Cotranslational integration and initial sorting at the endoplasmic reticulum translocon of proteins destined for the inner nuclear membrane

Suraj Saksena*, Yuanlong Shao†, Sharon C. Braunagel‡, Max D. Summers*‡§, and Arthur E. Johnson*†¶

Departments of *Biochemistry and Biophysics, †Entomology, and ‡Chemistry, and §Texas Agricultural Experiment Station, Texas A&M University, College Station, TX 77843; and ‡Department of Medical Biochemistry and Genetics, Texas A&M University System Health Science Center, College Station, TX 77843–1114

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Most membrane proteins in eukaryotic cells are cotranslationally integrated into the membrane of the endoplasmic reticulum (ER) at sites termed translocons (1–3). These proteins are then sorted and distributed to cellular locations where they function, typically, by means of vesicular trafficking to the Golgi compartments, other organelles, and the plasma membrane (4, 5). However, in some cases, newly synthesized membrane proteins are directed to the inner nuclear membrane (INM). Because the INM is contiguous with the ER membrane, it is generally presumed that, after leaving the translocon, membrane proteins destined for the INM diffuse through the ER membrane, the outer nuclear membrane, and the nuclear pore membrane to reach the INM (6). Proteins are then retained in the INM by binding to nuclear proteins or DNA that prevent their diffusion back into the ER membrane. This model for protein sorting to the INM is termed the “diffusion–retention” model (6).

The envelope proteins of the baculovirus occlusion-derived virus (ODV) also integrate into the ER and transit to virally induced intranuclear membranes for envelope assembly. A minimum sequence required to direct proteins to the INM was determined by using the viral envelope protein ODV-E66 (E66), and its N-terminal 33 aa were found to be sufficient to traffic fusion proteins to the INM and ODV envelope with an efficiency similar to wild-type protein (7). This sequence has therefore been termed an INM sorting motif (SM) (8). The SM consists of 18 hydrophobic amino acids that form a transmembrane sequence (TMS) with positively charged amino acids that are located four to eight residues from the TMS on the nucleoplasmic or cytoplasmic face of the membrane. Notably, the SM also directs proteins to the INM in the absence of infection (8). Furthermore, a comparison of the viral SM sequence with the sequences of cellular proteins revealed that well-characterized cellular INM proteins have an SM-like sequence, even though the TMSs may be oriented in opposite directions in the bilayer and may be inserted into the bilayer by different mechanisms (8). Hence, fusion proteins containing either viral or cellular SM sequences may constitute reasonable substrates for examining the molecular mechanisms that mediate protein movement into the INM.

Although diffusion–retention could explain ODV envelope protein trafficking, transport to the INM may be regulated by something more complex than passive diffusion and retention. The 33-residue E66 SM sequence was crosslinked to FP25K and BV/ODV-E26 (E26), thereby demonstrating that these two viral proteins are adjacent to the SM while it is still in the ER membrane (8). Moreover, when the gene coding for FP25K was deleted from the viral genome, trafficking of E66 during infection seemed to be blocked at the nuclear envelope: E66 accumulated in punctate regions associated with the ONM and was not detected in the INM or within the virally induced intranuclear membranes (9). Thus, the trafficking of E66 to the INM may involve the active participation of other proteins.

We have here used the viral and cellular SM-like sequences to address three issues that are critical to our understanding of protein sorting to the INM. First, we have used photochemical and chemical crosslinking to detect and identify proteins that are located adjacent to, and presumably interact with, nascent INM proteins at different stages of integration. Second, to determine whether viral and host INM proteins use the same sorting machinery and are sorted in the same way, we have compared the photoadducts obtained with two host and two viral INM proteins. Third, to ascertain at what point, if any, during integration the SM sequence directs INM membrane proteins into a different pathway than that taken by proteins destined for other cellular membranes, we have compared the photocrosslinking of nascent chains directed to either the INM or another membrane. The combined data reveal that viral and host INM proteins interact similarly with translocon proteins, but they interact differently with the translocon than do most other membrane proteins. Thus, the translocon seems to discriminate between INM and other membrane proteins while they are nascent.

