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Manipulation of Membrane Protein Topology on the Endoplasmic Reticulum by a Specific Ligand in Living Cells

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Almost all integral membrane proteins in the secretory pathway are cotranslationally inserted into the endoplasmic reticulum membrane. Their membrane topology is determined by their amino acid sequences. Here we show that the topology can be manipulated by a factor other than the amino acid sequence. A dihydrofolate reductase (DHFR) domain was fused to the N-terminus of the type I signal-anchor sequence of synaptotagmin II, which mediates translocation of the preceding portion. The DHFR domain was translocated through the membrane in COS7 cells and a transmembrane (TM) topology was achieved. When a DHFR ligand, methotrexate, was added to the culture medium, translocation of the DHFR domain was suppressed and both ends of the signal-anchor sequence remained on the cytoplasmic side. In contrast, translocation of the DHFR domain fused after the signal peptide, which translocates the following region, was not affected by the ligand. The topology-altered fusion protein was anchored to the membrane in a high salt-resistant state, and partially extracted from the membrane under alkali conditions. We concluded that the topology of membrane proteins can be manipulated by a *trans*-acting factor, even in living cells.

Key words: DHFR, endoplasmic reticulum, membrane protein, membrane topology, methotrexate, signal sequence.

Abbreviations: DHFR, dihydrofolate reductase; EndoH, endoglycosidase H; ER, endoplasmic reticulum; H-segment, hydrophobic segment; MTX, methotrexate; PDI, peptidyl disulfide isomerase; SA-I, type I signal-anchor; SA-II type II signal-anchor; SytII, synaptotagmin II; TM, transmembrane.

Membrane proteins in the secretory pathway in eukaryotic cells are synthesized by membrane bound ribosomes and cotranslationally integrated into the endoplasmic reticulum (ER) membrane (*1*). The ER targeting of the ribosome is mediated by a signal recognition particle, which recognizes the hydrophobic segments (H-segment) of signal sequences on the nascent polypeptide chains (*2*). On the ER membrane, the H-segment is transferred to the protein translocation channel, the so-called translocon (*3*). The translocon consists of a Sec61 complex (Sec61 $\alpha\beta\gamma$) and some accessory proteins; *e.g.*, a translocating polypeptide chain-associated membrane protein and a translocon-associated protein complex (*4*).

ER targeting and membrane insertion are determined by the H-segment of the signal sequence (*5*, *6*). Either the N- or C-terminal side of the H-segment is translocated through the translocon to yield the TM-topology. A signal peptide and a type II signal anchor (SA-II) sequence mediate translocation of the following C-terminal portion, while a type I signal-anchor (SA-I) sequence mediates translocation of the preceding N-terminal domain (*6*, *7*). The signal peptide is processed on the lumenal side by signal peptidase. Signal-anchor sequences are not cleaved to become anchoring segments. The longer H-segment tends to translocate the N-terminal portion, and positively-charged residues inhibit the translocation. When positive charges are introduced on the N-terminal side of the SA-I sequence, translocation of the N-terminal side is inhibited and the C-terminal side is translocated through the membrane (*8*, *9*). In the case of synaptotagmin II (SytII), positive charges just behind the H-segment are also essential for translocation of the N-domain (*10*). Thus, the positive charges on both sides of the signal sequences play a critical role in determination of the topology. The positive charges also have significant inhibitory effects during the stop-transfer process (*11*, *12*). In addition to positive charges, some specific amino acid residues have critical topogenic functions at specific positions. For example, specific amino acid residues with high turn-propensity indices (such as proline) are essential for breaking the long H-segment to form two TM segments (*13*). Similar residues with high turn-propensity indices are required between the N-domain and the H-segment of SytII for N-domain translocation (*14*). All of these factors are on the polypeptide chain and should therefore be called *cis*-acting topology determinants.

We explored the possibility of whether factors other than *cis*-acting elements manipulate membrane topology. The ability to regulate membrane topology in cells would be a novel tool for protein engineering as well as for analysis of membrane protein topogenesis. For this purpose, we utilized a dihydrofolate reductase (DHFR) domain, whose conformation stability can be regulated by the well-known ligand methotrexate (MTX). Eilers and Schatz reported a cell-free mitochondrial import system in which

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the mouse DHFR domain fused to the C-terminus of the mitochondrial targeting signal was efficiently imported into mitochondria, but the MTX-bound form was not (*15*). In this study, we examined the utility of the DHFR domain as a means of manipulating membrane protein topology. The mouse DHFR domain was fused to the N-terminus of SA-I protein mouse SytII and then transiently expressed in cultured cells. The translocation was suppressed by adding MTX to the culture medium and the topology switched from the TM-topology to the loop conformation, in which both ends are on the cytoplasmic side. We propose that membrane protein topology is ligand-manipulable, even in living cells. The ligand can be termed a *trans*-acting factor, while amino acid sequences act as *cis*-acting factors.

