Endoplasmic reticulum proteins involved in glycosylphosphatidylinositol-anchor attachment

Photocrosslinking studies in a cell-free system

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Assembly of glycosylphosphatidylinositol (GPI-anchored proteins requires translocation of the nascent polypeptide chain across the endoplasmic reticulum (ER) membrane and replacement of the C-terminal signal sequence with a GPI-moiety. The anchoring reaction is carried out by an ER enzyme, GPI transamidase. Genetic studies with yeast indicate that the transamidase consists of a dynamic complex of at least two subunits, Gaa1p and Gpi8p. To study the GPI-anchoring reaction, we used a small reporter protein that becomes GPI-anchored when the corresponding mRNA is translated in the presence of microsomes, in conjunction with site-specific photocrosslinking to identify ER membrane components that are proximal to the reporter during its conversion to a GPI-anchored protein. We generated variants of the reporter protein such that upon in vitro translation in the presence of $N^\omega$-(5-azido-2-nitrobenzoyl)-lysyl-tRNA, photoreactive lysine residues would be incorporated in the protein specifically near the GPI-anchoring site. We analyzed photoproducts resulting from UV irradiation of the samples. We show that proproteins can be crosslinked to the transamidase subunit Gpi8p, as well as to ER proteins of molecular mass ≈ 60 kDa, ≈ 70 kDa, and ≈ 120 kDa. The identification of a photoproduct between a proprotein and Gpi8p provides the first direct evidence of an interaction between a proprotein substrate and one of the genetically identified transamidase subunits. The ≈ 70-kDa protein that we identified may correspond to the other subunit Gaa1p, while the other proteins possibly represent additional, hitherto unidentified subunits of the mammalian GPI transamidase complex.

Keywords: endoplasmic reticulum; glycosylphosphatidylinositol; photocrosslinking; transamidase; translocon.

We are interested in the mechanism by which glycosylphosphatidylinositol (GPI) anchors are attached to endoplasmic reticulum (ER)-translocated proteins bearing a C-terminal, GPI-directed signal sequence [1–3]. Attachment of a GPI anchor occurs rapidly upon completion of translation and ER translocation, and involves replacement of the C-terminal signal sequence in the proprotein with a preformed GPI anchor. The reaction occurs in the lumenal leaflet of the ER.

GPI-anchoring has been reproduced in vitro using endogenous or newly translocated membrane proteins as substrates for GPI anchors [4–9]. Based on analyses of these cell-free systems it is clear that GPI anchors are attached via a transamidation reaction mechanism where small nucleophiles, such as hydrazine, can substitute for the GPI moiety [8,10]. Genetic approaches in yeast and mammalian cells have led to the identification of two gene products (Gaa1p and Gpi8p) that appear to be required for GPI-anchoring [11–17]. The two proteins probably exist as a complex in the ER, as immunoprecipitation experiments using epitope-tagged versions of Gaa1p and Gpi8p demonstrate a physical association between the two proteins after detergent solubilization of membranes [15]. Furthermore, it is likely that the complex is dynamic (dissociable) as dominant negative constructs of Gpi8p can displace endogenous Gpi8p to yield inactive enzyme [16]. However, the exact function of these proteins, the nature of their functional interaction with each other and with the proprotein and GPI substrates, the stoichiometry of the proteins in the transamidase complex, and, indeed, whether the transamidase complex contains other protein subunits remain to be determined.

In order to begin to address some of these issues, we adopted a site-specific photocrosslinking approach that would allow us to identify ER proteins that appear in close proximity to polypeptide chains during the process of ER translocation and GPI-anchoring. This approach has been used successfully in a number of laboratories to identify components of the ER protein translocon [18–21], and to study the mechanism of translocation and membrane integration of polypeptides in a variety of membrane systems. Using this strategy we expected that we would be in a position to demonstrate a physical association between an ER-translocated polypeptide and the genetically identified GPI anchor.
components, and also to uncover additional proteins (possibly other subunits of the transamidase) involved in the processing reaction.

