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A sugar chain at a specific position in the nascent polypeptide chain induces forward movement during translocation through the translocon

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Nascent polypeptide chains synthesized by membrane bound ribosomes are cotranslationally translocated through and integrated into the endoplasmic reticulum translocon. Hydrophobic segments and positive charges on the chain are critical to halt the ongoing translocation. A marginally hydrophobic segment, which cannot be inserted into the membrane by itself, can be a transmembrane segment depending on its downstream positive charges. In certain conditions, positive charges even 60 residues downstream cause the marginally hydrophobic segment to span the membrane by inducing the segment to slide back from the lumen. Here we systematically examined the effect of a core sugar chain on the fate of a marginally hydrophobic segment using a cell-free translation and translocation system. A sugar chain added within 12 residues upstream of the marginally hydrophobic segment prevents the sliding back and promotes forward movement of the polypeptide chain. The sugar chain apparently functions as a ratchet to keep the polypeptide chain in the lumen. We propose that the sugar chain is a third topology determinant of membrane proteins, in addition to a hydrophobic segment and positive charges of the nascent chain.

Keywords: glycosylation/membrane protein/protein topogenesis/translocation/translocon.

Abbreviations: AP, acceptor peptide; EndoH, endoglycosidase H; ER, endoplasmic reticulum; H-segment, hydrophobic segment; mH-segment, marginally hydrophobic segment; OSTase, oligosaccharyl transferase; ProK, proteinase K; RM, rough microsomal membranes; TM, transmembrane.

Polypeptide chains synthesized by membrane bound ribosomes are cotranslationally translocated across and/or integrated into the endoplasmic reticulum (ER) membrane via a protein-conducting channel, the so-called translocon (*1*). Its core portion is the Sec61 complex, comprising Sec61α, Sec61β and Sec61γ (*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 through which a wide variety of hydrophilic polypeptide chains can move. The pore is formed by 10 transmembrane (TM) helices and opens laterally to allow the translocating polypeptide to contact the membrane lipid. Oligosaccharyl transferase (OSTase) and signal peptidase flank the translocon. ER membrane proteins, such as translocating chain-associated membrane protein and translocon-associated protein complex, are also suggested to flank the Sec61 complex (*6*) and to promote the maturation of multispanning membrane proteins (*7*).

The ER-targeting and membrane insertion are initiated by a signal sequence emerging from the ribosome. The function of the signal sequence is primarily determined by its hydrophobic segment (H-segment), which is recognized by a signal recognition particle and subsequently by the translocon. During insertion into the membrane via the translocon, the orientation of signal sequences is influenced by their flanking positively-charged amino acid residues (*8–10*). If positive charges are rich on the N-terminal side of the H-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-terminus side is translocated through the translocon and the segment forms the N(lumen)/C(cytosol) orientation. The signal sequence itself serves to generate motive force for the N-terminal domain translocation (*11*, *12*). After the initiation of insertion into the translocon, the ongoing movement of polypeptide chains through the translocon is interrupted by the H-segment, which is functionally termed the 'stop-transfer sequence' (*13*). In this process, the hydrophobicity of the segment is the primary determinant and the flanking positive charges affect the stop-translocation (*13–15*). Simple partitioning of sufficient H-segments into the lipid phase through the translocon is a major and rational motivation for the stop-translocation and membrane insertion of the TM segments (*16*, *17*).

Even a marginal hydrophobic segment (mH-segment) that is insufficient for membrane insertion by itself, however, can span the membrane when accompanied by its downstream positively charged residues. Such an mH-segment is retained in a water environment, even though the segment spans the membrane (*18*). In certain cases, the positive charges are

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effective even when they are separated from the H-segment by more than 60 residues. The mH-segment is temporarily exposed to the lumen and then slides back to the translocon even in the presence of various chaperons in the lumen. The molecular mechanism for the sliding back remains unknown. When the exposed polypeptide chain contains an N-glycosylation site, it is accessible to OSTase in the lumen and in some cases glycosylated polypeptide chain does not slide back and is fully translocated. We hypothesized that a sugar chain might function as a ratchet, which retains the mH-segment in the lumen. The following reports rationalized the hypothesis; the N-terminal signal-anchor sequence can be reoriented by its downstream TM segment, and such reorientation is prevented by glycosylation of the segment initially exposed to the lumen (*19*). Translocation intermediates of secretory proteins whose C-terminus is retained in the ribosome can be released from the rough microsomal membranes (RM) into the cytosol. The release is also prevented by glycosylation in the lumen (*20*).

Here we examined the effect of a core sugar chain on the fate of the mH-segment of the nascent chain using a cell-free translation and translocation system. The sugar chain added within 12 residues upstream of the mH-segment induced forward movement of the polypeptide chain, whereas sugar chains at the upstream site more than 12 residues showed no stimulation effect. When glycosylation was inhibited by an acceptor peptide, translocation was not induced. The sugar chain sequestrates the mH-segment from the membrane and eventually promotes the forward movement of the chain. We point out that this sugar chain can also be a topology determinant, in addition to a hydrophobic segment and positive charges.

Materials and Methods

Materials

RM (*21*) and rabbit reticulocyte lysate (*22*) were prepared as previously described. RM was treated with EDTA and then with *Staphylococcus aureus* nuclease as previously described (*21*). Castanospermine (Calbiochem), the acceptor peptide (AP) (N-benzoyl-Asn-Leu-Thr-N-methylamide; Quality Controlled Biochemicals), proteinase K (ProK) (Merck), endoglycosidase H (EndoH) (New England Biolabs) and the DNA manipulating enzymes (Takara and Toyobo) were obtained from the indicated sources.

