Membrane integration of Sec61α: a core component of the endoplasmic reticulum translocation complex

Bruce C. KNIGHT and Stephen HIGH[1]

School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester, M13 9PT, U.K.

The Sec61 complex is a central component of the endoplasmic reticulum (ER) translocation site. The complex consists of three subunits: Sec61α, Sec61β and Sec61γ, at least two of which (α and β) are adjacent to nascent proteins during membrane insertion. Another component of the translocation machinery is the translocating chain-associating membrane (TRAM) protein, which is also adjacent to many nascent proteins during membrane insertion. Sec61α functions as the major component of a transmembrane channel formed by oligomers of the Sec61 complex. This channel is the site of secretory protein translocation and membrane protein integration at the ER membrane. Sec61α is a polytopic integral membrane protein, and we have studied its biosynthesis and membrane integration *in vitro*. Using a cross-linking approach to analyse the environment of a series of discrete Sec61α membrane-integration intermediates, we find: (i) newly synthesized Sec61α is adjacent to known components of the ER membrane-insertion site, namely Sec61α, Sec61β and TRAM, and thus the integration of Sec61α appears to require a pre-existing Sec61 complex; (ii) a site-specific cross-linking analysis indicates that the first transmembrane domain of Sec61α remains adjacent to protein components of the ER-insertion site (specifically TRAM and Sec61β) during the insertion of at least three subsequent transmembrane domains; and (iii) the membrane integration of Sec61α requires ER targeting by the signal-recognition particle.

INTRODUCTION

Membrane proteins are generally integrated into the endoplasmic reticulum (ER) by the same machinery that translocates secretory proteins across it [1,2]. The first stage of this process usually requires the targeting of the protein to the ER by the signal-recognition particle (SRP). SRP acts to present the nascent chain to the ER translocation/insertion machinery [3,4]. The Sec61 complex is a heterotrimer composed of Sec61α, Sec61β and Sec61γ [5,6]. The trimeric Sec61 complex and the dimeric SRP receptor are essential for both secretory protein translocation and membrane protein integration [5,7]. In many cases, the translocating chain-associating membrane (TRAM) protein is either essential for, or stimulatory to, translocation and integration [5,7,8]. The Sec61α subunit is believed to form the major part of a transmembrane channel, through which nascent polypeptides can be translocated (see [4] for recent review). Electron microscopy studies have shown that purified detergent-solubilized mammalian Sec61 complexes form cylindrical oligomers with a diameter of approx. 8.5 nm and a central pore of 2.0 nm [9]. It has been proposed that these structures, which probably contain 3–4 Sec61 heterotrimers, represent protein-conducting channels that span the ER membrane [9]. The internal diameter of an active ER-translocation site containing translocating chains is estimated to be 4–6 nm, making it the largest pore observed in any membrane that must maintain a permeability barrier [10].

A number of cross-linking studies have been used to identify the ER proteins responsible for the translocation of secretory proteins and the insertion of single-spanning membrane proteins [11–13]. The integration of polytopic proteins is less well-characterized, although two recent studies [14,15] indicate that these proteins use the same ER translocation machinery as secretory and single-spanning membrane proteins. The crucial difference between secretory-protein translocation and membrane-protein integration is the lateral exit of the membrane-protein transmembrane domain and its stable insertion into the lipid bilayer. A number of models have been proposed describing this insertion process (see [4] for recent review). One suggests that polytopic membrane proteins remain in the ER translocon until the completion of protein synthesis and the termination of translation [16]. A single-spanning membrane protein also remained associated with the translocation site throughout its synthesis, although its interaction with the protein components of the translocon altered significantly during this process [17]. An alternative model suggests that transmembrane domains can exit laterally from the translocon into the lipid bilayer during biosynthesis, and that the completion of protein synthesis is not essential for this process [15,18]. Hence the detailed mechanism of membrane-protein integration, and in particular the insertion of polytopic proteins, remains open to question. Mammalian Sec61α, the central component of the ER-translocation channel, is itself a multiple-spanning integral membrane protein [11] with a predicted ten transmembrane domains, as established for the *Saccharomyces cerevisiae* homologue Sec61p [19]. In this study, we have examined the membrane insertion of Sec61α using truncated mRNAs to generate 'integration intermediates', which remain trapped within the ER-insertion site as ribosome-bound peptidyl tRNAs [20]. Hetero- and homo-bifunctional cross-linking reagents were then used to identify cross-linking partners of these various nascent Sec61α chains. On the basis of this study, we conclude that: (i) Sec61α integration requires a pre-existing Sec61 complex; (ii) the first transmembrane domain of Sec61α remains adjacent to ER-insertion-site components during the integration of at least three subsequent transmembrane regions (TMs); and (iii) the targeting of nascent Sec61α to the ER is SRP-dependent.

