Amino acid residues before the hydrophobic region which are critical for membrane translocation of the N-terminal domain of synaptotagmin II

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Abstract We examined the fine structure of the type I signal-anchor sequence of synaptotagmin II, which has a 60-residue N-terminal domain followed by a hydrophobic region (H-region), focusing on the hinge region between the N-terminal and the H-regions. It was found that the charged or highly polar residues support the translocation of the N-terminal domain through the endoplasmic reticulum membrane at specific positions in the hinge. The residue requirement correlated with the turn propensity scale for transmembranes. It is suggested that a certain conformation, likely helical hairpin, in the hinge is critical for N-terminal domain translocation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Membrane topology; Signal sequence; Membrane protein; Membrane integration; Synaptotagmin

1. Introduction

The majority of membrane proteins in the secretory pathway are synthesized by ribosomes bound to the endoplasmic reticulum (ER) membrane and integrated into the lipid bilayer [1]. Completed proteins are then sorted to their final destinations via vesicle transport. Thus, at the synthesis step, the molecules should be correctly targeted to the ER membrane, each transmembrane segment should be inserted into the membrane in the correct orientation, and extracellular portions of hydrophilic sequences should be translocated through the hydrophobic interior of the membrane. Various protein factors are responsible for this process. The hydrophobic region (H-region) of signal sequence emerging from the free ribosome is recognized by a signal recognition particle (SRP). The SRP receptor on the ER membrane mediates release of the signal sequence from the SRP [2]. The Sec61 complexes form an aqueous channel, so-called translocon, through which polypeptide chains penetrate into the membrane [3,4].

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Abbreviations: ER, endoplasmic reticulum; N-domain, amino-terminal domain; H-region, hydrophobic region; RM, rough microsomal membranes; SA-I, type I signal anchor; Syt II, synaptotagmin II; SRP, signal recognition particle

To understand the structural formation of membrane proteins, it is essential to clarify how membrane topology is established at the ER membrane. So-called topogenic sequences of the nascent polypeptide chain regulate the topogenic process. The type I signal-anchor (SA-I) sequence is a unique signal sequence that mediates translocation of its amino-terminal domain (N-domain), while the other signal sequences, signal peptide and type II signal-anchor sequence, mediate translocation of the following portion. The SA-I sequence has been identified in the microsomal cytochrome P450 family [5], synaptotagmin family [6], and NADPH-cytochrome P450 reductase [7], etc. The first H-region of G-protein-coupled receptor family proteins, which possess seven transmembrane segments [8], is also thought to be an SA-I sequence. During insertion of the H-region into the translocon, the flanking sequences on both sides affect the orientation on the membrane [9–11]. For the integration of the SA-I sequence, the amino-terminal portion should not interfere with ER targeting or translocation. Positive charges in and stable folding properties of the N-domain inhibit translocation [12–14]. In addition to these characteristics of the N-domain, the length of the H-region is a critical factor; shorter H-regions cannot pull the N-domain into the membrane [10,11]. In polytopic membrane proteins, an internal SA-I sequence with an ability to pull the amino-terminal portion into the translocon can confer a transmembrane disposition onto a preceding segment, resulting in a topology where even a hydrophilic segment spans the membrane [15].

We previously examined the topogenesis of an SA-I protein, synaptotagmin II (Syt II), which possesses a 60-residue extracellular N-domain, followed by a H-region of 27 residues, and then a 335-residue cytoplasmic domain (Fig. 1). Syt II exists mainly on synaptic vesicles in brain cells and is thought to be involved in Ca^{2+}-regulated exocytosis [16–19]. The synthesis of Syt II proceeds for up to 110 residues on the free ribosome [6]. Then the hydrophobic sequence is recognized by the SRP and the ribosome-nascent polypeptide chain complex is targeted to the ER membrane. On the membrane, the H-region is transferred to the translocon. The N-domain is translocated through the translocon into the lumen. The translocation is supported by some of the sequences flanking the H-region. When the positively charged residues following the H-region are deleted, the carboxy-terminal portion instead of the N-domain translocates through the membrane, resulting in an inverted topology [6]. During the integration process, dramatic conformational changes probably occur near the N-termi-

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nal portion. The presence of at least one of the three proline residues, Pro 62, Pro 64, and Pro 65, at the hinge region between the N-domain and the H-region is critical [6]. When all of these residues were mutated to alanines, the H-region was firmly anchored to the membrane but both the amino- and carboxy-terminals were exposed on the cytoplasmic side.