Our experiments utilized a small reporter protein, prepromini-placental alkaline phosphatase (prepprominiPLAP), lacking N-glycosylation sites and containing N-terminal and C-terminal signal sequences for ER targeting and GPtdIns attachment [5]. Work by Udenfriend and colleagues showed that preprominiPLAP becomes GPtdIns-anchored in a cell-free translation-translocation system [5,6]. We generated preprominiPLAP variants such that upon \textit{in vitro} translation in the presence of $N^\epsilon$-(5-azido-2-nitrobenzoyl)-Lys-tRNA (εANB-Lys-tRNA), photoreactive lysine residues were incorporated at various specific sites in the protein near the GPtdIns-attachment site. We expected that upon photolysis, the (pro)miniPLAP reporter would be crosslinked to ER proteins in its immediate vicinity and that these proteins would be likely to be involved in the translocation and GPtdIns-anchoring of the reporter. An important advantage of this method in comparison to the detergent-solubilization and coimmunoprecipitation strategies mentioned above is that \textit{in situ} associations are detected. Our analyses provide the first evidence of a physical interaction between the genetically identified transamidase component Gpi8p and a proprotein substrate, and suggest that the transamidase contains additional subunits.

**MATERIALS AND METHODS**

**Plasmids and transcription**

The recombinant vector pGEM-4Z miniPLAPωSer-179 (a gift from S. Udenfriend [5]) was used to generate the various miniPLAP constructs (Fig. 1). Substitution of lysine codons with arginine or histidine codons, as well as introduction of new lysine codons within the miniPLAP coding region, was done by overlap extension mutagenesis using PCR. The PCR products were digested with EcoRI and HindIII and subcloned into the pGEM-4Z vector (Promega). The resulting plasmids (termed pmnP X-Lys) contain sequences encoding miniPLAP variants (residues $-17$ to $+209$) with appropriately positioned lysine codons as well as a HindIII restriction site placed adjacent to the termination codon.

To generate chimeric miniPLAPs that would yield mRNAs more suitable for translation in the wheatgerm system, the miniPLAP N-terminal sequence coding fragment was replaced with a PCR-amplified fragment (derived from pGEM-4Z/111p [20,22]) containing the 5'-UTR and N-terminal signal sequence of bovine preprolactin. The preprolactin sequence was introduced by overlap extension PCR. PCR-amplified fragments were digested with BstXI and HindIII, and subcloned into the pGEM-4Z vector. The

![Fig. 1. Schematic representation of protein constructs.](image-url)

(A) Sequence of the hybrid protein, p11, containing the N-terminal signal sequence of preprolactin (residues $-22$ to $+3$ of the mature protein) fused to residues $+5$ to $+209$ of preprominiPLAP. Lysine residues in the sequence are highlighted. The N-terminal signal sequence (amino acids $-22$ to $-1$) and C-terminal GPtdIns signal sequence (amino acids $178$ to $207$) are highlighted. The GPtdIns-attachment site ($\omega$ amino acid; $\omega178$) is indicated. (B) Schematic representation of constructs derived from p11. The N-terminal signal sequence is in gray and the GPtdIns signal sequence is hatched. Lysine residues in p11 are indicated by the tick marks. These lysine residues were converted to arginine or histidine residues (except for K96 in construct p6) as described in Materials and methods, and new lysine residues were introduced at indicated sites to generate p1, p2, p3, p5, p6, and p7. Proteins p5 and p7 have additional extensions of 43 amino acids (shown in white; sequence 208RSVPWOSCPPPEFSACPPVVLPEGQPVVPPESSPPESTLRGPA250) Proteins p6, p5 and p7 were truncated in the coding region (indicated by a vertical squiggle) to create integration intermediates attached to ribosomes. (C) Proposed membrane organization of p2, p6, and p5. The predicted localization of lysine residues is based on the assumption that 40 amino acids are buried in the ribosomal tunnel, and 30 amino acids are required to span the membrane bilayer in an $\alpha$-helical conformation. In this scenario, the $\omega$ site is depicted at the luminal surface of the ER membrane. An alternative possibility for p2 would be to have the $\omega$ site projecting by seven amino acids into the ER lumen such that the membrane-associated portion of the GPtdIns signal corresponds only to the hydrophobic stretch (amino acids 186–207).
resulting plasmids, termed P111/nmPLAP (p1-p11), contain sequences encoding miniPLAP variants with a preprolactin 5′-UTR and N-terminal sequence (encoding residues +22 to +83 of the mature protein), followed by a mNP sequence (encoding amino acids +84 to +209 of miniPLAP).

