Supporting Text

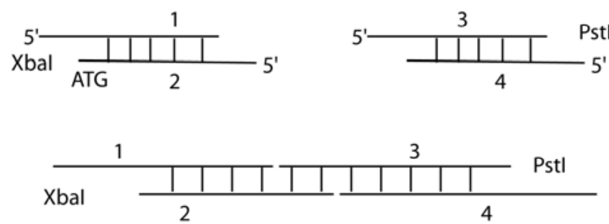
Supporting Methods

Plasmids and mRNA. The DNA sequence of each of the following plasmids was determined before use to ensure accuracy.

ODV-E25. The viral envelope protein gene occlusion-derived virus (ODV)-E25 was PCR-amplified from the *Autographa californica* nucleopolyhedrovirus (strain E2) *Hin*dIII C fragment and cloned into pBlueScript II KS.

*LBR***.** The lamin B receptor (LBR)-GFP fusion clone was kindly provided by J. Ellenberg (1), and this was used as a template to PCR a truncated version of LBR. The LBR1 sequence is: MFGGVPGVFLIMFGLPVFLFLLLMCKQKDPPVATM….GFP, where the second residue (F) corresponds to residue 208 in native LBR, the amino acids added in cloning are underlined, and "M….GFP" at the C terminus represents the GFP sequence.

*Nurim***.** Two complementary sets of oligonucleotides were synthesized. Oligonucleotides 1 and 2 contained a 5' *Xba*I site followed by the nurim sequence. The second set of oligonucleotides contained overlapping regions with the first, the sequence of nurim through amino acid 55 and a 3' *Pst*I site. Each set was annealed in equimolar ratios; then, the two sets were allowed to anneal to each other and were cloned into pGEM 4Z. See schematic diagram below. The same technique was used to add amino acids 55-92. The final nurim clone contains a conservative amino acid change at position 17 (L→M). The nurim sequence was obtained from GenBank no. AF143676 (2).



Truncated mRNAs coding for nascent chains of defined lengths were transcribed *in vitro* using SP6 RNA polymerase as before with PCR-produced DNA fragments of the desired length (3).

Microsomes. Canine signal recognition particle (SRP), column-washed microsomes (CRM), and high salt- and EDTA-washed microsomes (EKRM) were prepared as before (4). To prepare Sf9 microsomes, Sf9 cells were infected with the E2 strain of AcMNPV [multiplicity of infection (moi) = 10] and collected 33 h postinfection. Thirty grams of cells were diluted with 4 ml per gram of cell mass of buffer A [50 mM triethanolamine (pH 7.5)/50 mM KOAc/6 mM Mg(OAc)2/1 mM EDTA/1 mM DTT/0.5 mM phenylmethylsulfonyl fluoride] and was homogenized with 10 strokes using a motor-driven drill homogenizer, avoiding foam formation and heating. The homogenate was centrifuged for 10 min at 1,000 × *g*. The supernatant was recentrifuged for 10 min at 10,000 × *g*. Crude rough microsomes were collected by centrifugation of the 10,000 × *g* supernatant for 2.5 h at 140,000 × *g* (Beckman Ti50.2 rotor at 40,000 rpm) through a cushion of 1.3 M sucrose in buffer A. The pellets were resuspended by manual homogenization in a Dounce homogenizer in buffer B (250 mM sucrose/50 mM triethanolamine (pH 7.5)/1 mM DTT) to a concentration of 50 A280 units/ml. The microsomes were active, as judged by their ability to target and translocate preprolactin in rabbit reticulocyte lysate at levels comparable with canine pancreatic microsomes (data not shown).

Translation, Photolysis, Immunoprecipitation, and Analysis. SRP photocrosslinking experiments were done as described in *Methods* in the text, except that microsomes were omitted from the translation and immunoprecipitation conditions were the same for both SRP54-specific and translocating chain-associated membrane protein (TRAM)-specific antibodies (3).