Construction of model proteins

In the following DNA construction procedure, DNA fragments obtained by polymerase chain reaction using primers including the appropriate restriction enzyme site (indicated in parentheses) were ligated into plasmid vectors that had been digested with the restriction enzymes. At each junction, the six bases of the restriction enzyme site were designed to encode two codons. Model constructs were based on rat serum albumin as described previously (*18*). For reference, the residue numbers of rat serum albumin in the database (NCBI, NP599153) are indicated in the figures. We confirmed that all the newly created glycosylation sites were good substrates for OSTase in the absence of the LA8-segment. The cDNA fragment encoding M^{1}-H^{319} of rat serum albumin (including the Kozak sequence at the 5′-end region and the DNA sequence encoding the Val-stop codon at the 3′-end; XbaI/ApaI) was subcloned into a pRcCMV (XbaI/ApaI). The restriction enzyme sites of NheI and Aor51HI, which encode AS and SA, and the DD-segment were created between V^{166} and F^{173}. Oligonucleotides encoding the 8-residue repeated Leu-Ala sequence (LA8) were inserted between the NheI and Aor51HI sites. Potential glycosylation (g) sites and 6-lycine (6K) clusters were introduced at various positions as indicated in each figure, whose positions relative to the LA8-segment are indicated in the parentheses. Glycosylation sites were created by the Quick change method (Stratagene) at the indicated positions, as follows: g(−19), S^{150}FQ to NST; g(−16), E^{153}NP to NST; g(−15), N^{154}PT to NST; g(−14), P^{155}TS to NST; g(−13), T^{156}SF to NST; g(−12), S^{157}FL to NST; g(−11), F^{158}LG to NST; g(−10), L^{159}GH to NST; g(−9), G^{160}HY to NST; g(−7), Y^{162}LH to NST; g(−6), L^{163}HE to NST; g(−5), H^{164}EV to NST; g(+1), S^{169}ADD to NSTA; g(+15), E^{183}KY to NST; g(+25), C^{193}TE to NST; g(+40), DA^{208}VKE to ANSTA; g(+91), A^{259}TD to NST. The 6K-clusters were inserted just after the position indicated in each figure. Standard polypeptides were made by creating a termination codon (TAG) at the indicated positions of the 6K(+60) model protein. The positions are defined as their location relative to the LA8-segment throughout this article; e.g. Ter(+60) stands for a termination codon created 66 residues downstream of the LA8-segment. All the constructed DNAs were confirmed by DNA sequencing.

Cell-free transcription and translation

Cell-free transcription and translation were performed essentially as previously described (*23*), except that translation reactions with RM contained castanospermine to prevent heterogeneity of the products due to trimming of the core sugar chain. Plasmid DNA was linearized with XhoI and then transcribed with T7-RNA polymerase. The RNA was translated in the reticulocyte lysate cell-free system for 1 h at 30°C in either the absence or presence of RM. The translation reaction included 110 mM potassium acetate (KOAc), 0.8 mM magnesium acetate ($Mg[OAc]_2$), 32% reticulocyte lysate, castanospermine (20 μg/ml) and 15.5 kBq/μl [^{35}S]-EXPRESS protein-labelling mix (Perkin Elmer). Where indicated, the AP (80 μg/ml) dissolved in DMSO was included. After the translation reaction, aliquots were treated with ProK (200 μg/ml) for 1 h on ice. Where indicated, aliquots were treated with EndoH under denaturing conditions according to the manufacturer's instructions. Radiolabelled polypeptide chains were analysed by SDS–PAGE, visualized with an imaging analyser (BAS1800, Fuji Film), and quantified using Image Gauge software (Fuji Film). The translocation percentage was estimated as the percentage of the diglycosylated product among non-glycosylated, monoglycosylated and diglycosylated products. For the translocation chase experiment, translation reactions were performed for 20 min, terminated with 2 mM cycloheximide and then the reaction mixture was incubated further for the indicated period. Aliquots (10 μl) of the chase reaction were sampled and subjected to SDS–PAGE. Where indicated, aliquots were treated with ProK.

Results

A sugar chain induces forward movement of the mH-segment

To examine the effects of positive charges and a sugar chain on the movement of the mH-segment, a cluster of 6 lysines (6K-cluster), potential glycosylation sites and a mH-segment were introduced into model proteins (Fig. 1A). The model protein comprised rat serum albumin as a backbone, an N-terminal signal peptide and LA8-segment as the mH-segment. The potential glycosylation sites were introduced at either 19 residues upstream (−19) or 1 residue downstream (+1) of the LA8-segment, and at 91 residues downstream (+91) of the LA8-segment. The 6K-cluster was inserted either 11 residues (+11) or 60 residues (+60) downstream of the LA8-segment. In this article, the positions of the 6K-cluster and the glycosylation site in relation to the LA8-segment are indicated in parentheses; e.g. g(−19, +91) stands for the glycosylation sites located at 19 residues upstream and at 91 residues downstream of the LA8-segment; 6K(+11) stands for the 6K-cluster at 11 residues downstream of the