Abbreviations: ER, endoplasmic reticulum; INM, inner nuclear membrane; ODV, occlusion-derived virus; ODV-E26; ANB-Lys-tRNAamb,N9-(5-azido-2-nitrobenzoyl)-Lys-tRNAamb; SRP, signal recognition particle; TRAM, translocating chain-associated membrane protein; LBR, lamin B receptor.

*To whom correspondence should be addressed: Texas A&M University System Health Science Center, 116 Reynolds Medical Building, 1114 TAMU, College Station, TX 77843–1114. E-mail: aejohnson@tamu.edu.

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chains, which suggests that sorting is initiated very early during the integration process.

Methods

Materials. Plasmids containing the E66, E66G, and E66SM (this plasmid was termed “SM-cassette” in ref. 8) coding sequences have been described, as has the plasmid coding for Lep1 (10). Microsomes and mRNAs coding for E25, Nur1, and lamin B receptor (LBR) 1 were prepared as described in Supporting Text, which is published as supporting information on the PNAS web site. N(5-azido-2-nitrobenzoyl)-Lys-tRNAamb (εANB-Lys-tRNAamb) and truncated mRNAs were prepared as before (10–12).

Photocrosslinking. In vitro translations (50 µl; 26°C; 40 min) were performed in wheat germ cell-free extract in the presence of 40 nM canine signal recognition particle (SRP), 100 µCi of [35S]Met (1 Ci = 37 GBq), 8 equivalents of canine microsomes, and 72 pmol of εANB-Lys-tRNAamb as indicated (refs. 10 and 13 and Supporting Text). Samples were photolyzed on ice for 15 min by using a 500-W mercury arc lamp as before (13). After photolysis, microsomes were pelleted (4°C, 3 min, 100,000 rpm, Beckman TL A 100 rotor) through a 120-Å long flexible lysine side chain, so a 1.5-Å difference in helix could not explain the dramatic differences in photocrosslinking magnitude of photocrosslinking from adjacent positions in the TMS was randomly oriented and not bound to the translocon, parallel samples of E66 mRNAs containing amber codons at positions 10, 11, 12, or 13 were translated in the presence of SRP, microsomes, and εANB-Lys-tRNAamb, resulting in a 70-residue nascent chain (Fig. 1A). After photolysis, the extent of photocrosslinking of each E66 derivative to both Sec61α and TRAM was determined by immunoprecipitation by using affinity-purified antibodies specific for Sec61α and translocating chain-associated membrane protein (TRAM) (SDS/PAGE analyses of the total samples did not reveal any other major photoadducts, and no photoadducts were observed in the absence of light or ANB; data not shown). As shown in Fig. 1B, E66 reacts covalently with Sec61α primarily by means of probes positioned at residues 10 and 12. Probes at positions 11, 13, and, to a lesser extent, 10 photocrosslink to TRAM. As we have observed and discussed elsewhere (10), the great disparity in the magnitude of photocrosslinking from adjacent positions in the TMS of the SM reveals that it is not oriented randomly within the translocon, nor is the SM TMS free to rotate in or next to the translocon. Instead, the SM TMS seems to be bound in a fixed orientation by one or more translocon proteins. If the SM TMSs were randomly oriented and not bound to the translocon, then one would expect to see a symmetric photocrosslinking pattern in which each probe position reacts more or less equally with a given target protein (note that the probes are at the end of a 12-Å-long flexible lysine side chain, so a 1.5-Å difference in bilayer depth of probes located at adjacent residues in the TMS helix could not explain the dramatic differences in photoadduct
formation for adjacent probe locations seen in Fig. 1B). Thus, after SRP-dependent targeting, the TMS of the E66 SM sequence seems to occupy and be bound to a specific site within the translocon.