MATERIALS AND METHODS

Materials—MTX (Sigma Chemical Co., St. Louis, MO), anti-DHFR monoclonal antibodies (BD Biosciences, NJ), anti–C-terminal cytoplasmic region of calnexin (CNX, SPA860; StressGen, San Diego, CA), anti-PDI (SPA890; StressGen), and EndoH (New England Biolabs, MA) were obtained from the sources indicated. The anti-SytII antibodies were a generous gift from Dr. Fukuda, RIKEN (*16*).

DNA Construction—Fusion proteins were subcloned downstream of the cytomegalovirus-promoter of pRcCMV (Invitrogen, Carlsbad, CA). To construct the DHFR and SytII fusion protein, the DNA fragment encoding mouse DHFR was isolated from plasmid pSD(69) (*17*) by PCR using primers 5′-TTCCAAGCTTCCACCATGTCGAGATC-CGGCATCATG-3′, which contained a *Hin*dIII site, a Kozak sequence, and an extension sequence from the plasmid (underlined), and 5′-GGAAGAATTCGTCTTTCTTCTCG-TAGACTT-3′ (*Eco*RI site is underlined), and then digested with *Hin*dIII and *Eco*RI. SytII DNA was isolated from pSytII-03 (*10*) by PCR using the following primers: 5′-TCCGAATTCAGAAACATCTTCAAGAGGAAC-3′ (*Eco*RI site is underlined) and 5′-TAGCTCTAGATCACGTCAG-TGTCC-3′ (*Xba*I site is underlined), and then digested with *Eco*RI and *Xba*I. The two DNA fragments were subcloned between the *Hin*dIII and *Xba*I sites of pRcCMV to obtain pD-S. The DHFR cDNA was mutated to obtain a glycosylation site (Ile8Thr) and MTX binding mutants (Cys7Ser, Ser42Cys, and Asn49Cys) as previously described (*18*).

To obtain the signal peptide-DHFR fusion protein, the DNA fragments encoding the N-terminal sequence of human NHE6 (Met^1–Gly^{46}) (*19*) and the DHFR (Met^1–Asp^{187}) were joined using the overlap extension method (*20*). The obtained fusion DNA was digested with *Hin*dIII and *Xba*I, and then subcloned into a pRcCMV vector that had been digested with the same enzymes.

Cell Culture and Transfection Experiments—COS7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co., D6429) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) under an atmosphere of 5% CO_2 at 37°C. For transfection, COS7 cells were seeded into 3.5-cm wells (1.4×10^5 cells/well). After culturing for 16–24 h, the cells were transfected with an expression plasmid using FuGene6 reagent (Roche, Nutley, NJ). For DHFR-SytII fusion proteins, 54 ng of expression plasmid and 1 μg of vacant pRcCMV were mixed with 6 μl of FuGene6 reagent in 200 μl of DMEM. The amount of the expression plasmid was limited to avoid junk products caused by overexpression. The DNA-liposome complex was incubated with the cells according to the instruction manual. Cells were harvested 24 h after transfection. The expression time was also limited to minimize the toxic effects of overexpression. Where indicated, 5 μl of a MTX stock solution in DMSO that had been appropriately diluted was added 8 h before harvesting the cells.

The cells in the culture dish were washed twice with PBS and then scraped off with a cell scraper (Corning, NY) in 1 ml of ice cold PBS. Cells were precipitated at $2,000 \times g$ for 5 min, and then resuspended in small aliquots (10 μl) of PBS and mixed with 200 μl of sample buffer for SDS-PAGE; 6% SDS, 6 mM EDTA, 50 mM Tris/HCl (pH 6.8), 0.1 M dithiothreitol, and 20% glycerol. The mixture was immediately heated at 98°C for 5 min, and then briefly sonicated in a clear TPX tube with Bioruptor (Cosmo-Bio Co. Ltd., Tokyo, Japan) to break genomic DNA. Aliquots (20 μl) were subjected to SDS-PAGE analysis. SytII fusion proteins were detected by immunoblotting using rabbit antiserum against the SytII cytoplasmic domain and horseradish peroxidase–conjugated anti-rabbit IgG antibodies (Bio-Rad). The blot-membrane was blocked with 2% skim milk in PBS and then washed with 1% Tween20 and PBS. When anti-SytII antiserum was used, the washing buffer for the final step comprised 0.01% SDS, 150 mM NaCl, and 10 mM Na-phosphate (pH 7.4). Protein bands were visualized by ECL reagent (Amersham Biosciences UK Limited) and a bio-image analyzer (LAS1000mini; Fuji Film, Tokyo Japan).

