Discrete Cross-linking Products Identified during Membrane Protein Biosynthesis*  

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We have investigated the molecular details of the membrane insertion of the multiple-spanning membrane protein opsin. Using heterobifunctional cross-linking reagents the endoplasmic reticulum (ER) proteins adjacent to a series of defined translocation intermediates were determined. Once the nascent opsin chain reaches a critical minimum length Sec61β is the major ER component adjacent to the polypeptide. Using a homobifunctional reagent, the cross-linking partners from a single cysteine residue in the nascent chain were analyzed. This approach identified chain length-dependent cross-linking products between nascent opsin and a 21-kDa ribosomal protein, followed by Sec61β and finally with Sec61α. Our data support a model where the sequential transmembrane domains of a multiple-spanning membrane protein are integrated at an ER insertion site similar to that mediating the insertion of single-spanning membrane proteins.  

Membrane proteins are targeted to the endoplasmic reticulum (ER) membrane by a signal sequence which is usually a stretch of up to 20 apolar amino acid residues (1). The targeting process involves the recognition and binding of these signals by the signal recognition particle (SRP) (2). At the membrane, SRP interacts with the SRP receptor and, in a GTP-dependent manner, presents the nascent chain to the translocation/insertion machinery (2, 3).  

Cross-linking studies have been used to identify ER proteins responsible for the translocation of secretory proteins and the insertion of single-spanning membrane proteins (reviewed in Ref. 4). Using a photocross-linking approach, Sec61α was identified as a major cross-linking partner of the secretory protein preprolactin (5–7) and type I and type II membrane proteins (8–11). Sec61α was also identified as a major cross-linking partner of secretory and membrane proteins using bifunctional cross-linking reagents (10, 12, 13). Sec61α is part of a protein complex, together with Sec61γ and Sec61α (14, 15), and reconstitution studies showed that this Sec61 complex plus the SRP receptor are essential components for secretory protein translocation and membrane protein insertion (14, 16). Cross-linking also identified a second component of the translocation machinery, the translocating chain-associating membrane protein (TRAM) (8, 9, 11, 17–19). Reconstitution studies showed that TRAM is required for the efficient insertion/translocation of a subset of membrane and secretory proteins (14, 16, 19). While initial studies allowed cross-linking from a number of positions within the nascent chain, more recently site-specific cross-linking techniques have been developed, allowing the environment of particular regions of the nascent chain to be investigated (6–8, 20, 21).  

Previous cross-linking studies have concentrated on secretory proteins, and simple, single-spanning membrane proteins. In this study we have analyzed the biosynthesis of opsin, a 39-kDa multiple-spanning membrane protein which is targeted to the ER membrane by SRP (22, 23). Truncated mRNAs were used to generate “translocation intermediates” which remain associated with the ER translocation site due to the presence of the ribosome at the C terminus of the truncated polypeptide (24). Hetero- and homobifunctional cross-linking reagents were then used to identify cross-linking partners of the inserting nascent chain. With heterobifunctional reagents the only component of the ER translocation site cross-linked to nascent opsin chains was Sec61α. This adduct was only observed when the nascent chain reached a critical minimum length. In contrast, using the homobifunctional reagents we observed discrete chain length-dependent cross-linking products between the nascent chain and a 21-kDa ribosomal protein, followed by Sec61β, and finally Sec61α.  

EXPERIMENTAL PROCEDURES  

Materials—The plasmid coding for opsin, pGEM3OP, was kindly provided by Reid Gilmore, University of Massachusetts, Worcester, MA. BioHI, used to generate the 155-amino acid truncation of opsin and the Endoglycosidase H (recombinant fusion protein) (Endo H) were from New England Biolabs (Hitchin, Herts). [35S]Methionine was supplied by Amersham (Amersham, Bucks) and T7 RNA polymerase by Promega (Southampton, Hants). All cross-linking reagents were purchased from Pierce & Warriner (Chester, Cheshire) and Apollo Chemicals Ltd. (Stockport, Cheshire). The concanavalin A-Sepharose was purchased from Pharmacia Biotech (St. Albans, Herts). All other chemicals were purchased from BDH/Merck (Poole, Dorset) and Sigma (Poole, Dorset). The antibodies specific for SRP54, Sec61β, TRAM, and Sec61α (under denaturing conditions), were a gift from Bernhard Dobberstein, ZMBH, Heidelberg, Germany. The Sec61α antisera used under “native” conditions, and the Sec61γ antisera, were both raised by Research Genetics Inc. (Huntsville, AL) using peptides encoding the C-terminal (α) and N-terminal (γ) 12 amino acids of the published sequences.  