To assess the generality of these results, we examined the proximity to the translocon of nascent ODV-E25 (E25), a viral membrane protein that is also sorted to the INM. As above, an amber stop codon was substituted into position 10, 11, 12, or 13 (Fig. 1A) to yield constructs designated E25-A10, etc. When truncated mRNAs coding for 70 residues of each of these nascent chains were translated in parallel and then photolyzed, the photocrosslinking patterns were very similar to those of nascent E66. Sec61/H9251 was in close proximity to residues 10 and 12 in the E25 TMS, whereas residues 11 and 13 were adjacent to TRAM (Fig. 2C). Thus, the locations of the E66 and E25 TMS sequences within the translocon are indistinguishable early in integration. Furthermore, both viral TMSs were clearly adjacent to TRAM:

Fig. 1. Photocrosslinking of viral INM-directed TMSs to translocon proteins. (A) The N-terminal sequences of E66 and E25 are shown with the TMS underlined, as are the N-terminal sequences of the constructs containing the first TMS of LBR (LBR1), nurim (Nur1), and Lep (Lep1). In each case, an amber codon was substituted for the codon shown at position 10, 11, 12, or 13 (boxed in figure) to position the photoreactive probes at a single nascent chain location in each sample. The probes extend from different sides of the TMS a-helix surface as shown in the helical wheel representation. (B) Integration intermediates containing 70-residue E66-A10, E66-A11, E66-A12, or E66-A13 nascent chains were photolyzed, immunoprecipitated with antibodies to Sec61/H9251 (lanes 1–4) or TRAM (lanes 5–8), and analyzed by SDS/PAGE. Photoadducts to Sec61/H9251 and TRAM are indicated by the closed circle and arrowhead, respectively. The relative extent of photoadduct formation was quantified by comparing each photoadduct band intensity with that of the most intense photoadduct band in the gel (assigned 100%). (C) Integration intermediates containing 70-residue E25-A10, E25-A11, E25-A12, or E25-A13 nascent chains were photolyzed and analyzed as in B.
an average of 11% of the nascent chains photocrosslinked to translocon proteins, and approximately half of the photoadducts contained TRAM.

The E66 TMS also photocrosslinks to phospholipids in the translocon, as shown by the presence of photoadducts in these samples that are sensitive to treatment with phospholipase A2 (data not shown). In this respect, the environment of the E66 TMS in the translocon does not differ from that which has been reported for other TMSs (10, 15–17).

Mammalian INM TMS Proximity to Translocon Proteins. The unexpected photocrosslinking of viral TMSs to TRAM raised two questions. Is the close association with TRAM a property of viral proteins, or is this close association a property of INM-directed proteins, but not other membrane proteins? To ascertain which of these two possibilities, if either, is correct, the same approach was used to examine two mammalian proteins that localize in the INM: LBR and nurim. Both LBR and nurim are multispanning membrane proteins, but the first TMS and the flanking charged amino acids of both LBR (18) and nurim (19) are important for directing the protein to the INM. Thus, constructs that retained these features were generated and termed LBR1 and Nur1, respectively. After cotranslational insertion into the membrane, the LBR1 sequence adopts an orientation opposite to that of the first TMS in native LBR (6, 20); however, this difference in TMS orientation has no detectable effect on protein sorting to the INM because both LBR and LBR1 are each directed to the INM (S. T. Williamson, S.C.B., and M.D.S., unpublished results). Derivatives of LBR1 and Nur1 were then prepared by substituting an amber stop codon for a codon at positions 10, 11, 12, or 13 (Fig. 1A).

When 70-residue nascent chains of the LBR1 constructs were translated, targeted, and photolyzed, SDS/PAGE analyses of the TRAM- and Sec61-specific immunoprecipitates revealed that the photocrosslinking was asymmetric and the pattern was very similar to that seen with the viral INM TMSs. Sec61α was adjacent to positions 10 and 12 in the LBR TMS, whereas positions 11, 13, and, to a lesser extent, 10 were in close proximity to TRAM (Fig. 2A). As was seen with the viral INM-directed proteins, 11–12% of the total LBR1 and Nur1 nascent chains synthesized were photocrosslinked to TRAM plus Sec61α. The first LBR TMS and the viral TMSs therefore seem to occupy similar sites in the translocon.

