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A Few Positively Charged Residues Slow Movement of a Polypeptide Chain across the Endoplasmic Reticulum Membrane

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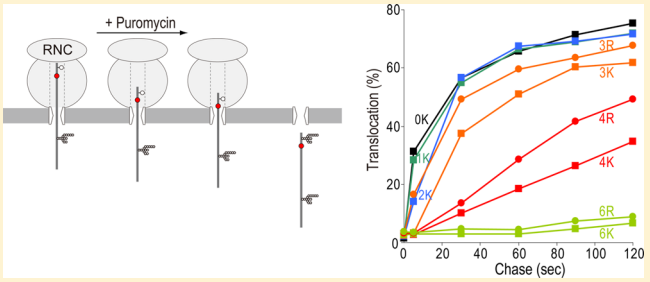
Supporting Information

ABSTRACT: Many polypeptide chains are translocated across and integrated into the endoplasmic reticulum membrane through protein-conducting channels. During the process, amino acid sequences of translocating polypeptide chains are scanned by the channels and classified to be retained in the membrane or translocated into the lumen. We established an experimental system with which the kinetic effect of each amino acid residue on the polypeptide chain movement can be analyzed with a time resolution of tens of seconds. Positive charges greatly slow movement; only two lysine residues caused a remarkable slow down, and their effects were additive. The lysine residue was more effective than arginine. In contrast, clusters comprising three residues of each of the other 18 amino acids had little effect on chain movement. We also demonstrated that a four lysine cluster can exert the effect after being fully exposed from the ribosome. We concluded that as few as two to three residues of positively charged amino acids can slow the movement of the nascent polypeptide chain across the endoplasmic reticulum membrane. This effect provides a fundamental basis of the topogenic function of positively charged amino acids.



Polypeptide chains synthesized by membrane-bound ribosomes are cotranslationally translocated across and integrated into the endoplasmic reticulum (ER) membrane via a protein-conducting channel, the so-called translocon.[1] The core of the translocon is the Sec61 complex.[2,3] Based on studies of the crystal structures of archaeal[4] and bacterial[5] SecY complexes, which are homologues of the eukaryotic Sec61 complex, a single SecY molecule forms an aqueous pore. The pore is formed by 10 transmembrane helices and can laterally open to allow contact between the translocating hydrophobic polypeptide and the membrane lipid. The functioning mammalian Sec61 complex also has a similar structure.[6] Oligosaccharyl transferase[7] flanks the translocon. Translocating chain-associated membrane protein and translocon-associated protein complex are also suggested to flank the Sec61 complex[8] and to promote the maturation of multispanning membrane proteins.[9]

The ER-targeting and membrane insertion are initiated by a signal sequence. The function of the signal sequence is primarily determined by its hydrophobic segment, which is recognized first by a signal recognition particle and then by the translocon. Upon insertion into the translocon, the orientation of the hydrophobic sequence is influenced by the flanking positively charged amino acid residues.[10−12] If positive charges are rich on the N-terminal side of the hydrophobic segment, the N-terminus is retained on the cytoplasmic side and the segment forms the N(cytosol)/C(lumen) orientation. On the other hand, if the C-terminal side is rich in positive charges, the N-terminal side is translocated through the translocon and the segment forms the N(lumen)/C(cytosol) orientation as a type I signal-anchor sequence. The hydrophobic region of the signal sequence provides an energetic force for translocation of the hydrophilic portion.[13,14]

After initiation of the translocation, the continuous movement of polypeptide chains destined for the ER lumen is interrupted by hydrophobic segments.[15] The hydrophobic sequence is functionally termed the "stop-transfer sequence". In this process, simple partitioning of sufficiently hydrophobic segments into the lipid phase through the translocon is a major and rational motivation for the stop-translocation and membrane insertion of the transmembrane segments.[16,17] Positive charges on the nascent chain enhance the stop translocation.[15,18,19] The positive charges can be effective in vitro even when they are separated from the hydrophobic segment by more than 60 residues. In such cases, a marginally hydrophobic segment that had been translocated into the ER lumen can be retrieved to the membrane by the 60-residue downstream positively charge clusters, indicating that the positive charges not only fix the transmembrane segment at the membrane but also induce retrograde movement of the polypeptide chain.[20,21] Furthermore, we demonstrated that a cluster of 10 positively charged residues momentarily arrest the

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movement, even in the absence of a hydrophobic segment.[22] Interestingly, the charge cluster can slowly move after the momentary stalling. Depending on the strong topogenic influence of positive charges, even an internal transmembrane segment with a flanking positive charge exerts its preferred orientation and influences the membrane topology of its flanking segment,[23,24] for example, even in a start-transfer context, when positive charges are flanking a hydrophobic segment that has a type I signal-anchor function inserting its upstream N-terminal portion. Via this mechanism, somewhat less-hydrophobic transmembrane segments can be formed as a part of multispanning membrane proteins. The unique topogenic features of the positively charged and hydrophobic residues have been elucidated. However, there has been no kinetic analysis concerning the function of these residues on the movement through the translocon; it is critically important to determine how individual residues influence the movement of chains through the translocon.