For EndoH treatment under denaturing conditions, 20-μl aliquots of the sample used for the SDS-PAGE analysis were directly subjected to EndoH treatment for 60 min at 37°C. The reaction was terminated by heating at 98°C for 5 min.

Membrane Extraction—For subcellular fractionation of COS7 cells, the cells were cultured and transfected in 10-cm dishes. Cells were resuspended in 1 ml of HES-buffer [50 mM HEPES (pH 7.5), 1 mM EDTA, 0.25 M sucrose, and complete protease inhibitor mixture (Roche)], and then homogenized by passage through 27-gauge needles for 15 strokes (30 s/stroke). The homogenates were centrifuged at 2,500 rpm for 5 min and the supernatants were further centrifuged at 40,000 rpm for 10 min in a micro-ultracentrifuge (S45A rotor; Hitachi High-Technologies Co, Tokyo). The microsomal membranes were re-homogenized with 1 ml of buffer containing 5 mM HEPES (pH 7.5) and a protease inhibitor mixture. Membranes (100 μl) were diluted to 1 ml and then adjusted to final concentrations of 0.5 M NaCl, 0.2 M Na_2CO_3, 0.2 M NaOH, and 1% Triton X-100 (Tx-100), as previously described (*21*). Where indicated, the Na_2CO_3 solution was adjusted to pH 12.5 with NaOH. The mixture was incubated on ice for 15 min and then centrifuged at 73,000 rpm (S120AT2 rotor; Hitachi High-Technologies Co, Tokyo) at 4°C for 10 min. The proteins in the supernatant were precipitated with trichloroacetic acid. The protein precipitates and the membrane precipitates were solubilized with 200 μl SDS-PAGE buffer and then subjected to immunoblotting analysis.

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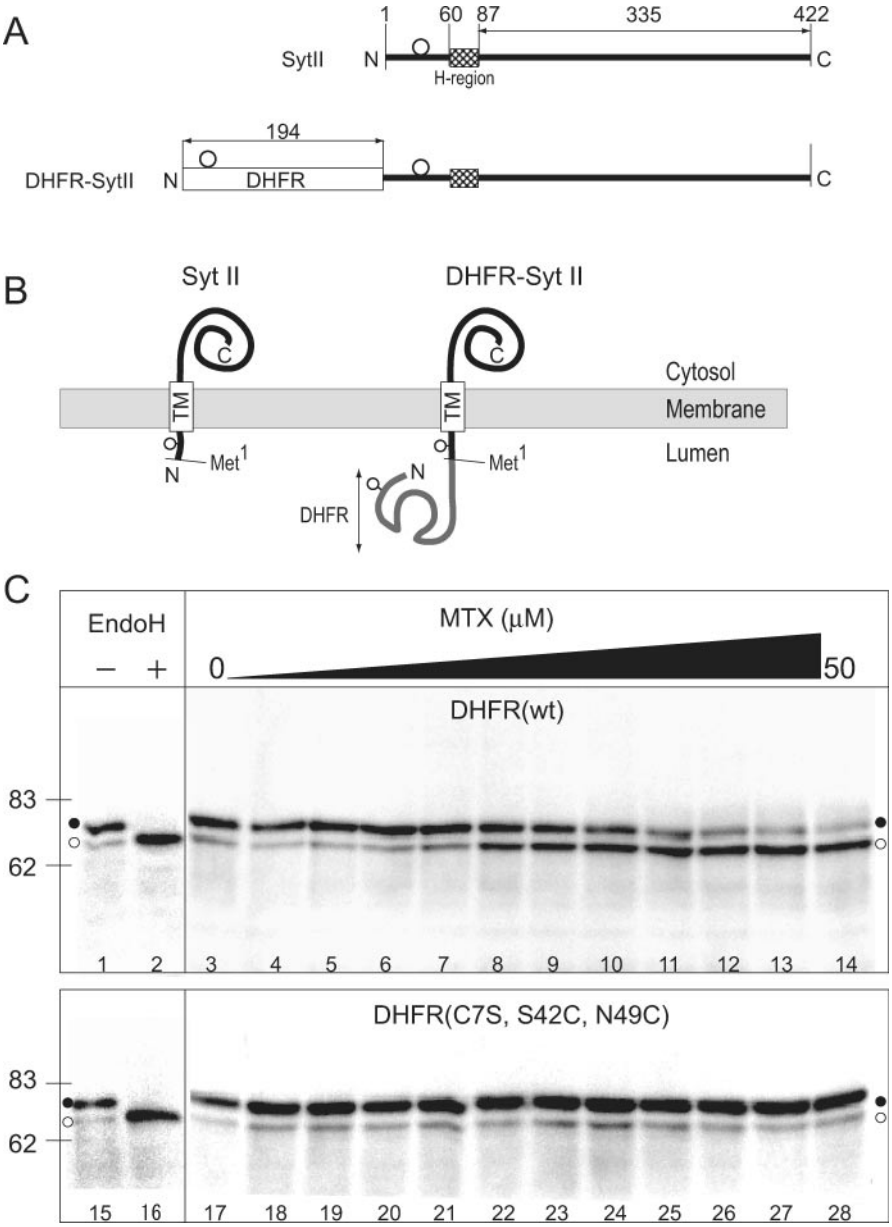

