Ribophorin I Associates with a Subset of Membrane Proteins after Their Integration at the Sec61 Translocon*

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The biosynthesis of membrane proteins at the endoplasmic reticulum (ER) involves the integration of the polypeptide at the Sec61 translocon together with a number of maturation events, such as N-glycosylation and signal sequence cleavage, that can occur both during and after synthesis. To better understand the events occurring after the release of the nascent chain from the ER translocon, we investigated the ER components adjacent to the transmembrane-spanning domain of a well characterized fragment of the amyloid precursor protein. Using individual cysteine residues as site-specific cross-linking targets, we found that several ER components can be cross-linked to the fully integrated polypeptide. We identified strong adducts with both the ribophorin I subunit of the oligosaccharyltransferase complex and the 25-kDa subunit of the signal peptidase complex. Focusing on the association with ribophorin I, we found that adduct formation occurred exclusively after the exit of the nascent chain from the Sec61 translocon and was unaffected by the N-glycosylation status of the associated precursor. Only a subset of newly made membrane proteins associated with ribophorin I in vitro, and we could recapitulate a specific association between the amyloid precursor protein fragment and ribophorin I in vivo. Taken together, our data suggest a model where ribophorin I may function to retain potential substrates in close proximity to the catalytic subunit of the oligosaccharyltransferase and thereby stochastically improve the efficiency of the N-glycosylation reaction in vivo. Alternatively ribophorin I may be multifunctional and facilitate additional processes, for example, ER quality control.

Most newly synthesized membrane and secretory proteins arrive at the ER via the signal recognition particle-dependent targeting pathway and are delivered to the Sec61 translocon that mediates their integration into the lipid bilayer (1, 2). During and immediately after their insertion newly synthesized membrane proteins are associated with a number of accessory components that facilitate processes related to biosynthesis, most notably the signal peptidase complex (SPC) and the oligosaccharyltransferase (OST) complex (3). The SPC mediates the catalytic cleavage of N-terminal signal peptides present on both secretory and some integral membrane proteins. In the case of the secretory protein preprolactin, probes located in its signal peptide were shown to cross-link to two of its subunits, SPC18 and SPC21 (4). More recently, the transmembrane (TM) domains of tail-anchored and signal-anchored proteins were found to be transiently adjacent to the SPC25 subunit following membrane insertion, consistent with the SPC scanning hydrophobic transmembrane regions for adjacent cleavage sites as they exit the Sec61 complex (5).

The OST mediates the transfer of high mannosyl oligosaccharides from a dolichol carrier to suitable -Asn-(Thr/Ser)- acceptor sites in nascent chains (6). In vitro studies have shown that the mammalian OST is closely associated with the ER translocon (7), consistent with the addition of N-linked glycans during and immediately after translocation. Biochemical studies have built upon the well defined yeast OST complex to show that its mammalian equivalent has different isoforms that contain several distinct subunits, namely ribophorin I, ribophorin II, OST48, Dad1, one of either STT3A or STT3B, and a loosely associated mammalian equivalent of the Saccharomyces cerevisiae OST3p/OST6p subunits (8). A major breakthrough in our understanding of OST function has been compelling evidence that the STT3 subunit recognizes consensus sites for N-glycosylation and most likely provides the catalytic center of the OST mediating glycosylation of polypeptide chains (8–10). In the first study to link ribophorin I with the N-glycosylation at the ER, it was suggested that its transmembrane domain might function as a dolichol binding site (11) and thereby act to bring the lipid-linked high mannosyl form of the glycan in close proximity to the OST complex and any potential protein substrates. More recent studies indicated that ribophorin I may form all or part of the active site of the OST complex (12, 13), although it seems more likely this function is mediated by STT3, and hence the precise role of ribophorin I and the other subunits of the OST remains unclear (8, 9).

In this study, we extended our previous analysis of membrane protein biosynthesis at the ER (5, 14, 15) to include a simple, single spanning membrane protein that has an atypical fate within the cell. During the maturation of the amyloid precursor protein (APP), specific proteolytic cleavage events generate peptides that are strongly implicated in the pathology of Alzheimer’s disease (16). In this case, we used the biologically relevant APP-C99 fragment as a model to study mem-
brane protein integration and maturation in vitro. APP-C99 is generated in vivo by the β-secretase (β-site APP-cleaving enzyme)-mediated proteolytic cleavage of full-length APP, and it is this fragment that is the substrate for the γ-secretase, which cleaves it within its transmembrane region to generate the Aβ peptides that are a key feature of Alzheimer’s disease (17).

