Passenger Protein Determines Translocation Versus Retention in the Endoplasmic Reticulum for Aromatase Expression

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ABSTRACT

Aromatase protein is overexpressed in the breasts of women affected with cancer. In the endoplasmic reticulum (ER), signal sequence and signal anchors (SAs) facilitate translocation and topology of proteins. To understand the function of type-I SAs (SA-I), we evaluated translocation of aromatase, whose signal anchor follows a hydrophilic region. Aromatase SA-I mediates translocation of a short N-terminal hydrophilic domain to ER lumen and integrates the protein in the membrane, with the remainder of the protein residing in the cytosol. We showed that lack of a signal peptidase cleavage site is not responsible for the stop-transfer function of SA-I. However, SA-I could not block the translocation of a full-length microsomal secretory

protein and was cleaved as part of the signal sequence. We propose that interaction between the translocon and the region after the signal anchor plays a critical role in directing the topology of the protein by SA-I. The positive charges in the signal sequence helped it to override the function of signal anchor. Thus, when signal sequence follows SA-I immediately, the interaction with the translocon is perturbed and topology of the protein in ER is altered. If signal sequence is placed far enough from SA-I, then it does not affect membrane integration of SA-I. In summary, we conclude that it is not just the SA-I, but also the region following it, which together affect function of aromatase SA-I in ER.

Introduction

Protein translocation across eukaryotic membranes is the most critical step in biogenesis, as protein function depends on the correct subcellular localization. The cotranslational translocation pathway plays a central role in the simultaneous synthesis and transport or integration of proteins into the endoplasmic reticulum (ER) membrane. Although soluble and secretory proteins fully cross the ER membrane to reach the lumen, integral membrane proteins embed in the ER membrane through one or more of their transmembrane segments. Furthermore, *N*-glycosylation and signal peptide cleavage are cotranslational modifications of proteins in the ER, performed by oligosaccharyltransferase (OST) and signal peptidase complex, respectively (Hegde and Bernstein, 2006; Kelleher and Gilmore, 2006). However, all the proteins use the same machinery, i.e., a protein-conducting channel formed by Sec61 complex called translocon, for their transport across the membrane. The cotranslational translocation pathway requires the protein to have a signal sequence at the N terminus. Signal sequences of secretory proteins and type I membrane proteins, characterized by a stretch of 7–25 mainly apolar amino acid residues, are proteolytically removed during or at the end of

protein transport (Walter and Johnson, 1994). Signal sequences directly target the nascent polypeptides to sites of translocation at the ER membrane and dictate the topology of the protein (Walter and Johnson, 1994; Keenan et al., 2001; Egea et al., 2005). The presence of a stop-transfer sequence blocks further translocation of membrane proteins, facilitating their insertion into the lipid bilayer. The N-terminal hydrophobic segments of microsomal cytochromes P450 (P450s) work as a type-I signal anchor (SA-I) (Nelson and Strobel, 1988) and have the combined topogenic functions of a signal sequence and a stop-transfer sequence (Sakaguchi et al., 1987; Monier et al., 1988; Szczesna-Skorupa et al., 1988). SA-Is generate integral membrane proteins with a $N_{\text{exoplasmic}}/C_{\text{cytoplasmic}}$ ($N_{\text{exo}}/C_{\text{cyt}}$) orientation in the ER membrane (Monier et al., 1988). Aromatase is a glycosylated microsomal cytochrome P450 protein (Shimozawa et al., 1993). Aromatase protein has a SA-I; however, a short hydrophilic domain precedes aromatase's signal anchor, making it an atypical member of the P450 family (Chen and Zhou, 1992). Aromatase catalyzes the formation of estrogens. Breast tumors express greater levels of aromatase (Killinger et al., 1987; Miller and O'Neill, 1987; Reed et al., 1989; Silva et al., 1989), leading to a manyfold increase in intratumoral estradiol compared with plasma and subsequent disease development (Thijssen and Blankenstein, 1989) (Fig. 1A). As a result, aromatase has become an attractive breast cancer therapeutic target, underscoring the need to better understand how the cell regulates its activity.

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ABBREVIATIONS: DHFR, dihydrofolatereductase; endo H, endoglycosidase H; ER, endoplasmic reticulum; MM, microsomal membrane; MTX, methotrexate; $N_{\text{exo}}/C_{\text{cyt}}$, $N_{\text{exoplasmic}}/C_{\text{cytoplasmic}}$; OST, oligosaccharyltransferase; P450, cytochrome P450; PK, proteinase K; pPL, prolactin; SA-I, signal anchor type-I; SCC, side-chain-cleavage enzyme; SRP, signal recognition particle.