Fig. 1. **Effect of MTX on the DHFR-SytII fusion protein topology.** (A) The mouse synaptotagmin II (SytII) molecule possesses a 60-residue lumenal domain, a 27-residue TM segment (polka-dotted box), and a 335-residue cytoplasmic domain. DHFR and mutated DHFR were fused to the N-terminus of SytII. In the fusion constructs, two glycosylation sites (circles) exist in the DHFR domain and in the lumenal domain of SytII. The amino acid numbers of the SytII molecule are indicated. (B) The membrane topology of the model proteins is illustrated. The N-terminal domain is translocated through the membrane by the SA-I function of the following hydrophobic TM segment. (C) The fusion proteins were transiently expressed in COS7 cells for 24 h after transfection. In the last 8 h of the expression period, MTX was added to the culture medium at the indicated concentrations. A total cell-lysate was subjected to SDS-PAGE (4% stacking and 7% separating gels) and immunoblot analysis. The fusion proteins were detected with rabbit antiserum against the SytII cytoplasmic domain. Some aliquots were treated with EndoH (+ lane). At the left side of the panel, the positions of pre-stained molecular weight markers are indicated. Closed and open circles indicate glycosylated and unglycosylated forms, respectively. The mutated DHFR domain with three mutations (C7S, S42C, and N49C) was glycosylated, even in the presence of MTX.

RESULTS

Translocation of the N-Terminal DHFR Domain was Suppressed by MTX—The N-terminal portion of SytII is in the ER lumen and the following large domain is on the cytoplasmic side (Fig. 1). To examine the possibility that translocation of the long N-terminal domain can be regulated in living cells, the mouse DHFR was fused to the N-terminus of SytII (Fig. 1, A and B). The construct contained the 194 N-terminal residues of the DHFR domain, the 60 residues of the hydrophilic extracellular domain, the 27 residues of the hydrophobic TM segment, and the 335 residues of the cytoplasmic domain. Within the construct, a glycosylation site was newly created near the N-terminus of the DHFR domain by point mutation (Ile8Thr) in addition to the endogenous glycosylation site that was located 29 residues before the H-segment (Fig. 1, A and B, circles). The fusion protein was transiently expressed in COS7 cells under the control of the CMV-promoter for 24 h. The expressed molecules in the total cell lysate were detected by immunoblotting using antibodies against the cytoplasmic domain of SytII (Fig. 1C). A single major band and a weak smaller one were detected (Fig. 1C, lane 1). The larger band was shifted down to the smaller one on EndoH treatment (Fig. 1C, lane 2). The EndoH-sensitive sugar chain is acquired in the ER lumen, thus the N-terminal DHFR domain was translocated through the ER membrane and glycosylated.