Using an in vitro system combined with cytostatic-mediated site-specific cross-linking, we investigated the ER components that are associated with APP-C99**, a derivative of APP-C99 with a 2-amino acid N-terminal extension, during both its membrane integration and subsequent maturation (see Fig. 1A). A preliminary scanning approach showed that this model protein is cross-linked to distinct subsets of ER components dependent upon the location of the cytochrome probe within the nascent polypeptide. Among these various adducts, strong cross-linking from a cytochrome located on the cisternal side of the TM to ribophorin I and SPC25 was observed. The association of newly synthesized membrane proteins with these components was concomitant with their lateral exit from the Sec61 translocon, and we speculate that ribophorin I may act to maintain potential substrates in close proximity to STT3, the presumptive catalytic subunit of the ribophorin I may act to maintain potential substrates in close proximity to STT3, the presumptive catalytic subunit of the ribosome. Samples were then plated immediately (5) or incubated for a further 10 min at 30 °C. Membrane-associated intermediates were isolated by centrifugation through a high salt/sucrose cushion (250 mM sucrose, 500 mM KOAc, 5 mM Mg(OAc)₂, 50 mM Hepes-KOH, pH 7.9) at 130,000 × g for 10 min at 4 °C. The resulting membrane pellet was resuspended in a low salt/sucrose buffer (250 mM sucrose, 100 mM KOAc, 5 mM Mg(OAc)₂, 50 mM Hepes-KOH, pH 7.0) and incubated at 30 °C for 10 min either in the presence of the cytochrome bermine-associated cross-linking reagents or in the equivalent dimethyl sulfoxide (Me₂SO) solvent control. The reaction was quenched by the addition of 2-mercaptoethanol to 10 mM and left for 10 min on ice, samples were then either analyzed directly or following immunoprecipitation in the presence of SDS as described previously (5). All samples were treated with RNase A (7 μg/translation reaction) to remove any RNA that remained attached to the stalled polypeptide chains prior to SDS-PAGE (23).

**Isolation of Ribosomal Subunits**—Membrane-associated translation intermediates were solubilized in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% (w/v) C₆E₈ detergent, and 1 mM phenylmethylsulfonyl fluoride; layered over a sucrose cushion (250 mM sucrose, 100 mM KOAc, 5 mM Mg(OAc)₂, 50 mM Hepes-KOH, pH 7.9) with 0.1% (w/v) C₆E₈, and centrifuged at 213,000 × g for 20 min to pellet ribosomal subunits and associated products. The location of ribosomal subunits was confirmed by immunoblotting using primary antisera recognizing the ribosomal proteins L23a and L35 in combination with the Western Lightning system (PerkinElmer Life Sciences).

**Sucrose Gradient Analysis**—Following in vitro translation and puromycin treatment, membrane fractions were isolated and treated with 50 μM BMH as described above. The membrane fraction was then reisolated by centrifugation at 130,000 × g for 10 min at 4 °C and solubilized in 0.5 ml of gradient buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% (w/v) C₆E₈, 1 mM phenylmethylsulfonyl fluoride). The resulting material was clarified by centrifugation at ~13,000 × g for 15 min, the supernatant was layered onto a 10-ml 5–25% (w/v) sucrose gradient (in gradient buffer) poured over a 0.5-ml 50% (w/v) chloroform/methanol solution, and centrifuged at 40,000 × g for 18 h at 4 °C. After centrifugation, 1-ml fractions were removed carefully from the gradient, and the samples were analyzed by SDS-PAGE and/or immunoblotting.

**Ribophorin I-Membrane Protein Association**

**Experimental Procedures**

**Materials**—General chemicals and reagents were obtained from Merck or Sigma, bismaleimidohexane (BMH) was from Prochum (Rockford, IL), and octaethylene glycol monododecyl ether (C₆E₈) was from Fluka (Gillingham, UK). All sera are rabbit polyclonal unless otherwise stated. Those recognizing ribophorin II, Daf3, and OST48 were made by Eurogentec (Seraing, Belgium). Antisera specific for Sec61α, Sec61β, and ribophorin I were gifts from R. Zimmerman (University of the Saarland, Homburg, Germany), B. Dobberstein (Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany), and I. Ivessa (University of Vienna, Vienna, Austria), respectively. Antisera recognizing the ribosomal proteins L23a and L35 were a gift from M. Pool (University of Manchester, Manchester, UK). The W02 mouse monoclonal antibody recognizing the N terminus of APP-C99 was kindly provided by M. Shearman and D. Beher (Medical Radiation Biology, University of Marlow, UK).

**cDNA Constructs**—The wild type signal peptide present on the SPAC7T derivative of APP (18) was exchanged for a variant from bovine prolactin (Fig. 1A). It should be noted that the first two residues of the mature prolactin sequence (Fig. 1C) were then replaced with DpnI (100 units/ml) for 1 h at 37 °C to remove any methylated parental plasmid DNA prior to transcription with SP6 or T7 primers as described previously (19). All resulting PCR products were subsequently introduced by PCR during the preparation of the DNA template for transcription.

**Transcription**—DNA templates for the in vitro transcription of mRNAs lacking a stop codon were generated by PCR using appropriate primers as described previously (19). All resulting PCR products were treated with DpnI (100 units/ml) for 1 h at 37 °C to remove any methylated parental plasmid DNA prior to transcription with SP6 or T7 RNA polymerase as described by the manufacturer (New England Biolabs, Hitchin, UK). All RNA transcripts were purified with an RNaseasy mini kit (Qiagen, Crawley, UK) before use in translation reactions.

