Assessment of Topogenic Functions of Anticipated Transmembrane Segments of Human Band 3*

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From the ‡Department of Molecular Biology, Graduate School of Medical Science and the §Department of Clinical Chemistry and Laboratory Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812-8582, Japan

Band 3 protein is a typical multispanning membrane protein whose membrane topology has been extensively studied from various protein chemical approaches. To clarify the membrane topogenesis of this multispanning protein on the endoplasmic reticulum, the topogenic functions of the anticipated transmembrane segments were individually assessed in an in vitro system using two series of model proteins in which each segment was placed in either a “stop-transfer” context or a “translocation initiation” context. They were expressed in a cell-free system containing rough microsomal membranes, and their topologies were evaluated by taking advantage of either sensitivity to protease or accessibility to N-glycosylation. We found that some segments seem to possess insufficient topogenic functions for membrane integration: the second transmembrane segment (TM2) is insufficient for the stop-transfer sequence, and TM3, TM5, and TM7 are not sufficient for the translocation initiation. In contrast to these phenomena, we herein demonstrate that TM2 shows an efficient stop-transfer function when it is near the preceding TM1 and suggest that TM3, TM5, and TM7 are followed by TM segments with a strong topogenic function to form N\textsubscript{exo}/C\texttextsubscript{cyt} topology, via which the preceding segments are integrated into the membrane. From these results, we propose that the interactions between the TMs should be operative during membrane integration, and that the segments with a weak topogenic function are given a transmembrane orientation by their following TMs.

Membrane proteins on the secretory pathway in eucaryotic cells are integrated into the membrane at the endoplasmic reticulum (ER)\textsuperscript{1} and acquire their final membrane topology. Almost all the membrane proteins are integrated into the membrane during protein synthesis. Several sequences of their nascent polypeptide chains (the so-called “topogenic sequences”) regulate the cotranslational insertion to define the final membrane topology (1–3). Signal sequences are responsible for ER targeting and for initiating the translocation. The signal sequences have been classified into three classes consisting of a signal peptide and two signal-anchor sequences (type I and type II signal-anchor sequences (SA-I and SA-II), respectively; Refs. 2 and 4). Signal peptide and SA-II mediate the translocation of their following portion showing N\textsubscript{cyt}/C\textsubscript{exo} orientation. Signal peptide is processed by signal peptidase, whereas SA-II is not cleaved to become a membrane-anchoring segment. In contrast, SA-I mediates the translocation of its amino-terminal portion and leaves the following portion on the cytoplasmic side of the membrane. All three signal sequences emerging from the ribosomes are recognized by the signal recognition particle and target the ribosome to the ER membrane. The signal sequences are released from the signal recognition particle by signal recognition particle receptor and then enter into the protein translocation channel in the ER membrane forming loop structure. The stop-transfer sequence (St) is a hydrophobic segment that interrupts the ongoing protein translocation initiated by signal peptide and SA-II (5, 6). The cotranslational integration of the membrane proteins is mediated by the protein translocation channel (or the so-called translocon) consisting of the Sec61p complex and translocating chain-associated membrane protein (TRAM) (7, 8).

Although the topogenic functions of these sequences in simple single spanning membrane proteins have been established, their contribution to the membrane topogenesis of multispanning proteins remains to be clarified. According to a hypothesis that has been widely accepted (9, 10), nascent polypeptide on the ribosomes is targeted by the amino-terminal signal sequence in a signal recognition particle-mediated fashion, and then the following hydrophobic segments are sequentially integrated into the membrane from the N terminus while showing the alternative functions for either translocation initiation or stop-translocation (11); after the synthesis of cytoplasmic domain, the following transmembrane segment (TM) initiates the translocation of the following portion (internal SA-II), and the next transmembrane segment is supposed to stop the ongoing translocation (St; Ref. 9). This model, however, is not always applicable; in some contexts, certain transmembrane segments are left outside the membrane (12, 13), and some TM segments in the translocation initiation context cannot mediate the translocation of the following portion (14, 15). In this study, we aim to clarify the topogenic functions of individual TMs of a multispanning membrane protein, band 3.

Band 3 is a major multispanning membrane protein in erythrocytes, and its topology has been extensively studied from various approaches (16, 17). It consists of two domains: (a) an amino-terminal cytoplasmic domain of about 400 amino acid residues that bind to the cytoskeleton to regulate the cell shape of the erythrocytes, and (b) the carboxyl-terminal half, which is a transmembrane domain responsible for the anion-exchanging activity. A current model of the membrane topology of human band 3 deduces 14 TMs (Fig. 1; Ref. 18). We herein elucidate
constructs for the translocation initiation assay (Fig. 3A).