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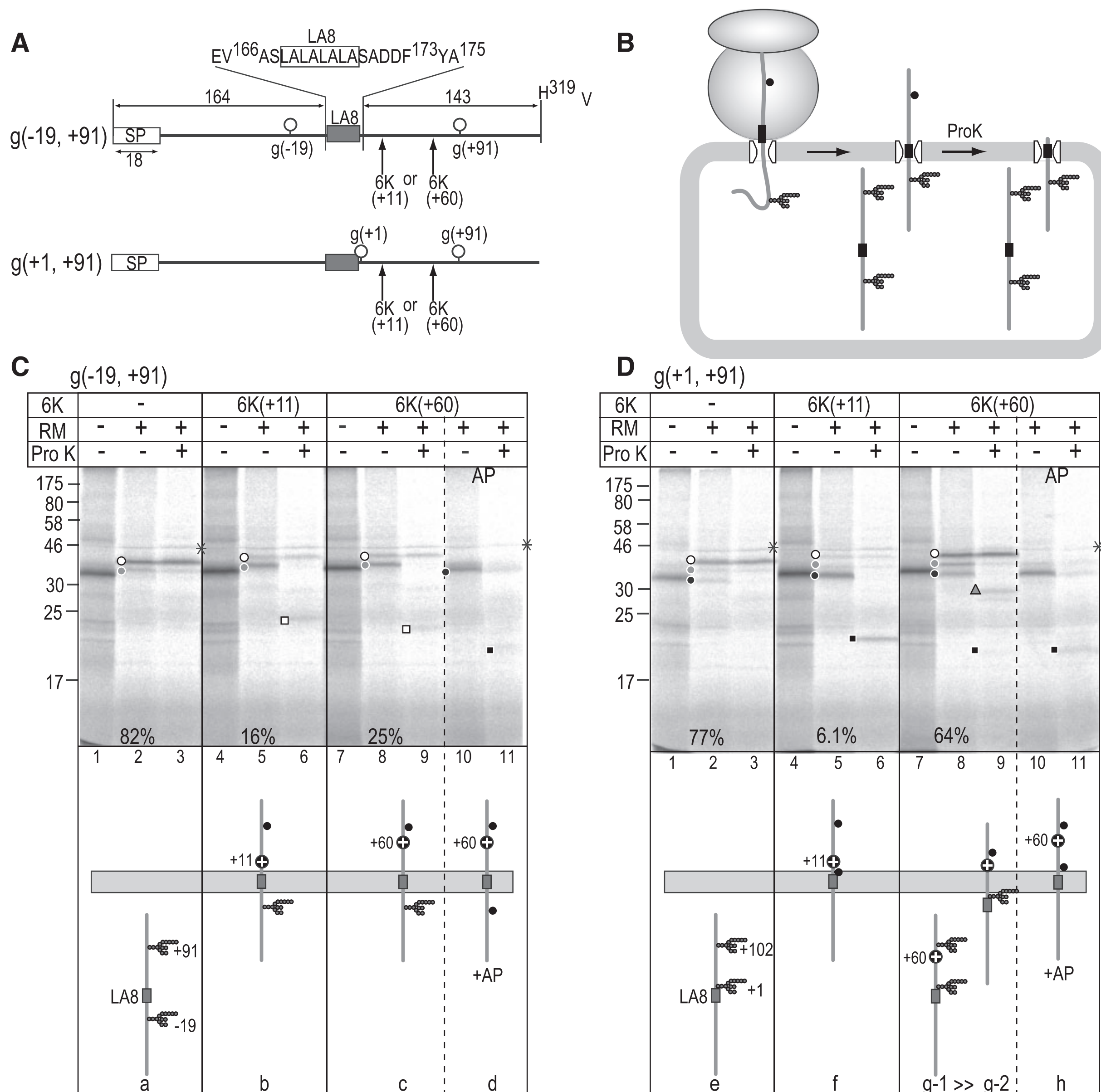

Fig. 1 A sugar chain induces the forward movement of the polypeptide chain. (A) Model proteins include a signal peptide (SP) at the N-terminus, LA8-segments in the middle portion, a cluster of six lysines (6K), and potential glycosylation sites (open circles) at the indicated positions. The superscripted numbers indicate the residue numbers of the original rat serum albumin polypeptide. The positions relative to the LA8-segment of the glycosylation sites and 6K-cluster are indicated in parentheses. (B) The membrane topology of the nascent polypeptide chain was assessed based on the glycosylation status and ProK-treatment. N-terminal SP initiates cotranslational translocation and the potential site is glycosylated in the lumen. If the polypeptide is fully translocated, it is diglycosylated. If translocation is stalled, the second glycosylation site is not glycosylated and the product is monoglycosylated. ProK degrades the polypeptide chain exposed to the cytoplasmic side of the membrane. Glycosylated (forks) and non-glycosylated (closed circles) potential sites were indicated. (C) Membrane translocation and topology assay of the g(−19, +91) model. The model proteins were translated in a cell-free system in the absence or presence of RM. An aliquot of the translation product was treated with ProK. Diglycosylated forms (open circles), monoglycosylated forms (grey dots), and non-glycosylated forms (black dots) are indicated. ProK resistant monoglycosylated (open squares) and non-glycosylated forms (closed squares) are indicated. The non-specific product of the endogenous mRNA is indicated (asterisks). Percent of diglycosylated form among glycosylated and non-glycosylated forms is indicated as an estimation of translocation per cent (%). An aliquot was synthesized in the presence of a sugar chain acceptor peptide (AP). In the absence of a 6K-cluster, the polypeptide chain was mainly translocated (case a). In the presence of the 6K-cluster, the products mainly spanned the membrane by the LA8-segment (case b, c, and d). An attached sugar chain is indicated by fork. The illustrations are not in scale: to clearly indicate relative positions of mH-segment, positive charge cluster, and sugar chains, we expanded the membrane region. (D) Membrane translocation and topology assay of the g(+1, +91) model. The ProK-resistant 30-kDa fragment is indicated (gray triangles). In the absence of a 6K-cluster, the majority of the polypeptide chain was fully translocated and diglycosylated (case e). In the presence of 6K(+11), the product spanned the membrane by the LA8-segment and was barely glycosylated (case f). In the presence of 6K(+60), the product was glycosylated not only at g(+1) but also at g(+91) (case g-1). ProK did not degrade the diglycosylated form (case g-1), whereas it partially degraded the monoglycosylated form and generated a membrane protected fragment (case g-2). A sugar chain at the g(+1) site induced translocation of polypeptide chain (case g-1).