**Translation and Cross-linking**—A rabbit reticulocyte lysate system (Promega), supplemented with canine pancreatic microsomes (20) at a final concentration of 1.5–2.9 A₂₆₀/ml as an ER membrane source, was used for membrane protein synthesis unless otherwise stated, incubations were performed at 30 °C for 1 h in the presence of [³⁵S]methionine (0.82 μCi/μl) and 50 mM ascorbic acid to reduce the formation of nonspecific background adducts via radical formation (21, 22). Aurintricarboxylic acid was then added to a final concentration of 100 μM to inhibit further initiation, and the incubation continued for another 10 min at 30 °C. Translation was then terminated by the addition of cycloheximide to 2 mM to stabilize ribosome bound integration intermediates or of puromycin and EDTA to 2 and 50 mM, respectively, to release the nascent chain from the ribosome. Samples were then plated immediately (5) or incubated for a further 10 min at 30 °C. Membrane-associated intermediates were isolated by centrifugation through a high salt/sucrose cushion (250 mM sucrose, 500 mM KOAc, 5 mM Mg(OAc)₂, 50 mM Hepes-KOH, pH 7.9) at 130,000 × g for 10 min at 4 °C. The resulting membrane pellet was resuspended in a low salt/sucrose buffer (250 mM sucrose, 100 mM KOAc, 5 mM Mg(OAc)₂, 50 mM Hepes-KOH, pH 7.0) and incubated at 30 °C for 10 min either in the presence of the cytochrome bermine-associated cross-linking reagents or in the equivalent dimethyl sulfoxide (Me₂SO) solvent control. The reaction was quenched by the addition of 2-mercaptoethanol to 10 mM and left for 10 min on ice, samples were then either analyzed directly or following immunoprecipitation in the presence of SDS as described previously (5). All samples were treated with RNase A (7 μg/translation reaction) to remove any RNA that remained attached to the stalled polypeptide chains prior to SDS-PAGE (23).

**Sample Analysis**—All samples were analyzed on 14% SDS-polyacrylamide Tris-glycine gels except those shown in Figs. 3, A and B (12%), and Fig. 6C (10%). The resulting gels were dried and then visualized using a Fuji BAS 1800 PhosphorImager system (Fuji Photo Film Co. Ltd., Tokyo, Japan).
RESULTS

Molecular Interactions of Newly Synthesized APP-C99—The primary aim of this study was to investigate the biosynthesis and maturation of a simple single spanning membrane protein and to analyze the ER proteins in close proximity to its TM domain after integration was complete (see Fig. 1A). For this purpose, we selected a well characterized C-terminal domain of APP, the APP-C99 polypeptide, as a useful model for studying this process in vitro. This fragment is of biological significance since it is equivalent to the region of APP that is generated in vivo by the action of the β-secretase, and the predicted transmembrane domain (underlined). Four versions of APP-C99 with residues 25, 40, 49, and 59 of the mature sequence altered to a cysteine (bold italics) were generated for use in cross-linking experiments.

Identification and Characterization of Specific Cross-linking Partners—The APP-C99 polypeptide lacks any sites for N-glycosylation of single cysteine residues for use as site-specific cross-linking probes (5, 15, 19). The wild type preprolactin signal sequence contains a single cysteine residue, and to avoid any cross-linking to ER components from this location either before or after cleavage from the APP-derived polypeptide (4), this residue was altered to a glycine. Using this APP-C99 derivative (denoted APP-C99') as a starting point, single cysteine residues were then introduced at four different locations within and flanking the TM region (see Fig. 2). These proteins were then synthesized as ribosome-bound integration intermediates at the ER membrane and subjected to cysteine-specific cross-linking with BMH either before or after a 10-min puromycin treatment to release the chains from the ribosome. In each case, discrete BMH-dependent adducts could be seen with the probes at residues 49 and 59 appearing to generate the most adducts (see Fig. 2). Of particular note was the observation that several adducts appeared to remain after the puromycin-dependent release of the nascent chain from the ribosome (Fig. 2, lanes 3, 4, 11, 12, 15, and 16, filled circles and asterisks). In each case, some higher molecular weight products could be seen in the absence of BMH treatment. These most likely represent a combination of peptidyl-tRNA species (25), in vitro ubiquitination of the APP-C99' chain (26), and radical-mediated adduct formation that can occur in the reticulocyte system (21, 22). Nevertheless it was clear that discrete BMH-dependent adducts could be observed with variants of APP-C99', particularly with the APP-C99'[Cys49] and APP-C99'[Cys59] (Fig. 2, lanes 11, 12, 15, and 16).