Prolactin Fusions—TM1 (Thr375–Arg432, NocIXhol), TM1–2 (Thr375–Gln457, NocIXhol), and TM1–3 (Thr375–Ile626, NocIXhol) were ligated with mature prolactin fragment (Xhol/XbaI) on pCITE2b (NocIXbaI) to yield pTM1–2–2, and pTM1–3–2, respectively. TM1 (Thr375, NocIXhol), mature prolactin fragment SaUManI, TM2 (Gly428–Gln527, EcoRI/Xhol), and mature prolactin fragment Xhol/XbaI were sequentially ligated to yield pTM1–200–2–2. In all of these constructs, Thr377 was mutated to create the NcoI site. To monitor the translocation, the reporter of prolactin was mutated to include the N-glycosylation site by point mutagenesis (T9ON).

Constructs for the Orientation Assay—The glycosylated loop of band 3 (Asp657–Try662, Noc/I/EcoRI), inserted fragment (EcoRI/Xhol), and mature prolactin (Xhol/XbaI) was sequentially ligated into pCITE2b (NocIXbaI). The Asp657 residue was changed to create an initiation codon and the NcoI site. In these cases, both the flanking cytoplasmic and extracellular loops were included in the inserted TMs (TM1 (Ala400–Gly436), TM4 (Pha737–Arg828), TM6 (Leu626–Arg652), and TM8 (Gln625–Gly701)).

In Vitro Transcription Translation and Protease Treatment

Both in vitro transcription and a topology assay were carried out as described previously (19). Plasmids were linearized by ScaI and then transcribed using T7 RNA polymerase. The RNAs were translated in reticulocyte lysate in either one- or presence of rough microsomal membranes (RM). After translation, the aliquots were treated with proteinase K (200 μg/ml) and/or endoglycosidase H.

RESULTS

The major objective of this study was to examine whether or not the anticipated TMs of band 3 function individually during the cotranslational integration process. To this end, each TM with either an extracellular or a cytoplasmic loop was positioned either in the St or the internal SA-II context as described below. Four loops (L2, L9, L11, L13) consisting of less than 5 residues were not examined.

Assessment of the Stop-Transfer Function—The assessment of a stop-transfer function was carried out essentially as described by Kuroiwa et al. (5, 6) using systematically constructed model proteins (Fig. 2A). Translocation was initiated by the amino-terminal signal peptide. If the inserted TM shows the St function, then the carboxyl-terminal half is exposed on the cytoplasmic side of the membrane, where the carboxyl-terminal PL domain is sensitive to externally added proteinase K (Fig. 2B). If the inserted TM does not show the St function, the nascent polypeptide should be translocated into the luminal space, where it becomes fully resistant to the externally added protease (Fig. 2B).

Typical results of the assay are shown (Fig. 2C). When the model proteins were synthesized in the absence of RM, single bands were observed with the expected molecular weight (lanes 1, 4, and 7). When synthesized in the presence of RM, they were processed into mature forms (lanes 2, 5, and 8). After proteinase K treatment, truncated fragments (a) were newly observed in addition to the mature forms (b; lanes 3, 6, and 9). Both of the membrane-protected forms were degraded by proteinase K treatment in the presence of detergent (data not shown).

Assessment of the Translocation Initiation Function—To assess the internal SA-II function of the anticipated TMs, we constructed another series of model proteins (Fig. 3A). The H1 loop of the TM was replaced with the H1 segment including an N-glycosylation consensus site (Noc/I/EcoRI) to obtain the

the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each TM and demonstrate that those of individual TMs are not always high enough to explain the topogenic functions of each.
segment translocated the amino-terminal domain, which is glycosylated in the ER lumen (13, 20). If the inserted TM initiates the translocation of the following domain, then the carboxyl-terminal PL domain becomes proteinase K resistant (Fig. 3B).

The typical results of the assay are shown (Fig. 3C). When the model proteins were expressed in the absence of RM, single bands with the expected molecular weight were observed (Fig. 3C, lanes 1, 4, and 7). Upon being synthesized in the presence...
Efficient Membrane Integration of TM2 Requires the Existence of Flanking TM1—TM2 and even the TM2–3 segment seemed to be insufficient for the stop-transfer sequence when they were assessed in the model constructs in which those were separated 200 residues from the amino-terminal signal peptide (Fig. 2). We then examined the topogenic properties of the amino-terminal TMs in the original context using three fusion constructs (Fig. 5). The extent of this glycosylated form indicates the membrane insertion of the H1 portion. After the proteinase K treatment, substantial amounts of truncated forms were protected (c-form; lanes 3, 6, and 9).