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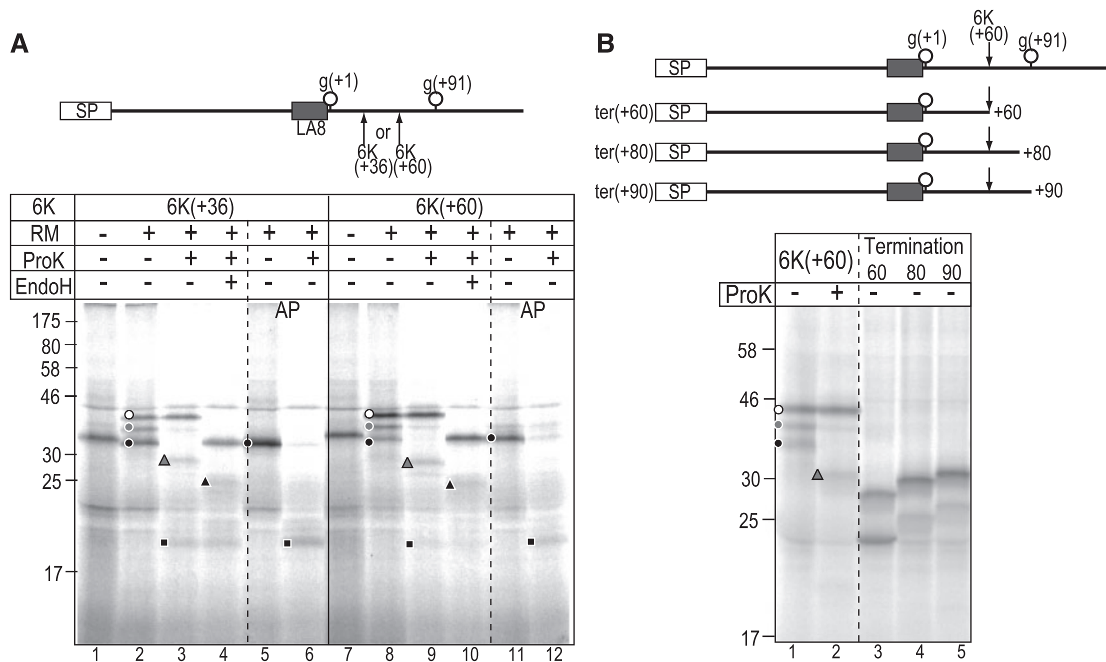

Fig. 2 The 30-kDa ProK-resistant fragment is a translocation intermediate. (A) Model proteins were expressed. Where indicated, the reaction included RM and/or AP. Aliquots were treated with ProK and/or EndoH. The ProK-resistant 30 kDa-fragment (grey triangles) and its de-glycosylated form (black triangles) are indicated. The 30-kDa fragment was not detected when glycosylation was suppressed by AP (lanes 6 and 12). (B) Standard polypeptides, in which the termination codon was inserted into the indicated positions, were translated in the presence of RM. The model protein with 6K(+60) was expressed in the presence of RM and an aliquot was treated with ProK. The 30-kDa fragment showed mobility between the ter(+80) and ter(+90) models. The fragment was glycosylated at the first glycosylation site, but the C-terminal portion of 60–70 residues remained on the cytoplasmic side (Fig. 1, case g-2).

LA8-segment. The model proteins were expressed in a cell-free translation system in the presence of RM. The N-terminal signal peptide triggers translocation of the following polypeptide chain. If the polypeptide is fully translocated, it is diglycosylated (Fig. 1B). If translocation is stalled at the translocon between the glycosylation sites, the g(+91) site is not glycosylated. Membrane topology was also probed with ProK treatment, which degrades the polypeptide chain exposed on the cytoplasmic surface of the membrane.

First, a model protein with g(−19) and g(+91) sites was examined (Fig. 1C). When synthesized in the presence of RM, the model protein without a 6K-cluster became 4 or 2 kDa larger than the non-glycosylated form that was synthesized in the absence of RM. The non-glycosylated form was rarely observed in the presence of RM, indicating that the ER targeting is efficient in this system. These larger forms were converted to the non-glycosylated form by EndoH treatment (data not shown), confirming that the 4 and 2 kDa larger forms were diglycosylated and monoglycosylated forms, respectively. The per cent of the diglycosylated form among the glycosylated forms was used to estimate translocation per cent (%). When treated with ProK, the diglycosylated form was not degraded (lane 3). These results indicated that in the absence of the 6K-cluster, the nascent chain was largely translocated into the lumen and the LA8-segment by itself is insufficient for stop-translocation. On the other hand, in the presence of 6K(+11) or 6K(+60), the monoglycosylated form was the major product (Fig. 1C, lanes 5 and 8). Upon ProK-treatment, the monoglycosylated form was degraded and membrane protected ProK-resistant fragment was observed, indicating that the LA8-segment with the 6K-cluster spans the membrane (Fig. 1C, case b-c). The ProK cleaves predominantly around 13–15 residues downstream of the LA8-segment, so that the ProK-resistant fragment of the 6K(+11) model is larger than that of the 6K(+60) model (*18*).

To test the effect of the sugar chain position, the g(−19) site was moved to g(+1) (Fig. 1B). In the absence of the 6K-cluster, the product was largely diglycosylated. Only trace amounts of the processed non-glycosylated form were observed (Fig. 1D, lane 2). Again, the LA8-segment alone was translocated through the membrane. In the presence of 6K(+11), the major product was the processed non-glycosylated form (Fig. 1D, lane 5). The diglycosylated form was present in only trace amounts and was resistant to ProK (lane 6), whereas the major non-glycosylated product was converted to the ProK-resistant fragment. The 6K(+11) caused the LA8-segment to span the membrane, but did not allow for glycosylation of the g(+1) site. In surprising contrast, the model with 6K(+60) was efficiently diglycosylated (Fig. 1D, compare lanes 5 and 8). The diglycosylated form was resistant to ProK, indicating that the major product was fully translocated into the lumen. The 6K(+60) allowed not only the g(+1) site, but also g(+91) site to be glycosylated. A 30-kDa ProK-resistant fragment was also reproducibly observed in addition to the diglycosylated fully ProK-resistant form. Details of