Abbreviations used: BMH, bis-maleimidohexane; ER, endoplasmic reticulum; NTCKO, N-terminal cysteine knockout; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SRP, signal recognition particle; TM, transmembrane region; TRAM, translocating chain-associating membrane.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Restriction enzymes were obtained from New England Biolabs (Hitchin, U.K.), and *Pfu* DNA polymerase was from Stratagene (Cambridge, U.K.). RNAsin was from Pharmacia (St. Albans, U.K.), [^{35}S]methionine was from NEN–Dupont (Stevenage, U.K.), and cycloheximide and 7-methylguanosine 5′-monophosphate were supplied by Sigma (Poole, U.K.). All other chemicals were supplied from BDH/Merck (Lutterworth, U.K.) and Sigma (Poole, U.K.). Wheat-germ lysate, SRP and canine pancreatic rough microsomes were prepared as previously described [21]. All cross-linking reagents were obtained from Pierce and Warriner (Chester, U.K.) and Apollo Scientific (Stockport, U.K.). The Sec61α cDNA was a gift from T. A. Rapoport (Harvard Medical School, Boston, MA, U.S.A.). Antibodies for SRP54 and TRAM were a gift from B. Dobberstein (ZMBH, Heidelberg, Germany). The Sec61α and Sec61β antisera were a gift from R. Zimmermann (University of Saarland, Hamburg, Germany). The Sec61γ antisera was raised by Research Genetics Inc. (Huntsville, AL, U.S.A.) using a 12-amino-acid peptide at the N-terminus from the published sequence.

Transcription and translation

An *Nco*I/*Sal* I fragment from the coding region of Sec61α cDNA [11] was cloned into the pSPUTK vector (Stratagene, Cambridge, U.K.) using the *Nco*I site of the Kozak optimal initiation region. DNA templates for the transcription of different truncated Sec61α mRNAs [wild-type and N-terminal cysteine knockout (NTCKO)] were prepared by PCR [14,22] using high-fidelity *Pfu* DNA polymerase. Primers were designed to generate N-terminal Sec61α-derived polypeptides of 96 and 136 amino acids upon translation. The upstream primer was 50 bases from the 5′ end of the SP6 RNA polymerase promoter and had the sequence 5′-TTA GAA CGC GGC TAC AAT TA-3′, antisense primers were designed to make truncations of 96 and 136 amino acid residues. For the 96-amino-acid truncation, the antisense primer 5′-CAG CCA AAA GCT GCA TGA TG -3′ was used, and for the 136-amino-acid truncation, the antisense primer 5′-CAT TCC CGT CAT CAC ATA TA -3′ was used. The PCR products were purified directly from the reaction mixture using the Wizard PCR purification kit (Promega, Southampton, U.K.). The template for 207-amino-acid-truncated and full-length versions of Sec61α were made by cleavage of the Sec61α pSPUTK plasmid using the restriction endonucleases *Eco*RI and *Sal*I respectively.

The N-terminal cysteine (Cys^{13}) was changed to a serine (Ser^{13}) by site-directed mutagenesis to create an NTCKO mutant of Sec61α using the Stratagene Quick-Change Site-Directed Mutagenesis kit (Stratagene, Cambridge, U.K.). The following primers were used to make the change: sense 5′-GTT ATC AAG CCT TTC AGT GTC ATC CTG CCA G-3′ and antisense 5′-CTG GCA GGA TGA CAC TGA AAG GCT TGA TAA C-3′.

DNA templates were transcribed using SP6 RNA polymerase, as described by the manufacturer (Promega). The resulting RNA was translated in a wheat-germ translation system at 26 °C [23], supplemented with [^{35}S]methionine and canine pancreatic microsomes, to generate integration intermediates [14]. Translation initiation was inhibited after 10 min by the addition of 4 mM 7-methylguanosine 5′-monophosphate, and chain elongation was allowed to continue for a further 10 min, until translation was inhibited by the addition of 2 mM cycloheximide.

Cross-linking of membrane-integration intermediates

After translation in the presence of canine pancreatic microsomes, the samples were layered over 750 μl high salt/sucrose cushion [250 mM sucrose/500 mM potassium acetate/5 mM magnesium acetate/50 mM Hepes/KOH (pH 7.9)] in Beckman TLA 100.2 tubes. The samples were spun for 10 min at 100000 ***g*** and 4 °C and the resulting pellet (membrane-associated intermediates) was resuspended in 200 μl low salt/sucrose buffer [250 mM sucrose/100 mM potassium acetate/5 mM magnesium acetate/50 mM Hepes/KOH (pH 7.9)]. Cross-linking was performed with either bis-maleimidohexane (BMH) or succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) dissolved in DMSO. BMH is a homobifunctional reagent specific for the free thiols of cysteine residues. SMCC is a heterobifunctional reagent, which reacts with suitable amino groups (primarily the side chains of lysine residues) and with free thiol groups. Both BMH and SMCC are membrane-permeable.

Membrane-associated integration intermediates were incubated at 26 °C for 10 min with either 1 mM BMH, 1 mM SMCC or DMSO (control) and quenched with 100 mM glycine, 5 mM 2-mercaptoethanol (SMCC-treated samples) or with 5 mM 2-mercaptoethanol alone (BMH-treated samples). A fraction of the sample (one-tenth) was removed for extraction with 100 mM Na_2CO_3 (to enrich for integral-membrane proteins) [24]. Before immunoprecipitation, samples were heated to 95 °C for 5 min in the presence of 1 % (w/v) SDS, diluted with four vol. of immunoprecipitation buffer [10 mM Tris/HCl (pH 7.6)/140 mM NaCl/1 mM EDTA/1 % (v/v) Triton X-100], and the relevant antisera (anti-SRP54, anti-Sec61α C-terminus, anti-Sec61β N-terminus, anti-Sec61γ N-terminus and anti-TRAM C-terminus) added. Following overnight mixing at 4 °C and the addition of Protein A–Sepharose for 2 h, the samples were washed with immunoprecipitation buffer, and the resulting beads heated to 95 °C for 5 min in sample buffer [50 mM Tris-HCl (pH 6.8)/50 mM dithiothreitol/4 % (w/v) SDS/0.1 % (w/v) Bromophenol Blue and 10 % sucrose]. Samples were analysed using SDS/PAGE (12 % gels), and exposed overnight to an imaging plate for visualization on a Fujix BAS-2000 Bioimaging system.