We previously demonstrated that a cluster of a large number of positively charged residues arrests the polypeptide chain movement through the translocon.[22] To examine the kinetic effect of small numbers of positive charges on the movement, we developed an experimental system to assess the movement kinetics with a subminute time resolution. In the system, polypeptide movement can be completed within 60 s and the effect of each amino acid residue can be assessed with a time resolution of tens of seconds.

EXPERIMENTAL PROCEDURES

Materials. RM[25] and rabbit reticulocyte lysate[26] were prepared as previously described. RM were treated with EDTA and then with *Staphylococcus aureus* nuclease (Roche) to remove endogenous mRNAs, as previously described.[25] Puromycin (Sigma-Aldrich), DNA-manipulating enzymes (Takara and Toyobo), RNaseA (Nippon Gene), and PEG-maleimide [methoxy-PEG-$(CH_2)_3$NHCO$(CH_2)_2$-MAL, MW 2000 SUNBRIGHT ME-020MA, NOF Corporation, Japan] were obtained from the indicated sources.

Construction of Model Proteins. Model proteins were based on rat serum albumin as previously described.[20] DNA fragments encoding them were subcloned between the *Xba*I and *Apa*I sites of pRc/CMV (ver. 1, Invitrogen) and placed downstream of the T7-RNA polymerase promoter. The models included the signal peptide at the N-terminus and the N-glycosylation sites at N^{67} and N^{180}. The amino acid sequence around the second glycosylation site (N^{180}) was optimized for efficient glycosylation by OSTase (E^{177}LLYYAEKY^{185} to AQQNSTAAY). To create a *Bam*HI site at the187th residue, the amino acid sequence was changed to V^{186}DP. The nucleotide and amino acid sequence of the basal model protein is indicated in Figure S1, Supporting Information. The insertions of the positively charged-clusters (Figure 2), the three-residue clusters (Figure 4), and the leucine clusters (Figure 6) between S^{168} and S^{169} were made by the method of Kunkel[27] or the QuickChange method (Stratagene). For the cysteine modification experiments (Figure 5A,B), the upstream region encoding M^1–S^{168} was replaced with a DNA fragment in which all of the cysteine codons were exchanged with the alanine codon,[20] and a cysteine codon was inserted just after the three lysine cluster.

Cell-Free Transcription and Translation. The DNA fragments encoding the model proteins were transcribed under the control of the T7-RNA polymerase promoter. For the truncated RNAs, the template DNA fragments were generated by polymerase chain reaction (PCR) essentially as previously described.[28] The forward primer had a sequence at 50 bases upstream of the T7-RNA polymerase promoter of pRcCMV: GCAGAGCTCTCTGGCTAACT. Reverse primers corresponded to the sequence of N^{180}–Y^{185} and the following threonine codon (except Figure S2, Supporting Information). To make mRNA encoding the models of various C-terminal lengths (Figure 5), the reverse primers corresponded to the C-terminal six codons and the subsequent threonine codon. For the RNA with the termination codon, the template DNA was also generated by PCR using a reverse primer corresponding to the N^{180}–Y^{185}–T–termination codon and subsequent 15-base extension. The extension after the termination codon was essential for the efficient release of the nascent chain by the termination codon.[28] The PCR was performed with KOD-DNA polymerase, and the produced DNA fragments were purified by agarose gel electrophoresis, band excision, and column purification using the DNA gel band purification system (Qiagen). The fragments were subjected to transcription with T7-RNA polymerase as previously described.[28] Cell-free translation with the reticulocyte lysate was performed as previously described,[29] except that translation reactions with RM contained castanospermine to prevent heterogeneity of the products due to trimming of the N-linked sugar chain. The translation reaction was performed for 15–20 min at 30 °C in either the absence or the presence of RM. The translation reaction included 100 mM potassium acetate, 1.2 mM magnesium acetate, 32% reticulocyte lysate, castanospermine (20 μg/mL), and 15.5 kBq/μL [^{35}S]-EXPRESS protein-labeling mix (PerkinElmer). The movement of the nascent chain from the RNC was initiated with 2 mM puromycin. The puromycin stock solution (20 mM) was dissolved in 100 mM HEPES/KOH (pH 7.4). Aliquots (5.5 μL) of the chase reactions were sampled at the indicated chase time and mixed with sample buffer (10 μL) containing RNaseA (40 μg/mL), and the mixture was incubated at 30 °C for 3–5 min and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Radiolabeled polypeptide chains were visualized with an imaging analyzer (BAS1800, Fuji Film) and quantified using Image Gauge software (Fuji Film). Translocation was estimated as the percentage of the diglycosylated product among monoglycosylated plus diglycosylated products.

PEG-Maleimide Reaction. After a 15 min translation reaction in the absence of RM, cycloheximide (2 mM) was added. The products were incubated with 10 mM PEG-maleimide for 6 min on ice. The stock solution of PEG-maleimide (100 mM) was dissolved in water immediately before use. The reaction was quenched with 100 mM dithiothreitol for 5 min on ice, mixed with RNase-sample buffer, incubated at 30 °C for 5 min, and subjected to SDS-PAGE. Where indicated, the PEG-maleimide reaction was performed after the 5 min puromycin treatment.