Construct Preparation—The coding region of bovine opsin was subcloned from pGEM3OP into the plasmid pGEM3z using an EcoRl/HindIII fragment. Most of the templates for the transcription of different truncated opsin mRNAs were prepared by PCR (25). The resulting opsin translocation intermediates were all efficiently membrane inte-
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grated, glycosylated, and immunoprecipitated with an anti-opsin monoclonal antibody. The upstream primer was 160 bases 5'- of the T7 RNA polymerase promoter and had the sequence 5'-GGCCCTTCTTCTGATCCCT-TAGCC-3', antisense primers were designed to make 5 truncations of 106, 127, 152, 157, and 150 amino acids. For the 106-amino acid truncation (OP106) the primer 5'-GGCCCTTCTTCTGATCCCT-TAGCC-3' was used, 127-amino acid truncation (OP127) 5'-GGACAGTGCAATTTCAC-3', 132-amino acid truncation (OP132) 5'-GGACACCAACCAAGGACCA-3', and for the 150-amino acid truncation (OP150) the primer 5'-CTCCCGAAGCCGAGGTGCT-3' was used. The PCR products were purified directly from the reaction mixture using the Wizard PCR Prep kit (Promega). The template for the 155-amino acid truncation (OP155) was made by cleavage of the plasmid within the coding region using the restriction endonuclease BanHI.

Site-directed Mutagenesis—The cysteine residue at position 140 was altered to a glycine using the Clontech Laboratories site-directed mutagenesis kit (Palo Alto, CA). Subsequent sequencing revealed two discrepancies from the published sequence (26), these were single base changes in the codons for amino acids 101 and 118 and did not alter the amino acid residue encoded. These changes were also present in the original plasmid.

In Vitro Transcription and Translation—Transcription of the purified RNA was carried out as described by the manufacturer (Promega, Southampton, Hants.). Translation of the resulting truncated rabbit reticulocyte lysate system (Promega, Southampton, Hants.) was carried out at 30 °C in the presence of [35S]methionine and canine pancreatic microsomes as described by the manufacturer. Translation initiation was inhibited after 15 min by the addition of 4 mM 7-methyloxazolone, 5'-monophosphate, and chain elongation allowed to continue for a further 10 min until translation was inhibited by the addition of 2 mM cycloheximide.

Cross-linking with Bisfunctional Reagents, Immunoprecipitation, and Sample Analysis—For cross-linking with bifunctional reagents, the membrane associated components were isolated by centrifugation through a high salt/sucrose cushion (250 mM sucrose, 500 mM KAc, 5 mM Mg(OAc)2, and 50 mM HEPES-KOH, pH 7.9) for 10 min at 4 °C and 55,000 × g (100,000 × g in a TLA100.2 rotor, Beckman instruments). The resulting pellet was resuspended in a low salt/sucrose buffer (250 mM sucrose, 100 mM KAc, 5 mM Mg(OAc)2, and 50 mM HEPES-KOH, pH 7.9), a sample taken for trichloroacetic acid precipitation, then digested with proteinase K (Promega, Southampton, Hants.) toethanol or 5 mM 2-mercaptoethanol alone (BMP and BMH). A second sample was removed for immunoprecipitation. In order to establish whether PCR-induced mutations had any effect; mRNAs derived from independent PCR reactions were translated and the cross-linking products were compared. No differences between independent experiments could be detected.

Denaturing immunoprecipitations were performed by heating the samples for 5 min at 95 °C in the presence of 1% SDS. Native immunoprecipitations were performed by releasing the ribosome from the nascent chain by incubation with 1 mM puromycin and 400 mM KCl for 10 min at 30 °C. Four volumes of immunoprecipitation buffer (10 mM Tris/HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100) were then added to all samples and aliquots were incubated overnight at 4 °C with the relevant antisera in the presence of 0.2 mg/ml phenylmethylsulfonyl fluoride and 1 mM methionine. Protein A-Sepharose was added for 2 h and samples processed as described previously (see Ref. 28). All samples were analyzed on 12% SDS-polyacrylamide gels and exposed overnight to a PhosphorImaging plate for visualization on a Fuji BAS-2000 PhosphorImaging system. Concanavalin A binding was performed as described by Krieg et al. (15).