Fig. 1. Topology of glycosylated aromatase. (A) Schematic representation of the steroid hormone synthesis pathway and the role of aromatase. (B) Cell-free transcription-translation synthesis (CFS) of [35 S]methionine-labeled aromatase with and without MM and treatment with endo H. (C) Aromatase CFS, with and without MM, treated with PK and PK plus Triton-X100. (D) Membrane integration assay where aromatase CFS (with and without MM) was treated with Na_2CO_3 and supernatant (S) was separated from the membrane fraction (P). (E) Top panel shows construction of N-terminal and internal deletion mutants (F and G) of aromatase. Bottom panel shows CFS with MM of these mutants. Δ represents deleted amino acids. Double red lines show that same number of amino acids were present in full-length (Ar) and all the mutant aromatase proteins. Downward arrowheads ($\blacktriangledown$) show Asn12 in cartoons and glycosylated aromatase in gels. (H) Effect of point mutations at 12th (asparagine, N to threonine, T) and 14th (threonine, T to alanine, A) amino acid residues on glycosylation. (I) Cartoon showing the topology of glycosylated aromatase in the ER. Yellow bar depicts endoplasmic reticulum membrane and green vertical bar represents SA-I of aromatase. Four-point star represents glycosylated Asn12 due to its luminal localization and red cross (X) represents Asn-180, which is nonglycosylated as it is in the cytosol.

The topology of proteins cotranslationally integrated into the ER stems from topogenic sequences in their primary structures (Blobel, 1980; Sabatini et al., 1982). The distribution of charged amino acids on either end of the signal sequence and the hydrophobicity of the apolar core of the signal sequence or signal anchor determine signal orientation (Sakaguchi et al., 1992b). The more positively charged flanking sequence is on the cytosolic side of the membrane due to the electrostatic interactions at or near the translocon (Heijne, 1986). Accordingly, mutations of charged residues flanking a signal sequence can affect its orientation significantly (Beltzer et al., 1991; Parks and Lamb, 1991, 1993; Andrews et al., 1992). Moreover, the tendency for translocation of the N terminus into the ER lumen increases with an increase in hydrophobicity of the N-terminal region (Sakaguchi et al., 1992b; Wahlberg and Spiess, 1997; Eusebio et al., 1998; Harley et al., 1998; Rösch et al., 2000). Signal sequences and signal anchors are interconvertible

by mutations that lead to changes in charge and hydrophobicity of these regions (Szczesna-Skorupa et al., 1988; Kuroiwa et al., 1991). Thus, the exact mechanism by which newly synthesized proteins translocate across the ER membrane remains unclear. It is still unknown why some proteins completely translocate across the ER bilayer whereas others stay partially embedded. Although the possibility exists that the nature of the protein chain being translocated affects the signal sequence/anchor function and that the position of these sequences affect their function, this has not been fully explored. Also, questions remain as to what the molecular basis is for the stop-transfer function of signal anchors.

In this study, we show that the SA-I cannot block the translocation of a passenger secretory protein. As well, we show for the first time that a passenger signal sequence could block the function of an upstream signal anchor.

Materials and Methods

Reagents. All reagents, unless specified otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

Construction of cDNAs. Full-length cDNA encoding human aromatase and other cDNAs were subcloned into the SP6 vector, downstream of the SP6 RNA polymerase promoter. Site-directed mutagenesis was performed by polymerase chain reaction amplification using Pfu-DNA polymerase. Cycles consisted of denaturation at 94°C denaturation, annealing at 55°C annealing and extension at 72°C extension, with a final extension for 5 minutes at 72°C. The polymerase chain reaction-amplified product was purified using gel electrophoresis and a QIAquick Gel extraction kit (Qiagen, Hilden, Germany). The purified amplified product was digested with BglII/EcoRI restriction enzyme (New England Biolabs, Ipswich, MA) and ligated to predigested SP6 vector with T4 DNA ligase (New England Biolabs). The ligation was then transformed to DH5 α competent cells and the plasmid was purified through QIAprep Spin Miniprep kit (Qiagen). The accuracy of the plasmids was checked by dideoxy chain termination method in both the strands from a commercial source (MC Laboratory, San Francisco, CA).

Cell-Free Transcription-Translation and Synthesis of Chimeric Proteins. A cell-free transcription/translation synthesis was performed to express proteins for in vitro experiments using wheat germ extract (Promega, Madison, WI) labeled with [³⁵S]methionine (MP Biomedicals, Solon, OH). The wheat extract provides ribosomes and cofactors for protein expression. The cDNAs of interest coupled to an SP6 promoter were added, along with SP6 polymerase (Promega), in the presence and absence of rough microsomal membranes (MM) from dog pancreas. One microliter rough microsomal membranes and signal recognition particle (SRP) (tRNA probes, College Station, TX), when indicated, was added per 12.5 μ l of translational reaction. The mixture was incubated at 22°C for 2 hours, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized through phosphorimager or by autoradiography. The figures were prepared from the same experiment or two identical experiments performed at two different times without changing the integrity of the figure.

