavior of hydrophobic segments.

Cholesterol affects the bilayer stiffness of the lipid bilayer and should therefore have direct or indirect effects on membrane protein conformations and modulate various channel activities. The physical properties of the membrane such as elasticity or stiffness modulate channel activities.[33−35] We expected that cholesterol would influence some translocon function but found more pleiotropic effects on protein translocation than expected.

Cholesterol inhibited movement of the lysine cluster, while the translocation of ordinary sequences (RSA polypeptide and N-terminal domain of SytII) was not affected. Because cholesterol does not possess any charge, it should affect the dynamic actions of the translocon pore. The translocation pore is surrounded by 10 TM helices of the Sec61α subunit. When the translocon pore is opened by a signal peptide and accommodates a translocating chain, movement of ordinary sequences through the translocon was not affected, indicating that the interior portion of the pore was not drastically affected. There are several ways that positive-charge movement can be influenced. First, cholesterol might induce a conformational change of the translocon subunits and affect the accessibility of the positive charges to the interaction partners of translocon subunits. Some of the charged residues on yeast Sec61p are involved in positive-charge recognition during membrane integration of hydrophobic segments.[36,37] Second, cholesterol might change the surface charge distribution of the membrane and modulate the electrostatic interactions between membrane lipids and the positively charged cluster. Third, cholesterol supplementation might affect the lateral gate of the Sec61 channel and enhance the accessibility of positive charges with surface negative charges of the membrane.

In contrast to inhibition of the positive-charge movement, cholesterol enhanced movement of the marginally hydrophobic segment (10LA segment), indicating that it inhibited the stop translocation. Cholesterol also inhibited the initiation of translocation by hydrophobic signal sequences in a sequence-dependent manner. Both the stop translocation and the start translocation functions seem to couple with interaction between the hydrophobic segment and the membrane lipid. Stop translocation and membrane insertion are primarily determined by partitioning of the hydrophobic segment into the lipid environment.[18,38] We previously demonstrated with SA-I (signal sequence) that the signal sequence provides a translocation motive force[14] and that the translocation motive force is provided by partitioning the hydrophobic segment into the membrane.[15] There are two possible explanations for the effect of cholesterol on the functions of hydrophobic sequences: an effect on the physical properties of the membrane and an effect on the functions of the translocon channel. First, membrane fluidity and stiffness are greatly affected by cholesterol. Those properties directly determine the diffusion or partition of hydrophobic segments into the lipid. In addition, membrane deformation should be restricted by the stiffness of the membrane, which is critical for membrane accommodation of the short transmembrane hydrophobic helix.[39] Membrane stiffness should inhibit adaptation of the bilayer to the short transmembrane segment and thus inhibit stop translocation of the short hydrophobic segment (10LA segment). In fact, cholesterol reduces the rate of incorporation of the short TM peptide into the negatively hydrophobic mismatched bilayer.[40] Second, the membrane properties should affect the dynamics of the translocon, especially the function of the lateral gate. Membrane stiffness should restrict the freedom of the lateral gate and impair contact of the hydrophobic segment with the membrane lipids. Furthermore, cholesterol would directly induce a conformational change in the translocon channel and affect the recognition of the hydrophobic segment by the translocon. Protein−protein interactions might be involved in the stop transfer process.[16] A pore ring mutation of yeast Sec61 affects the selective recognition of the TM segment.[41] If sensing of the hydrophobic segment by the translocon channel is impaired, the hydrophobic segments are more strictly sequestered from membrane lipids and readily translocated.

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ABBREVIATIONS

CHX, cycloheximide; ER, endoplasmic reticulum; LepB, leader peptidase B; MCD, methyl-β-cyclodextrin; ProK, proteinase K; RM, rough microsomal membrane; RSA, rat serum albumin; SA-I, type I signal anchor; SA-II, type II signal anchor; SAv, streptavidin; SBP, streptavidin binding protein; SytII, synaptotagmin II; TM, transmembrane.

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