Truncated miniPLAP constructs were generated by PCR using pUC/M13 forward primer paired with an oligonucleotide directed against a defined region in miniPLAP and containing a HindIII restriction site. C-terminal miniPLAP extension mutants were derived from P111/nmPLAP by PCR-directed substitution of the stop codon with an arginine codon, followed by PCR amplification using pUC/M13 forward primer with an external primer containing a HindIII restriction site. The nucleotide sequences of the subcloned fragments were confirmed by dideoxy sequencing.

The resulting HindIII linearized DNAs were transcribed in vitro with SP6 RNA polymerase (Promega) in the presence of m7G(5′)ppp(5′)G (New England Biolabs). mRNAs were purified using the RNaIid purification reagents (BIO101).

Cell culture and DNA transfection

The Thy1null mouse thymocyte cell line BW5147.3 was maintained in suspension culture as previously described [23]. HeLa cells were grown in 150 cm² cell culture dishes under the same conditions. For transfection experiments cells were grown to 80% confluency in 175 cm² dishes. FLAG-tagged Gaa1p or FLAG-tagged Gpi8p expression constructs (subcloned into a PME-Pyori18SF-expression vector [15]) were generously provided by T. Kinoshita (Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Japan). Transfection of expression vectors was performed using fugegene-6 transfection reagent (Boehringer Mannheim) following the protocol provided by the manufacturer. 24–36 h after transfection, cells were rinsed with NaCl/IP, and used for microscope preparation. Microsomes from mouse thymoma cells, or HeLa cells expressing Gaa1p-FLAG and Gpi8p-FLAG, were prepared as described previously [23].

GPtdIns-anchoring in a cell-free system

P111/nmPLAP (p1-p11) mRNAs were translated using a nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of rough microsomes and [35S]methionine according to previously published procedures [5,23]. Translation-translocation was carried out for 20–120 min at 26 °C. Proteinase K treatment was performed on ice for 20 min using the protease at a final concentration of 100 µg mL⁻¹. Proteinase K treatment was performed on ice for 20 min using the protease at a final concentration of 100 µg mL⁻¹. Proteolysis was stopped by adding phenylmethylsulfonyl fluoride (3 mM final concentration, from a 150-mM stock in ethanol) and membranes were recovered by centrifugation through a 0.5-m sucrose cushion (Beckman TLA 100.1 rotor, 200 000 g, 30 min, 4 °C). The membrane pellet was resuspended in SDS-sample buffer and analyzed by SDS/PAGE and fluorography.

Photocrosslinking and analysis

Translation and photolysis were performed following previously described protocols [20,22]. Briefly, mRNAs were translated in vitro in 25–100 µL of a reaction mixture containing wheatgerm extract, microsomes derived from the cell lines described above, single recognition particle (SRP) prepared from dog pancreas, eANB-Lys-tRNA and [35S]methionine. Translations were performed for 20–90 min at 26 °C. After translation, samples were placed on ice and photolyzed (15 min at 0 °C) [20]. To test for the light dependence of crosslinking, control reactions were not photolyzed or assay mixtures were prepared using pre-irradiated eANB-Lys-tRNA. Following photolysis, samples were diluted two- to threefold with buffer A (0.25 mM sucrose, 10 mM Hepes/NaOH, pH 7.5) and pelleted through a 0.5-m sucrose cushion in a Beckman airfuge. The microsomal pellets were analyzed directly or after being subjected to immunoprecipitation. Immunoprecipitations were carried out on 100-µL reactions. Sec61α was immunoprecipitated as described elsewhere [22]. For samples containing FLAG-tagged Gaa1p and Gpi8p, the membrane pellets from a 100-µL translation were solubilized at 65 °C for 30 min in 50 µL 100 mM Tris/HCl (pH 7.5) containing 0.2% (w/v) SDS. The samples were then diluted to 750 µL with IP buffer [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% (w/v) Triton X-100] and 25 µL of anti-FLAG M2-agarose affinity gel (Sigma) was added. Before use the agarose beads were preincubated with 15% (w/v) BSA for 2 h at room temperature. The samples were then rocked overnight at 4 °C. The samples were then washed three times with 750 µL of IP buffer and twice with IP buffer without detergent. SDS/PAGE analysis of photocrosslinked products was done using 7–12.5% or 10–15% acrylamide gradient gels. The radioactivity in dried gels was quantified by phosphorimager. The efficiency of immunoprecipitation was ≈25% with anti-Sec61α Ig and ≈75% with anti-FLAG Ig.