Glycosylation, Proteolysis, and Membrane Integration Assay. Aliquots of the translation product were incubated at 100°C for 10 minutes with 10 $\times$ glycoprotein denaturing buffer, and then the denatured samples were treated with endoglycosidase H (endo H; New England Biolabs) at 37°C for 1 hour following manufacturer's protocol (the reagents and buffers were supplied by the manufacturer). For proteolysis, aliquots of translated product were treated with 1 μ g/ml of proteinase K (PK) at room temperature for 15 minutes. Proteolysis was terminated by the addition of phenyl-methylsulfonyl fluoride to a final concentration of 2 mM and 2 $\times$ sodium dodecyl sulfate sample buffer, and the mixture was transferred to a boiling water bath. For membrane integration study, microsomes were collected with ultracentrifugation at 109,000g (Beckman TL-100.2; Brea, CA) at 4°C for 30 minutes. The supernatant was collected, and the pellet was treated with freshly prepared 100 mM sodium carbonate solution (pH, 11.4) on ice for 15 minutes. The samples were ultracentrifuged to separate soluble fraction from the membranous fraction. In experiment with methotrexate (MTX), 100 nM was used as the final concentration. All radioactive electrophoresed bands were visualized through phosphorimager and by autoradiography.

Results

Aromatase Is an Integral Membrane Protein with a $N_{\text{exo}}/C_{\text{cyt}}$ Topology. Microsomal P450s reside in the cytosol but are integrated into the ER membrane by their membrane anchors. *N*-Glycosylation occurs in the ER lumen and so, although P450 proteins have putative *N*-glycosylation

sites, they typically remain unglycosylated. However, the N terminus of the P450 protein aromatase is atypical compared with other microsomal P450s in that its signal anchor region follows a hydrophilic domain. Glycosylation of aromatase, which has putative sites at Asn12 and Asn180, in the presence of MMs (Shimozawa et al., 1993) signify that, unlike other P450 proteins, these sites are accessible to OST. OST, located within the ER lumen, adds oligosaccharides to Asn-X-Ser/Thr acceptor tripeptides (Dalbey et al., 1997). Cell-free translation of aromatase in the absence of MM showed a protein band (Fig. 1B). When rough microsomes were added to the translation mixture, we observed an additional protein with a decreased mobility (Fig. 1B), indicating that targeting aromatase to the ER results in its modification. We next treated the translated product with endo H (Fig. 1B). Endo H is a highly specific endoglycosidase that cleaves asparagine-linked mannose-rich oligosaccharides (Sakaguchi et al., 1992a). As expected, endo H completely digested the higher molecular weight band (Fig. 1B), confirming the glycosylation of aromatase. We treated an aliquot of the cell-free translated aromatase with PK in presence and absence of detergent (Triton X-100) (Fig. 1C). PK completely digested both the glycosylated and nonglycosylated protein (Fig. 1C), suggesting that the ER membrane did not protect aromatase. Addition of detergent during proteolysis had a protective effect on aromatase, because PK-mediated digestion was significantly reduced in the presence of Triton X-100 (Fig. 1C). The result suggests that detergent may act as a chaperone (Hegde et al., 1998), forming a structure that prevents PK from completely proteolyzing aromatase.

To examine the extent of integration of aromatase into the ER membrane, aliquots of translated product, with and without MM, were incubated with freshly prepared Na₂CO₃ and then ultracentrifuged to separate the membrane pellet from supernatant fractions (Fig. 1D). Na₂CO₃ breaks protein-protein interactions (Li and Shore, 1992) but not lipid-protein interactions, so all the peripheral membrane and soluble luminal proteins are recovered in the soluble fractions and membrane integrated proteins in the pelleted membrane (Chuck and Lingappa, 1992). In the absence of MM, washing with buffer as a control and alkali treatment resulted in recovery of the protein predominately in the soluble fraction (Fig. 1D). Addition of MM led to the recovery of glycosylated aromatase in the pellet fraction, both in buffer and alkali-treated samples, suggesting that the protein needed a membrane environment for integration (Fig. 1D, right-hand side).

To confirm the specificity of Asn glycosylation, we generated multiple aromatase N-terminal deletion mutants. The deletion constructs N Δ 50, N Δ 40, N Δ 30, and N Δ 20 did not appear glycosylated (Fig. 1E). However, we observed glycosylation, albeit at lower levels, of N Δ 10 (Fig. 1E). These results suggest that Asn12 was the only site glycosylated in aromatase. To confirm that only Asn12 is glycosylated, we generated the internal deletion mutants N Δ 11–40, N Δ 11–30, and N Δ 11–20 and found that these lacked glycosylation when translated in the presence of MM (Fig. 1F). We further narrowed the region to N Δ 12–14, N Δ 12–15, and N Δ 12–16 and again saw no glycosylation (Fig. 1G). To confirm further the specificity of amino acids, we made point mutations by substituting a polar to a nonpolar residue and from a bulky to a small or neutral amino acid. First, we mutated the 12th amino acid, asparagine to threonine, and, second, we mutated

the 14th amino acid, threonine to alanine. Identical cell-free transcription translation experiments in the presence of microsomal membrane showed no glycosylation (Fig. 1H). Therefore, of the two potential glycosylation sites, Asn12 and Asn180, only Asn12 was glycosylated and hence localized to the lumen.