The variety of internal SA-II function was observed (Fig. 3D). Odd-numbered segments TM3, TM5, TM7, and TM11, which were supposed to be an internal SA-II, showed an insufficient function of internal SA-II, thus suggesting that these segments are integrated into the membrane by a mechanism that differs from the conventional one. It is highly likely that TM11 should be a stop-transfer sequence as described below and also as suggested previously (21).

The results of Fig. 3C clearly demonstrate that the combined segment TM9–10 should be a better internal SA-II than TM9 alone. TM10 alone did not initiate translocation at all. It is also concluded that TM10 did not interfere with the translocation initiated by TM9 but even enhances the SA-II function of TM9. Because TM9–10 were not interrupted by any charged residues, we thus reasoned that the two TMs functioned as a single topogenic unit of an internal SA-II (Fig. 6).

DISCUSSION

Each anticipated TM of human band 3 is assessed for the topogenic functions of internal SA-II and St, which are postulated to be responsible for the topogenesis of the multispanning membrane proteins. We found TMs to possess unequal topogenic functions. Several segments showed either insufficient or no supposed topogenic functions. The stop-transfer function of TM2 is affected by the distance from the preceding TM1. Some
Membrane Topogenesis of Band 3

FIG. 5. N\textsubscript{exo}/C\textsubscript{cyt}-topogenic function of even-numbered segments. A, the constructs for examining the preferred orientation of each TM. The glycosylated loop of band 3 (Asp\textsuperscript{626}-Trp\textsuperscript{662}), each segment including both flanking loops, and mature prolactin were fused sequentially. B, the expected membrane topology of the constructed proteins. If the segment shows N\textsubscript{exo}/C\textsubscript{cyt} SA-I function, the protein is glycosylated and is not resistant to protease. If it shows N\textsubscript{exo}/C\textsubscript{cyt} SA-II function, the PL domain becomes resistant to protease \(K\), but the N terminus is not glycosylated. C, an in vitro assay of the topologies of the constructs. The constructs were expressed in vitro in the absence (−) or presence (+) of RM. Aliquots were treated by protease \(K\) (PK+). TM8 gave a diglycosylated form, because the segment included another glycosylated loop of band 3. The mono- and diglycosylated forms were indicated by single and double dots, respectively. The protease \(K\)-resistant forms were indicated by arrowheads. D, quantitation of the orientation of the segments. The glycosylated (\(g\)) and nonglycosylated (\(ng\)) forms and the protease \(K\)-resistant form (\(r\) form) were quantitated by image analysis, and the topogenic functions were calculated from the formulas (SA-I efficiency) = \((g\ form) \times 100/([g\ form] + [ng\ form])\) and (SA-II efficiency) = \((r\ form) \times k^{-1} \times 100/([g\ form] + [ng\ form])\), where \(k\) is the protease \(K\) protection efficiency of the translocated PL domain (see Fig. 3). The number of methionines in each protected form was compensated. Note that the \(ng\ form\) includes polypeptides that had not been targeted to the membrane as well as those with SA-II topology.

segments could not show the supposed internal SA-II function, but those were followed by highly hydrophobic segments with a de novo N\textsubscript{exo}/C\textsubscript{cyt} topogenic function. These facts could not be explained by the conventional model.

TM2 partially interrupts the ongoing translocation (only 40%) when it is placed 200 residues away from the preceding signal peptide (Fig. 2). In contrast to this fact, the stop-transfer action of TM2 improved when it was located near the TM1 segment, as in the case of the original band 3 molecule (Fig. 4). This result demonstrated that the stop-transfer action of the segment depends on its location. When the connecting loop is long, the preceding segment should be allowed to exit from the protein translocation channel (22). If the loop becomes shorter, the signal-anchor sequence should still be in the translocation channel. The existence of the preceding hydrophobic segment within the translocon would affect the translocation of the following portion; the presence of the preceding segment may affect the character of the translocon, or the two segments may directly interact with each other, so that translocation of the latter segment would easily stop within the translocon. Under such a context, the segment with a lower hydrophobicity can thus be integrated into the membrane.

A protein chemical analysis has already demonstrated that segments TM1 to TM3 of band 3 are stabilized in the membrane via an interaction between the peptide segments but not via protein-lipid interaction, because these segments were readily extracted from the erythrocyte membrane by alkali denaturation and subsequent protease treatment (16). These alkali-extractable transmembrane segments should be surrounded by other transmembrane segments that interact directly with the membrane lipids. This fact supports the idea that a direct interaction between TM1 and TM2 exists during the membrane insertion step. These segments are likely to be assembled within the translocon and are released into the hydrophobic environment as pointed out by Borel and Simon (23). The structure requirements that define such interaction are now under investigation.