RESULTS AND DISCUSSION

Experimental design

In order to use photocrosslinking methods to identify ER proteins involved in processing polypeptide chains to GPtdIns-anchored proteins, preproproteins were engineered with strategically placed lysine residues, and photoactive probes were incorporated into these proteins by translating the corresponding mRNA in the presence of eANB-Lys-tRNA. The use of this chemically modified aminocyclitRNA results in the incorporation of a photoreactive lysine into the corresponding mRNA in the presence of eANB-Lys-tRNA. The use of this chemically modified aminocyclitRNA results in the incorporation of a photoreactive lysine into the nascent chain wherever in-frame lysine codons occur in the mRNA [18–21]. The preproprotein that we chose for these experiments is preprominiPLAP, a model protein engineered from placental alkaline phosphatase [5]. To ensure efficient translation and proper ER targeting in a wheatgerm translation system supplemented with mammalian cell microsomes, the 5′-UTR and N-terminal signal sequence of preprominiPLAP were replaced with the corresponding sequence of a preprolactin mutant previously shown to be effective for this purpose [20]. The preprominiPLAP variant thus generated is referred to as protein p11 (Fig. 1A). Variants of p11 (termed p1, p2 and p3) were also generated in which all the lysine residues in p11 were systematically replaced with arginines or histidines, and new lysine residues were introduced at
specific locations near the GPtdIns-attachment site (serine 178, the ω site) (Fig. 1B).

An additional set of p11-derived proteins was produced with the aim of creating translocation intermediates that remain bound to ribosomes. In order to do this the mRNAs were truncated in the coding region, resulting in the elimination of the stop codon [18]. One such intermediate, p6 (Fig. 1B), corresponds to a protein that is truncated N-terminal to the ω site, and is therefore expected to stay within the Sec61 translocation channel while remaining bound to the ribosome (Fig. 1C). To generate fully assembled intermediates containing a functional GPtdIns-directing signal sequence, truncated mRNAs were prepared that corresponded to C-terminally extended derivatives of p11. Based on estimates that the ribosomal cleft protects 20-30 residues are required to span the membrane bilayer in α-helical configuration, we prepared truncated mRNAs that encoded proteins with a 43 amino-acid extension C-terminal to the GPtdIns signal sequence. The length of this extension should be sufficient to expose the GPtdIns-modification site to the lumen of ER for further processing events. The proteins p5 and p7 depicted in Fig. 1B represent variants of p3 (Fig. 1B) with an extra 43 amino acids at the C-terminus. Additionally, the p7 protein differs from p5 in that a leucine residue (L194) in the hydrophobic stretch of the GPtdIns signal sequence is replaced by a lysine residue.