Furthermore, glycosylation of aromatase in the absence of first 10 amino acids from the N terminus shows that translocation of aromatase into the ER did not require the N-terminal hydrophilic sequence. However, the observed weaker glycosylation suggests that its absence possibly interfered with the accessibility of OST to the glycosylation site. Thus, the observed glycosylation of aromatase in the presence of MM, complete accessibility to PK, and resistance to alkali treatment indicate that aromatase is an integral membrane protein with $N_{\text{exo}}/C_{\text{cyt}}$ topology in the ER (Fig. 1I).

Aromatase N-Terminal 40 Residues Control Translocation of Passenger Proteins. We further addressed the role of the aromatase N terminus on mediating translocation by replacing the signal sequence of preprolactin (pPL), a secretory protein with a cleavable 30 residue signal sequence (Stern and Jackson, 1985), with increasing lengths of the aromatase N terminus (first 12, 20, 30, 40, and 50 residues) (Fig. 2A). A hydrophobic amino terminus is indicative of a membrane anchor in microsomal P450s (Chen and Zhou, 1992). However, in aromatase, a more hydrophilic domain of ~20 residues precedes the hydrophobic region (Chen and Zhou, 1992). Thus we wanted to confirm that it is actually the hydrophobic region following the short N-terminal hydrophilic domain that is working as SA-I. We left the four amino acids preceding the cleavage site in pPL intact (Fig. 2A). In the presence of microsomal membrane, pPL undergoes processing to form the lower molecular weight protein, PL, which is resistant to protease digestion in the absence of detergent (Nicchitta and Blobel, 1989). However, we found that none of the aromatase-pPL fusion proteins underwent processing to a lower molecular weight protein (Fig. 2B). This result (Fig. 2B) confirms that the aromatase N terminus works as a signal anchor, thereby blocking the ability of the pPL signal peptidase cleavage site to reach the lumen and undergo processing.

Furthermore, we observed that the fusion proteins containing the first 12, 20, and 30 amino acids of aromatase were not glycosylated (Fig. 2B), whereas the fusion protein containing the first 40 aromatase amino acids was glycosylated (Fig. 2, B and C). Thus we conclude that the first 40, but not 30, amino acids of aromatase can act as a signal anchor, placing the N-terminal domain in ER lumen and halting the translocation of the passenger protein pPL. Aromatase N terminus from residues 20–40 is the hydrophobic region (Chen and Zhou, 1992) and thus working as SA-I. Moreover, the crucial region of the aromatase signal anchor appears to be located between 31 and 40 amino acid residues.

To confirm our above finding, we similarly fused the N-terminal 40 residues of aromatase with the cytosolic protein dihydrofolatereductase (DHFR) (Fig. 3A, top panel). DHFR is a 25-kDa cytosolic protein with 187 residues that has been widely used as a passenger protein to various organelles (Hurt et al., 1985). Translation of the fusion protein [1-40Ar+1-187DHFR] in the presence of MM resulted in its glycosylation, whereas incubation with endo H resulted in deglycosylation (Fig. 3A, bottom panel). To confirm our results, we translated

Fig. 2. Effect of aromatase SA-I on passenger protein translocation. (A) Fusion proteins of aromatase (black bar) and pPL (white bar). Signal sequence of pPL, except the four residues preceding the signal peptidase cleavage site, was replaced with increasing lengths of aromatase N terminus (first 12, 20, 30, 40, and 50 residues). Downward arrowheads (▼) show Asn12 in aromatase and downward arrow (↓) is signal peptidase cleavage site in pPL. (B) Cell-free transcription-translation (CFS) with and without MM of fusion proteins in presence and absence of PK. (C) CFS with and without MM of aromatase and pPL fusions in presence and absence of endo H.

the fusion protein in the presence and absence of MTX, a specific ligand of DHFR (Blakley and Cocco, 1985) (Fig. 3B). MTX treatment did not affect glycosylation of the fusion protein (Fig. 3B). We next fused the first 40 residues of aromatase with a full-length mitochondrial-targeting cytochrome P450 protein, side-chain-cleavage enzyme (SCC), generating 1-40Ar+1-521SCC (Fig. 3C, top panel). SCC's first 39 residues work as a cleavable N-terminal sequence that mediates translocation into the mitochondria (Chung et al., 1986). However, translation in the presence of MM resulted in

Fig. 3. Effect of aromatase signal anchor on passenger protein translocation. (A) Top panel shows the construction of fusion protein of aromatase first 40 residues (black bar) with full-length DHFR (white bar). Bottom panel shows cell-free transcription-translation (CFS) of fusion protein with and without MM treated with endo H. (B) 1-40Ar+1-187DHFR CFS with and without MM in presence and absence of MTX. (C) Top panel shows fusion protein of aromatase first 40 residues (black bar) with full-length SCC (white bar). Bottom panel shows CFS with and without MM treated with endo H. Downward arrowheads ($\blacktriangledown$) represent Asn12 in cartoons and glycosylated proteins in gels. Right arrow ($\rightarrow$) shows the precursor (p) form of the fusion protein.

a glycosylated fusion protein, sensitive to endo H and resistant to cleavage to a lower molecular weight protein (Fig. 3C, bottom panel). Thus, the results confirm that the aromatase N-terminal 40 residues work as the signal anchor.