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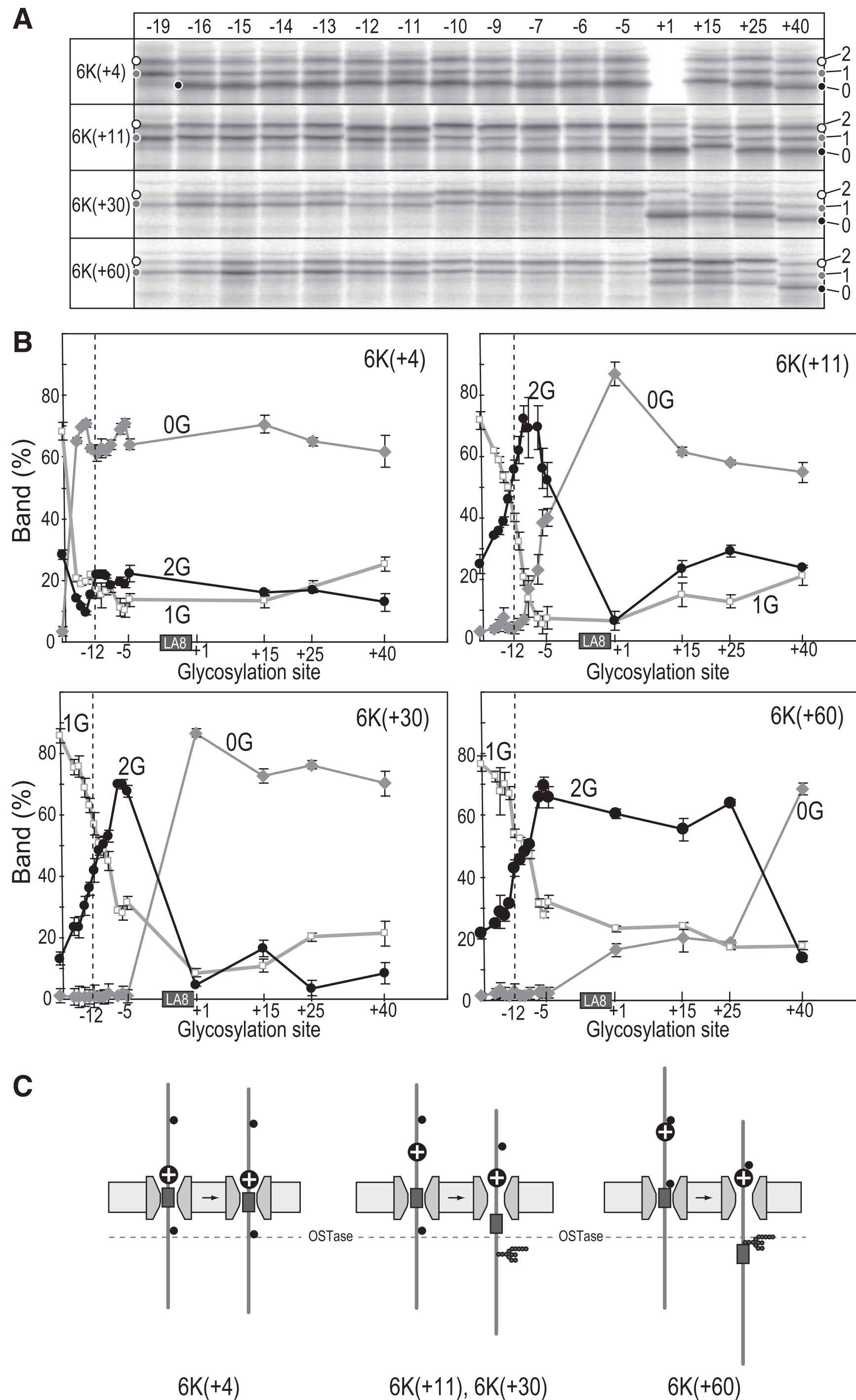

Fig. 3 Effect of the sugar chain position on translocation. (A) The models in which the first glycosylation sites were scanned, the second site was fixed at g(+91), and the 6K-cluster was included at the indicated positions, were translated in the presence of RM. Diglycosylated (open circles), monoglycosylated (grey dots), and non-glycosylated (black dots) forms are indicated. Numbers of the sugar chain are indicated at the right side of the panel. Because the g(+1) site of the 6K(+4) model was hardly glycosylated even in the absence of the LA8-segment, this model was not examined. (B) Each form was quantified and the percentage of each band among the total product was calculated and indicated. The assay was repeated three times and the averages are indicated with SD. (C) Schemes indicating the topology of the 6K-cluster, LA8-segment, and glycosylation sites. In the case of 6K(+4), all the glycosylation sites except g(−19) were hardly accessible to OSTase. As the 6K-cluster shifted downstream, the more downstream potential sites became glycosylated. An attached sugar (forks) and non-glycosylated potential sites (closed circles) are indicated.

the unexpected large ProK-resistant fragment are addressed in the next section. Apparently, the g(+1) sugar chain caused full translocation of the product, despite the presence of the LA8-segment and 6K-cluster.

To examine whether the sugar chain itself at g(+1) is critical for the translocation, glycosylation of the nascent chain by OSTase was inhibited by an acceptor peptide (AP). It is a tri-peptide (Asn-Leu-Thr) of the sugar acceptor sequence. In the presence of AP,

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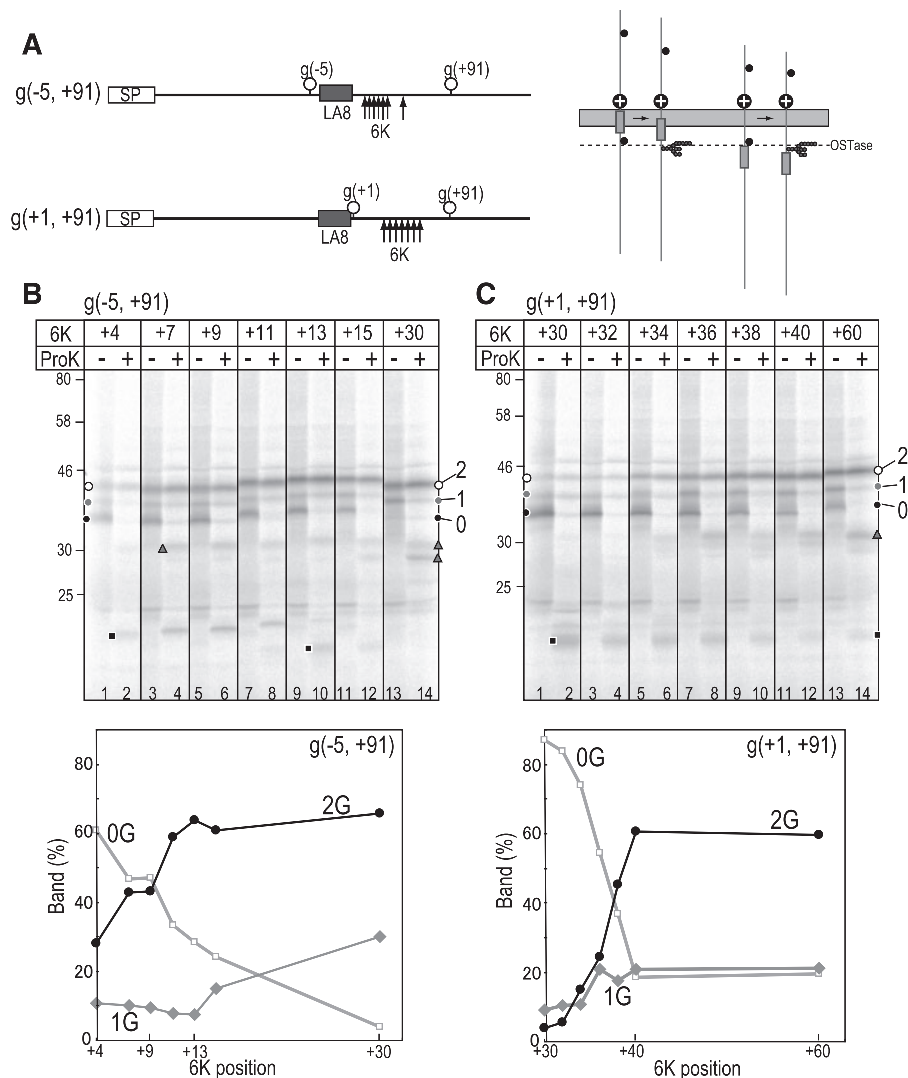