SRP-dependence assay

Full-length Sec61α mRNA was translated for 1 h in wheat-germ lysate [23] supplemented with sheep pancreatic microsomes [25] in the presence or absence of approx. 20 nM canine SRP. After translation with sheep pancreatic microsomes, samples were layered over 750-μl-high salt/cushion in Beckman TLA 100.2 tubes. These were spun for 10 min at 100000 ***g*** and 4 °C. The resulting pellet (membrane-associated intermediates) was resuspended in 100 μl low salt/sucrose buffer. The sample was then split into three aliquots. These were incubated at 26 °C for 30 min, either alone or with 0.5 mg/ml trypsin in the presence or absence of 0.1 % (v/v) Triton X-100. All samples were then heated immediately to 95 °C for 5 min in sample buffer. Samples were analysed using SDS/PAGE (12 % gels), and exposed overnight to an imaging plate for visualization on a Fujix BAS-2000 Bioimaging system.

RESULTS

Discrete cross-linking products are observed with wild-type Sec61α integration intermediates

In order to analyse the molecular environment of Sec61α during membrane insertion, truncated mRNAs were used to prime *in vitro* synthesis. In the absence of a stop codon, the resulting truncated proteins remain attached to the ribosome as peptidyl tRNA species and, in the presence of canine pancreatic micro-

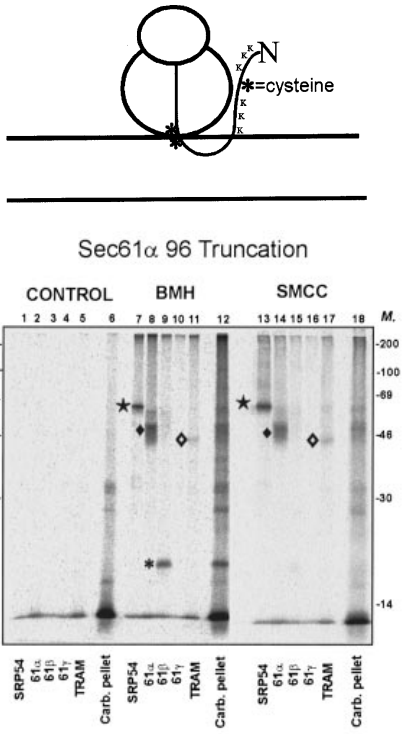

Figure 1 Immunoprecipitation of Sec61α-96 cross-linking products obtained with DMSO (control), BMH or SMCC using antisera recognizing SRP54, Sec61α, Sec61β, Sec61γ or TRAM, as indicated

Adducts with SRP54 (star in lanes 7 and 13), Sec61α (◆ in lanes 8 and 14), Sec61β (* in lane 8) and TRAM (◇ in lanes 11 and 17) are shown. Products found in the membrane pellet remaining after extraction with alkaline sodium carbonate buffer (carb. pellet; lanes 6, 12 and 18) are also included. A diagram of the integration intermediate is shown above the SDS/polyacrylamide gel. Cysteine residues at position 13, 45 and 46 are indicated by an asterisk, and lysine residues (positions 4,10,21,25,29 and 31) are also shown (K).

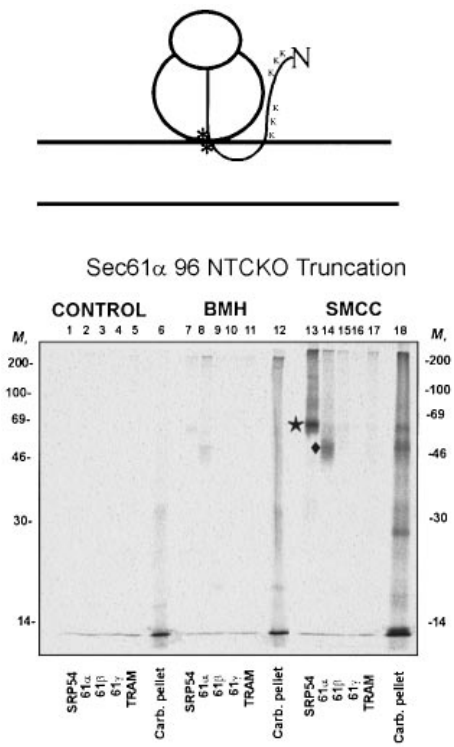

Figure 2 Immunoprecipitation of Sec61α-96 NTCKO cross-linking products obtained with DMSO (control), BMH or SMCC

Adducts with SRP54 (star in lane 13) and Sec61α (◆ in lane 14) are indicated. Products found in the membrane pellet remaining after extraction with alkaline sodium carbonate buffer (carb. pellet; lanes 6, 12 and 18) are also shown. A diagram of the integration intermediate is labelled as in Figure 1.

somes, form integration intermediates that are trapped at particular stages of membrane insertion [20].