Fig. 1. Co-translational integration of a model single spanning membrane protein. A, following targeting to the ER, the nascent polypeptide chain is inserted into the translocon, and the signal peptide (SP) is cleaved by the SPC. The protein can be artificially trapped at the translocon as a ribosome-bound integration intermediate (23) or released from the ribosome by puromycin treatment to allow complete insertion and the potential association of the newly made protein with post-translocon components of the ER (indicated by an arrow (cf. Ref. 18), and the predicted transmembrane domain (underlined). B, the amino acid sequence of the APP-C99' precursor consists of the signal sequence from bovine preprolactin (bold), 2 residues of the mature prolactin sequence (italics) that will remain after signal sequence cleavage as indicated by an arrow (cf. Ref. 18), and the predicted transmembrane domain (underlined). Four versions of APP-C99 with residues 25, 40, 49, and 59 of the mature sequence altered to a cysteine (bold italics) were generated for use in cross-linking experiments.
linked glycosylation, and a preliminary analysis of the adducts shown in Fig. 2 was carried out by treating them with endoglycosidase H to establish whether any of the cross-linked components were N-glycosylated (data not shown). This analysis suggested that the ~80-kDa adduct represented the cross-linking of the ~14-kDa APP-C99 polypeptide to an ER protein of ~66 kDa (assuming the migration of the adduct reflects the true migration of its two constituents) and showed that this ER protein most likely had a single N-linked glycan that could be removed by endoglycosidase H digestion (data not shown). On this basis, the prominent ~66-kDa cross-linking partner of APP-C99[Cys59] (Fig. 2, lanes 15 and 16, asterisk) was identified by immunoprecipitation as ribophorin I (Fig. 3, A and B, lane 5). The authenticity of the BMH-dependent adducts obtained with APP-C99[Cys59], including that with ribophorin I, was underlined by the observation that antisera specific for both N- and C-terminal regions of APP-C99 immunoprecipitated the major cross-linking products (Fig. 3, A and B, cf. lanes 2, 3, 4, and 5). Ribophorin I is an N-glycosylated (~67-kDa) single spanning, type I membrane protein that forms part of the OST complex (11, 27). Its cytosolic tail has two cysteine residues that would present ideal targets for cross-linking from the single cysteine present in the APP-C99[Cys59] polypeptide (cf. Fig. 2, schematics).

During subsequent analysis of the cross-linking partners of APP-C99[Cys59], we carried out a more detailed analysis of the adducts obtained before and after the puromycin-dependent release from the ribosome. As previously reported (28), adds with subunits of the Sec61 translocon were seen only when a cycloheximide-stabilized integration intermediate was used and were lost after puromycin treatment (cf. Fig. 3, A and B, lane 6). Thus, puromycin treatment allows the efficient integration of the trapped integration intermediates into the ER membrane. In contrast, adducts with ribophorin I (Fig. 3, A and B, lane 5) and partners with approximate molecular masses of ~14 and ~24 kDa (Fig. 3, A and B, lanes 2–4, indicated by a cross and filled circle, respectively) were present in the cycloheximide-stabilized sample and remained after puromycin treatment.

As in some previous studies (15), BMH-dependent adducts of APP-C99[Cys59] with the Sec61α subunit were not readily apparent in the total products but were easily distinguished after immunoprecipitation (cf. Fig. 2, lanes 13 and 15, with Fig.
The cross-linking of Cys 59 of the ribosome-bound integration intermediate of APP-C99/H11032 to Sec61/H9251 is consistent with previous studies showing that single cysteine probes located 40 residues or more from the peptidyltransferase center of the ribosome are in close proximity to the ER translocon (15). As with Sec61/H9252 (cf. Fig. 3), adducts with Sec61/H9251 were lost upon puromycin treatment of the integration intermediates but remained if the incubation was carried out in the presence of cycloheximide (Fig. 4A, lanes 3, 4, 7, and 8).

Once again (cf. Fig. 3, A and B), BMH-dependent cross-linking to ribophorin I was detected with both the cycloheximide-treated integration intermediates and the puromycin-released chains (Fig. 4B, lanes 3, 4, 7, and 8). Using immunoprecipitation screening, we were able to identify the ~24-kDa cross-linking partner of APP-C99[Cys59] as the 25-kDa subunit of the SPC (Fig. 3, A and B, lanes 2–4, filled circle; Fig. 4C, lane 3).

Ribophorin I and SPC25 Are Associated Exclusively with Nascent Chains That Have Been Released from the Ribosome—To date, our cross-linking analysis indicated that nascent APP-C99' chains were only associated with subunits of the Sec61 translo-
con when they were present as cycloheximide-stabilized integration intermediates, while ribophorin I and SPC25 were adjacent to both cycloheximide-stabilized and puromycin-released chains (Figs. 2–4). However, this interpretation is only valid if the artificial, ribosome-bound integration intermediates used to trap the APP-C99’ chains at the ER translocon are completely stable and retain all of the chains in this form. While no significant loss of cross-linking to Sec61 lanes 1–5, pellet fraction at all time points (Fig. 5B, cf. lanes 3 and 4), this assay reports the environment of only a fraction of the total nascent chains present. Furthermore previous studies have shown that artificial peptidyl-tRNA complexes such as that used here to generate APP-C99’ integration intermediates have shown them to have differing stabilities (23), raising the possibility that even after cycloheximide stabilization a proportion of the nascent chains may be released from the ribosome, exit the Sec61 translocon, and associate with distinct ER components.