RESULTS

Topology of the Nascent Opsi Translocation Intermediate—In this study opsin “translocation intermediates” were used to investigate the insertion of a model multiple-spanning protein into the ER membrane using cross-linking techniques. A translocation intermediate is a truncated nascent chain which, due to the lack of a stop codon in the truncated mRNA, is not released from the ribosome and becomes trapped in the translocation machinery (24).

Prior to cross-linking analysis, the topology of the 155-amino acid long opsin truncation (OP155cko) was established using a protease protection assay (Fig. 1a). Nascent chains protected against protease digestion were detected by immunoprecipitation with an antibody specific for the N terminus of opsin. Proteinase K digestion of OP155 after puromycin release resulted in a large protected fragment which migrated slightly faster than the undigested form (OP155–2CHO). A fraction of the proteinase K-treated material had a smaller molecular weight after Endo Hf digestion, this large protected band shifted to migrate with a mobility similar to the nonglycosylated nascent chain (OP155) (Fig. 1a, lane 3, asterisk). A fraction of the proteinase K-treated material had a smaller molecular weight after Endo Hf digestion, this large protected band shifted to migrate with a mobility similar to the nonglycosylated nascent chain (OP155) (Fig. 1a, lane 3, asterisk). A fraction of the proteinase K-treated material had a smaller molecular weight after Endo Hf digestion, this large protected band shifted to migrate with a mobility similar to the nonglycosylated nascent chain (OP155) (Fig. 1a, lane 3, asterisk). A fraction of the proteinase K-treated material had a smaller molecular weight after Endo Hf digestion, this large protected band shifted to migrate with a mobility similar to the nonglycosylated nascent chain (OP155) (Fig. 1a, lane 3, asterisk).
Sec61a. No cross-linking of Sec61β alone was visible (cf. Fig. 3), however, a high molecular weight product was specifically immunoprecipitated with anti-Sec61β serum under denaturing conditions (Fig. 2a, lane 22, unfilled arrow). This product must therefore include the nascent chain, Sec61β and at least one unidentified protein. A high molecular weight product was also immunoprecipitated with antisera recognizing TRAM (Fig. 2a, lane 23, broad band), again suggesting multiple cross-linking.

Bifunctional reagents similar to MBS but with shorter (N-hydroxysuccinimidyld iodoacetate) and longer (succinimidyl 4-(p-maleimidophenyl) butyrate) spacer arms were also used. Both gave very similar results to MBS showing that the length of the spacer arm did not affect the cross-linking partners detected (data not shown). However, when S-MBS, a water soluble analogue of MBS was used, fewer cross-linking products were visible (Fig. 2b). SRP54 was still a cross-linking partner of all three nascent chains (Fig. 2b, lanes 4, 12, and 20, open arrows), but with OP155cko, only Sec61a was detected (Fig. 2b, lanes 18 and 21, closed arrow). None of the higher molecular weight species immunoprecipitated with antisera against Sec61β and TRAM were visible (Fig. 2b, lanes 22 and 23) suggesting that penetration of the bilayer by the cross-linking reagent was necessary to obtain these products, but not for cross-linking to Sec61a.

Our principal conclusion from this initial analysis is that Sec61a is only seen as a major cross-linking partner when the opsin nascent chain is 155 amino acids in length. This result was unaffected by either the length of the spacer arm, or the solubility of the reagent used. The simplest interpretation of these results is that cross-linking of nascent opsin to ER membrane components occurs primarily from cysteine residue 110 of the nascent chain, and that this residue is only close to Sec61a when it has entered the plane of the membrane (cf. Fig. 1c).

Membrane Integration of Nascent Opsin—To further investigate ER proteins adjacent to cysteine residue 110, two cysteine specific cross-linking reagents with different length spacer arms, bismaleimidopropane (BMP, 11 Å) and bismaleimidohexane (BMH, 16.1 Å), were used. All known components of the ER translocation site have one or more cysteine residues (5, 15, 17), therefore the use of cysteine-specific homobifunctional reagents does not in principle restrict the cross-linking partner.