**Fig. 2.** Cell-free processing of p11 variants to GPtdIns-anchored proteins. (A) Full-length mRNAs corresponding to each of the indicated proteins were translated using a rabbit reticulocyte lysate in the presence of thymoma cell microsomes for 90 min at 26 °C. Samples were then placed on ice, treated with proteinase K and analyzed by SDS/PAGE (12.5% gel) and fluorography. Processed proteins correspond to N-terminal signal sequence cleaved pro-pX and GPtdIns-anchored pX/GPtdIns (X is 1, 2, 3, or 11, as indicated). Of the four proteins shown, only p1 (lane 1) is not converted to p1/GPtdIns. (B) mRNAs corresponding to the proteins p7 and p5 were translated in an SRP-supplemented wheatgerm lysate in the presence of thymoma microsomes for 90 min at 26 °C (p5 and p7 contain 43 amino-acid extensions C-terminal to the GPtdIns signal sequence (Fig. 1B), and lack stop codons). The p5/GPtdIns product was identified by predicted gel mobility and also via experiments in which the assays, when supplemented with hydrazine [8,10], yielded p5-hydrazide (a characteristically fast-moving product) rather than p5/GPtdIns. The p7 protein cannot be processed to a GPtdIns-anchored form. The predicted membrane topology of ribosome-attached p5 is shown in Fig. 1C.

**Translocation and processing of p11 and p11 variants**

When preprominiPLAP mRNA is translated in the presence of ER-derived thymoma microsomes, two protease-protected (membrane translocated) proteins are generated, corresponding to N-terminal-signal-sequence-cleaved prominiPLAP and mature, GPtdIns-anchored miniPLAP [5,10,23]. To determine if the changes that we made in the primary structure of preprominiPLAP (Fig. 1B) affected its ability to be GPtdIns-anchored, we translated the different mRNA variants using both rabbit reticulocyte lysate and wheatgerm translation systems in the presence of thymoma microsomes. Assays were carried out for 90 min at 26 °C (Fig. 2A, 2), and the production of protease-protected GPtdIns-anchored proteins was monitored.

As shown in Fig. 2A (lane 4) substitution of the preprominiPLAP signal sequence with a lysine-free preprolactin signal sequence (to yield protein p11) had no effect on GPtdIns-anchoring; roughly 50% of translocated and proteinase K-protected p11 (pro-p11) was converted to a ~25-kDa band corresponding to GPtdIns-anchored p11 (p11/GPtdIns). The efficiency of conversion corresponds to that reported previously for wild-type miniPLAP [6]. When the assays were carried out in the presence of hydrazine [10], the yield of the GPtdIns-anchored miniPLAP product was reduced and a new, lower molecular-mass band (~23 kDa), corresponding to miniPLAP-hydrazide was formed (data not shown).

All the lysine residues in p11 were replaced with arginine or histidine, and new lysine residues were introduced to generate p11 variants (Fig. 1B). The effect of these alterations on GPtdIns-anchoring varied depending on the location of the new lysine residues. Introduction of a lysine residue (replacing arginine) at the ω +6 position in otherwise lysine-free protein p2 had no effect on GPtdIns-anchoring (Fig. 2A, lane 2), while exchange of threonine to lysine at the ω -2 position (protein p1) completely inhibited the conversion of the proprotein to a GPtdIns-anchored form (Fig. 2A, lane 1). However, the double substitution resulting in the introduction of lysine residues at both ω +6 and ω -2 (protein p3), allowed GPtdIns-anchoring albeit at a somewhat lower efficiency (Fig. 2A, lane 3).

Kinetic analyses established that the proproteins were converted to GPtdIns-proteins on a time scale similar to that originally described for prominiPLAP. GPtdIns-anchored p2 and p3 were first detected ≤20 min after initiating the translation-translocation reaction, and the yield of these GPtdIns-anchored proteins increased steadily until the assay was terminated at 90 min (Fig. 3C). These results resemble those reported originally by Kodukula et al. [5,6] and confirm that the lysine substitutions in p2 and p3 have little or no effect on the kinetics and efficiency of processing of these polypeptides to GPtdIns-anchored proteins.