Since Sec61α is a core component of the ER-translocation site, it was possible that it used a novel membrane-integration mechanism that avoided any requirement for a pre-existing Sec61 complex. Therefore, in order to analyse the membrane insertion of the Sec61α integration intermediates, bifunctional cross-linking reagents were added. This allowed the covalent cross-linking of the radiolabelled nascent Sec61α chains to adjacent ER proteins, thereby enabling their identification. When a 96-amino-acid N-terminal fragment of Sec61α was used to generate a membrane integration intermediate, strong cross-linking products with known components of the ER-insertion site were observed (Figure 1, lanes 12 and 18). When the bifunctional maleimide reagent BMH was used, cross-linking could only occur from the three cysteine residues present in Sec61α 96 to available cysteine residues present in adjacent ER proteins. When these cross-linking products were analysed by immunoprecipitation [14], adducts with Sec61α, Sec61β and TRAM could be identified (Figure 1, lanes 8, 9 and 11), although no adducts were seen with Sec61γ. When a heterobifunctional reagent, SMCC, was used, cross-linking between cysteines and lysines could now occur. Since Sec61α 96 contains six lysine residues in addition to the three cysteine residues, cross-linking with SMCC increased the opportunities for adduct formation dramatically. Nevertheless, SMCC-dependent cross-linking of Sec61α 96 also identified Sec61α and TRAM as the major components present in the adducts formed with this integration intermediate (Figure 1, lanes 14 and 17). No cross-linking to either Sec61β or Sec61γ was detected with SMCC.

Importantly, control experiments confirmed that in the absence of any added cross-linking reagents, no artifactual cross-linking products were observed in the wheat-germ translation system that was used (Figure 1, lanes 1–6). In a previous study, we observed that such non-specific adducts can occur when a reticulocyte lysate translation system is used [26]. The BMH- and SMCC-dependent cross-linking products were dependent upon the presence of a ribosome-bound integration intermediate, because when Sec61α 96 integration intermediates were treated with puromycin before cross-linking, the adducts were no longer observed (results not shown).

In addition to the ER-integration site components, both BMH and SMCC revealed strong cross-linking to the 54 kDa subunit of SRP. These products are most likely owing to the incomplete release of SRP-bound nascent chains [14], and suggested immediately that the targeting of nascent Sec61α to the ER membrane was SRP-dependent. This possibility was subsequently investigated in detail (see Figure 7).

Cysteine deletion at position 13 reveals distinct regions of Sec61α 96 cross-link-specific components

Previous studies have clearly illustrated that the location of a probe within the nascent chain can influence the ability to cross-

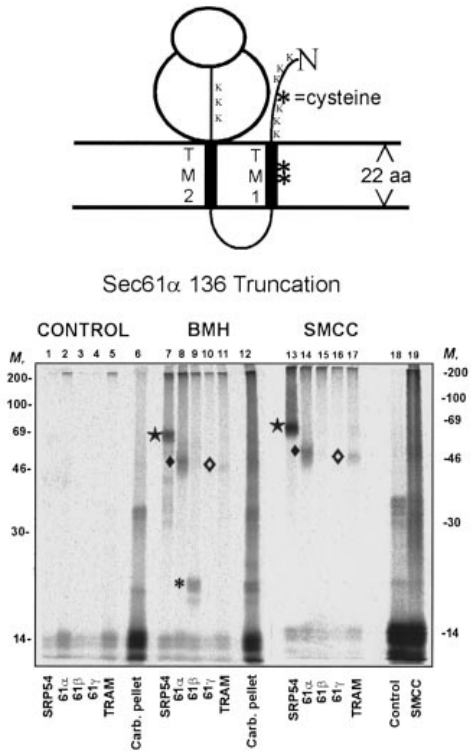

Figure 3 Immunoprecipitation of Sec61α-136 cross-linking products obtained with DMSO (control), BMH or SMCC

Products are labelled as described in the legend to Figure 1. A diagram of the integration intermediate is included with transmembrane domains (TM), and lysine residues (positions 4, 10,21,25,29,31,98,107 and 117) are indicated (K).

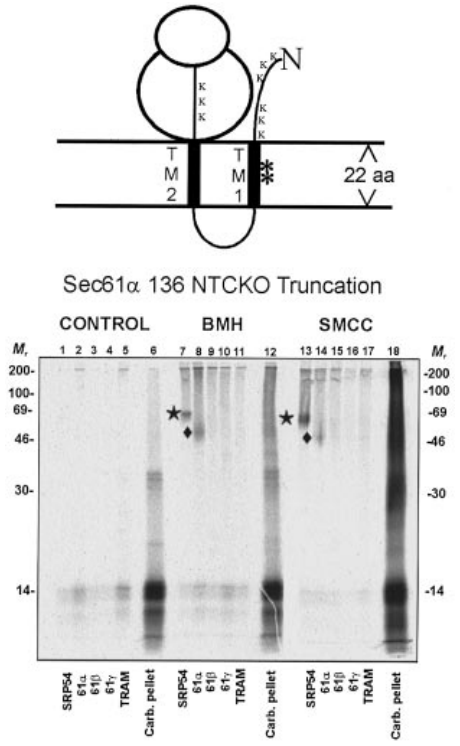

Figure 4 Immunoprecipitation of Sec61α-136 NTCKO cross-linking products obtained with DMSO (control), BMH or SMCC