The Concluding Region of Aromatase SA-I Sequence Is Indispensable for Its Function. We have now established that the first 40 residues of aromatase can target passenger proteins to the ER; however, the crucial part of the signal anchor lies between residues 31 and 40, as the first 30 residues could not mediate glycosylation. Sequence analysis of aromatase from various species (Chen and Zhou, 1992) and the three-dimensional crystal structure of aromatase purified

from human placenta (Ghosh et al., 2009) suggest that residues 21–42 integrate into lipid, forming a transmembrane segment. On the basis of the evidence, we hypothesized that residues 21–42 of aromatase serve as the signal anchor region and prepared a series of internal deletion mutants to test this. In the first set of mutants, we kept the N-terminal hydrophilic domain spanning residues 1 to 20 and the glycosylation site (Asn12) intact (Fig. 4A, top panel). When translated in the presence of MM, N Δ 21–50 and N Δ 21–40 (Fig. 4A, bottom panel) were not glycosylated, indicating that the region is crucial for translocation and thus glycosylation of aromatase. To determine the specificity of this region, we divided residues 21–40 into two equal parts, generating the mutants N Δ 21–30 and N Δ 31–40 (Fig. 4, A and B, top panels). When these mutants were translated with MM, we observed glycosylation of the N Δ 21–30 protein (Fig. 4A, bottom panel) but not of the N Δ 31–40 protein (Fig. 4B, bottom panels). The results of deletion mutant confirmed that residues 31 to 40 are necessary to place aromatase N terminus in ER lumen. Mutant proteins N Δ 41–60 and N Δ 51–60 further supported our conclusion, because both these mutant proteins were glycosylated in the presence of MM (Fig. 4C, bottom panel), suggesting that residues 41 and higher did not contribute to the glycosylation of aromatase. We next generated additional mutants, N Δ 26–35, N Δ 36–45, and N Δ 46–55 (Fig. 4D, top panel). Assays revealed the glycosylation of both N Δ 26–35 and N Δ 46–55, but not of N Δ 36–45 (Fig. 4D, bottom panel), indicating that residues from the 36th position in the signal anchor region are most crucial for aromatase SA-I function. The hydrophobic regions of the cytochrome P450 signal anchors are ~20 amino acid residues in length, much shorter than other type I signal anchor proteins, such as mouse synaptotagmin II (Kida et al., 2000). Nevertheless, deleting a major part of the signal anchor region had no effect on the glycosylation of aromatase. From above results, we infer that the last five residues of aromatase signal anchor (36–40) are critical for its function. This was indeed the case, as N Δ 36–40 mutant protein was not glycosylated in the presence of MM (Fig. 5A, bottom panel). However, it is possible that deletion of these last five amino acids affect positioning enough to inhibit glycosylation, even if the insertion and topology are in correct orientation. To confirm this, we did sodium carbonate extraction of N Δ 36–40 mutant protein in the presence and absence of MM (Fig. 5B). The mutant protein was recovered in the soluble fraction (Fig. 5B). The result corroborated our inference that residues 36 to 40 are indispensable for membrane integration and thus glycosylation of aromatase, even if rest of the signal anchor remains intact. We next prepared mutant proteins with point substitutions to investigate the individual residues in region N36–40. The region (36–40 residues) consists of the hydrophobic amino acids LLVWN, which we sequentially replaced with the hydrophobic residues VVLFQ (Fig. 5C, top panel). All the mutants were found to be glycosylated in the presence of MM (Fig. 5C). It appears that the hydrophobicity of this short region may support the correct orientation of aromatase in the ER membrane. So, absence of the last five residues of SA-I (N36–40) blocks aromatase glycosylation, even when rest of the SA-I remained intact, making it the most critical region of SA-I. To determine whether residues 36–40 are also crucial for interaction with SRP, we translated N Δ 36–40 in cell-free system in presence and absence of SRP (Fig. 5D, left panel). SRP arrests the

Fig. 4. Effect of internal deletions in signal anchor on aromatase glycosylation. (A–D) Top panels show the construction of the deletion mutant proteins of aromatase. V, deleted region in the mutants; ▼, Asn12 in aromatase; //, same number of amino acids were present in full-length (Ar) and all the mutant proteins. Bottom panels show the translation of mutants in presence of MM. Downward arrowheads (▼) show glycosylated aromatase.

translation of protein with membrane-insertion signals in cell-free system (Holland and Drickamer, 1986; Spiess and Lodish, 1986). We saw that the translation of NΔ36–40 protein was arrested by the addition of SRP (Fig. 5D), confirming that SRP does not need residues 36–40 for its interaction with the signal anchor of aromatase. Addition of SRP to the translation system did not affect the translation of globin (Fig. 5D), as globin is a cytosolic protein with no signal peptide sequence. Translation of full-length aromatase was also reduced in presence of SRP (Fig. 5D), confirming that the signal anchor of aromatase interacts with SRP. In summary, our current results show that aromatase translocates in ER by using its signal anchor to open the lateral gate of translocon to get membrane integrated (Fig. 5E).