When the Nur1 TMS sequence was examined, the photocrosslinking targets were the same, but the photocrosslinking efficiencies at different probe locations differed significantly from those of other INM TMSs. The extent of photocrosslinking to TRAM from positions 11 and 13 of Nur1 was similar to those observed with LBR1 (Fig. 2A and B), whereas photocrosslinking to Sec61α was most efficient from position 12 and much less efficient from position 11 (Fig. 2B). Only a trace of photocrosslinking to Sec61α was obtained from position 10, in contrast to the trace photocrosslinking obtained from position 11 in the other three INM TMSs (Figs. 1 B and C and 2.4). The asymmetry of the photocrosslinking to Sec61α and TRAM shows that the first nurim TMS is bound in a fixed orientation within the translocon. Furthermore, the nurim TMS clearly occupies a site in the translocon adjacent to TRAM. But the differences in photocrosslinking yields from different probe positions indicate that the nurim TMS does not interact with the translocon in exactly the same way as the viral TMSs.

Nascent Chain Length Dependence of Photocrosslinking. We have observed that TMSs of non-INM proteins are retained in the translocon even when the length of the nascent chain is far in excess of that required to release the TMS into the lipid bilayer (10, 13). Moreover, the lengthening of the nascent chain did not substantially alter the asymmetric photocrosslinking patterns obtained with different TMSs (10). To assess the retention of INM TMSs in the translocon, we monitored the photocrosslinking of E66SM-A10 as a function of nascent chain length in parallel incubations (E66SM was created by fusing the N-terminal 33 aa of E66 to a lysine-free sequence described in ref. 8). After photolysis, the samples were split for immunoprecipitation with antibodies specific for either Sec61α or TRAM. The extent of E66SM-A10 (Fig. 3) and E66SM-A12 (data not shown) photocrosslinking to Sec61α decrease rapidly as the nascent chain lengthens. At 70 residues, the TMS has just emerged from the ribosome, and lengthening the chain by 5–17 residues results in a substantial decrease in nascent chain photocrosslinking to Sec61α. By the time the nascent chain reaches 100 residues, the E66 TMS has moved away from Sec61α.

In contrast, the E66 TMS was adjacent to TRAM until later in the process. The extent of E66SM-A11 (Fig. 3) and E66SM-A13 (data not shown) photocrosslinking to TRAM was the same for nascent chain lengths of 75–87, but photoadducts were still visible when the nascent chain reached 120 residues (Fig. 3). Thus, during its passage through the translocon, the E66 SM seems to remain adjacent to TRAM for a longer period than to Sec61α.

SM Sequence Crosslinking to Non-Translocon Proteins. Another approach that can be used to identify proteins that associate with the SM sequence is chemical crosslinking. Because the E66SM construct contains only two lysine codons near the C-terminal end of the SM sequence (Fig. 5A), the crosslinking of the E66SM substrate to another protein by means of bis(sulfosuccinimidyl) suberate, a lysine-specific homobifunctional reagent, would require a Lys residue on the target protein to be in close proximity to a Lys residue in the substrate. Because the sample contains many proteins with surface-exposed lysine amino groups, the detection of a specific covalent complex would be explained most reasonably by the association of the substrate SM sequence with a particular target protein.

By using this approach, we showed that the SM sequence in full-length E66SM was chemically crosslinked to viral proteins FP25K and/or E26 (8). This crosslinking was observed either in nuclei from insect Sf9 cells that had been infected with a recombinant baculovirus expressing E66SM or when E66SM was
Discussion
Several important conclusions can be drawn from the data reported here. First, the asymmetric photocrosslinking to TRAM and to Sec61α of each TMS examined here reveals that each is bound to a protein(s) within the mammalian translocon (cf. ref. 10). Second, the proteinaceous environment within the translocon is very similar for both cellular and viral INM TMSs. Third, the proteinaceous environment within the translocon differs markedly for INM TMSs and for TMSs directed elsewhere. Fourth, an integrated viral SM sequence is crosslinked to two viral proteins after being released from the translocon in ER microsomes purified from infected insect cells. Fifth, this study confirms the widely held presumption that some INM-directed proteins are targeted to the ER membrane by SRP and integrated cotranslationally into the translocon. Other INM proteins may be inserted posttranslationally as C-tail-anchored proteins (21). Taken together, these results strongly suggest that some membrane protein sorting to the INM is initiated within the translocon early in the integration process, is protein-mediated, and involves substrate recognition by and interaction with a sequence of proteins that function as sorting factors to facilitate the movement of proteins to the INM.