Fig. 4 Effects of the positive charge position on translocation. (A) The 6K-cluster was scanned as indicated in the model. The extent of glycosylation was determined by the 6K-cluster position. (B and C) Models were expressed in the presence of RM. Aliquots were treated by ProK. Each form of the products was quantified and the per cent among the total products was calculated.

glycosylation was greatly inhibited (Fig. 1D, lane 10) and the product was largely degraded by ProK. The 30-kDa ProK-resistant fragment was not observed, but the amount of the ProK-resistant membrane protected fragment was increased. Taken together, the 6K(+60) allowed for glycosylation of the g(+1) site and the sugar chain of g(+1) induced full translocation (Fig. 1D, case g-1).

The 30-kDa ProK-resistant polypeptide was derived from the translocation intermediate

To address the nature of the unexpected 30-kDa ProK-resistant fragment, we performed the same experiments using another model, 6K(+36). The large fragment was reproducibly observed with the 6K(+36) model and the 6K(+60) model (Fig. 2A, lanes 3 and 9). In the case of 6K(+36), the processed non-glycosylated form was increased. The ProK-resistant fragment was also increased with the 6K(+36) model. EndoH treatment after ProK digestion caused the 30-kDa form to shift down (lanes 4 and 10), indicating that the fragment was also glycosylated. When synthesized in the presence of AP, the 30-kDa ProK-resistant fragment was not observed, but the amount of the ProK-resistant fragment increased (lanes 6 and 12). Thus, the 30-kDa fragment possessed at least one sugar chain, but the C-terminal portion was on the cytoplasmic side and sensitive to ProK. The intermediate was due to the g(+1) sugar chain.

To estimate the precise length of the 30-kDa ProK-resistant fragment compared to standard polypeptides, we introduced a termination codon downstream of the LA8-segment and expressed them *in vitro* in the presence of RM (Fig. 2B). The 30-kDa fragment was observed between models with ter(+80) and ter(+90), indicating that it had been cleaved around 20 residues downstream of the 6K(+60)-cluster. The ProK-resistant fragment was same size as that of 6K(+36) model. Highly likely, only one site should be accessible to ProK in these cases. These results indicated that the 30-kDa ProK-resistant fragment is derived from the translocation intermediate,

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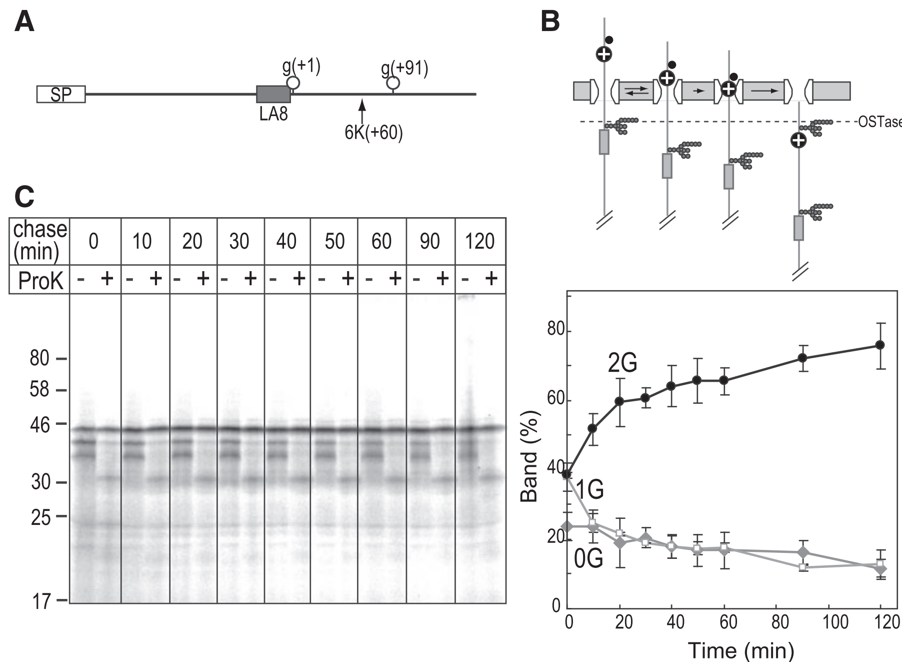

Fig. 5 Time course of sugar-driven translocation. (A) Model protein used. (B) Possible topology of the monoglycosylated form. Forward movement is stalled by the 6K-cluster, and sliding back is prevented by the sugar chain (forks). The rate-limiting process is entrance of the 6K-cluster into the translocon. (C) The model protein was translated in the presence of RM for 20 min. After the reaction was terminated by cycloheximide, incubation was continued for the indicated period. An aliquot was sampled and treated with ProK where indicated. Percent of each form was quantified. The experiments were performed more than three times and the averages are indicated with SD.

where the g(+1) site was glycosylated and the LA8-segment was in the lumen, but the C-terminal portion was on the cytoplasmic side (Fig. 1D, case g-2).