Passenger Signal Sequence Overrides Upstream-Placed Signal Anchor. The underlying mechanism of signal anchor function remains unclear. Although SA-I functions as a classic signal sequence and as a stop-transfer sequence (Sakaguchi et al., 1987; Monier et al., 1988; Szczesna-Skorupa et al., 1988), the lack of a signal peptidase cleavage site is not responsible for the stop-transfer function of signal anchors. To better understand the function of SA-I, we investigated whether it could convert the topology of a full-length secretory protein, such as pPL, by blocking the translocation of its signal sequence. We fused 40 and 50 residues of the N terminus of aromatase in front of full-length pPL (Fig. 6A, top panel), generating 1–40 and 1–50Ar+1-229pPL. When these fusion proteins were translated in the cell-free system with MM, we observed the appearance of lower molecular

weight proteins (Fig. 6, C and D). Processing to a lower molecular weight protein can only happen if proteins translocate across the ER membrane and reach the lumen where signal peptidase cleave the signal sequence to generate lower molecular weight proteins. Because the aromatase signal anchor lacks a signal peptidase cleavage site, the observed lower molecular weight proteins likely resulted from processing at the cleavage site in pPL. The identical molecular weight of the processed fusion proteins supports this possibility and suggests that the N terminus of aromatase, which is a signal anchor, could not block the translocation of the passenger full-length pPL. Instead, the signal anchor of aromatase was cleaved as part of the signal sequence in these fusion proteins, indicating that the pPL signal sequence overrode the function of the aromatase signal anchor. Thus, the signal sequence converted the SA-I to a signal sequence, resulting in a soluble protein.

To determine the general applicability of our findings, we next generated a fusion protein consisting of membrane-anchor signal peptide of CYPIIC2 (Szczesna-Skorupa et al., 1988), followed by full-length pPL (1-26 CYPIIC2+1-229 pPL; Fig. 6A, bottom panel). CYPIIC2 also has a type-I signal anchor and integrates into the membrane with a $N_{\text{exo}}/C_{\text{cyt}}$ topology (Szczesna-Skorupa et al., 1988). When the negative charge in the signal anchor of CYPIIC2 was replaced with positive charges, the mutant protein behaved similar to a secretory protein (Szczesna-Skorupa et al., 1988). Translation of the CYPIIC2-pPL fusion protein in the presence of MM also resulted in the appearance of a protein

Fig. 5. Effect of deletions in signal anchor on aromatase glycosylation. (A) Top panel shows the construction of deletion mutant (NΔ36–40) of aromatase. V, deleted region in the mutant; ▼, Asn12; //, same number of amino acids were present in full-length (Ar) and mutant aromatase protein. Bottom panel shows the translation of full-length aromatase (Ar) and NΔ36–40 in presence and absence of MM. Downward arrowheads (▼) show glycosylated aromatase. (B) Membrane integration assay of NΔ36–40, where cell-free transcription-translation (with and without MM) was extracted with Na_2CO_3 and supernatant (S) was separated from the membrane fraction (P). (C) Top panel shows effect of point mutations in the region 36–40 on glycosylation of aromatase. L, leucine; V, valine; W, tryptophan; F, phenylalanine; N, asparagine; Q, glutamine. Downward arrowhead (▼) shows glycosylated aromatase. Bottom panel shows densitometric analysis of glycosylated protein bands in various mutants. (D) Left panel shows translation of aromatase, NΔ36–40, and globin in presence and absence of SRP. Right panel shows the densitometric analysis intensity of protein bands in presence and absence of SRP. (E) Model presenting possible mechanism of insertion of aromatase in the ER membrane to attain a N_{exo}/C_{cyt} topology. Once the aromatase signal anchor (green bar) is synthesized it interacts with the translocon. The signal anchor opens the lateral channel toward the lipid bilayer. Next, the protein is integrated in the membrane with N terminus in the lumen and C terminus in the cytosol. Red circle shows Asn12, red four-point star shows glycosylated Asn, and red cross (X) shows nonglycosylated Asn site.

with a lower molecular weight (Fig. 6E). Thus, CYPIIC2 signal anchor also could not block the translocation of the downstream pPL signal sequence, resulting in its cleavage. The results (Fig. 6, C and E) confirm that a secretory signal sequence can override type-I signal anchors when placed downstream to the latter, and thus, type-I signal anchors cannot block translocation of secretory signal sequences. To confirm that the lower molecular weight protein is a secretory protein, we treated the newly synthesized fusion protein (1-40Ar+1-229pPL) with sodium carbonate (Fig. 6F). In the presence of MM, the lower molecular weight protein was

completely recovered in the soluble fraction, confirming our results.