To assess the SRP dependence of sorting motif (SM) targeting to microsomes (cotranslational targeting), truncated mRNAs coding for the N-terminal 200 aa of E66G were translated as described in *Methods* in the text, except that the 25-μl incubations

lacked N^{ε}-(5-azido-2-nitrobenzoyl)-Lys-$tRNA^{amb}$ (εANB-Lys-$tRNA^{amb}$) and contained 5 μCi (1 Ci = 37 GBq) of [^{35}S]Met, 8 equivalents of CRMs or EKRMs, and additional SRP as indicated. To assess posttranslational targeting, the E66G translation intermediates were prepared in the same way, except that CRMs or EKRMs were not included. Nascent chains were released from the tRNA and ribosomes by puromycin (2 mM, 26°C, 30 min), and then 8 equivalents of CRMs or EKRMs were added and incubated at 26°C for 30 min. The microsomes in each sample were sedimented through a sucrose cushion as above, resuspended directly into sample buffer, and analyzed by SDS/PAGE (3).

Supporting Results

Targeting of the SM to the Endoplasmic Reticulum (ER) Membrane Occurs Cotranslationally and Is SRP-Dependent. Full-length E66 has been observed to insert into membranes posttranslationally, although at low efficiency (5). Hence, it was first necessary to ascertain whether E66 is normally targeted to the ER membrane in an SRP-dependent manner and integrated cotranslationally. Thus, E66 was modified by extending its N terminus to position a consensus glycosylation acceptor sequence sufficiently far (18 residues) from the nonpolar transmembrane sequence (TMS) to be glycosylated when the -N-S-T- is translocated into the ER lumen (6). The resulting construct, here termed E66G (Fig. 5*A*), has also been used to determine the normal orientation of E66 in the inner nuclear membrane (INM) (7).

The targeting to the ER membrane of most ribosome-nascent chain complexes synthesizing eukaryotic membrane proteins is SRP-dependent (8). When E66G was translated in the presence of canine column-washed rough ER microsomes (CRMs) containing SRP, much of the nascent E66G was glycosylated (Fig. 5*B*, lane 1). However, when the CRMs were added after nascent E66G had been released from the ribosomes, no E66G was glycosylated (Fig. 5*B*, lane 2), thereby showing that the signal sequence-containing nascent E66G could not be targeted and translocated posttranslationally. The SRP dependence of this targeting was further examined using microsomes that had been stripped of their SRP and residual ribosomes by washing in EDTA and high salt

(EKRMs). E66G proteins synthesized either in the presence (Fig. 5*B*, lane 3) or in the absence (Fig. 5*B*, lane 4) of EKRMs were not glycosylated but were glycosylated if SRP was included with EKRMs in the incubation from the beginning (Fig. 5*B*, lane 5). Thus, the targeting, translocation, integration, and glycosylation of E66G is SRP-dependent and occurs cotranslationally.

The above results suggest that the SM sequence acts as a signal sequence to target ribosome-nascent chain complexes（RNCs) synthesizing E66G to the translocon. If true, one would predict that the SM sequence would bind to SRP, and that this association could be detected by nascent chain photocrosslinking to the 54-kDa subunit of SRP (SRP54) (9). An amber stop codon was substituted into the nonpolar core of E66 at position 12 to create a construct designated E66-A12 (Fig. 5*A*; other amber codon-containing constructs are identified similarly), and a truncated mRNA transcribed from this DNA was translated in the presence of SRP and either εANB-Lys-tRNA^{amb} or unmodified Lys-tRNA^{amb}, but in the absence of microsomes to block the targeting pathway at the RNC•SRP intermediate. Upon illumination, an ≈61-kDa photoadduct containing the 70-residue nascent chain and a larger protein was formed (Fig. 5*C*, lane 4), and this target protein was shown to be SRP54 by immunoprecipitation with SRP54-specific antibodies (Fig. 5*C*, lane 5). As expected, no photoadduct was observed in the absence of light (Fig. 5*C*, lane 2) or probe (Fig. 5*C*, lane 1). Furthermore, a nascent chain lacking the SM sequence (termed E66-Δ33), but containing a photoreactive probe at position 12, did not photocrosslink to SRP54 (Fig. 5*C*, lane 3), thereby showing the requirement of the SM sequence for nascent chain binding to SRP.