To examine whether the DHFR ligand MTX can affect translocation of the DHFR domain, MTX was added to the culture medium during the last 8 h of the expression period. The glycosylation of the DHFR domain was suppressed depending on the MTX concentration (Fig. 1C, lanes 3–14). The total amounts of the glycosylated and unglycosylated forms were not affected. The DHFR-SytII fusion protein possesses two glycosylation sites in the

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N-terminal domain and three in the cytoplasmic domain. When the C-terminal domain was translocated through the membrane, the glycosylation sites were efficiently glycosylated, as previously reported (*10*, *14*). The complete suppression of the glycosylation, thus, indicated that not only the N-terminal DHFR domain but also the C-terminal portion was on the cytoplasmic side of the membrane.

We also used a mutant DHFR domain, in which three residues were mutated to prevent MTX binding. The glycosylated form of this mutant was also detected (lane 15). MTX had no significant effect on the translocation of the mutated DHFR (lanes 17–28). Thus, the effect of MTX was specific for the wild-type DHFR domain, and did not involve by nonspecific inhibition of the protein translocation mechanism of the ER membrane.

When MTX was added at earlier time points, the glycosylated form decreased (data not shown), indicating that the trace amount of the glycosylated form detected following incubation for 8 h in the presence of MTX (in lane 14) was a pre-existing molecule. These data clearly indicated that the DHFR-SytII fusion protein showed the loop-topology on the membrane (*cf.* Fig. 5) in the presence of MTX, and that the topology of the N-terminal DHFR domain was manipulated by MTX in the culture medium.

MTX Does Not Affect Translocation of the DHFR Domain Following the Signal Peptide—Next, we examined the effect of MTX on DHFR domain translocation mediated by the N-terminal signal peptide (Fig. 2). The signal peptide of Na^+/H^+ exchanger isoform 6 (*19*) was fused to the N-terminus of the DHFR domain (Fig. 2A). The resulting construct (H1-DHFR) was expressed in COS7 cells and a total cell lysate was subjected to immunoblotting using anti-DHFR monoclonal antibody (Fig. 2B). H1-DHFR was detected as a single band on the blot sheet (Fig. 2B, lane 1). When treated with EndoH, the band clearly shifted down by 3 kDa (Fig. 2B, lane 2). The glycosylation was not affected by a high concentration of MTX (Fig. 2B, lane 4). These data demonstrated that the DHFR domain was translocated and glycosylated in the lumen. The DHFR domain following the signal peptide was translocated even in the presence of MTX. This is clear evidence that the nascent chain following the signal peptide does not fold before translocation. This finding is consistent with the fact that a signal peptide targets a ribosome to the ER translocon where the nascent polypeptide chain directly enters the translocon channel from the ribosome tunnel. It was again confirmed that MTX was not toxic to the translocation machinery and that the MTX effect was due to stabilization of the DHFR domain.

Membrane Anchoring Property of the Topology-Altered Form—The above data indicate that the DHFR-SytII fusion construct exhibits two different membrane topologies on the ER membrane depending on the presence of MTX (*cf.* Fig. 5, a and b). In order to determine the membrane anchoring properties of the membrane proteins with the unique loop-topology, we examined the extractability with various reagents. Membrane vesicles prepared from a post-nuclear supernatant of COS7 cells were extracted under high-salt (0.5 M NaCl), alkaline (0.1 M NaOH), and detergent (1% Tx100) conditions, and the supernatant (S) and membrane precipitate (P) were analyzed by immunoblotting (Fig. 3). The SytII fusion construct showing the TM topology in the absence of MTX was recovered in the precipitate, even with NaOH (Fig. 3, lane 5), and was extracted in the supernatant with the detergent (Fig. 3, lane 6). On the other hand, the topology-altered form

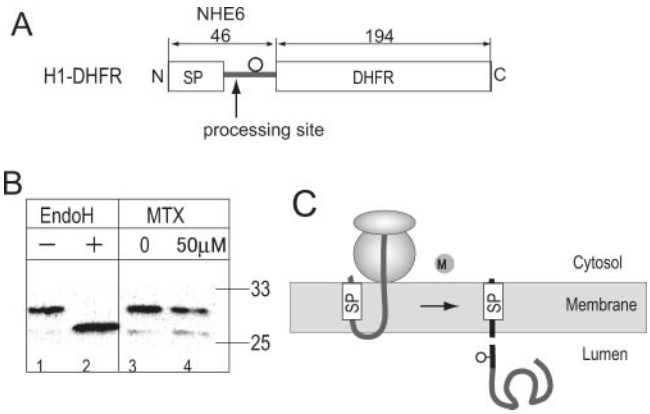

Fig. 2. **Effect of MTX on signal peptide-mediated translocation of the DHFR domain.** (A) The 46-residue N-terminal segment of human Na^+/H^+-exchanger isoform 6 (NHE6) containing a signal-peptide (SP) and a lumenal segment including a glycosylation site (open circle) were fused to the N-terminus of DHFR. (B) The proteins were expressed in the presence or absence of 50 μM MTX. Aliquots were treated with EndoH. Proteins were analyzed by SDS-PAGE (12% separating gel), and then subjected to immunoblotting with anti-DHFR monoclonal antibody. (C) The DHFR domain following the signal peptide (SP) was translocated through the ER membrane, processed, and glycosylated, even in the presence of MTX (M).