Signal sequence is characterized by being more positively charged compared with signal anchor and mutations in charges interconvert signal sequence and signal anchor (Szczesna-Skorupa et al., 1988; Kuroiwa et al., 1991). To get a deeper insight into our above observation, we mutated the positive charges in the signal sequence of pPL in the fusion protein 1-40Ar+1-229pPL. The amino acid changes are presented schematically (Fig. 7A). The mutated proteins were then translated in the presence and absence of MM (Fig. 7B). We

Fig. 6. SA-I did not block signal sequence-mediated translocation. (A) Construction of fusion protein of aromatase SA-I (first 40 and 50 residues, black bar) with full-length pPL (white bar). Similarly, SA-I of CYP11C2 (black bar) was fused with full-length pPL (white bar). Downward arrowhead (▼) shows Asn12 in aromatase and downward arrow (↓) is the signal sequence cleavage site in pPL. Cell-free transcription-translation (CFS) with and without MM of full-length pPL (B) and fusion proteins (C–E) in presence and absence of PK is shown. Upward arrow (↑) shows the processed soluble secretory protein. F, Membrane integration assay of 1-40Ar+1-229pPL, where CFS (with and without MM) was extracted with Na_2CO_3 and supernatant (S) was separated from the membrane fraction (P).

observed that the processing of these mutated proteins were very similar, except the mutant protein KKR to DDD (Fig. 7B). The processing of the KKR to DDD mutant protein into the ER as a soluble protein was significantly inhibited, confirming that positive charges of signal sequence played a major role for overriding the signal-anchor function (Fig. 7C).

To understand the mechanistic detail of signal anchor, we performed a competition experiment between the signal sequence and signal anchor. We placed signal sequence of pPL at distance of 10 and 160 amino acids from the signal anchor, generating 1-50Ar+1-229pPL and 1-200Ar+1-229pPL (Fig. 7D, top panel). We next studied the translocation of the fusion proteins after translation in the presence and absence of MM (Fig. 7D, bottom panel). The results show that the fusion protein 1-50Ar+1-229pPL was processed to a lower molecular weight protein (Fig. 7D, bottom panel). However, fusion protein 1-200Ar+1-229pPL (Fig. 7D, bottom panel) showed reappearance of glycosylation. Glycosylation of 1-200Ar+1-229pPL fusion protein can happen if the signal anchor of aromatase is integrated into the membrane. Membrane-integration of signal anchor in fusion protein 1-200Ar+1-229pPL is possibly because the signal sequence was far apart to interfere with the interaction between signal anchor and the translocon. Therefore, by delaying the translation of the signal sequence, the signal anchor got enough time to perform its function as stop-transfer (Fig. 7E).

Discussion

We embarked on this study to better understand the mechanism of signal anchor-mediated targeting of newly synthesized proteins to the ER. Unlike signal sequences, SA-Is work as both membrane-integration and stop-transfer signals (Sakaguchi et al., 1987; Monier et al., 1988). In addition, a recent report describes the ability of SA-I to mediate the translocation of a preceding amino-terminal domain (Kida et al., 2005). Although many families of proteins have topogenic SA-I regions, it most commonly occurs in the microsomal cytochrome P450 family (Kida et al., 1998).

The specificity of signal sequence and anchor activity depends on the balance between the hydrophobic segment and the N-terminal charges (Sakaguchi et al., 1992b). Signal anchors are uncleavable signal sequences. Although the absence of a signal peptidase cleavage site is important (Holland and Drickamer, 1986; Zerial et al., 1986), studies have shown that the length and hydrophobicity of the hydrophobic core (Spiess and Lodish, 1986) as well as the lack of positive charge (Szczesna-Skorupa et al., 1988) may also contribute to the lack of cleavage of SA-I. Not surprisingly, modifications that alter the balance between hydrophobicity and charge can convert signal anchors to cleaved signal sequences, if provided with a cleavage site (Szczesna-Skorupa et al., 1988; Kuroiwa et al., 1991). In our study, we showed that the SA-I mediated the translocation of the preceding hydrophilic domain into the ER lumen, leading to its glycosylation. Furthermore, we found that part of the protein integrated into the ER membrane, whereas the rest resided in the cytosol. In the absence of the signal anchor, the hydrophilic domain did not translocate the ER membrane. We also concluded that the first 40 residues of aromatase were sufficient to work as a signal anchor, based on the ability of this peptide to target cytosolic and mitochondrial passenger proteins to the ER. However, the signal anchor of aromatase could not block the translocation of an immediately following full-length secretory protein. In this case, the signal anchor was cleaved as part