As discussed elsewhere (10), the binding of a substrate TMS to a translocon protein is revealed by the nonrandom and asymmetric photocrosslinking of the TMS to TRAM and/or Sec61α from different photoreactive probe locations that encircle the middle of the cylindrical TMS α-helix. This high-resolution approach for characterizing a TMS’s environment within the translocon has so far been applied to 11 TMSs in this (Figs. 1 and 2, and Fig. 7, which is published as supporting information on the PNAS web site) and our previous study (10). Because each of these 11 TMSs was not free to rotate and randomize its orientation relative to TRAM and Sec61α in the translocon, each TMS binds to a translocon protein(s). This interaction presumably plays a direct (but as-yet undefined) mechanistic and/or regulatory role in the integration process.

When the translocon environments of the viral E66 and E25 TMSs were examined, the photocrosslinking of these two TMSs to TRAM and Sec61α was found to be indistinguishable (Fig. 1). In addition, a similar photocrosslinking pattern was obtained when the proximity of the first TMS of mammalian LBR to translocon proteins was investigated (Fig. 2A). The close similarity of the photocrosslinking results for these three INM proteins seems more than coincidental. Furthermore, the first TMS of mammalian nurim also photocrosslinked to TRAM and Sec61α from the same probe positions as the other three TMSs, although the extent of Sec61α photocrosslinking from positions 10 and 11 of Nur1 was reversed from that of the other three substrate TMSs (Fig. 2B). Most striking, however, was the observation that 4 of 4 INM TMSs were efficiently photocrosslinked to TRAM. Furthermore, these TMSs were in very similar, but not identical, proteinaceous environments or sites within the translocon based on the photocrosslinking-detected proximities of different TMS surfaces to Sec61α and TRAM.

In contrast, of the 10 total non-INM-directed TMSs whose photocrosslinking to Sec61α and TRAM has been examined by different groups, only the two native TMSs of the vesicular stomatitis virus G (VSVG) protein (10, 13) and the Ii invariant chain (15) were crosslinked to TRAM by means of photoreactive probes in the middle of the TMS. Non-photocrosslinked to TRAM. Furthermore, these TMSs were in very similar, but not identical, proteinaceous environments or sites within the translocon based on the photocrosslinking-detected proximities of different TMS surfaces to Sec61α and TRAM.

It is, of course, possible that INM and non-INM TMS-binding sites within the translocon overlap to some extent. Consistent with the close juxtaposition of potentially different interaction sites within the translocon, both nascent secretory and nascent membrane proteins have previously been photocrosslinked to TRAM, usually from sites that flank the TMS or the nonpolar signal sequence core (e.g., refs. 1 and 22). On the other hand, because of variations in probe length, reactivity, location, and target atom, one must be cautious in extrapolating from a crosslink to a specific structural arrangement between two macromolecules. For example, the second TMS in opsin was chemically crosslinked to TRAM (23), but no crosslinking to TRAM was detected when photoreactive probes were positioned in the middle of the second opsin TMS in a chimeric protein (10). Thus, high-resolution experiments using multiple different probes will be necessary to assess the extent to which the INM and non-INM TMS binding sites in the translocon overlap spatially and dynamically.

Based on photocrosslinking data obtained with charged Lep1 mutants, Heinrich et al. (17) proposed that TRAM interacts with TMSs that were charged or hydrophilic. Yet, of the four INM-
directed TMSs found adjacent to TRAM during integration, none contains a charged amino acid (Fig. 1A). Furthermore, based on the White–Wimley values (ΔG_{swc} − ΔG_{wli}) for quantifying amino acid movement from the aqueous to the nonpolar phase, the free energies of transfer of the four INM TMSs range from −3.0 kcal/mole for the first nunir TMS to −10.5 kcal/mole for the E66 TMS (a more negative number indicates a more hydrophobic sequence) (8, 24). Thus, even the E66 TMS, which is the most hydrophobic of the 11 TMSs that we have thoroughly examined to date, is positioned adjacent to and photocrosslinks to TRAM during cotranslational integration. It therefore seems that TRAM functions in a different or an additional role than that proposed by Heinrich et al. (17).