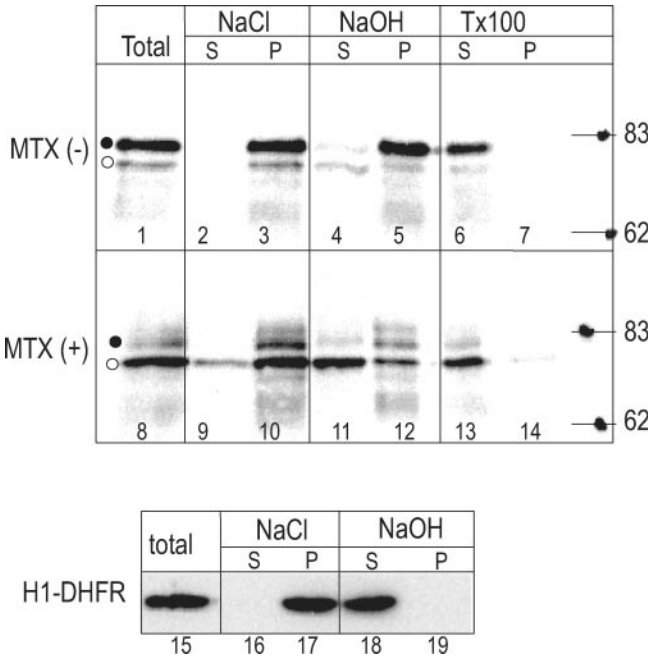

Fig. 3. **Membrane anchoring of the topology-altered form.** DHFR-SytII was expressed in the absence (–) or presence (+) of MTX, and then membranes were obtained by ultracentrifugation. Membranes were treated under high salt (0.5 M NaCl), alkali (0.1 M NaOH), and detergent (1% Tx100) conditions, and separated into supernatant (S) and membrane precipitates (P) by ultracentrifugation. Both fractions were analyzed by immunoblotting analysis. The same procedures were performed with the H1-DHFR fusion protein.

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synthesized in the presence of MTX was extracted with a NaOH solution (Fig. 3, lane 11). Under NaOH alkaline conditions, closed membrane vesicles are fragmented, and content proteins and peripheral membrane proteins are released in soluble forms (*22*). When the processed and glycosylated H1-DHFR was examined under the same conditions as the control, it was not extracted from the membrane under high salt conditions (Fig. 3, lane 17), but was completely extracted with the NaOH solution, as expected (Fig. 3, lane 18), indicating that the NaOH solution completely extracted soluble proteins in the lumen.

Milder alkali conditions were then examined (Fig. 4). Even under mild conditions with sodium carbonate, closed membrane vesicles are converted to open membrane sheets, and lumenal proteins and peripheral membrane proteins are released in soluble forms (*23*). Only the integral membrane proteins interacting with the membrane via a hydrophobic interaction were recovered in the precipitate. DHFR-SytII synthesized in the absence of MTX was recovered in the membrane precipitate (lanes 3, 5, 7, and 9). An integral membrane protein, endogenous calnexin, was also recovered to the membrane (lanes 21, 23, 25, and 27), while a soluble protein in the lumen, PDI, was completely extracted in the soluble fraction with all concentrations (lanes 29, 31, 33, and 35). The topology-altered DHFR-SytII was partially extracted under mild alkaline conditions (lanes 11–16). These results indicated that the topology-altered form was targeted to the ER membrane and anchored in a high salt-resistant manner, but could be readily extracted from the membrane under alkaline conditions, in contrast to the proteins with the TM-topology.