**Processing of ribosome-bound intermediates**

When in vitro translation-translocation assays are programmed with mRNAs truncated in the coding region, fully assembled translocation intermediates are created in which the polypeptide chain remains bound to the ribosome [20]...
(Fig. 1C, variant p5, for example). As the GPTdIns signal sequence is typically 18–32 amino acids long, under normal circumstances translation has to be completed and the nascent chain has to be released from the ribosome before GPTdIns attachment can proceed. However, it has been suggested that an internally located GPTdIns signal sequence can be recognized by the transamidase machinery as chimeric proteins with the GPTdIns signal sequence in the middle of the polypeptide chain can be processed, albeit inefficiently, to mature GPTdIns-anchored proteins [26,27]. We took advantage of this possibility to determine if translation termination is a prerequisite for GPTdIns-anchoring, and whether the GPTdIns signal sequence could be recognized by the transamidase when the polypeptide chain remained bound to the ribosome. In order to do this, translocation intermediates (p5 and p7, Fig. 1B) containing an internal GPTdIns signal sequence were generated as described above, and their processing to mature GPTdIns-anchored proteins was examined.

The p5 and p7 polypeptide chains contain a 43 amino-acid extension following the C-terminal GPTdIns signal sequence and are attached to the ribosome (Fig. 1B and C). Fig. 2B shows that p5 is recognized and converted to a GPTdIns-anchored protein (identified by its characteristic gel mobility and via experiments using hydrazine [10] in which p5-hydrazide rather than p5/GPTdIns was formed), while p7 with a lysine residue at o + 16 is not. The lack of processing seen with p7 is consistent with previously reported data indicating that changes in the profile of the hydrophobic segment of the GPTdIns signal sequence have a direct effect on GPTdIns-anchoring [28–32]. We conclude that ribosome-bound translocation intermediates with a functional, albeit internal, GPTdIns signal sequence can be recognized and processed to GPTdIns-anchored proteins. While this conclusion would suggest that the transamidase is able to recognize ribosome-bound proteins and is therefore positioned close to the translocon, closer examination of Fig. 2B indicates a potential complication. In addition to the radiolabeled bands corresponding to pro-p5 and p5/GPTdIns, Fig. 2B (lane 2) contains a number of other bands of intermediate molecular mass. These bands might represent processing intermediates that have been proteolytically trimmed in their extra-membrane C-terminal extension sequences, while still retaining their GPTdIns signal sequence. Thus it is possible that p5 is dislodged from the ribosome (and consequently, the translocon) by proteolysis at sites in the extra-membrane C-terminal extension; once release is effected, the trimmed p5 protein with its intact GPTdIns signal sequence could be recognized and processed by a distally located transamidase. In an attempt to test this idea, we used two approaches to dislodge the ribosome-attached proprotein and improve the efficiency of the putative proteolytic event. In the first approach we stopped translation-translocation assays after 30 min, stripped off ribosomes by high-salt/EDTA treatment on ice and trypsinized the membranes before continuing the incubation at 26 °C. The results showed that while ~30% of pro-p5 was converted to a faster migrating proteolytically trimmed product, there was no detectable increase in production of p5/GPTdIns (data not shown). In the second approach we treated samples with puromycin to release ribosome-bound chains; again, there was no improvement in the yield of GPTdIns-anchored protein.
Fig. 3 shows photocrosslinking experiments with the proteins p2 and p3, carried out over the time course of GpDIns-anchoring in the translation-translocation system (note the increasing production of p2/GpDIns and p3/GpDIns, starting 20 min after initiation of translation-translocation; quantitative data on the production of proproteins and GpDIns-anchored proteins are shown in Fig. 3C and D). For both proteins, a crosslinked product of \( \approx 66 \) kDa was detected in samples that were photolyzed after a translation-translocation reaction at least 10 min (Fig. 3, lanes 6–8 and 10–12), correlating well with the kinetics of processing to p2/GpDIns and p3/GpDIns. The \( \approx 66 \)-kDa photoadduct was not seen if pre-irradiated \( \varepsilon \)ANB-Lys-tRNA was used in the translation mixture (Fig. 3B). The molecular mass of the photoadduct suggests a crosslink between pro-p2 or pro-p3, and a target protein of \( \approx 40 \) kDa (Table 1). The time course over which the crosslink is detected suggests that the \( \approx 40 \)-kDa protein is in the immediate vicinity of the proproteins as they are translocated as in panel A, except that the samples were photolyzed after a 40-min incubation. The samples were analyzed by SDS/PAGE (10–15% gradient gel) and fluorography. Gel lanes containing molecular mass markers are indicated as M. Photoadducts in the vicinity of the 66-kDa marker as well as an adduct of mass \( \approx 140 \) kDa are indicated by various symbols (see Table 1 for a summary of photoadducts). The gel-migration positions of p2, p2/GpDIns, p6 and p7 are indicated by the white arrowheads. (B) The polypeptides p2 and p5 were translated-translocated as in panel A, except that the samples were photolyzed after a 40-min incubation. The samples were analyzed by SDS/PAGE (7–12.5% gel) and fluorography. Photoadducts are indicated by marks and can also be seen in Fig. 6B, lanes 1 and 3.