Position effect of the sugar chain for forward movement of polypeptide chain

The g(+1) sugar chain induced forward movement of the polypeptide chain, whereas the g(−19) sugar chain did not. We next systematically examined the effect of the sugar chain position on the translocation (Fig. 3). The g(−19) site was moved to various downstream positions. All the created potential glycosylation sites were confirmed to be efficient substrates of OSTase when the LA8-segment was deleted (data not shown). With the g(−19) model, irrespective of the 6K position, an intense monoglycosylated form were observed, indicating that the g(−19) site was glycosylated in all cases, whereas the translocation was stalled.

In contrast, when the glycosylation site moved to more downstream positions, the glycosylation status varied widely depending on the positions of the 6K-cluster. In the case of the 6K(+4) series, models with the g(−16) or the more downstream glycosylation sites were mainly non-glycosylated, and there were little monoglycosylated and diglycosylated forms. The glycosylation status was essentially the same among all the examined glycosylation sites (Fig. 3B). The models with the LA8-segment and the 6K(+4)-cluster were diglycosylated by nearly 30%, indicating that the product spanned the membrane by 70%. The models with the more downstream 6K-cluster showed a variety of glycosylation status. As the glycosylation site moved up to g(−7), the yield of the monoglycosylated form decreased and that of the diglycosylated form increased. The position showing the half maximal value of diglycosylation was g(−13)-g(−12), which was similar among the three models. In the case of the 6K(+11) and 6K(+30) models, the diglycosylated form was decreased and the non-glycosylated form was increased according to the glycosylation site. Peak diglycosylation occurred at the g(−9)-g(−7) site for the 6K(+11) model and at the g(−7)-g(−5) site for the 6K(+30) model. In the case of the 6K(+60) series, even the models with g(+1), g(+15) and g(+25) sites could be diglycosylated, whereas that with the g(+40) site was rarely glycosylated. The sugar chains at a specific position induce full translocation of the nascent chain.

Position of the K-cluster determines accessibility to OSTase

To address the precise dependency of the 6K-cluster position, we performed scanning of the cluster using the models with either g(−5, +91) or g(+1, +91) sites (Fig. 4). In both cases, the non-glycosylated form decreased and the diglycosylated form increased as the 6K-cluster moved downstream. The small ProK-resistant fragment spanning membrane by the LA8-segment similarly decreased. The extent of the 30-kDa ProK-resistant fragment was correlated with that of the diglycosylated form. Access of the first glycosylation sites to OSTase in the lumen caused glycosylation of these sites and the sugar chain induced further translocation of the polypeptide chain. The fate of the mH-segment (LA8-segment) is greatly

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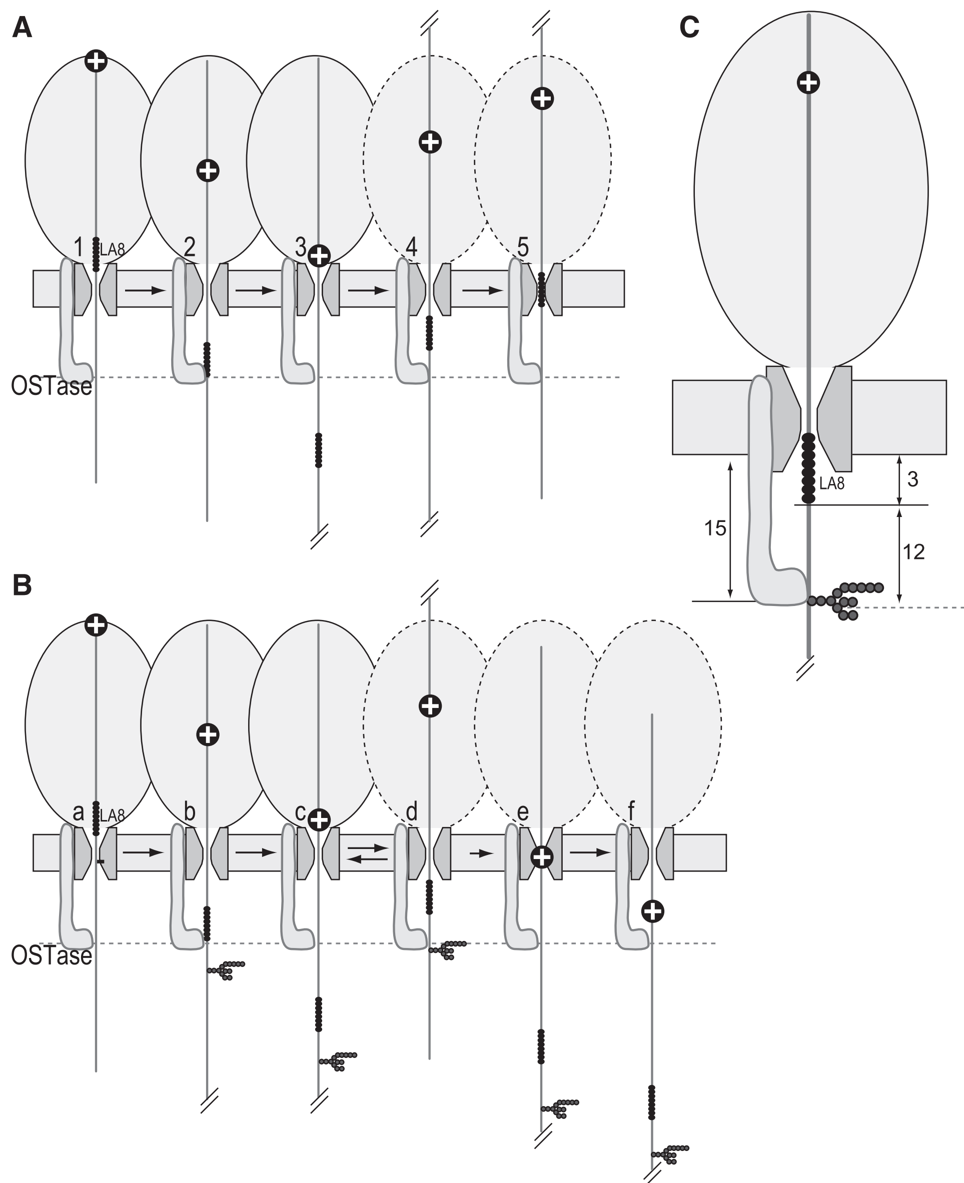