Fig. 7. SA-I did not block signal sequence-mediated translocation. (A) Construction of mutant proteins from 1-40Ar+1-229pPL. Three positive charges (at positions 4, 9, and 12) in the signal sequence of pPL was subsequently mutated as shown and named accordingly. Downward arrowhead (▼) represents Asn12 in aromatase and vertical double arrowhead (‡) shows signal sequence cleavage site in pPL. Black plus (+) sign shows positive amino acids and red negative sign (-) shows negative amino acids in pPL. D, K, and R represent aspartic acid, lysine, and arginine, respectively. (B) Translation of 1-40Ar+1-229pPL and its mutants in presence and absence of MM. Upward arrow (↑) shows the processed soluble secretory protein. (C) A model of the mechanism of translocation when SA-I is followed immediately by a full-length secretory protein. Cartoon shows nascent chain being synthesized on cytosolic ribosome. As the signal sequence (cyan collate) follows the SA-I (green bar), it destabilizes the closed state of the translocon because of repulsion between the positive charges of signal sequence and the plug of translocon. It leads to premature opening of the pore toward the luminal exit, potentially allowing the translocation of the protein in the ER lumen. This premature opening halts the opening of lateral exit, and thus the SA-I could not get membrane integrated and subsequently did not block the translocation of the passenger protein. In this way, the pPL signal sequence directed the translocation of both flanking protein domains, and once inside the lumen, signal peptidase (black scissors) cleaved the signal sequence generating a soluble protein. (D) Top panel shows construction of fusion protein of aromatase (first 50 and 200 residues, black bar) with full-length pPL (white bar). Asn12 in aromatase [downward arrowhead (▼)] and signal sequence cleavage site in pPL [downward arrow (‡)] is represented schematically. Bottom panel shows translation of fusion proteins in presence and absence of MM. Glycosylated protein is shown by downward arrowhead (▼), and upward arrow (↑) shows the processed soluble secretory protein. (E) A model of the mechanism of translocation when SA-I is separated by a distance from a full-length secretory protein. Once the aromatase signal anchor (green bar) is synthesized, it interacts with the translocon. As signal sequence (cyan collate) is still not completely translated, SA-I gets enough time to open the lateral channel of translocon and undergoes membrane-integration. Therefore, the protein now has a $N_{\text{exo}}/C_{\text{cyto}}$ topology, with its N terminus glycosylated and following signal sequence residues in the cytosol. Red circle shows Asn12 and red four-point star shows glycosylated Asn.

of the signal sequence, and the signal anchor no longer functioned as a stop-transfer sequence, which resulted in the generation of a soluble secretory protein. This outcome suggests that the signal sequence of the passenger secretory

protein altered the topological function of the signal anchor.

The possibility exists that the complete change in the signal anchor function that we observed in the fusion protein may

have resulted from altered interaction with the translocon. The translocon mediates translocation across and integration of proteins into the endoplasmic reticulum membrane (Johnson and van Waes, 1999; Osborne et al., 2005). The translocon consists of Sec61 complex with 10 transmembrane domains that form a hydrophilic channel with a lateral exit site. The central hydrophilic pore is blocked by a central constriction and a plug domain on the luminal side. The plug on the luminal side needs to open and the ring must enlarge to allow protein translocation (Tam et al., 2005; Saparov et al., 2007). Furthermore, the translocon has been shown to open laterally toward the lipid bilayer, and the degree of opening is likely to vary according to the hydrophobic property of the signal and signal anchor sequence (Martoglio et al., 1995) because of a direct interaction between membrane integrating sequences (transmembrane segments) and the lipid phase responsible for recognition of these regions at the translocon (Hessa et al., 2005). The loss of SA-I function that we observed when SA-I is followed by a signal sequence immediately may have resulted from the destabilization of the closed state of translocon pore (Fig. 7C). Destabilization could lead to a premature opening of the pore toward the ER lumen (Junne et al., 2007), allowing translocation to occur rather than the transfer of the signal anchor to the lipid bilayer. Secretory signal sequences are characterized by positive charges and signal anchors by negative charges. Mutations leading to a net positive charge in a signal anchor can subsequently convert it to a signal sequence (Szczena-Skorupa et al., 1988; Kuroiwa et al., 1991). Thus, our results strongly suggest that negatively charged signal anchors interact with translocon to keep its luminal exit in a closed position (Figs. 5E and 7E). The signal anchor then interacts with the lateral exit site, resulting in transfer to the lipid phase. This association of SA-I with the translocon may explain why the addition of a cleavage site after the aromatase signal anchor did not result in translocation of the protein to the lumen where it can be cleaved. However, when the positive charge of a signal sequence follows the signal anchor, the possibility exists that the luminal end plug opens to reduce repulsion of the more positively charged end of the signal sequence (Fig. 7C) and thus mediating translocation in the ER lumen. Significantly reduced processing to soluble protein in the mutant protein (KKR to DDD) supports our hypothesis.

Our results also suggest that time may elapse before the membrane anchor is transferred from the proteinaceous channel to the lipid phase, and thus the signal sequence was able to override the effect of the flanking domains, leading to their translocation in the ER lumen. Had the membrane integration occurred before the signal sequence opened the luminal exit of the translocon, the protein would have not been able to translocate (Fig. 7E). Thus, our results show that insertion of the membrane anchor into the lipid bilayer play a very critical role in the stop-transfer function of signal anchors. Furthermore, it is not only the charges or the hydrophobicity of sequences that decide the topology of proteins in the ER but also the amino acid sequences following them. To our knowledge, this is the first report showing that a passenger signal sequence could override a signal anchor, thereby regulating the topology of the protein.

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Authorship Contributions

Participated in research design: Kaur, Bose.

Conducted experiments: Kaur.

Performed data analysis: Kaur, Bose.

Wrote or contributed to the writing of the manuscript: Kaur, Bose.

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