We have here compared the photocrosslinking targets of probes that are in different constructs but are positioned the same number of residues from the N terminus. Another approach would be to compare the photocrosslinking targets for probes positioned the same number of amino acids from another reference point, such as the N-terminal residue in the putative TMS. But, because only small variations in photocrosslinking patterns were observed with E66, E25, LBR1, and Nur1 TMSs (Figs. 1 and 2), it seems that the differences are not due to variations in length and location (i.e., the number of residues between the N terminus and the putative start of the TMS) shown in Fig. 1A. Moreover, the photocrosslinking of Lep1 TMS does not photocrosslink to TRAM (Fig. 7).

What, then, is the key structural determinant of the SM that is recognized by the translocon? The sample size is still too small to provide a clear answer to this question, but there are some interesting clues in the results presented here. First, as was noted above, we have here examined constructs containing only the first TMSs of LBR and nurin because these TMSs are important in terms of sorting to the INM (18, 19) and we wished to facilitate comparisons with the INM-directed viral proteins that contain only a single TMS. Yet, the orientation of the LBR1 TMS in the bilayer is opposite to the bilayer orientation of the same TMS in native LBR. Strikingly, and unexpectedly, both LBR1 and native LBR are sorted and targeted to the INM (S. T. Williamson, S.C.B., and M.D.S., unpublished results). Because the LBR TMS is directed to the INM, it seems that the differences in TMS length and the putative start of the TMS) shown in Fig. 1

Moreover, both E66 and E25 are directed to the INM, even though E25 lacks the cytosolic positive charge that is characteristic of an SM sequence. Because the E66 and E25 TMSs photocrosslink to TRAM and Sec61α identically (Fig. 1), it seems that the positive charge that flanks the TMS is not involved in positioning an INM TMS at a particular site within the translocon. Moreover, both E66 and Lep1 have positive charges flanking the TMS, yet their TMSs occupy different locations in the translocon (Figs. 1B and 7). It therefore seems that the properties of the TMS itself dictate where it moves in the translocon, whereas the positive charge may be important for interactions that occur after leaving the translocon, such as recognition and/or association with FP25K and/or E26 (Fig. 4, and Fig. 8, which is published as supporting information on the PNAS web site). These results, taken together, strongly suggest that the characteristic structural elements that identify a protein as an INM protein and initiate sorting to the INM are located within the TMS itself. Further experiments with multiple INM-directed TMSs and derivatives will be required to determine what TMS features correlate with INM sorting.

The distinctive photocrosslinking pattern observed with INM TMSs indicates that INM and non-INM TMSs occupy distinctly different sites within the translocon, which in turn suggests that INM-directed TMSs are first identified and sorted by components of the translocon. Because INM TMSs are adjacent to both Sec61α and TRAM (Fig. 1 and 2), either or both could be actively involved in recognizing and sorting INM TMSs; future experiments will clarify their involvement. Also, because an INM TMS remains adjacent to TRAM longer than to Sec61α (Fig. 3), it is possible that TRAM may be involved in the hand-off of INM TMSs to the next participant in the putative INM sorting pathway. Upon leaving the translocon, the viral INM-directed proteins interact with other viral proteins that are required for sorting to the INM. Because viruses typically appropriate host mechanisms to achieve their objectives, the identification of both soluble (FP25K) and membrane-associated (E26) viral proteins that are predicted as required for the sorting of viral proteins to the INM strongly suggests that a similar host protein(s) may also be involved in INM sorting. It will require investigating INM substrate transfer from the translocon to other proteins to further clarify the participants and mechanisms that accomplish membrane protein sorting to the INM.

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