Adducts with SRP54 (star in lanes 7 and 13) and Sec61α (◆ in lanes 8 and 14) are indicated. Products found in the membrane pellet remaining after extraction with alkaline sodium carbonate buffer (carb. pellet; lanes 6, 12 and 18) are also shown. The diagram of the integration intermediate is labelled as in Figure 3.

link to neighbouring proteins [13,27]. In Sec61α 96, BMH-dependent cross-linking can occur from two distinct regions within the integration intermediate, i.e. from the lone cysteine residue at position 13 and the two cysteine residues at positions 45 and 46. We examined the BMH-dependent cross-linking pattern of a Sec61α 96 translocation intermediate, where cysteine residue 13 had been changed to a serine (denoted by Sec61α 96 NTCKO). The effect of this change was dramatic, because the BMH-dependent adducts were now almost completely absent (Figure 2, lanes 7–9 and 11). By using SMCC, which allowed cross-linking to occur from additional sites within the nascent chain, strong adducts between Sec61α 96 NTCKO and both endogenous Sec61α and SRP54 were now observed (Figure 2, lanes 13 and 14). We therefore conclude that with the short integration intermediate (Sec61α 96), nearly all of the cysteine-dependent cross-linking occurs from residue 13 present in the cytosolic N-terminal domain. Cysteine residues 45 and 46 are predicted to be near the ribosome/membrane junction, and appear to be incapable of significant cross-linking to the proteins of the ER-insertion site.

The synthesis of subsequent transmembrane domains does not alter the adjacent neighbours of the N-terminal cytosolic domain of Sec61α

A key question with regard to the membrane integration of polytopic proteins is the fate of the N-terminal region of the polypeptide, whilst the remainder of the protein is being synthesized. In particular, does the first transmembrane domain laterally exit the ER-insertion site upon insertion of the second transmembrane domain, or is lateral-exit dependent upon the completion of protein synthesis [4]?

In order to investigate the environment of the N-terminal region of Sec61α at different stages of its biosynthesis, we investigated the cross-linking partners of longer Sec61α translocation intermediates with increasing numbers of transmembrane domains. By comparing the wild-type and NTCKO forms of the nascent Sec61α 96 chains, we were able to establish the cross-linking partners of Cys^{13} specifically. When the chain length of the integration intermediate is increased from 96 to 136 amino acids, the first transmembrane domain is predicted to be fully inserted, with the second transmembrane domain having already entered the plane of the membrane. The cross-linking partners of wild-type Sec61α 136 were essentially identical to those seen with Sec61α 96. BMH-dependent cross-linking to Sec61α, Sec61β and TRAM (Figure 3, lanes 8, 9 and 11) and SMCC-dependent cross-linking to Sec61α and TRAM (Figure 3, lanes 14 and 17) was observed. Again, no cross-linking to Sec61γ was evident (Figure 3, lanes 10 and 16).

In contrast to Sec61α 96 NTCKO (see Figure 2), when Sec61α 136 NTCKO was analysed for BMH-dependent cross-linking, clear adducts with endogenous Sec61α were now observed (Figure 4, lane 8). Presumably, by increasing the chain length of the integration intermediate, cysteine residues 45 and 46 have now completely exited the ribosome, and entered the plane of the membrane, enabling cross-linking to adjacent components (cf. [14]). Cross-linking with SMCC also gave adducts between

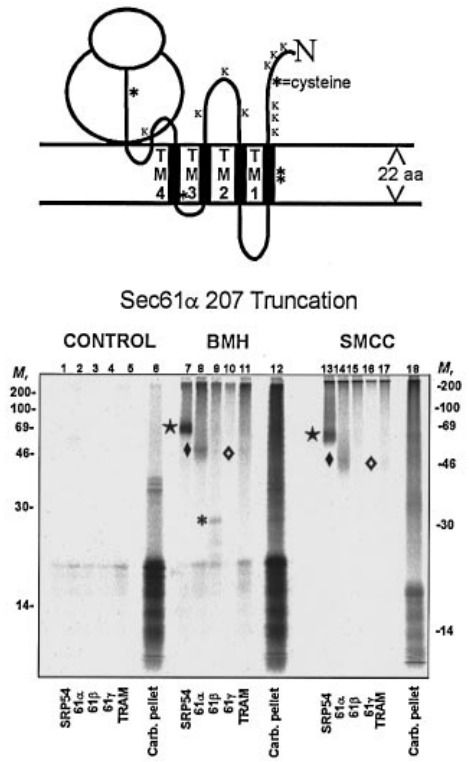

Figure 5 Immunoprecipitation of Sec61α-207 cross-linking products obtained with DMSO (control), BMH or SMCC

Products are labelled as described in the legend to Figure 1. The diagram of the integration intermediate is labelled as described in Figure 3, except that additional cysteine residues (positions 148 and 188) and an additional lysine (position 171) are included.