Examination of the detailed structural requirements of the hinge region in the present study revealed that (i) charged or highly polar residues, irrespective of the charges, in this region have a critical role in N-domain translocation, (ii) the turn propensity scale for transmembrane segments determined by Monne et al. [20,21] correlates well with the requirements, and (iii) these residues have their role at the specific sites.

2. Materials and methods

The plasmid, pSyt II, in which Syt II cDNA was subcloned between *Hin*dIII and *Xba*I sites of pRcCMV (Invitrogen), was described previously [6]. Rabbit reticulocyte lysate [22] and rough microsomal membranes from dog pancreas (RM) [23] were prepared as previously described. All mutants were constructed using the method of Kunkel [24]. The Gene32 protein (Amersham Pharmacia) was included into the reactions of T4 DNA polymerase and T4 DNA ligase to enhance chain elongation as described previously [25]. The accuracy of mutagenesis was confirmed by DNA sequencing. Sequences of oligonucleotides used are available from the author upon request. In vitro transcription and translation were performed essentially as described previously [6,26]. The protein bands were visualized with an image analyzer (BAS2000 and FLA2000, Fuji) and quantified using Image-Gauge software (Fuji).

3. Results

3.1. Importance of amino acid residues with high turn propensity between N-domain and H-region

For membrane topogenesis of the Syt II molecule, the 60-residue N-domain should be translocated through the membrane (Fig. 1A,B). Studies using truncated mRNAs without termination codons revealed that when the nascent polypeptide chain is only 127 residues long, the N-domain is translocated through the membrane and Asn 32 is glycosylated [6]. The glycosylation is a useful marker for the correct integration, because molecules correctly integrated were almost completely glycosylated. When Syt II was synthesized in a cell-free system in the absence of RM, it produced a single polypeptide (Fig. 1C, lane 1). Upon synthesis in the presence of RM, the major band shifted to a larger form (lane 2). Endoglycosidase H treatment shifted the larger bands down to smaller ones, confirming that the larger bands were glycosylated polypeptides (data not shown). In contrast, the mutant in which the three prolines between the N-domain and the H-region were changed to alanines (AAA mutant) gave only trace amounts of the glycosylated form (lane 4). We previously reported that the AAA mutant is anchored to the membrane in a form resistant to alkaline extraction, with both terminals on the cytoplasmic side of the membrane (Fig. 1B) [6]. When the third alanine (Ala 65) of the AAA mutant was changed to tyrosine (AAY mutant), the N-domain translocation was partially recovered (lane 6). Only one proline at position 65 (AAP mutant) was also sufficient for N-domain translocation [6].