To address this issue directly, we first established conditions that could be used to efficiently solubilize the membrane-associated integration intermediates and isolate ribosomes and any associated nascent chains. We found that if the membrane fraction was treated with the non-ionic detergent C_{12}E_{8} and loaded onto a sucrose cushion containing C_{12}E_{8}, the samples could be separated by centrifugation to give a pellet highly enriched in ribosomes and a supernatant almost completely devoid of ribosomes (Fig. 5A, cf. lanes 1–4). The efficient pelleting of the ribosomes, as evidenced by the immunodetection of the two ribosomal subunits L23a and L35, was unaffected by the length of the cell-free translation reaction (Fig. 5A, cf. lanes 1 and 2).

Having confirmed that ribosomes could be efficiently recovered in this way, the location of the various BMH-dependent cross-linking products obtained with cycloheximide-treated integration intermediates of APP-C99(Cys\(^{59}\)) was determined. By using translation reactions stopped at different time points across a 1-h incubation, we also established whether the use of short translation times had any effect on the relative proportion of ribosome-bound chains (23). The samples were fractionated into a ribosomal pellet and a ribosome-depleted supernatant, and the adducts of APP-C99’(Cys\(^{59}\)) with Sec61β (Fig. 5B), ribophorin I (Fig. 5C), and SPC25 (Fig. 5D) were recovered by immunoprecipitation from each fraction at each time point. In all cases, at only 5 min into the time course relatively little APP-C99’(Cys\(^{59}\)) had been synthesized (data not shown). Hence no significant adducts were detected in these samples (Fig. 5B, C, and D, lanes 1 and 6). At later time points adducts were clearly visible, and those with Sec61β were largely in the pellet fraction at all time points (Fig. 5B, lanes 1–5) confirming that cross-linking to Sec61β is primarily a feature of ribosome-bound integration intermediates that have remained attached to the ribosome during the solubilization and isolation procedure detailed above (cf. Fig. 5A).

In contrast, even from the earliest time point at which cross-links could be detected, adducts with ribophorin I were found almost exclusively in the supernatant fraction (Fig. 5C, lanes 6–10), and their intensity increased across a 1-h time period consistent with an increasing proportion of the nascent APP-C99’ chains being released from the ribosome across this incubation period. This presumably occurs via the hydrolysis of the peptidyl-tRNA bond that retains the nascent chain at the ribosome (23). The behavior of the adducts formed with SPC25 was similar to those seen with ribophorin I, and these products were also seen only in the supernatant fractions and became stronger across the 1-h incubation period (Fig. 5D, lanes 6–10). Taken together, these data indicate that only APP-C99’ chains that have been released from the ribosome can associate with ribophorin I and confirm that this is the case even when cycloheximide is used to “stabilize” the ribosome nascent chain complex.

**Association of APP-C99’ with Ribophorin I Is Independent of N-Glycosylation.**—Ribophorin I is a subunit of distinct oligosaccharyltransferase complexes present in the ER membrane that mediate N-glycosylation (6). While the truncated form of APP used in this study lacks any sites for N-glycosylation, the full-length protein contains two N-linked glycans in its large extracellular domain. It was therefore possible that the addition of N-linked glycans acted as a “release signal” for ribophorin I association with APP-C99’. We investigated this possibility by introducing a site for N-glycosylation into the short N-terminal region of APP-C99’ and determining its effect upon the association of the polypeptide with ribophorin I. The artificial site for N-glycosylation was efficiently used as evidenced by the sensitivity of the resulting product to digestion with PNGase F (Fig. 6, A and B, cf. lanes 3 and 4, APP-C99’-1CHO and APP-C99’). However, the efficient N-glycosylation of APP-C99’ did not prevent its association with ribophorin I (Fig. 6, A and B, cf. lanes 2, 5, and 6).

Although the N-glycosylation of the APP-C99’ derivative was efficient, a small fraction of non-glycosylated precursor was present and might in principle be responsible for the cross-linking to ribophorin I that was observed. To exclude this possibility, we analyzed the adducts of APP-C99’ and APP-C99’-1CHO following digestion with PNGase. We found that, when resolved on a low percentage polyacrylamide gel, the size of the APP-C99’-1CHO-ribophorin I adduct was clearly larger than that with APP-C99’ (Fig. 6C, cf. lanes 2, 3, 6, and 7). Upon the removal of N-linked glycans with PNGase, the apparent mobility of the two adducts was the same (Fig. 6C, cf. lanes 4 and 8). Hence, while the APP-C99’(Cys\(^{59}\))-ribophorin I adduct contains only a single N-linked glycan contributed by ribophorin I, the majority of APP-C99’-1CHO(Cys\(^{59}\))-ribophorin I adducts have two glycans, one from ribophorin I and the other from the glycosylated APP-C99’ polypeptide. We therefore conclude that the association of ribophorin I with APP-C99’ is unaffected by the N-glycosylation of the polypeptide.