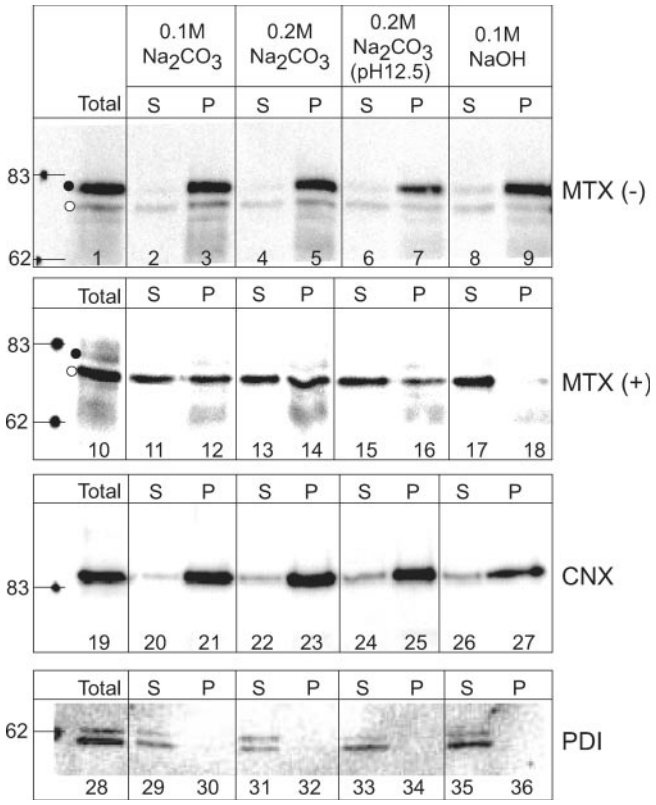

Fig. 4. **Effect of alkaline conditions on the extraction.** DHFR-SytII was expressed in the absence or presence of MTX, and membrane vesicles from the cells were treated with the indicated solutions. After incubation for 15 min on ice, membranes were precipitated by ultracentrifugation. DHFR-SytII, calnexin (CNX), and PDI were detected by immunoblotting.

DISCUSSION

The long N-terminal DHFR domain can be translocated by the following SA-I and TM topology was achieved in living cells (Fig. 5a). The translocation was arrested by MTX, which has a specific effect on the folding of the DHFR domain, but does not affect the protein translocation machinery. In the presence of MTX, the TM-form was replaced by the topology-altered form (b). The data clearly indicate that the N-terminal domain preceding the SA-I sequence takes on the folded active structure before being translocated. The translocation of the N-terminal domain occurs from the C-terminus to the N-terminus (*e.g.*, C-to-N translocation), but not from the N-terminus to the C-terminus (N-to-C translocation). The polypeptide chain is proposed to be maintained in the translocation-competent conformation via various molecular chaperones before translocation (*24*). The nascent chains are thought to be in the unfolded conformation. The DHFR domain can, however, be folded into the active structure even in living cells.

We demonstrated that the N-terminal domain is translocated just after the H-segment emerges from the ribosome (*10*). The translocation was completed when 130 of the 422 residues of the SytII molecule were synthesized. The DHFR domain should, thus, fold before the H-segment emerges from the ribosome. The cotranslational folding occurs as previously suggested (*25*, *26*). The three-dimensional structure of the human DHFR molecule indicates that the 10-residue N-terminal segment directly interacts with the 130th to 140th residues, forming a parallel β-strand (PDB ID, 1U72). The 40-residue C-terminal segment does not firmly interact with the internal side of the DHFR domain. The C-terminus is followed by a 60-residue N-terminal segment of SytII and then by a 27-residue H-segment (Fig. 1). The DHFR domain should fold before the H-segment emerges from the ribosome after the 127 N-terminal residues emerge from the ribosome. Thus, the DHFR domain folding should occur within the time span for polypeptide elongation of the 127 residues. The rate of polypeptide chain elongation in living cells is roughly estimated to be five residues per second in cultured cells (*25*, *27*). Within at most 25 s, the DHFR domain folds and binds MTX.