RESULTS

Experimental System. The objective of this work was to examine the effect of each amino acid residue on the rate of movement of nascent polypeptide chains across the ER membrane. To this end, a model protein was constructed that comprises the N-terminal 186 residues of the secretory protein rat serum albumin with the N-terminal signal peptide and two potential glycosylation sites (Figure S1, Supporting Information). To uncouple the movement from the chain

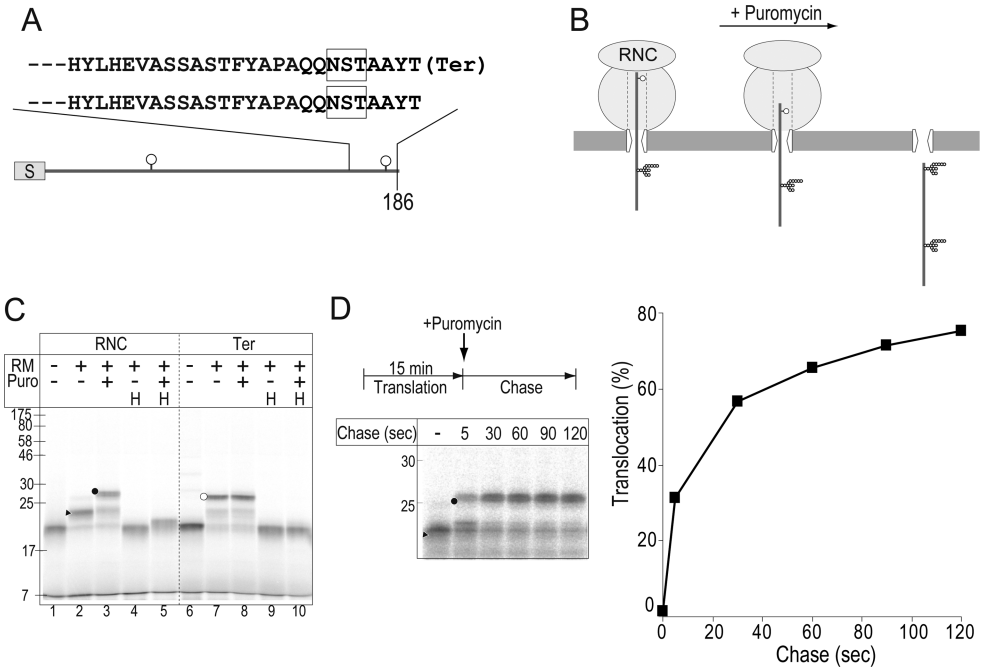

Figure 1. Movement assay. (A) Model protein was based on rat serum albumin, including the N-terminal signal peptide (S) and glycosylation sites at N^{67} and N^{180} (circles). Amino acid sequences of the C-terminal portion and the second glycosylation site (boxed) are indicated. The RNAs were truncated at the Y^{185}-codon followed by the Thr-codon (the original V^{186} was replaced with Thr for efficient release by puromycin) or included the termination codon (Ter). (B) The nascent chain ribosome complex (RNC) is formed by translation of the truncated RNA in the presence of RM. The N-terminal portion of the nascent chain is in the lumen and the first glycosylation site is glycosylated (fork), whereas the C-terminal portion is retained in ribosome and the second site is not glycosylated. Upon puromycin treatment, the nascent chain is released, translocated across membrane, and diglycosylated (forks). The diglycosylation is used to estimate chain movement. (C) Puromycin-induced release and movement of the polypeptide chain. The mRNAs with (Ter) and without (RNC) termination codon were translated in the presence or absence of RM for 20 min. Aliquots were chased in the presence of puromycin (Puro) for 5 min. Where indicated, aliquots were treated with endoglycosidase H under denaturing conditions (H lanes). After SDS-PAGE analysis, radiolabeled proteins were visualized by image analysis. The monoglycosylated chain in the RNC (closed triangles), the diglycosylated chain with puromycin (filled circles), and free diglycosylated chain (open circles) are indicated. (D) Time course of glycosylation after the puromycin chase. After the 15 min translation reaction, the nascent chain was released by puromycin from the RNC. Aliquots were sampled before the chase and after the chase for the indicated time periods. The diglycosylated and monoglycosylated forms were quantified and the percent of diglycosylation was calculated and indicated in the right panel.

elongation in the ribosome, we used the ribosome nascent chain complex (RNC), which was obtained by translating a truncated RNA possessing no in-frame termination codon. When the truncated RNA is translated in a cell-free system in the presence of rough microsomal membranes (RM), the signal peptide initiates translocation through the membrane, the upstream portion is translocated into the lumen, and the C-terminus is retained in the ribosome as the peptidyl tRNA at the peptidyl transferase center (PTC; Figure 1A, RNC). In this situation, the first potential glycosylation site is accessible to oligosaccharyl transferase (OSTase), but the second site is not. When the nascent chain is released from the ribosome by puromycin, the polypeptide chain moves into the lumen, and the second site is glycosylated. Thus, the movement of the chain can be monitored by the glycosylation of the second site.