**Ribophorin I Adducts Are Present in Protein Complexes.**—At least three different oligosaccharyltransferase complexes have been identified within the ER membrane, and all of them have been reported to contain ribophorin I (6). To better understand the context of the ribophorin I cross-linking observed with APP-C99’(Cys\(^{59}\)), the translation reactions were treated with puromycin to release any nascent chains from the ribosome (cf. Fig. 5). The resulting membrane-associated BMH-dependent adducts were then analyzed by centrifugation through a sucrose gradient containing 1% C_{12}E_{8}, a detergent known to preserve the OST complex in an active form (11). When these gradient fractions were analyzed, we found that, although the majority of the APP-C99’(Cys\(^{59}\)) chains were in the lighter fractions 2–4 (Fig. 7A), the bulk of the APP-C99’(Cys\(^{59}\))-ribophorin I adducts were located further into the gradient, particularly at fractions 5–9 (Fig. 7B), migrating with markers of ∼250 to ∼600 kDa (Fig. 7 and data not shown).

In a parallel experiment, the distribution of three subunits of the OST complex across similar sucrose gradients was analyzed following the treatment of microsomes with 50 μM BM and solubilization in 1% C_{12}E_{8} and A-glycosylation (6). The bulk of ribophorin II was in fractions 3–7 (Fig. 7D), and the bulk of OST48 was in fractions 4–10 (Fig. 7E). Taken together, these data indicate that the ribophorin I subunits that can be cross-linked to APP-C99’(Cys\(^{59}\)) polypeptides are present in the context of larger...
oligomeric complexes. These complexes are most likely equivalent to one or more of the distinct OST isoforms as defined by Kelleher et al. (6).

Post-translocon Association with Ribophorin I Is Not Unique to APP-C99 but Is Restricted—To establish whether the association of APP-C99(Cys59) with ribophorin I reflected a specific post-translocon interaction with a particular nascent chain or a more general facet of membrane protein synthesis, its association with other single spanning integral membrane polypeptides was investigated. For these studies individual TM regions

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**Fig. 5. Ribophorin I and SPC25 are associated exclusively with APP-C99 chains released from the ribosome.** Membrane-associated integration intermediates of APP-C99(Cys59) were isolated after synthesis for between 5 and 60 min followed by cycloheximide treatment as indicated. BMH was added to the integration intermediates, and, after quenching with 2-mercaptoethanol, the samples were solubilized in buffer containing C12E8. The ribosome-bound chains and their associated adducts were then isolated by pelleting through a sucrose cushion, leaving free chains and their associated adducts in the supernatant fraction. The resulting pellet and supernatant fractions were then either analyzed for the presence of ribosomal proteins L23a and L35 by immunoblotting (A), or specific cross-linking products with Sec61β (B), ribophorin I (RI) (C), and SPC25 (D) were recovered by immunoprecipitation from each fraction at each time point. A 40-kDa product was consistently detected in the pellet fraction (labeled N/S in B, C, and D). We believe that this most likely represents the cross-linking of APP-C99(Cys59) to a ribosomal protein that is recovered nonspecifically during immunoprecipitation (15).
derived from the polytopic membrane protein opsin were used (29), each containing a single cysteine residue in their cytosolic domains equivalent to that in APP-C99/Cys\(^{59}\). In the case of OP91/TM1HA[Cys\(^{65}\)], which uses opsin TM1 for both ER targeting and subsequent membrane integration, puromycin-treated nascent chains showed no cross-linking to either the Sec61\(\alpha\) or Sec61\(\beta\) subunits of the Sec61 complex (Fig. 8A, lanes 3 and 4) confirming their efficient exit from the ER translocon. In contrast, the puromycin-treated OP91/TM1HA[Cys\(^{65}\)] chains were cross-linked to ribophorin I (Fig. 8A, lane 6). Several other BMH-dependent cross-linking products were also seen after puromycin treatment (Fig. 8A, lane 2), but an adduct

|          | APP-C99' | APP-C99'.1CHO |
|----------|----------|---------------|
| αRI      | -        | -             |
| PNGase F | -        | -             |
| BMH      | -        | -             |
| Puromycin| -        | -             |

**Fig. 6.** The association of APP-C99' with ribophorin I is unaffected by N-glycosylation. Membrane-associated integration intermediates of APP-C99'[Cys\(^{59}\)] (A and C) or a version with a consensus site for N-glycosylation (B and C) were incubated with 2 mM cycloheximide (lanes 1–5) or 2 mM puromycin and 50 mM EDTA (lane 6) for 10 min at 30°C, and the samples were then treated with 50 μM BMH or an Me\(\text{2}SO\) control as indicated. In A and B, the total products before or after BMH treatment (lanes 1 and 2) or adducts with ribophorin I recovered by immunoprecipitation (lanes 5 and 6) were analyzed, and the sensitivity of the nascent chain to digestion with PNGase was determined (lanes 3 and 4) using a 14% polyacrylamide gel. In C, the total membrane-associated products of APP-C99' and APP-C99'.1CHO are shown with and without BMH treatment (lanes 1, 2, 5, and 6). Ribophorin I adducts were also immunoprecipitated and analyzed before (lanes 3 and 7) or after (lanes 4 and 8) treatment with PNGase. In this case, samples were resolved on a 10% polyacrylamide gel to better distinguish alterations in the mobility of the ribophorin I adducts (RI) resulting from differences in the number of N-linked glycans attached to the cross-linking products.
with a C-terminal HA epitope tag was expressed, and the cells were then solubilized in buffer containing 1% C12E8, a non-ionic detergent known to preserve OST subcomplexes that retain enzymatic activity in vitro (6, 11) and to maintain the APP-C99' (Cys59)/ribophorin I association detected in this study even in the absence of covalent cross-linking between the two partners (data not shown). The solubilized, HA-tagged APP-C99' chains were recovered by immunoprecipitation using an HA-specific monoclonal antibody, and the resulting material was analyzed by Western blotting. HA-tagged APP-C99' (Cys59) was specifically recovered from transfected but not from mock-transfected cells (Fig. 9A, cf. lanes 1 and 2). More significantly, when the HA-tagged APP-C99' (Cys59) was analyzed for cellular components that were co-immunoprecipitated with the protein, a significant amount of ribophorin I was found to be present (Fig. 9B, lane 1) but was absent in the mock-transfected sample that expressed no APP-C99' (Cys59)/HA (Fig. 9B, lane 2).