When opsin translocation intermediates were isolated and incubated with the cross-linking reagent BMP, specific cross-linking products were observed dependent upon the length of the translocation intermediate (Fig. 3). OP106 contained no cysteine residue, and no cross-linking products were observed (Fig. 3, lanes 1–8). OP127, OP132, and OP137 all formed cross-linking products of apparent molecular mass ~42 kDa. Sub-
trating the contribution of the nascent chain, this indicated each opsin translocation intermediate was cross-linked to a protein of ~21 kDa (Fig. 3, lanes 10, 18, and 26, asterisk) which was not a known component of the ER targeting or translocation machinery. In contrast, OP137, OP150cko, and OP155cko all showed discrete cross-linking products with Sec61β (Fig. 3, lanes 30, 38, and 46, diamond). The first obvious evidence of Sec61α cross-linking products was only obtained when OP155cko was used (Fig. 3, lane 45, closed arrow). The Sec61α cross-linking product was a broad doublet similar to that seen with MBS and S-MBS. In addition to the nascent chain-Sec61β adduct, a high molecular weight product identical to that observed with MBS was also seen (Fig. 3, lane 46, unfilled arrow, cf. Fig. 2a, lane 22). Analysis using BMH gave similar results, again showing that the cross-linking partners were not limited by the spacer arm length of the cross-linking reagent used (data not shown).

A number of opsin translocation intermediates are cross-linked to SRP54 (Figs. 2, a and b, and 3). Using a flotation assay (30) to separate membrane targeted nascent chains from non-targeted chains, we analyzed the OP155-SRP54 adducts and found these products fractionated with non-targeted nascent chains. In contrast, the Sec61α and Sec61β cross-linking products were present in the membrane fraction (data not shown). We conclude that the SRP54 adducts arise from ribosome-bound opsin chains which are pelleted with the membrane fraction prior to cross-linking, but which have not interacted productively with the ER microsomes used (see also results of protease protection above). Such incomplete release of SRP54 by canine pancreatic microsomes has been previously reported (10).

The reticulocyte lysate translation system has been reported to generate cross-linker-independent adducts under some circumstances (31). When control immunoprecipitations were performed without adding cross-linking reagents, a weakly labeled OP155-SRP54 adduct was observed (data not shown). No such products were immunoprecipitated with any of the other antisera used in this study confirming that the 21-kDa protein, Sec61β and Sec61α are bona fide cross-linking partners.

The 21-kDa Cross-linking Partner of Short Opsi n Translocation Intermediates Is a Ribosomal Protein—Cross-linking of OP127, OP132, and OP137 with BMP resulted in a single major cross-linking partner of 21 kDa (Fig. 3, lanes 10, 18, and 26, asterisk) which was not a known component of the ER insertion machinery (see Fig. 3, lanes 11–16, 19–24, and 27–32) but was immunoprecipitated with a monoclonal antibody specific for opsin (data not shown). Cysteine residue 110 of OP127 is predicted to be deeply buried in the ribosome (Fig. 1c) and if the first transmembrane domain of OP127 is correctly inserted into the membrane the nascent chain should be glycosylated and hence any cross-linking products sensitive to Endo H digestion (Fig. 4). Endo H digestion does indeed increase the mobility of both the OP127 cross-linking product (Fig. 4, lane 4, asterisk) and the diglycosylated nascent chain (indicated with a dot). Thus, a correctly inserted and glycosylated OP127 translocation intermediate is cross-linked to a 21-kDa protein. The 21-kDa component was still a cross-linking partner in the absence of membranes (Fig. 4, lane 6), indicating that the 21-kDa protein is of ribosomal origin. The cross-linking product was not immunoprecipitated by antibodies recognizing the β subunit of the nascent polypeptide-associated complex, a previously identified 21-kDa ribosome-associated protein (30, 32).