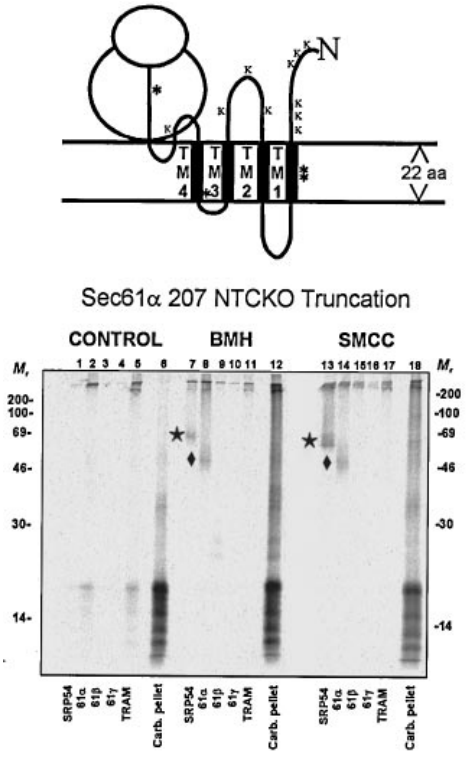

Figure 6 Immunoprecipitation of Sec61α-207 NTCKO cross-linking products obtained with DMSO (control), BMH or SMCC

Products are labelled as described in the legend to Figure 2. The diagram of the integration intermediate is labelled as in Figure 5.

Sec61α 136 NTCKO and endogenous Sec61α. In contrast to the wild-type protein, no cross-linking of Sec61α 136 NTCKO to either Sec61β or TRAM was observed with either reagent. We therefore conclude that Cys[13] is solely responsible for the cross-linking of both Sec61α 96 and Sec61α 136 to Sec61β and TRAM.

By increasing the chain length of the integration intermediate even further to 207 amino acids, the environment of a nascent Sec61α polypeptide chain with four putative transmembrane domains was investigated. When a consensus site for N-linked glycosylation is engineered into the first lumenal loop of this intermediate, it can be utilized by the ER-lumenal oligosaccharyl transferase (results not shown). This confirmed that the complete integration of transmembrane domains one and two of Sec61α 207 had indeed occurred. The 207-amino-acid intermediate contains two additional cysteine residues at position 148 (within transmembrane domain four) and position 188 (which is 19 amino acids from the ribosomal peptidyl-transferase site, and should therefore be buried deep within the ribosome) [13]. The wild-type Sec61α 207 was cross-linked to Sec61α (Figure 5, lanes 8 and 14), Sec61β (Figure 5, lane 9), and weakly to TRAM (Figure 5, lanes 11 and 17), as previously observed with Sec61α 96 and Sec61α 136. Analysis of the Sec61α 207 NTCKO mutant showed that even with this integration intermediate, the N-terminal cysteine residue at position 13 was primarily responsible for cross-linking to Sec61β and TRAM (compare Figure 5, lanes 9, 11 and 17 with Figure 6, lanes 9, 11 and 17). We therefore conclude that even after four out of a probable total of ten transmembrane domains of Sec61α have been inserted, the N-terminal cytosolic domain remains adjacent to both Sec61β and TRAM.

Sec61α targeting to the ER is SRP-dependent

In all of the cross-linking experiments described above, SRP54 was identified as a major cross-linking partner of the nascent chains. It is not unusual for such systems *in vitro* to be inefficient, resulting in a significant proportion of chains that bind to SRP, but are not correctly targeted to the ER membrane [14]. Whilst this reproducible SRP54 cross-linking strongly suggested that the ER targeting of Sec61α required SRP, we tested this proposition experimentally. Sheep pancreatic microsomes have extremely low levels of endogenous SRP [25], and we therefore used these to test the efficiency of Sec61α-membrane integration in the presence and absence of additional SRP. Hence full-length Sec61α was synthesized in a wheat-germ translation system supplemented with sheep pancreatic microsomes, and in the presence and absence of additional purified canine SRP. It has long been known that the endogenous wheat-germ SRP cannot supplement the requirement for canine SRP in order to obtain efficient targeting to canine rough microsomes, and this has also been found to be the case for sheep microsomes [25]. In the absence of canine SRP, full-length Sec61α was not protease-protected (Figure 7, lane 5) indicating no membrane integration had taken place. However, when the translation system was supplemented with canine SRP, a significant fraction of the full-

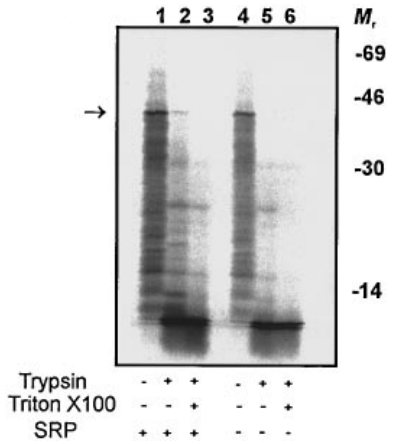

Figure 7 Targeting of Sec61α in sheep pancreatic microsomes

Full-length Sec61α (arrow indicates position on gel) was translated in the presence (lanes 1–3) or absence (lanes 4–6) of added canine SRP. The membrane-associated material was then incubated with trypsin in the presence or absence of Triton X-100, as indicated.

length Sec61α was protease-protected when the membranes were intact (Figure 7, lane 2, arrow) but not when the membranes were disrupted with Triton X-100 (Figure 7, lane 3). We conclude that the membrane integration of Sec61α is dependent upon SRP for targeting to the ER.

DISCUSSION

Since Sec61α is a core component of the ER membrane-insertion site, this raised the interesting question of how Sec61α itself becomes membrane-integrated. Using an *in vitro* system to reconstitute this process, we found that nascent Sec61α chains were cross-linked to several known components of the classical ER targeting and membrane-insertion machinery. Hence cross-links with SRP54, Sec61α, Sec61β and TRAM were observed.