When the mRNA with a termination codon was translated in the presence of RM, the newly synthesized nascent chain was diglycosylated, which was 4-kDa larger than the product synthesized in the absence of RM (Figure 1C, lanes 6 and 7). On the other hand, when the mRNA did not contain the termination codon (RNC), the product remained monoglycosylated, even after 20 min of translation (Figure 1C, lane 2). When treated by puromycin, the monoglycosylated form was converted to the diglycosylated form (Figure 1C, lane 3). The puromycin-adduct form (filled circles) of the nascent chain was slightly larger than that without it (open circles). The larger products were shifted to the smaller ones by endoglycosidase H treatment, confirming that the larger one was the glycosylated form (Figure 1C, lanes 4, 5, 9, and 10). Upon glycosidase treatment, the mobility difference between the puromycin-adduct form and the free form became more apparent (Figure 1C, lanes 4 and 5). The conversion from the monoglycosylated form to the diglycosylated form is a convenient probe of polypeptide chain movement from the ribosome into the lumen. To optimize the release reaction, we examined the effect of the C-terminal amino acid residues (Figure S2, Supporting Information) and found that cysteine and threonine residues were best for puromycin release among the 20 amino acid residues. Because cysteine was used for the chemical modification experiment, we selected the threonine residue as the C-terminal residue in this work.

We examined the time course of the diglycosylation (Figure 1D). The monoglycosylated form was converted to the diglycosylated form within 60−80 s. Initial movement monitored by the glycosylation was more than 30% within the initial 5 s, indicating that the movement can be monitored with a time resolution of at least tens of second. More than 90% nascent chain remained as the monoglycosylated form after 5 min incubation in the absence of puromycin (data not shown). We followed the rapid movement through the translocon, and expected to assess the weak slowdown effect caused by a cluster of a few amino acid residues.

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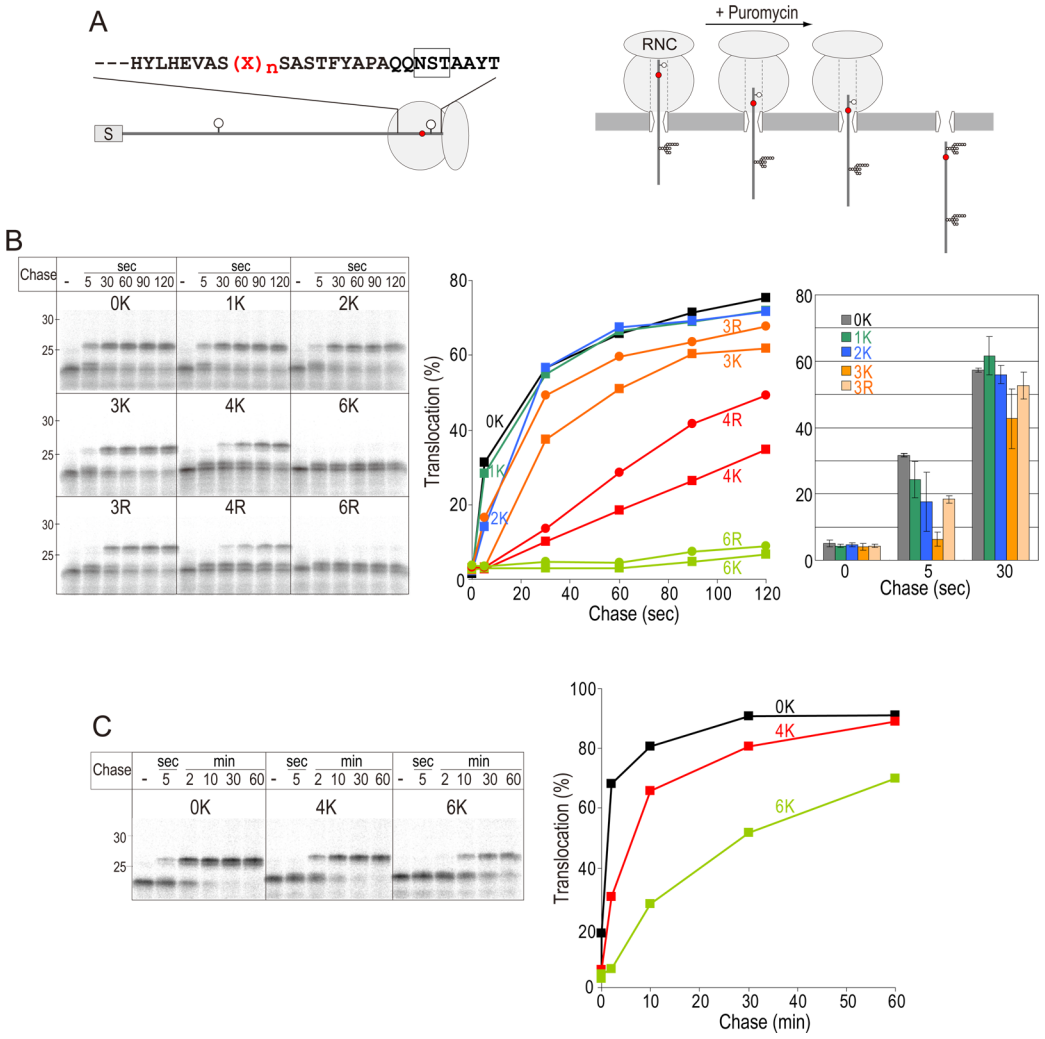

Figure 2. Effect of positive charge clusters on the movement. (A) Clusters of various numbers (n) of lysine and arginine residues (X, red dots) were inserted 18 residues upstream of the C-terminus. Movement was expected to be slowed by the positive charges as indicated in the right panel. (B) Time course of diglycosylation of model proteins after puromycin chase. The truncated RNAs were translated in the presence of RM for 15 min, and the movement assay was performed as in Figure 1. The quantified data were indicated in the center panel. The short period chase experiments were performed three times and the averages are indicated with SD in the right panel. (C) The chase reaction of the indicated models was continued for up to 60 min.