To further address the specificity of the association between APP-C99' (Cys59)/HA and ribophorin I, we investigated whether other major ER components were also associated with this material after immunoprecipitation in buffers containing 1% C12E8. Although cross-linking of APP-C99' (Cys59) to SPC25 had been detected (cf. Figs. 4C and 5D), SPC25 did not co-isolate with APP-C99' (Cys59)/HA under these conditions (Fig. 9C, lane 1) despite a clear signal from the total material before immunoprecipitation (Fig. 9C, lane 3). Likewise no Sec61β subunit was co-precipitated with the HA-tagged APP-C99' (Cys59)/HA polypeptide (data not shown). These data indicate that during the biosynthesis and maturation of APP-C99' (Cys59)/HA in transfected mammalian cells, the protein displays a specific association with ribophorin I such that the two components can be co-precipitated when the cellular membranes are solubilized with a suitable non-ionic detergent. Taken together, the results described above identify a specific interaction between certain newly synthesized membrane proteins and ribophorin I that occurs both in vitro and in vivo.

**DISCUSSION**

During its integration at the ER membrane, we observed cross-linking of ribosome-bound APP-C99' integration intermediates to the translocon components Sec61α and Sec61β and found that these adducts were lost upon puromycin treatment, which promotes the lateral exit of the protein from the ER translocon into the lipid bilayer. This is entirely consistent with the Sec61 complex acting as the major site for the integration of both single spanning and polytopic membrane proteins (1, 2, 5, 31). The principal focus of this study was to examine the environment of a single TM-spanning protein after it has exited the Sec61 translocon and become integrated into the ER membrane. Using a single cysteine probe located at the cytosolic face of the TM region we identified adducts with a specific subset of ER components following their integration at the ER translocon.

**Ribophorin I Associates with APP-C99' in Vivo**—To date, our analysis of the association between ribophorin I and newly synthesized membrane proteins had been carried out in vitro. We wished to investigate the biological significance of this observation by investigating whether such an association was also observed in vivo. For this reason, we transiently expressed APP-C99' (Cys59) in cultured mammalian cells and used a co-immunoprecipitation approach to detect interacting cellular components (30). In this case, a version of APP-C99' (Cys59)
that at least some TM domains visit distinct environments even within the context of the ER translocon (15, 32). The two distinct environments we resolved in this study are consistent with a model proposed from the recent high resolution structure of the Sec61 complex where the TM domain would laterally exit the translocon via a relatively confined route between the rearranged TM domains of the Sec61/Sec61/HR925 subunit (31). We assume that cross-linking to ribophorin I and SPC25 represents a stage of biosynthesis beyond the point of putative TM exit.

To address the specificity of the adducts formed by fully integrated APP-C99/HR11032[Cys59], two other model single spanning membrane proteins with single cysteine probes located in equivalent positions were generated and analyzed by cross-linking. While by no means a comprehensive study, this comparison showed that different TM-spanning polypeptides were associated with different subsets of ER components. Hence opsin TM1 was cross-linked to ribophorin I but not to SPC25, while opsin TM5 was cross-linked to neither of these ER proteins but formed adducts with several unknown components. We conclude that after release from the Sec61 translocon, different newly integrated membrane proteins have the potential to associate with distinct ER components. Interestingly a study of N-terminal signal sequences suggested that information encoded by the signal sequence could influence the subsequent maturation pathway taken by the nascent polypeptide (33), and our data suggest that the same may also be true for TM domains.