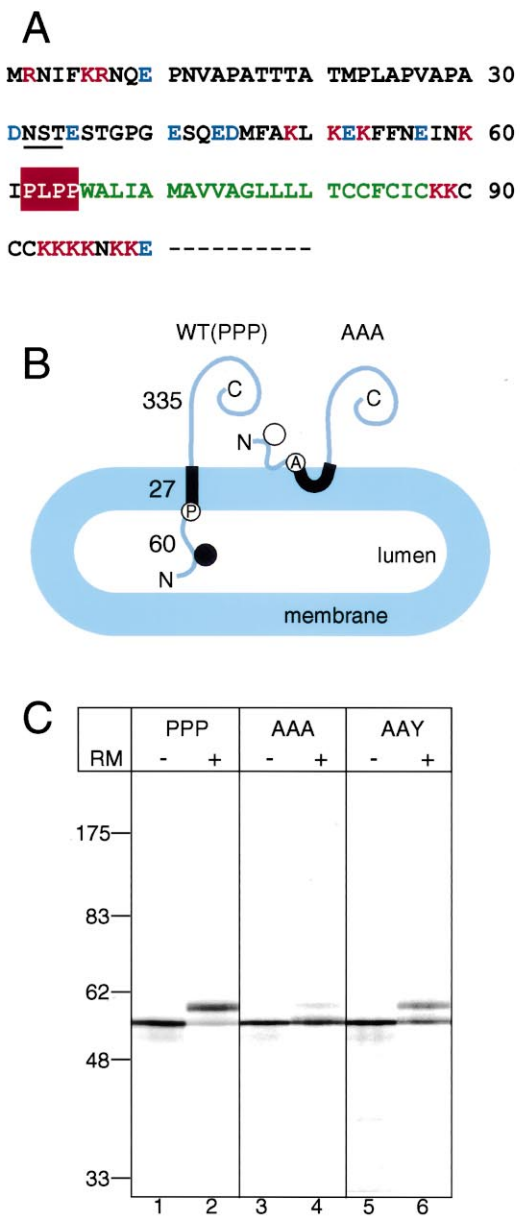

Fig. 1. Translocation of Syt II N-domain. A: Amino acid sequences of 100-residue N-terminal of mouse Syt II. Red, blue, and green characters indicate positively charged, negatively charged, and the hydrophobic transmembrane region (H-region), respectively. A potential glycosylation site in the 60-residue N-terminal hydrophilic domain (N-domain) is indicated by the underline. The hinge region critical for N-domain translocation is indicated by the red box. B: Membrane topology of mouse Syt II. Syt II consists of 60-residue N-domain, 27-residue H-region, and 335-residue C-terminal cytoplasmic domain. Asn 32 of the N-domain is glycosylated in the lumen (closed circle). The AAA mutant, in which three prolines between the N-domain and H-region were changed to alanines, is anchored to the membrane but the N-domain was not translocated. The critical proline residues are indicated by P. C: Glycosylation analysis in vitro. Wild-type (PPP), AAA, and AAY mutants were translated in a cell-free system in the absence (−) or presence (+) of RM. Products were analyzed using SDS–PAGE and subsequent image analysis. Molecular weight markers are shown on the left side.

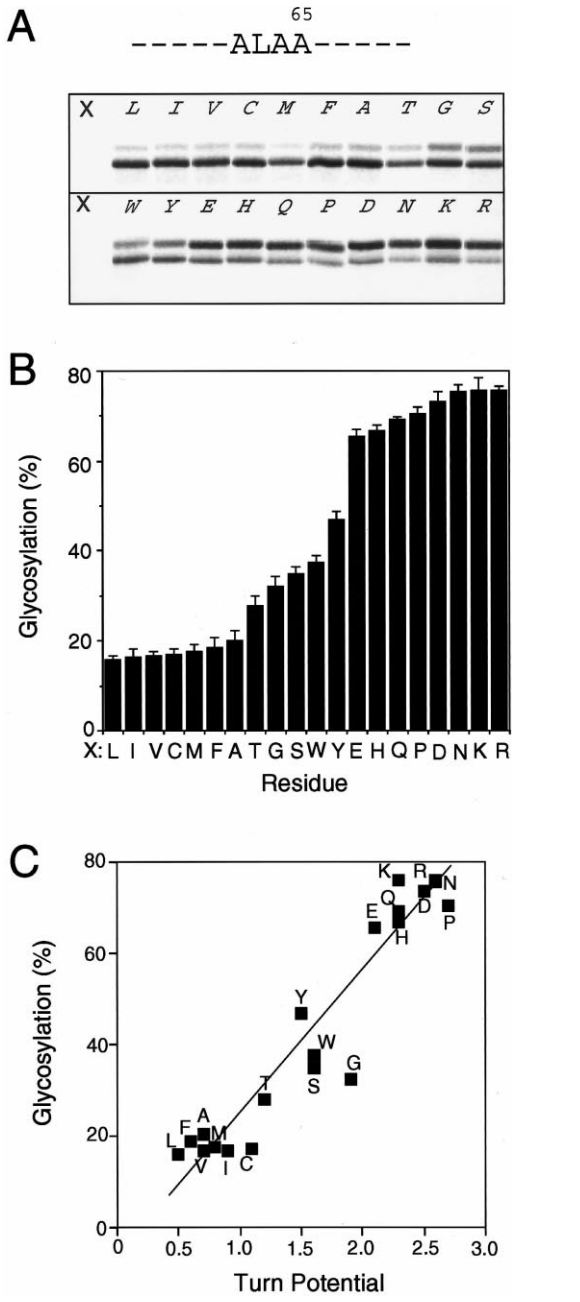

Fig. 2. The amino acid residue with a high turn propensity scale for transmembrane helices at position 65 is essential for N-domain translocation. A: Constructs in which residue 65 was changed with all naturally occurring amino acids were translated in the presence of RM and analyzed using SDS–PAGE. B: Quantified results of N-terminal translocation. The results shown in the figure represent the average of more than three experiments. Standard deviations (error bars) are also indicated. C: Correlation between N-domain translocation efficiency and the turn propensity scale (normalized values) by Monne et al. [21]. The correlation coefficient (r) is 0.95.