The Sec61β Cross-linking Product Is from a Stably Inserted Opsi n Translocation Intermediate—Cross-linking of OP137 with homobifunctional cysteine-specific reagents resulted in a discrete Sec61β-OP137 cross-linking product (Fig. 3, lane 30, and data not shown). This product could be immunoprecipitated with antisera against both opsin (Fig. 5, lane 2, diamond) and Sec61β (Fig. 5, lane 3). The cross-linking product bound to concanavalin A-Sepharose (Fig. 5, lane 4) and was specifically eluted by 0.5 M α-methyl-β-mannoside (data not shown) indi-

**Fig. 2.** a, cross-linking of different length nascent opsin chains to ER components using MBS. Opsin translocation intermediates were incubated in the presence of 1 mM MBS (+) or mock treated with dimethyl sulfoxide (−). After quenching with 100 mM glycine and 5 mM 2-mercaptoethanol, total products were trichloroacetic acid precipitated (lanes 1, 2, 9, 10, 17, and 18). The rest of the samples were solubilized in immunoprecipitation buffer and incubated overnight with antisera against known components of the ER targeting and translocation machinery. Cross-linking products to SRP54 (open arrow), to Sec61α (closed arrow), and a high molecular weight complex incorporating Sec61β (unfilled arrow) are indicated. A dot denotes the diglycosylated opsin nascent chain. b, cross-linking with S-MBS. Translocation intermediates were incubated with 0.12 mM S-MBS (27) or mock treated with water (−). After quenching, total products were trichloroacetic acid precipitated (lanes 1, 2, 9, 10, 17, and 18) or immunoprecipitated as above.
cating the presence of a carbohydrate moiety. Since canine Sec61b does not contain any potential N-linked glycosylation sites (15), the carbohydrate present on the cross-linking product is from the opsin nascent chain. Sec61b is therefore cross-linked to a correctly inserted OP137 translocation intermediate. The OP137-Sec61b cross-linking product was also found to be sensitive to Endo Hf digestion (data not shown).

**DISCUSSION**

The truncated opsin nascent chains used in this study were shown to insert into canine pancreatic microsomes and form bona fide translocation intermediates. Once the nascent chain was trapped in the translocation machinery it could be cross-linked to adjacent proteins.

Using heterobifunctional reagents, and nascent polypeptides ranging from 106 to 155 amino acid residues in length, SRP54 adducts were observed with all chain lengths. These products represent SRP-bound nascent chains which have failed to target to the membrane. In striking contrast, strong cross-linking of membrane translocation intermediates to Sec61a was only observed with OP155cko, the longest of the nascent chains studied. This result was independent of the spacer arm length and solubility of the heterobifunctional reagents used suggesting that the majority of cross-linking products obtained are from the -SH group of cysteine residue 110 in opsin to -NH2 groups in the adjacent ER protein (see Fig. 1c). We conclude that cysteine 110 is only sufficiently close to Sec61a for cross-linking when it is 45 amino acid residues from the peptidyl transferase site of the ribosome, i.e. within the plane of the membrane. This is consistent with Sec61a being the major protein constituent of the ER insertion and translocation site (4–7, 10, 12–14, 16).

A comparison of membrane permeable and membrane impermeable (water soluble) cross-linking reagents revealed a clear difference. Membrane permeable reagents generated discrete high molecular weight products specifically immunoprecipitated with antisera against Sec61β and TRAM. These “multiple” cross-linking products must represent OP155cko-Sec61β and OP155cko-TRAM products that are in turn cross-linked to other components that remain to be identified (see below). Such products were not seen when S-MBS was used although cross-linking to Sec61α was maintained. Hence, penetration of the bilayer is necessary to obtain the high molecular weight Sec61β and TRAM products. Thus, analysis with heterobifunctional reagents fully supports the view that Sec61α is the major protein component of an aqueous channel (7, 33, 34) through which the nascent opsin chain is inserted into the ER membrane. Sec61β and TRAM are adjacent to both the nascent

![Fig. 3. Cross-linking with the cysteine specific reagent BMP.](image)

OPSIN TRANSLATION INTERMEDIATES WERE INCUBATED WITH 1 mM BMP (+) OR MOCK TREATED WITH DIMETHYL SULFOXIDE (−). AFTER QUENCHING WITH 5 mM 2-MERCAPTOETHANOL, TOTAL PRODUCTS WERE TRICHLOROACETIC ACID PRECIPITATED (LANES 1, 2, 9, 10, 17, 18, 25, 26, 33, 34, 41, AND 42) OR IMMUNOPRECIPITATED AS INDICATED. AN ASTERISK INDICATES A NOVEL 21-KDA CROSS-LINKING PARTNER. FOR OTHER SYMBOLS, SEE LEGEND TO FIG. 2A.