**Photocrosslinking of full-length miniPLAP constructs (p2, p3) to ER proteins**

In order to identify ER membrane components that are in close proximity to the GpDIns signal sequence during translocation and processing, translation was carried out in the presence of \( \varepsilon \)ANB-Lys-tRNA. Messenger RNAs corresponding to the test proteins were used to program a translation-translocation system containing an SRP-supplemented wheat germ lysate, \( \varepsilon \)ANB-Lys-tRNA, [\( \text{\textsuperscript{35}} \text{S}\)]methionine, and thymoma microsomes. After translation-translocation, samples were photolyzed and analyzed directly by SDS/PAGE and fluorography.

Table 1. Summary of photocrosslinking results. Data are summarized from the Results section and Figs 3–6. The miniPLAP constructs used are described in Fig. 1B and C; p2 and p3 represent full-length polypeptides that are capable of acquiring a GpDIns anchor, p1 is a full-length polypeptide that cannot be GpDIns-anchored, and p5–p7 are ribosome-bound translocation intermediates of which only p5 can be GpDIns-anchored.

<table>
<thead>
<tr>
<th>MiniPLAP construct</th>
<th>Photoadduct (kDa)</th>
<th>Crosslinking partner (kDa)</th>
<th>Source of data (Figure number and symbol used)</th>
</tr>
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<tbody>
<tr>
<td>p2, p3</td>
<td>( \approx 66 )</td>
<td>( \approx 40 ) (Gpi8p)</td>
<td>3, 4A, 5, 6 (*)</td>
</tr>
<tr>
<td></td>
<td>( \approx 85 )</td>
<td>( \approx 60 )</td>
<td>6 (○)</td>
</tr>
<tr>
<td></td>
<td>( \approx 140–145 ) b</td>
<td>( \approx 115–120 )</td>
<td>3, 4A, 6 (○)</td>
</tr>
<tr>
<td>p1</td>
<td>( \approx 66 )</td>
<td>( \approx 40 ) (Gpi8p)</td>
<td>3 (*)</td>
</tr>
<tr>
<td></td>
<td>( \approx 100 )</td>
<td>( \approx 70 ) (Gaa1p?)</td>
<td>3 (▽)</td>
</tr>
<tr>
<td>p5</td>
<td>( \approx 72 ) (smear)</td>
<td>( \approx 40 ) (Gpi8p) c</td>
<td>4B, 5, 6 (*) d</td>
</tr>
<tr>
<td>p6</td>
<td>( \approx 60 )</td>
<td>( \approx 40 ) (Sec61α)</td>
<td>4A, 5 (●)</td>
</tr>
<tr>
<td>p7</td>
<td>( \approx 70 ) (smear)</td>
<td>( \approx 40 ) (Sec61α) c</td>
<td>4A, 5 (●)</td>
</tr>
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</table>

\( a \) The estimated molecular masses of the radiolabeled miniPLAP variants involved in the photoadducts are as follows: p1, p2, p3 \( \approx 26 \) kDa; p5, p7 \( \approx 31 \) kDa; p6 \( \approx 19 \) kDa. \( b \) The 145-kDa photoadduct is detected transiently in the course of the in vitro translation-translocation reaction; as translation-translocation ceases after \( \approx 20–40 \) min (see cessation of pro-pX production after 40 min in Fig. 3C), the reaction may be viewed as a pulse-chase experiment permitting detection of transient interactions. \( c \) The molecular mass of the crosslinking target was obtained by using the upper limit of the smeared adduct as visualized on the fluorogram. \( d \) Indicated as a square bracket in Fig. 6, lanes 2 and 4.