Although the odd-numbered TMs of band 3 molecule have been supposed to possess an internal SA-II function that mediates the translocation of their following portion, TM3 and TM5 showed an unexpectedly weak internal SA-II function (Fig. 3). TM3 did not mediate the translocation of the reporter, even in the original context (Fig. 4). Despite these observations, the third loop connecting TM3 and TM4 was demonstrated to be in the luminal space of the ER, because the glycosylated loop inserted between TM3 and TM4 was efficiently glycosylated (21). The loop connecting TM5 and TM6 has also been found in the lumen (21). To explain these discrepancies, we hypothesized that TM4 and TM6 should be an internal SA-I that possesses a N\textsubscript{exo}/C\textsubscript{cyt} orientation and promotes the integration of TM3 and TM5, respectively. Our results indicated this to be the case. Thus, it is strongly suggested that the odd-numbered segments (TM3, TM5, and TM7), which possess either no or low topogenic function, are integrated by the SA-I function of their following segments.
Consistent with the consideration above, Tam et al. (24) have reported that TM7 possesses partial SA-II activity, whereas the translocation of the loop between TM7 and TM8 was improved by the presence of TM8 (24). This observation supports our proposal that SA-I activity of TM8 contributes correct integration of its preceding region. Furthermore, it was demonstrated that fragments of band 3 membrane domain (e.g. TM9–14, TM8–14, and TM13–14) were correctly integrated into the membrane, indicating that there are unique topogenic sequences in these fragments (25, 26). In the case of the TM8–14 construct, the amino-terminal TM8 should be correctly inserted in a SA-I orientation. In the case of triple-spanning coronavirus M protein, each transmembrane segment was demonstrated to possess its intrinsic preferred orientation (27). The amino-terminal TM1 of cystic fibrosis transmembrane conductance regulator was inserted into the membrane depending on the following TM2 (28). Pairing of two transmembrane segments in the H1–ATPase was suggested to be essential for integration into the microsomal membrane (29). These findings also suggested that the transmembrane segment can contribute membrane insertion of its preceding segment.

We assessed the translocation initiation function of TM4, TM6, and TM8 in two different contexts (Figs. 3A and 5A). In the former construct, the loop connecting the H1 and the segment to be assessed was only 40 residues long, which is too short to allow the segments to form SA-I topology. In such a context, the presence of H1 itself dictates that the following TM cannot be inserted in a SA-I orientation. On the other hand, when they were placed near the N terminus, as in the latter case (Fig. 5A), they showed a strong SA-I function. In the original context of band 3 molecule, their preceding sequences including the TMs (TM3 and TM5) were sufficiently long and should be integration competent (as the glycosylated loop connecting TM7 and TM8). Thus, it is highly likely that in the original context of band 3, the even-numbered TMs mediate the integration of their preceding TMs.

The segment including both TM9 and TM10 was suggested to form one topogenic unit as SA-II. This conclusion is consistent with the report that the loop connecting these segments is on the luminal side of the membrane at least during the process of biosynthesis (Fig. 6; Ref. 21). Based on these findings, TM11 is most likely a stop-transfer sequence. TM12 was demonstrated to be left outside of the cytoplasmic side of the membrane (21), whereas our observations suggested TM12 to be a highly functional internal SA-II sequence. This discrepancy remains to be clarified.

Based on the abovementioned findings, we propose the following model of the topogenic process for band 3 (Fig. 6). TM1 is a SA-II that is responsible for ER targeting and for initiating the translocation of the following portion. TM2 interrupts the translocation by interacting with the closely positioned TM1. TM3, which has a weak topogenic function and low hydrophobicity, is integrated by the internal SA-I function of the following TM4. TM5 is also likely to be integrated by the SA-I function of TM6. TM7 has a moderate (but not sufficiently high) internal SA-II function to translocate the long hydrophilic loop with the N-glycosylation site. TM8 stops the translocation initiated by TM7 and also mediates the translocation of the glycosylated loop that had not been inserted by TM7. In this connection, the membrane topology in the TM7-loop-TM8 region is established by both the function of internal SA-I of TM8 and that of internal SA-II of TM7. TM9–10 functions as a single unit of internal SA-II as a whole. TM11 and/or TM12 is a stop-transfer segment. Some part of this segment may be left out of the hydrophobic core of the membrane.

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