Fig. 6 A sugar chain prevents the H-segment from sliding back and induces forward movement of the polypeptide chain. (A) In the absence of a flanking sugar chain, the mH-segment is translocated into the lumen (1–3) and the 6K-cluster causes the H-segment to slide back to translocon (4 and 5). Large subunits of ribosomes are schematically indicated by ovals. Those being released from the nascent chain are indicated by dotted ovals. (B) In the presence of a sugar chain (forks) flanking the mH-segment, the mH-segment exposed to the lumen is prevented from sliding back (a–d) and the polypeptide could be fluctuate (c and d). The sugar chain strictly suppresses the backward motion, but arrest by the 6K-cluster might occasionally be overcome during fluctuation (c–e). The polypeptide chain ends up moving toward the lumen (f). (C) The potential glycosylation site separated by 15 residues from the membrane is accessible to OSTase. The glycosylated chain can move freely at least beyond the OSTase. The OSTase, translocon, or its neighbouring proteins should hinder the glycosylated polypeptide chain from moving back.

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affected by the positions of positive charges and glycosylation sites.

Time-course of full translocation

A sugar chain at the g(−12) site or more downstream site promotes the movement of the polypeptide chain. We assessed the timing of the forward movement of the newly synthesized chain: whether it occurs after termination of the chain elongation (Fig. 5). The translation reaction of the model protein was performed for only 20 min and terminated with cycloheximide. The reaction mixture was further incubated for the indicated time period. After 20 min translation, a significant amount of the monoglycosylated form remained. The monoglycosylated form and non-glycosylated form were sensitive to ProK, while the diglycosylated form was resistant, indicating that the monoglycosylated form was a translocation intermediate. It was converted to the diglycosylated form during the chase period. The translocation was partially stalled until the polypeptide chain elongated to the C-terminus (Fig. 6B, case c-d) and the intermediate was post-translationally translocated into the lumen in a time-dependent manner.

Discussion

We systematically analysed the effect of a sugar chain on the behaviour of the mH-segment of a nascent polypeptide chain in the translocon. The fate of the mH-segment is critically affected by a sugar chain at a specific site in the nascent chain. Here we propose that the sugar chains are the third topology determinant in addition to hydrophobic segments and positive charges. In certain cases, the mH-segment (LA8-segment) can form a TM topology depending on its far downstream positive charges (Fig. 6A); it is tentatively exposed in the lumen, slides back to translocon, and ends up forming a TM topology. During the process, the sugar chain added within 12 residues upstream of the mH-segment promotes full-translocation through the translocon as follows (Fig. 6B). The LA8-segment is once translocated through the translocon into the ER lumen and the glycosylation site is accessible to OSTase in the lumen (Fig. 6B, case a-b). The following positive charges arrest the forward movement and induce the nascent chain to slide back (c-d). The extent of exposure is critically determined by the position of the 6K-cluster. As the 6K-cluster shifted downstream, the more downstream potential sites became glycosylated. The g(−12) site of the 6K(+4) model can hardly be accessible to OSTase and the model showed only 20% translocation. In the case of other models with more downstream 6K-cluster, the g(−12) site was accessible to OSTase and the sugar chain induced translocation (Fig. 3). If the sugar chain is added to the g(−12) site or to more downstream positions, the sugar chain prevents the LA8-segment from sliding back to the translocon (d). If glycosylation is suppressed by AP, the polypeptide chain can back up to the translocon. Because the sugar chain strongly blocks the slide back, the restriction of positive charges could probably be overcome by Brownian motion of the chain (e) and the chain moves forward (f). Apparently, the function of the sugar chain is to act as a ratchet. Even the cotranslational protein translocation is not only a simple vectorial movement during polypeptide chain elongation, but also includes bidirectional movement of the polypeptide chain in the translocon; cotranslational movement (a-c), translocation arrest and slide back (c-d), and forward movement (d-f).

The position effect of the sugar chain on the chain movement shows a clear threshold. The position for the half maximum is around the g(−12) site. Whenever the g(−12) site or the more downstream site is glycosylated, the LA8-segment is trapped in the lumen and the nascent chain is committed to be fully translocated (Fig. 6B). The sugar chain added at a more than 12 residues upstream of the LA8-segment, however, does not show such an effect. The threshold seemed constant irrespective of the position of the 6K-cluster. Because a potential glycosylation site separated by 15 residues from the membrane is accessible to OSTase (*24*, *25*), only a few residues of the LA8-segment are exposed from the translocon when the g(−12) site is glycosylated (Fig. 6C). The polypeptide chain with the sugar chain should move forward in the lumenal space and could slide back at least to the OSTase active site (Fig. 6B, case c-d). Thus, it is reasonable to hypothesize that steric hindrance for sliding back should be located at the translocon or its adjacent materials (OSTase or other accessory factors of the translocon). Actually, the translocon-associated protein complex has been observed as a large protrusion flanking the Sec61 complex (*26*, *27*).

Here we present the possibility that the fate of a polypeptide is greatly affected by the presence of a sugar chain. This could be a critical topology determinant of multi-spanning membrane proteins. If there is no sugar chain flanking the mH-segment, an mH-segment retained in the translocon by downstream positive charges could be stabilized by interacting with other TM segments to form a TM helix bundle. On the other hand, if there is a sugar chain at a certain position, the mH-segment no longer spans the membrane and is positioned in the lumen as an extra-membrane loop. Furthermore, it would greatly affect the context of the following hydrophobic segments and consequently their TM topology. During the evolution of multispanning membrane proteins, glycosylation sites flanking the mH-segments should be selected, as should positive charges.

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Conflict of interest

None declared.

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