These findings suggest that some specific residues have critical roles at position 65.

We examined the effect of other naturally occurring amino acid residues at position 65 on the N-domain translocation (Fig. 2). When the mutants were synthesized in the presence of RM, various levels of glycosylation were observed (Fig. 2A,B). An efficiency of 75% was considered the standard value of glycosylation of wild-type Syt II under the experimental conditions used, and the efficiency increased to almost 100% as the amount of RM was increased (data not shown). Polar residues, irrespective of their charges, resulted in N-domain translocation and proline, the helix-breaking residue, had the same effect. In contrast, hydrophobic residues resulted in a very low level of N-domain translocation. Quantification revealed a clear cut-off of the residue requirements. The efficiency of the given residue at position 65 to mediate the N-domain translocation correlates well with the turn propensity scale for transmembrane helices reported by Monne et al. [20,21] (Fig. 2C; correlation coefficient $r = 0.95$). This scale was experimentally estimated for the tendency of amino acid

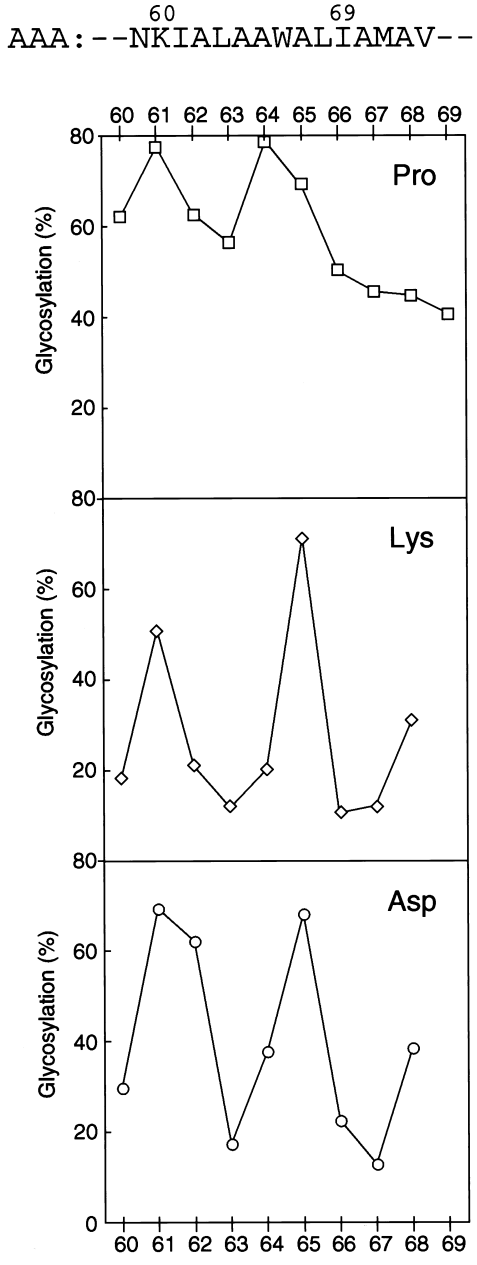

Fig. 3. Effect of positions of aspartic acid and lysine within the hinge region on the N-domain translocation. Proline, lysine, and aspartic acid were scanned from position 60 to 69. Scanning mutants were translated in the presence of RM and the N-domain translocation was quantified as in Fig. 2.