Movement Was Slowed by Positive Charges. To estimate the effect of a small number of positively charged residues on movement, small clusters of positively charged residues were introduced 18 residues away from the C-terminus (Figure 2A). Diglycosylation was clearly slowed by as few as two lysine residues (Figure 2B, right panel). Three lysine or arginine residues induced a marked slowdown. Clusters of four lysine or arginine residues drastically slowed chain movement. A cluster of six lysine or arginine residues did not move during the 120 s chase. Upon prolonged incubation, the four lysine and six lysine clusters translocated slowly and the movement continued for at least 60 min (Figure 2C). We concluded that a small number of positive charges caused a remarkable slowdown of chain movement through the translocon.

To examine for the possibility that the inserted positive charges did not slow the movement but suppressed the OSTase reaction in the lumen, we examined the effect of high salt conditions (0.5 M NaCl), which accelerates the movement of positive charges.[22] Under high-salt conditions, movement of the four lysine cluster was clearly increased (Figure 3), indicating that the glycosylation reaction was not the rate-limiting process of the overall reaction.

Kinetic Effect of Each Amino Acid Residue. We then examined the effects of each of the amino acid residues on chain movement (Figure 4). A cluster of three residues of each amino acid was inserted 18 residues away from the C-terminal. Each model protein was then subjected to the movement assay (Figure 4B). In the case of asparagine, a higher-glycosylated form was observed due to an N-glycosylation site that was generated by the insertion of the three asparagine cluster. The clusters of the other amino acids induced some minor effects on the movement (Figure 4B). The magnitude of these effects, however, was much lower than that of the positively charged amino acids. When the experiments were repeated, the effects were reproducibly observed and statistically remarkable (Figure 4C). It is clear that only the lysine and arginine residues had a drastic slowing effect and that the lysine residue was more effective than arginine.

Positive Charges Can Be Effective Outside of the Ribosome Tunnel. The positive charges possibly exert the

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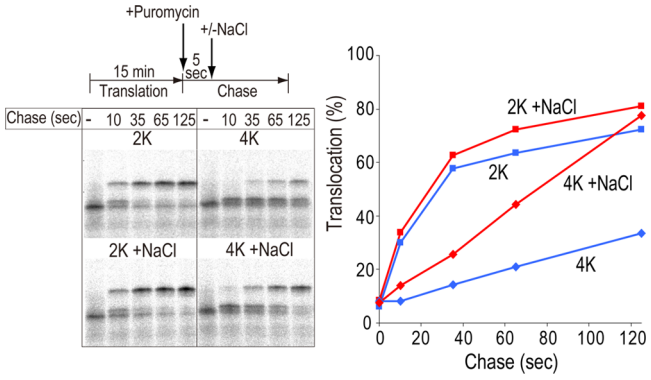

Figure 3. High-salt conditions accelerate the movement. Movement assay of the two lysine and four lysine models was performed. Where indicated, NaCl solution was added to 0.5 M after 5 s puromycin chase. The translocation rate of the four lysine model was apparently enhanced under high-salt conditions. The OSTase reaction in the lumen was not the rate-limiting factor of the movement assay.

slowing effect within the ribosome tunnel interacting with rRNA or protein subunits.[30,31] Alternatively, they are effective after exiting the ribosome tunnel. To address these two possibilities, we examined the effect of the C-terminal length on the movement behavior. First, to assess exit of the positively charged cluster from ribosome, we examined the effect of C-terminal length downstream of the three lysine cluster on the reactivity of the cysteine residue just after the three lysine cluster with a high-molecular weight SH-reagent, 2-kDa PEG-maleimide (Figure 5A,B). The cysteine residue would be reactive to the reagent only outside of the ribosome tunnel. For the maleimide-reaction experiment, all of the cysteine residues upstream of the second glycosylation site were changed to alanine. mRNAs truncated at various positions were translated in the absence of RM, and the products were subjected to the PEG-maleimide reactive assay. When the cysteine was within 28 residues of the C-terminus, the SH was hardly reactive (Figure 5B). When the nascent chain was released from ribosome by puromycin, the cysteine residues in the C-terminal region were fully reactive, indicating that the PEG-maleimide reaction distinguished the location of the three lysine cluster and the cysteine. When the length was 30−38 residues, it became moderately reactive but was not fully reactive. When the length was 40 residues, cysteine was largely reactive to PEG-maleimide, indicating that the length of 39 residues was the threshold for the full exit of the three lysine cluster from ribosome tunnel.