![Fig. 4. Characterization of the 21-kDa cross-linking partner of OP127.](image)

OP127 WAS SYNTHESIZED IN THE PRESENCE (LANES 1–4) OR ABSENCE (LANES 5 AND 6) OF MICROSMAL MEMBRANES. AFTER CROSS-LINKING WITH BMH, SAMPLES WERE ANALYZED DIRECTLY AFTER TRICHLOROACETIC ACID PRECIPITATION (LANES 1 AND 2) OR AFTER ENDO HF TREATMENT (LANES 3 AND 4). THE MATERIAL LOADED IN LANES 3 AND 4 IS EQUIVALENT TO THREE TIMES THAT LOADED IN LANES 1 AND 2. THE MF157 TRANSLATED WITHOUT MEMBRANES WAS INCUBATED IN THE ABSENCE (LANE 5) OR PRESENCE (LANE 6) OF 1 mM BMH FOR 10 MIN. AFTER QUENCHING THE RIBOSOME/NASCENT CHAIN COMPLEXES WERE ISOLATED AND ANALYZED DIRECTLY. OP DENOTES THE UNGLYCOXYLATED OPSEN CHAIN. FOR OTHER SYMBOLS SEE PREVIOUS FIGURE LEGENDS.
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The Sec61β cross-linking product is from stably inserted nascent chains. OP137 translocation intermediates were cross-linked to Sec61β using BMH and products immunoprecipitated with antisera specific for opsin (lanes 1 and 2) or Sec61β (lanes 3 and 4). Samples in lanes 1–3 were then analyzed directly. The products immunoprecipitated in lane 4 were eluted from the protein A-Sepharose and further analyzed for concanavalin A-Sepharose binding prior to electrophoresis. For symbols, see previous figure legends.

When the chain length of the translocation intermediate was increased to 137 amino acids, cross-linking to Sec61β was observed. Sec61β is part of the Sec61 complex composed of Sec61α, Sec61β, and Sec61γ (14) and is probably a tail-anchored membrane protein with a single cysteine residue present in the cytoplasmic N terminus (15). This is the first direct evidence that a membrane inserting nascent chain is adjacent to Sec61β. Cross-linking to Sec61β was shown to be from glycosylated, membrane inserted, translocation intermediates. Since the single cysteine residue present in OP137 is probably still within the ribosome (Fig. 1c) this suggests that the cytoplasmic domain of Sec61β extends into the channel of the large ribosomal subunit.

After initial cross-linking of the nascent chain to Sec61β alone, increasing the nascent chain length to 155 residues (OP155cko) allows cross-linking to both Sec61α and Sec61β. Sec61α is proposed to be the major protein component of the ER translocation site (5–7, 10, 12, 13). Thus, these data support the view that the integration of multiple-spanning membrane proteins occurs at a general ER translocation site, very similar to that which promotes the insertion of single-spanning membrane proteins and the translocation of secretory proteins (4, 35).

In addition to adducts between OP155cko and Sec61β alone, more complex high molecular weight products were observed with all cross-linking reagents used. These cross-linking products are not immunoprecipitated with antisera specific for Sec61α and therefore contain additional unidentified protein(s) which may be ribosomal (see above).

These results are consistent with the ribosome and the ER translocation site forming a continuous channel (33) where some ER protein components extend into the ribosomal channel (7, 36). Sec61α appears to be the major constituent of this membrane channel, our data suggest that Sec61β may also contribute to the environment. Alternatively, Sec61β may play a distinct role such as regulating the lateral exit of transmembrane domains from the ER translocation site into the lipid bilayer (4). Recent evidence has shown this to be a complex process which may involve TRAM (8).

The approach used in this study only enables the detection of proteins adjacent to the inserting opsin nascent chains. Recent photocross-linking analysis suggests that the translocation site is partly composed of phospholipid, perhaps promoting the lateral exit of hydrophobic transmembrane domains into the bilayer (20). Our results suggest that both Sec61α and Sec61β are significant components of the translocation channel, however, they do not preclude the possibility that lipid also makes a significant contribution to the site of opsin integration at the ER membrane. We are presently attempting to address this question.

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