Using sheep pancreatic microsomes, which have very low levels of endogenous SRP, we were able to show that the membrane insertion of newly synthesized Sec61α was completely dependent upon the addition of purified canine SRP. Thus the targeting of Sec61α to the ER membrane is SRP-dependent. Sec61α is a very hydrophobic integral membrane protein, and our data fit very well with previous studies indicating that the SRPs from *S. cerevisiae* [28], *Escherichia coli* [29–31] and chloroplast stroma [32] all show a marked preference for precursors with very hydrophobic ER-targeting signals, such as those typically found within integral membrane proteins.

After release of the nascent Sec61α chains from SRP, cross-linking to three known components of the ER membrane-insertion site, namely Sec61α, Sec61β and TRAM, were detected. Previous studies of polytopic membrane-protein integration found that integration intermediates of bovine opsin were cross-linked to Sec61α and Sec61β [14], as were integration intermediates derived from *E. coli* leader peptidase [15]. In contrast, neither opsin- nor the leader-peptidase-derived proteins produced discrete cross-linking products with TRAM [14,15]. This lack of TRAM cross-linking may reflect the relative positions of the probes within the proteins [13,27], and cross-linking to TRAM has certainly been observed during the integration of a single-spanning membrane protein [17]. The simplest interpretation of these cross-linking data is that Sec61α uses the 'typical' Sec61 complex for its integration into the ER membrane. Since the ER is never synthesized *de novo*, and at least a portion of the ER is inherited during cell division [33], a requirement for pre-existing Sec61 complexes in order to integrate newly synthesized Sec61α polypeptides does not pose particular problems for eukaryotic cells.

In order to analyse the membrane insertion of Sec61α in more detail, we constructed a cysteine deletion mutant (NTCKO), which was no longer capable of cysteine-dependent cross-linking from the cytoplasmic N-terminus of the integration intermediates. By comparing the cross-linking partners of the wild-type and mutant Sec61α nascent chains, we were able to establish a number of features about the insertion process. First, we found that cysteine residues 45 and 46, present in TM1 of nascent Sec61α, can only be cross-linked to endogenous Sec61α when they have fully exited the ribosomes and entered the plane of the membrane. This is in good agreement with our previous study of opsin integration, where a cysteine residue in a translocated lumenal loop could only be cross-linked to Sec61α from a similar location [14].

Secondly, essentially all of the cross-linking of nascent Sec61α with endogenous Sec61β and TRAM occurred from cysteine residue 13 of the N-terminus. The cross-linking of nascent Sec61α chains to the single cysteine residue of Sec61β present in its putative cytosolic domain is consistent with both the proposed topology of Sec61β [34] and previous studies [14,15]. The specific proximity of TRAM to regions N-terminal of the hydrophobic core of ER-targeting signals has also been previously observed for cleavable signal sequences [13,27], and hence our data are in accordance with these previous studies. Finally, we found that as the chain length of the Sec61α integration intermediate was increased, allowing an increasing number of TMs to be inserted into the membrane, specific cross-linking from cysteine residue 13 to Sec61β and TRAM was still observed. On this basis, we propose that the cytosolic N-terminus of newly synthesized Sec61α remains adjacent to protein components of the ER membrane-insertion site, namely Sec61β and TRAM, for a prolonged period during the membrane insertion of the nascent chain. The absence of any adducts with Sec61γ suggests that the relative position of this subunit renders it unavailable to cross-link from the nascent chain. A previous study, with bifunctional reagents [14], was also unable to demonstrate cross-linking to Sec61α, lending further support to this hypothesis.

Our data indicate that TM1, which is contiguous with the cytosolic N-terminus of nascent Sec61α, also remains close to the protein components of the ER-insertion site after the integration of up to three subsequent TMs. Thus our data are consistent with the proposal that the TMs of polytopic proteins do not completely integrate into the lipid bilayer until protein synthesis is complete [16], or at least a substantial portion of the polypeptide has been made. It may well be that the proteinaceous environment of the integration intermediate alters during the insertion of subsequent transmembrane domains. Such alterations have been observed by comparing different stages during the membrane insertion of a single-spanning protein [17]. Nevertheless, we detected no absolute changes in the cross-linking partners of the different integration intermediates used in this study.

Previous site-specific photocross-linking studies have established that the TMs of both single-spanning [18] and polytopic [15] membrane proteins have lateral access from the ER-insertion site to the phospholipid bilayer from an early stage of their membrane integration. Although in this study, we have not addressed the lateral access of the TMs to the lipid bilayer, these regions of Sec61α may well have such early access to the lipid phase. Nevertheless, our data are consistent with the N-terminal regions of polytopic integral membrane proteins remaining

adjacent to the protein components of the ER-insertion site during biosynthesis. It therefore remains possible that the ER membrane-insertion site acts to modulate the assembly of complex polytopic integral membrane proteins [4,16]. In the case of proteins with fewer transmembranous spans, or less complex structures, this may not be necessary [15].

On the basis of the work outlined in this report, there is no reason to propose that the biosynthesis and membrane integration of Sec61α differs significantly from that of any other polytopic integral membrane protein. However, the fate of Sec61α is unique, because it is destined ultimately to become a component of the Sec61 complex. It therefore remains a definite possibility that newly synthesized Sec61α subunits are biosynthetically incorporated into pre-existing Sec61 complexes, and therefore the membrane integration of Sec61α differs from most other integral membrane proteins. Only future studies will be able to establish whether or not this proves to be the case.