We then examined the effect of the downstream length on the movement of the four lysine cluster. The slowing effect decreased as the downstream length increased (Figure 5C). The four lysine cluster was, however, effective when the downstream length was more than 40 residues, indicating that it was effective after exiting the ribosome. The slow down was 50% of the level seen when the cluster is 18 residues from the PTC, suggesting that a significant part of the slow down might be due to the interaction with the ribosome. When the cluster was 60 residues from the PTC, the slowing effect essentially disappeared, indicating that the positively charged cluster must be within 60 residues of the PTC. The slowing effect is likely to occur at the interface of the ribosome translocon junction as well as within the ribosome.

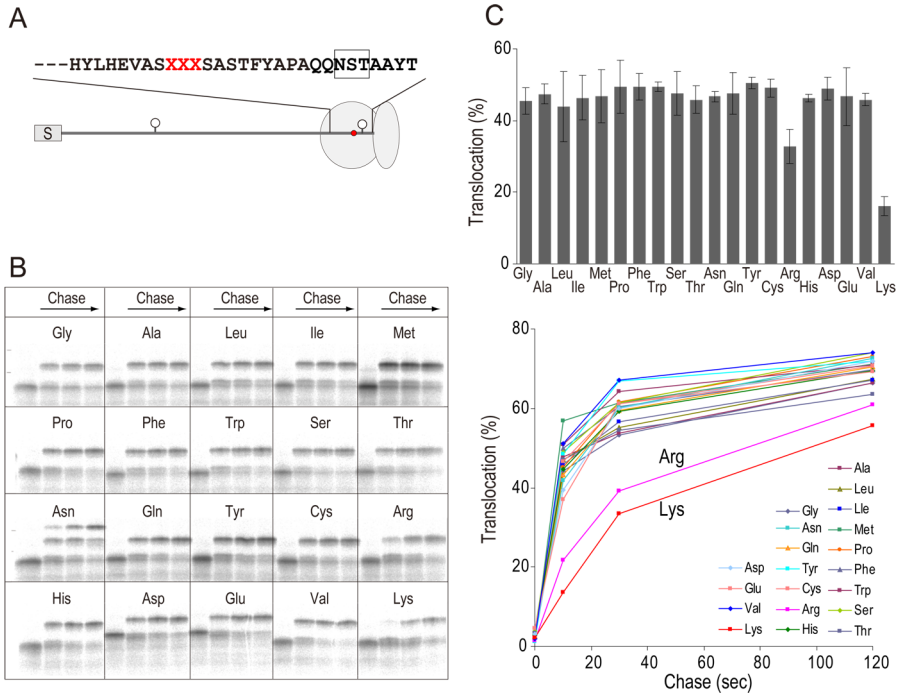

Figure 4. Positively charged residues slow the movement. (A) A three-residue cluster of each amino acid (red characters, red dot) was inserted 18 residues upstream of the C-terminus. (B) The movement assay was performed as in Figure 1. The reaction mixtures were sampled before and after the 10, 30, and 120 s puromycin chase. The diglycosylation percent is indicated in the right panel. Because a third glycosylation site was generated by the insertion of an asparagine cluster, the triglycosylated forms were also taken into account. (C) The effect of the three-residue clusters. The movement at the 20 s chase was analyzed, and the mean and standard deviation values (error bars) are indicated. Lysine and arginine reproducibly slowed the movement. Lysine is more effective than arginine.

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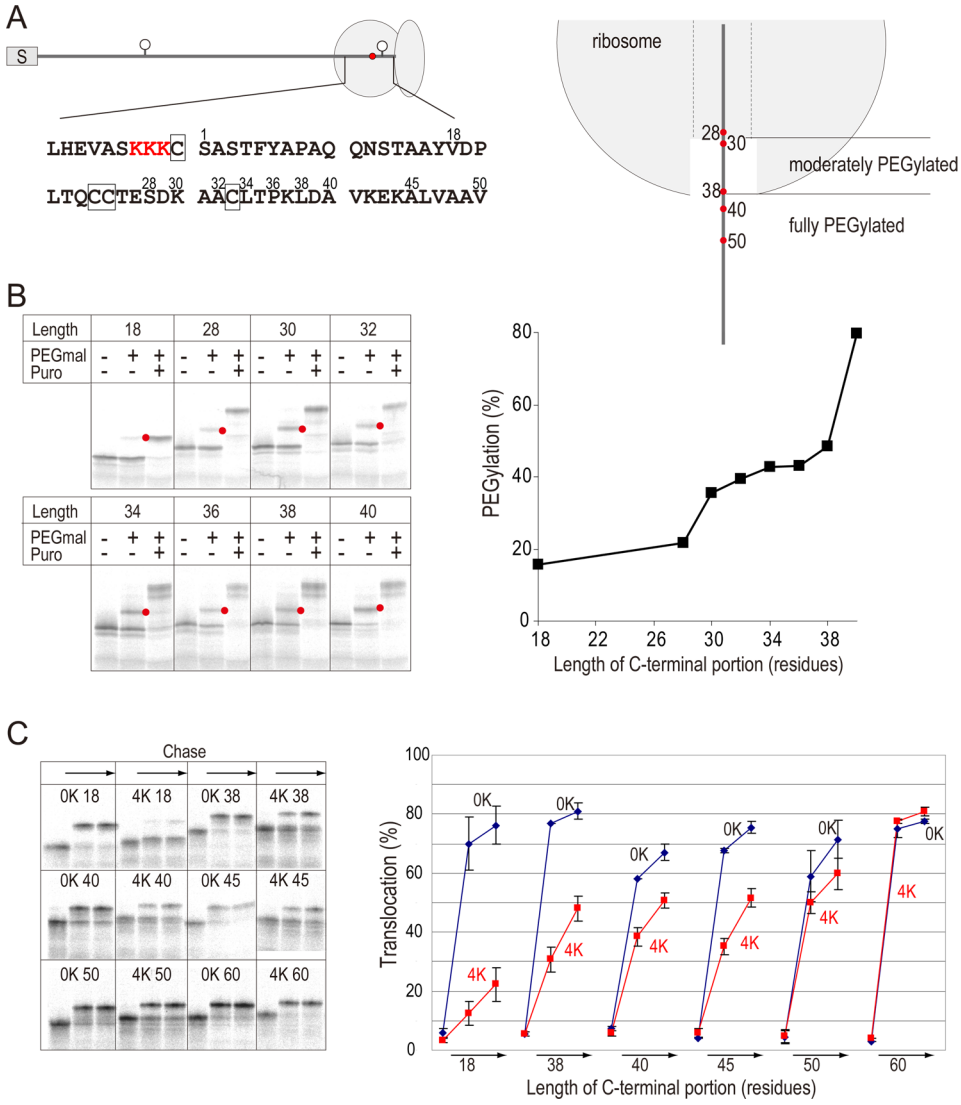