While we analyzed only a limited number of membrane proteins, it is tempting to speculate that the association of specific TM domains with ribophorin I is the result of particular features they possess. For example, while both APP-C99/HR11032 and opsin TM1 both contain multiple threonine residues toward their C termini, opsin TM5 has none. Whether the association of newly synthesized membrane proteins with ribophorin I is dependent upon such a simplistic motif or whether there are more subtle requirements such as the hydrophobic contacts proposed to form a dolichol binding site within the ribophorin I TM domain (11) remains to be established. In the case of SPC25, we have previously reported it as a cross-linking partner of newly synthesized membrane proteins and suggested that hydrophobic regions such as TM domains may be “scanned” by the signal peptidase complex for potential cleavage sites in the adjacent ER luminal domain (5). Our current data would support this hypothesis but suggest that either not all hydrophobic regions interact or that in some cases, such as

![Diagram](image-url)
and lysed in immunoprecipitation buffer containing 1% C12E8. APP-[Cys59] derivative in a mammalian expression vector (lane 1) or mock-transfected (lane 2) COS-7 cells were harvested 18 h after transfection and subjected to immunoprecipitation. A major issue was whether the association of certain membrane protein precursors with ribophorin I that we detected by cross-linking in vitro reflected a biologically significant interaction that occurred in living cells. To address this point we attempted to recapitulate the interaction in vivo by transiently expressing an epitope-tagged version of APP-C99’[Cys59], solubilizing the mammalian cells in a non-ionic detergent, immunoenriching the APP-C99’[Cys59]HA, and analyzing the resulting material for co-purifying components. We found that ribophorin I co-purified with APP-C99’[Cys59]HA, while SPC25 and Sec61β did not. Thus, ribophorin I can form detergent-stable complexes with membrane proteins synthesized at the ER consistent with a specific binding event occurring in vivo. Significantly two other studies have also provided evidence that ribophorin I is bound to membrane proteins made at the ER. In the first case the integral membrane protein angiotensin-converting enzyme was found to be co-isolated with ribophorin I during studies of its biosynthesis (34), and in the second case a recent study of the human cytomegalovirus protein US11 also identified ribophorin I in a stable complex with this protein (35). In this latter study, two other subunits of the OST complex, ribophorin II and OST48, also co-purified with the US11 protein consistent with our proposal that these associations are with a subcomplex of the OST.

Taken together, our data suggest a model where ribophorin I may act to stochastically delay potential membrane protein substrates in the proximity of the oligosaccharyltransferase and thereby increase their opportunity for N-glycosylation by the catalytic STT3 subunit (6, 8, 10). The association of potential membrane protein substrates with ribophorin I might also act to minimize any potential competition between protein folding and N-glycosylation (36). However, our limited data set indicates that an association with ribophorin I may not be essential for efficient N-glycosylation since the N-terminal domain of bovine opsin (residues 1–15) is efficiently glycosylated whether it is attached to a polypeptide that does associate with ribophorin I (opsin TM1) or one that does not (opsin TM5). The idea that ribophorin I may facilitate N-glycosylation rather than playing an essential role in this process is further supported by the fact that prokaryotes lack an obvious ribophorin I equivalent and carry out an N-glycosylation reaction that appears to be solely dependent upon an STT3 homologue (10).

Our data are consistent with a possible role for ribophorin I in substrate retention at the OST but provide no evidence that such an interaction directly influences the propensity of the substrate to be N-glycosylated. This and the relative stability of the in vivo association between ribophorin I and several different membrane proteins (34, 35) raise the possibility that ribophorin I and perhaps other subunits of the OST complex may have functions distinct from, or additional to, their as yet poorly defined contribution to N-glycosylation (6, 9). The human cytomegalovirus US11 protein is associated with the ER chaperones BiP and calnexin plus p97, a member of the AAA (ATPases associated with a variety of cellular activities) super-

![Figure 9: Ribophorin I interacts with membrane proteins in vivo.](https://example.com/figure9.png)

**Fig. 9.** Ribophorin I interacts with membrane proteins in vivo. COS-7 cells were transfected with an HA-tagged APP-C99’[Cys59] derivative in a mammalian expression vector (lane 1) or mock-transfected (lane 2) as shown (A–C). Cells were harvested 18 h after transfection and lysed in immunoprecipitation buffer containing 1% C12E8. APP-C99’[Cys59]HA and associated cellular components were then recovered by immunoprecipitation using a monoclonal antibody specific for the HA epitope tag. The resulting material was analyzed by Western blotting with antisera recognizing APP-C99’ (A), ribophorin I (RI) (B), or SPC25 (C). A fraction of the total cellular extract from the transfected cells (~1/10) used for immunoprecipitation was included as a control (lane 3). IP, immunoprecipitation.
family, in addition to ribophorin I, ribophorin II, and OST48 (35). This raises the intriguing possibility of a link between subunits of the OST complex and quality control processes at the ER (37, 38). Clearly a full understanding of the molecular basis for the association of ribophorin I with specific membrane proteins present in the ER will require a better understanding of ribophorin I function, and our future efforts will be directed toward this goal.

**Acknowledgments**—We thank Konrad Beyreuther for supplying the APP-C99 cDNA, Ervin Ivessa for anti-ribophorin I serum, Bernhard Dobberstein and Richard Zimmerman for anti-Sec61α and -Sec61β sera, Mark Shearman and Dirk Beher for the WO2 antiserum, and Martin Pool for the antibodies recognizing ribosomal proteins. We thank Neil Bulleid, Martin Pool, Markus Aebi, and Lisa Swanton for invaluable comments at various stages during the preparation of this manuscript.

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J. Biol. Chem. 2005, 280:4195-4206.
doi: 10.1074/jbc.M410329200 originally published online November 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410329200

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