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REFERENCES

1 Stirling, C. J., Rothblatt, J., Hosobuchi, M., Deshaies, R. and Scheckman, R. (1992) Mol. Biol. Cell **3**, 129–142
2 High, S., Andersen, S. S. L., Görlich, D., Hartmann, E., Prehn, S., Rapoport, T. A. and Dobberstein, B. (1993) J. Cell Biol. **121**, 743–750
3 Walter, P. and Johnson, A. E. (1994) Annu. Rev. Cell Biol. **10**, 87–119
4 High, S. and Laird, V. (1997) Trends Cell Biol. **7**, 206–210
5 Görlich, D. and Rapoport, T. A. (1993) Cell **75**, 615–630
6 Hartmann, E., Sommer, T., Prehn, S., Görlich, D., Jentsch, S. and Rapoport, T. A. (1994) Nature (London) **367**, 654–657
7 Oliver, J., Jungnickel, B., Görlich, D., Rapoport, T. A. and High, S. (1995) FEBS Lett. **362**, 126–130
8 Voigt, S., Jungnickel, B., Hartmann, E. and Rapoport, T. A. (1996) J. Cell Biol. **134**, 25–35
9 Hanein, D., Matlack, K. E. S., Jungnickel, B., Plath, K., Kalies, K-U., Miller, K. R., Rapoport, T. A. and Akey, C. W. (1996) Cell **87**, 721–732
10 Hamman, B. D., Chen, J-C., Johnson, E. E. and Johnson, A. E. (1997) Cell **89**, 535–544
11 Görlich, D., Prehn, S., Hartman, E., Kalies, K-U. and Rapoport, T. A. (1992) Cell **71**, 489–503
12 High, S., Andersen, S. S., Görlich, D., Hartmann, E., Prehn, S., Rapoport, T. A. and Dobberstein, B. (1993) J. Cell Biol. **121**, 743–750
13 Mothes, W., Prehn, S. and Rapoport, T. A. (1994) EMBO J. **13**, 3973–3982
14 Laird, V. and High, S. (1997) J. Biol. Chem. **272**, 1983–1989
15 Mothes, W., Heinrich, S. U., Graf, R., Nilsson, I., von Heijne, G., Brunner, J. and Rapoport, T. A. (1997) Cell **89**, 523–533
16 Borel, A. C. and Simon, S. M. (1996) Cell **85**, 379–389
17 Do, H., Falcone, D., Lin, J., Andrews, D. W. and Johnson, A. E. (1996) Cell **85**, 369–378
18 Martaglio, B., Hofmann, M. W., Brunner, J. and Dobberstein, B. (1995) Cell **81**, 207–214
19 Wilkinson, B. M., Critchley, A. J. and Stirling, C. J. (1996) J. Biol. Chem. **271**, 25590–25597
20 Gilmore, R., Collins, P., Johnson, J., Kellaris, K. and Rapiejko, P. (1991) Methods Cell Biol. **34**, 223–239
21 High, S., Flint, N. and Dobberstein, B. (1991) J. Cell Biol. **113**, 25–34
22 Nilsson, I., Whitley, P. and von Heijne, G. (1994) J. Cell Biol. **126**, 1127–1132
23 Steuber, D., Ibrahimi, D., Cutler, B., Dobberstein, B. and Bujard, H. (1984) EMBO J. **3**, 3143–3148
24 Fujiki, Y., Fowler, S., Shio, H., Hubbard, A. L. and Lazarow, P. B. (1982) J. Cell Biol. **93**, 103–110
25 Kaderbhai, M. A., Harding, V. J., Karim, A., Austen, B. and Kaderbhai, N. N. (1995) Biochem. J. **306**, 57–61
26 Oliver, J. D., Hresko, R. C., Mueckler, M. and High, S. (1996) J. Biol. Chem. **271**, 13691–13696
27 High, S., Martaglio, B., Görlich, D., Andersen, S. S. L., Ashford, A. J., Giner, A., Hartmann, E., Prehn, S., Rapoport, T. A., Dobberstein, B. and Brunner, J. (1993) J. Biol. Chem. **268**, 26745–26751
28 Ng, D. T. W., Brown, J. and Walter, P. (1996) J. Cell Biol. **134**, 269–278
29 Valent, Q. A., Kendall, D. A., High, S., Kusters, R., Oudega, B. and Luirink, J. (1995) EMBO J. **14**, 5494–5505
30 Ulbrandt, N. D., Newitt, J. A. and Bernstein, H. D. (1997) Cell **88**, 187–196
31 de Gier, J. W., Mansournia, P., Valent, Q. A., Phillips, G. J., Luirink, J. and von Heijne, G. (1996) FEBS Lett. **399**, 307–309
32 High, S., Henry, R., Mould, R. M., Valent, Q., Meacock, S., Cline, K., Gray, J. C. and Luirink, J. (1997) J. Biol. Chem. **17**, 11622–11628
33 Warren, G. and Wickner, W. (1996) Cell **84**, 395–400
34 Rapoport, T. A., Jungnickel, B. and Kutay, U. (1996) Annu. Rev. Biochem. **65**, 271–303

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