Figure 5. Lysine cluster fully exposed from the ribosome tunnel can slow movement. (A) To examine whether the lysine cluster exits the ribosome, a cysteine residue was inserted just after the three lysine cluster (red characters), and all of the cysteine residues upstream of the three lysine cluster were exchanged with alanine. Cysteine residues in the C-terminal portion are boxed. RNAs were truncated at the indicated positions. The SH groups are reactive (PEGylated) to the 2-kDa SH-reagent PEG-maleimide (PEGmal), only when they exit from the ribosome tunnel. Results in panel B are quantified in the right panel. (B) Each RNA was translated in the absence of RM for 15 min, and the reaction mixture was incubated with PEG-maleimide on ice for 6 min in the presence or absence of puromycin (Puro). The mono-PEGylated products (red dots) in the absence of puromycin increased as the C-terminus elongated. The mono-PEGylation percent (%) in the absence of puromycin is indicated in the right panel. (C) Four lysine and zero lysine model proteins with the C-terminal portion of indicated lengths were subjected to the movement assay. The reaction mixture was sampled before and after the 30 and 60 s chase. The experiments were performed more than four times with the four lysine model proteins and two times with the zero lysine model proteins, and the averages of diglycosylation (%) are indicated with SD in the right panel.

Effect of Hydrophobic Residues. Hydrophobic residues are the major topology determinants of membrane proteins. Thus, we examined the effects of various numbers of leucine residues. The leucine clusters were inserted instead of the positively charged clusters, and the models were subjected to the movement assay (Figure 6). The three leucine cluster had essentially no effect on chain movement, whereas longer clusters had a remarkable effect. The five leucine and seven leucine clusters caused a partial stop at an intermediate percentage of complete translocation (relative to the zero lysine cluster). The hydrophobic cluster reached a plateau value more rapidly than did the positively charged clusters. Even the six lysine cluster moved from 28% at 10 min to 70% at 60 min, whereas the five leucine cluster moved from 45% at 10 min to only 66% at 60 min. A nine leucine cluster was required to completely stop the movement. Even though hydrophobic clusters of the intermediate length were not simply recognized in an all-or-none fashion, they were statically retained at the membrane.

DISCUSSION

Here we described an experimental system to analyze the effects of clusters of small numbers of each amino acid residue on the rate of polypeptide chain movement through the ER translocon. Our findings indicated that small numbers of positively charged amino acid residues slow chain movement. Movement was slowed by as few as two lysine residues. Our previous observation indicated that a cluster of at least 8−10 positively charged residues momentarily stalls at the membrane

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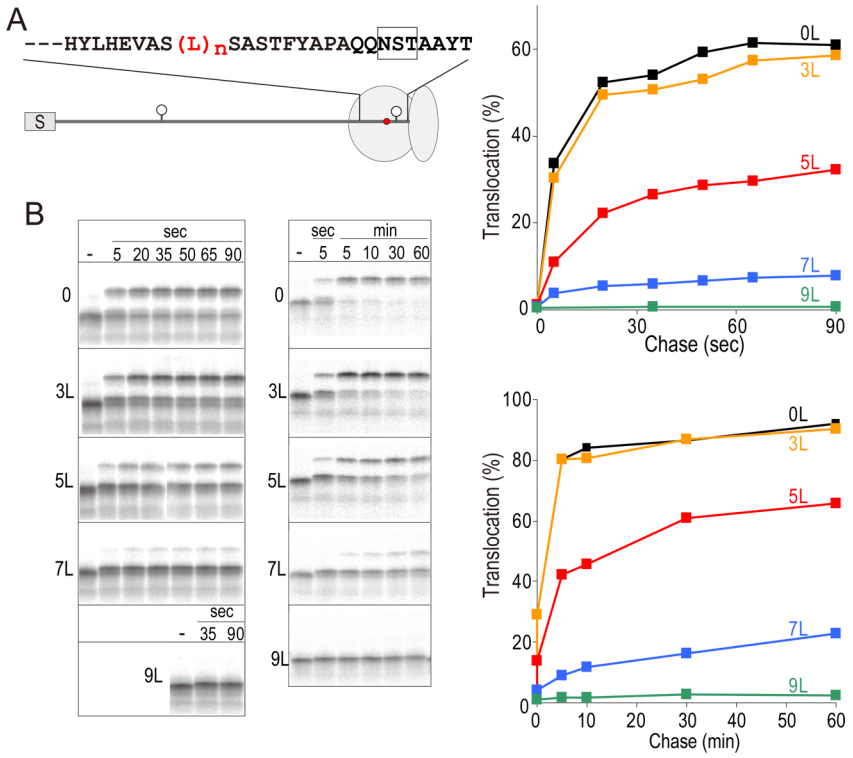