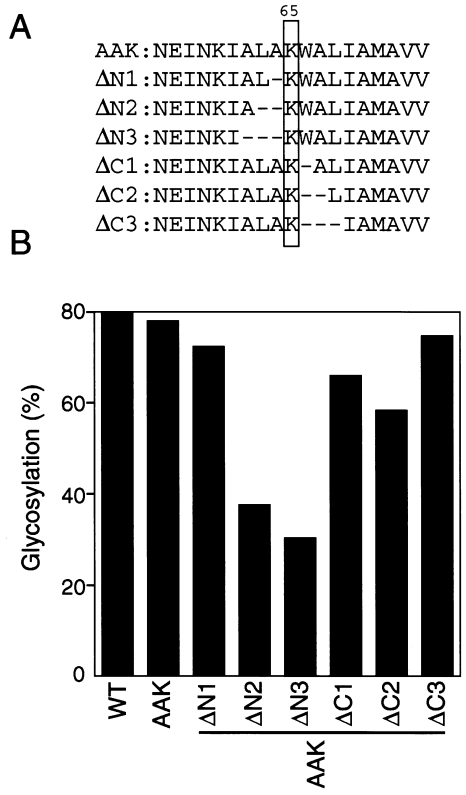

Fig. 4. Effect of deletions from the flanking region on N-domain translocation. A: One, two, and three residues were deleted from either the N-terminal or C-terminal flanking sequence of Lys 65, as indicated by the dashes. B: The N-domain translocation of each mutant was quantified as in Fig. 2.

residues by which the hydrophobic segment of 40 leucine residues can form a turn structure at the middle of the segment resulting in two transmembrane helices. The high correlation between the two scales might suggest that the helical hairpin-like structure is induced for N-domain translocation.

3.2. Position effect of aspartic acid, lysine, and proline

We then examined the position effect of three typical residues, aspartic acid, lysine, and proline, on N-domain translocation using the constructs in which these were scanned in the hinge region of the AAA mutant (Fig. 3). Proline scanning mutations did not result in a dramatic decrease in efficiency, whereas two peaks were observed at the positions separated by two residues. In contrast, scanning mutants of lysine and aspartic acid had clear position dependency with a distinct peak and bottom, indicating that these residues have crucial roles at specific positions. Because the deletion of up to six residues from the middle portion of the H-region had no effect on N-domain translocation [6], it is not likely that these mutations resulted in the translocation defects of the N-domain by affecting the hydrophobicity of the H-region. We concluded that the positions in the hinge region were not equivalent for the function of lysine or aspartic acid.

To clarify whether the strict position requirements were defined by the context in relation to the preceding or following portions, we deleted one, two, or three residues either from the N-terminal or from the C-terminal flanking regions of the Lys 65 construct (Fig. 4). The deletion of two or three residues from the N-terminal flanking region decreased N-domain translocation, whereas deletions of the C-terminal flanking sequence did not (Fig. 4B), suggesting that the preceding sequence defines the position requirement.

4. Discussion

The ribosome synthesizing Syt II is targeted to the membrane just after the N-domain and the H-region emerge from the ribosome. Translocation of the N-domain is initiated by the H-region, which penetrates into the translocon (Fig. 5). An unexpected finding in the present study is that a single amino acid residue in the hinge between the N-domain and the H-region greatly affects N-domain translocation: the AAA mutant of Syt II did not acquire the SA-I orientation, whereas the single amino acid change at position 65 into amino acid residues with a high turn propensity scale resulted in the correct membrane topology. These results indicate that specific residues with high turn propensity scale play the critical role for N-domain translocation at specific positions and suggest that the formation of a hairpin-like structure at the hinge is critical for translocation of the Syt II N-domain.

A group of amino acid residues with a low turn propensity at position 65 prevent translocation of the N-domain. At least three possible factors can direct the fate of the N-domain:

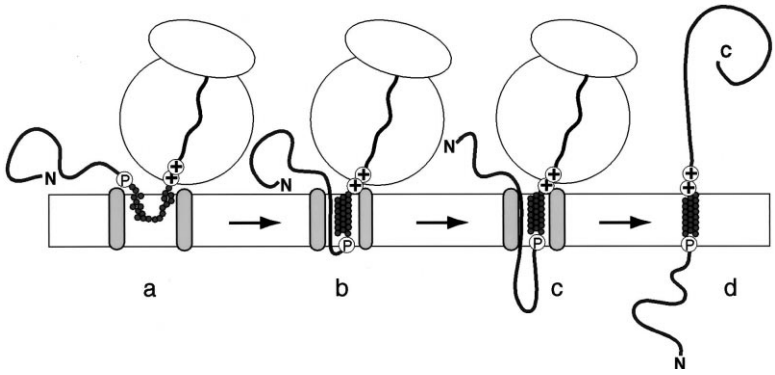

Fig. 5. Working model of secondary structure during N-domain translocation within the translocon. a: After the nascent polypeptide chain on the ribosome is targeted to the translocon on the ER membrane, the H-region is released from the SRP and then enters into the translocon. b: Formation of a hairpin structure is critical for the H-region to form a transmembrane helix. c,d: After the hairpin structure formation, some portion exits to the lumen and would be trapped by a molecular chaperone. The polypeptide chain is then pulled into the lumen by unknown factors.

(i) positively charged residues are well known determinants of orientation of the H-region of signal sequences and negatively affect N-domain translocation. Introduction of those residues into the amino-terminal side of SA-I often results in conversion of the orientation, exhibiting SA-II function [9,13,27,28]. Those residues are also frequently observed after the stop-transfer sequences and they inhibit polypeptide chain translocation at the entrance of the translocon [29,30]. (ii) Hydrophobicity and length of the H-region could be determinants of N-domain translocation. When the H-region of SA-I of cytochrome P450(1A1) was shortened, translocation of the N-domain was suppressed and the mutated segment became either a type II signal-anchor sequence or a signal peptide [9]. In the cases of the mutants used in this study, hydrophobicity should not be affected by these minor changes. We previously demonstrated that deletion of at most six residues from the center of the H-region had no influence on N-domain translocation [6]. (iii) Stable folding of the N-domain is also a candidate to suppress translocation of the N-terminus: stabilization of the folding state by N-domain ligands prevents translocation and fixes the N-domain on the cytoplasmic side [12]. The amino acid requirements at position 65 can be explained by the local secondary structure of the hinge region. We demonstrated that the AAA mutants were correctly targeted to the translocon and anchored in the membrane in an alkaline-resistant manner [6], indicating that SRP recognition, targeting to the ER, and partition of the H-region to the membrane environment are not affected by the mutation. An appropriate local structure, most likely a hairpin structure, should be adopted at the hinge to translocate the N-domain polypeptide.

When the residues were scanned along with the hinge between residues 60 and 69 of the AAA mutant, an interesting periodicity was observed, suggesting a helical conformation. The proline residue had a similar pattern to aspartic acid and lysine but the decrease due to the proline shift was not as dramatic as that due to the other two (Fig. 3). This suggests a difference of some intrinsic characteristics of these residues: aspartic acid and lysine probably do not have a strong tendency to form a turn structure but do not impede breaking of the helix in hydrophobic–hydrophilic interface, whereas the proline tends to break the α-helix and form a turn structure by itself. The former two can, thus, result in a hairpin structure only at the specific position. The position effect indicates that those residues have roles at the same face of the α-helix. The function of Lys 65 for mediating N-domain translocation was gradually suppressed by two or three residue deletions from its N-terminal flanking region, while deletions from the C-terminal flanking region did not have an effect (Fig. 4). The deletion of one to six residues from the central part of the H-region also did not affect the function of Lys 65 (data not shown). Thus, the position of Lys 65 is defined by the former sequence but not by the following H-region. The residue with high turn potential allows freedom of the amino-terminus of the H-region that is penetrating the translocon. Because the specific side of the H-region of the signal peptide interacts with a specific site of the translocon [31], the specific site of the N-domain is also likely to interact with a subunit of the translocon in this case. Such an interaction might explain the clear position dependency of the residue.

In contrast to the specific requirements at the hinge, the H-region does not require amino acid residues with high turn propensity, suggesting that the H-region forms a loose loop but not a tight hairpin structure while it is entering the translocon (Fig. 5). The turn structure at the hinge, however, is a prerequisite for the completion of the transmembrane helix of the H-region. What type of force drives further movement of the polypeptide chain of the N-domain? We hypothesize that the formation of the transmembrane helix of the H-region induces the movement of the N-domain like the lash of a whip. Once some residues of the N-domain are exposed to the lumen, they would be trapped and/or be pulled by a chaperone molecule into the lumen. Such factors driving the movement are currently being investigated in our laboratory.

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