Our previous observations with *in vivo* pulse labeling and *in vitro* protein synthesis procedures indicated that the large N-terminal DHFR domain translocation is not efficient; only 30% of newly synthesized DHFR-SytII molecules take on the TM topology (28). Efficient translocation of the DHFR-domain is observed only when the nascent chain is kept bound to the ribosome in a cell free system (*28*). The present observation on immunoblotting appears to be inconsistent. It is, however, highly likely that the non-glycosylated form on the cytoplasmic side is unstable in the absence of MTX, whereas the molecule

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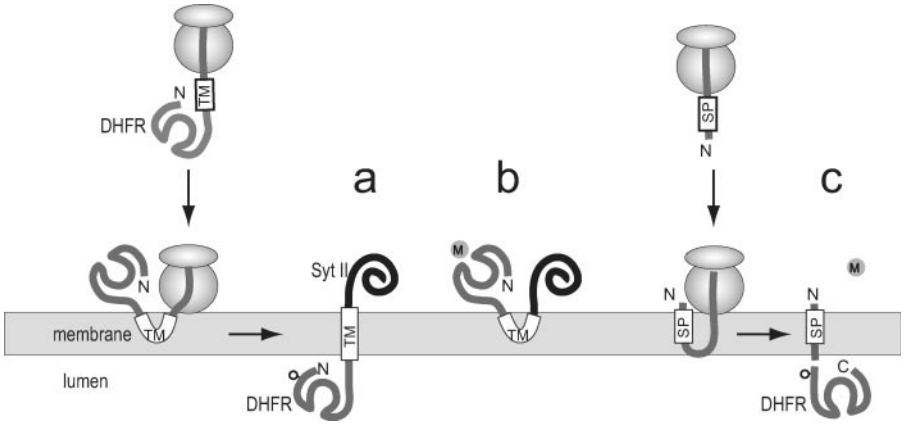

Fig. 5. **Manipulation of the membrane protein topology by a *trans*-acting factor.** The DHFR-SytII fusion protein is targeted to the ER membrane after the TM segment emerges from the ribosome. At this stage, the N-terminal DHFR domain is folded to be able to bind the ligand (M). In the absence of MTX, the N-terminal domain is translocated to the lumenal space (a). When MTX is added to the culture medium, the DHFR domain is stabilized and translocation is suppressed (b). When the DHFR follows the signal peptide (SP), it is translocated through the translocon, even in the presence of MTX (c). The membrane topology of the N-terminal DHFR domain can be manipulated by MTX.

whose N-terminal DHFR domain is in the lumen is more stable. In the presence of MTX, the DHFR domain should be stabilized even on the cytoplasmic side and was detected on immunoblotting. Although the mechanistic details of such selective stabilization are not known, the membrane topology of the stationarily existing DHFR-SytII protein can be regulated by MTX in living cells. Using this technique, the topology of membrane proteins can be manipulated without any changes in the amino acid sequence or additional transfection.

The topology-altered DHFR-SytII protein has a loop topology in which both ends of the H-segment are on the same side of the lipid bilayer (Fig. 5b). To our knowledge, few examples of such a loop-topology being decisively proven have been reported. Cytochrome P450, cytochrome b_5, *etc.*, are integral membrane proteins with a transmembrane anchoring domain. As an example, a SytII mutant, in which the proline residue just preceding the H-segment is mutated to alanine, cannot translocate the N-terminal hydrophilic segment of 60 residues nor the following C-terminal domain, but is integrated cotranslationally into the ER membrane via the translocon (*10*). The mutant molecule having the loop-conformation was released from the Sec61α translocon subunit and anchored in an alkaline-resistant manner (*10*). Two topologies have also been proposed for the membrane anchoring domain of cytochrome b_5; one is a TM topology that represents the authentic naturally occurring conformation and the other is a loop conformation on proteoliposomes of detergent-purified cytochrome b_5 (*29*, *30*). The latter conformation is highly likely to be a loosely bound form, which is transferable between membrane liposomes in a cell-free system (*29*, *30*). The loop conformation of cytochrome b_5 should be more readily extracted from the membrane than the TM conformation.

All the glycosylated form of DHFR-SytII fusion synthesized in the absence of MTX was EndoH-sensitive, indicating that it remained in the ER but was not modified in the Golgi apparatus into a complex form. The unglycosylated form synthesized in the presence of MTX showed the loop-topology and should be integrated into the membrane depending on the signal recognition particle-translocon system, as observed for the SytII topology mutant that showed the loop-topology (*10*). Although the precise intracellular localization of the topology-altered form is not known, the major conclusion regarding the topology alteration is not affected.

Our finding of topology-alteration suggests that membrane topology can be manipulated by a reagent (Fig. 5). The correlation between amino acid sequences and membrane topology is well-established; positive charges, folding properties, hydrophobic characteristics, *etc.* These should be called *cis*-acting determinants of membrane topology. Here we propose the concept of a *trans*-acting topology determinant as a new tool for protein engineering to be used to manipulate the membrane topology of engineered proteins in living cells, and to act as a switching device for various bioreactor and cellular functions.

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