Figure 6. Effect of short hydrophobic segment. (A) A cluster comprising various residues (n) of leucine (red characters) was inserted 18 residues from the C-terminus. (B) Movement assay of the model proteins. The truncated RNAs were translated in the presence of RM for 15 min, and the reaction mixture was sampled before and after the puromycin chase for the indicated time periods. Diglycosylation (%) is indicated in the right panel.

and then slowly moves forward.[22] Such a kinetic effect was confirmed in the present study with only a few lysine and arginine residues. In the previous analysis, we analyzed the movement of the positively charged clusters in a 300-residue polypeptide chain that had been synthesized and released from the ribosome by a termination codon. In that system, only a strong slowing effect could be detected. In this new experimental system, polypeptide chain movement could be analyzed synchronously, independent of the chain elongation and could thus be assessed with subminute time resolution. This system provides a convenient tool for analyzing translocon function and polypeptide chain movement.

The slowing effects were dependent on the number of the positive residues, throughout a wide range of numbers. Such drastic kinetic effects were not observed with three-residue clusters of the other 18 amino acids. Based on the present findings, we concluded that naturally occurring numbers of positive charges exert remarkable kinetic effects on movement through the translocon. The slowing effect of positive charges should be a fundamental basis for topogenic function. The effects allow the hydrophobic segment of the signal-anchor sequences to orient within the translocon and to be released into the membrane environment. With this effect, even internal hydrophobic transmembrane segments show their unique orientation depending on their own characteristics.[23,24]

As the positively charged cluster is moved within the ribosome toward the exit site and out of the ribosome, the slow down is affected. The clusters more than 40 residues away from the PTC were still effective for slowing movement, while the slow down is 50% of the level seen when the cluster is 18 residues from PTC. The previous biochemical experiments with PEG-mal in addition to our data demonstrated that the cysteine residue of a nascent chain 34 residues from PTC is already exposed from ribosome tunnel.[32] A protease domain elongating 36-residues from the PTC can cotranslationally fold, indicating that the domain is outside of the ribosome tunnel.[33] Proteinase digestion experiments demonstrated that nascent polypeptide included in the ribosome tunnel is less than 40 residues.[34] The positively charged residues can exert the slowing effect outside of the ribosome tunnel, although the interaction with the negatively charged RNA within the ribosome should be involved in the slowing effect. Clusters 50 residues away from the PTC had a weak effect and the clusters 60 residues from the PTC had no effect.

The interaction between the positive charges and the negative charges of the ribosome inhibits polypeptide chain elongation and translation.[30] A 12 lysine cluster stalls translation prior to reaching the termination codon[31] and further causes clearance of the polypeptide chain encoded by a nonstop mRNA at the ribosome.[35] Importantly, in our cell free system, the same amount of nascent chain could be subjected to movement analysis, suggesting that several inserted residues with a positive charge did not severely suppress polypeptide chain synthesis.

The kinetic effects of the positive charges and hydrophobic residues differ from each other. The effect of positive charges is additive, and the movement continued during the entire 60 min chase period, indicating that the slowing effect is a momentary trap. On the other hand, the effect of the hydrophobic residues showed some threshold and is likely a more permanent trap. The three leucine cluster elicited no slowing effect, but longer leucine clusters were effective. As previously proposed,[15−18] the effect of hydrophobic residues requires clustering of the residues, and the hydrophobic segment is likely recognized as a single unit to become a transmembrane segment. The movement of the five leucine and seven leucine clusters rapidly

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reached a plateau, and the five leucine segment did not move beyond 60% translocation after a 30 min chase. In contrast, the six lysine cluster continued to move even after a 30 min chase throughout the chase incubation. It is possible that a moderately hydrophobic segment is statically trapped at the translocon after release from the ribosome. Our recent observations indicated that the trapping state of moderately hydrophobic segments at the translocon is regulated by the ribosome synthesizing the translocating nascent chain.[36]

■ ASSOCIATED CONTENT

Supporting Information

Nucleotide and amino acid sequence of the basal model protein and data indicating that the cysteine and threonine residues were best for puromycin release experiments among the 20 amino acid residues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

†M.Y. and Y.O. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ER, endoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ; OSTase, oligosaccharyl transferase; PTC, peptidyl transferase center; PEG-mal, poly(ethylene glycol) maleimide; RM, rough microsomal membranes; RNC, ribosome nascent chain complex

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