Non-INM TMS Proximity to Translocon Proteins. To determine whether the crosslinking patterns shown in Figs. 1 and 2 were unique to INM-directed membrane proteins, two non-INM TMSs were examined using the same techniques. The first TMS of leader peptidase (Lep1) has been shown to photocrosslink, at least transiently (10-12) and in an asymmetric fashion (3), with Sec61α. We therefore determined whether Lep1 also photocrosslinked to TRAM. The Lep1 construct was substituted with a single amber stop codon at each of four adjacent codons in the first TMS (Fig. 1*A*), and a 70-residue

nascent chain of each of these constructs was translated, targeted, photolyzed, and examined as above. In contrast to the results obtained with INM-directed TMSs, Sec61α photocrosslinked to the Lep1 TMS from positions 11, 13, and, to a lesser extent, 10 (Fig. 7). But no photocrosslinking to TRAM was detected from any of the four probe positions (for comparison, the extent of TRAM photocrosslinking obtained in a parallel sample containing a 70-residue E66-A11 nascent chain is shown in lane 9 of Fig. 7). Thus, the Lep1 TMS appears to occupy a fixed position within the translocon, but this site differs markedly from that occupied by the INM-directed TMSs.

Similarly, four probe locations in the TMS of transferrin receptor (TfR) photocrosslink asymmetrically to Sec61α, but they do not photocrosslink at all to TRAM (ref. 3 and data not shown). Thus, the TfR TMS occupies a specific site in the translocon during cotranslational integration, but this site differs substantially from that occupied by the INM-directed TMSs.

Not Every Substrate Crosslinks to FP25K and E26. Lep1 contains lysines in approximately the same positions as E66SM, and hence it is pertinent to ask whether Lep crosslinks to FP25K or E26. No chemical crosslinks to FP25K (Fig. 8*A*, lane 1) or E26 (Fig. 8*A*, lane 3) were observed with nascent, puromycin-released Lep intermediates after integration into ER microsomes from infected cells. In contrast, efficient crosslinks to FP25K (Fig. 8*A*, lane 2) and E26 (Fig. 8*A*, lane 4) were seen with full-length E66SM after integration into infected ER microsomes and incubation with bis(sulfosuccinimidyl)suberate. The difference in the crosslinking efficiencies of Lep1 and SM were not based on differences in levels of translation (Fig. 8*B*). We therefore conclude that FP25K and E26 do not crosslink (and presumably do not bind to) every protein, but that they are selective in their association with substrates.

Nascent Chain-Length Dependence of E66SM Translation. The immunoprecipitation data in Fig. 4 reveal that only full-length E66SM was chemically crosslinked to FP25K and E26, a conclusion that is justified only if the number of E66SM polypeptides available for crosslinking in each sample was approximately the same. To demonstrate

the validity of this assumption, we removed equal aliquots from each sample in an E66SM bis(sulfosuccinimidyl)suberate chemical crosslinking experiment and analyzed the total translation products by SDS/PAGE. As shown in Fig. 6, the numbers of E66SM polypeptides translated in the parallel samples were comparable (within a factor of three when quantified using the PhosphorImager and correcting for the number of methionines per polypeptide). When this same gel was overexposed to reveal the extent of E66SM crosslinking to one of the two 25-kDa putative sorting factors, a radioactive crosslinked species was seen only in lane 5 with full-length E66SM. Similarly, the immunoprecipitation data of Fig. 4 also show that only full-length E66SM is able to crosslink to FP25K or E26.

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