compared with untreated samples (data not shown). These results argue against the proteolysis model proposed above. Although more work needs to be done to clarify this issue, we tentatively conclude that artificially generated ribosome-bound proproteins with an internal GpDIns signal sequence are substrates for the GpDIns transamidase. This suggests that although the transamidase normally processes proteins that have been discharged from the ribosome–translocon complex, it is also able to access ribosome-bound proteins.
being converted to GPtdIns-anchored proteins. This result is consistent with the idea that the ~40-kDa protein may be directly involved in the GPtdIns-anchoring reaction. The molecular mass of the target further suggests that it may be the transamidase subunit Gpi8p.

Another photoadduct of molecular mass ~145 kDa (implying a crosslink between pro-p2 or pro-p3 and a near neighbor target protein of ~120 kDa) was detected in samples that had been translated-translocated for 40 min (Fig. 3A, lanes 7 and 11). Fig. 3F shows that the yield of the ~145-kDa adduct is much lower in samples that were photolyzed prior to or after the 40-min time point, suggesting that the interaction between pro-p2 or pro-p3 and the ~120-kDa target occurs transiently, possibly after initiation of the GPtdIns-anchoring reaction. However, there was some variability in the optimal time for obtaining a crosslink to the ~120-kDa protein as in other experiments the ~145-kDa adduct was detected at 20 min (Fig. 4A, lane 7, open circle), but not later (90 min [Fig. 4 panel A, lane 8]). Translation/translocation in the in vitro system slows down or ceases after ~20–40 min (possibly because of degradation of the enzymatic machinery or mRNA in the translation mix, or occupancy of all translocons), resulting in a pulse-chase scenario where it would be possible to detect a transient interaction as a pulse of prominiPLAP interacts with the ~120-kDa protein en route to the transamidase.

Other potential crosslinked products (Fig. 3) running at ~33 kDa (double arrowhead) and ~46 kDa (arrowhead), were found not to be photoadducts as they were present in samples that received pre-irradiated eANB-Lys-tRNA (Fig. 3B) or did not receive either UV light or eANB-Lys-tRNA (data not shown).

**Photocrosslinking of a miniPLAP variant (p1) that cannot be GPtdIns-anchored**

We carried out similar photocrosslinking experiments with p1, a miniPLAP variant bearing a lysine residue at the ω−2 position that blocks its conversion to GPtdIns-anchored form. Fig. 3A (lanes 3–4) and Fig. 3E show that, similar to p2 and p3, a photoadduct of ~66 kDa is formed with p1; as discussed above, this adduct may represent a complex between p1 and Gpi8p. Fig. 3A also shows that the ~145-kDa adduct seen transiently with p2 and p3 is not detected with p1. However, in addition to the ~66-kDa adduct seen with all three constructs (p1, p2, p3), a ~100-kDa photoadduct was seen in the p1 sample that was not detected with p2 or p3 (Fig. 3A, lanes 3–4, and Fig. 3G). The molecular mass of the adduct suggests a crosslink between p1 and a target protein of ~70 kDa, similar in size to the transamidase subunit Gaa1p.

Although this experiment does not allow us to prove conclusively that the ~70-kDa crosslinking target is Gaa1p, our results are consistent with the suggestion that, despite the inability to be GPtdIns-anchored, p1 is associated with both the genetically identified transamidase subunits. Our interpretation is also consistent with the conclusions of Aceto et al. [31] who showed that certain modifications of the GPtdIns signal sequence in the vicinity of the ω-site not only resulted in lack of GPtdIns-anchoring but also inhibited the processing of wild-type protein in a dose-dependent manner. Why is the ~100-kDa adduct not seen with p2 and p3? One explanation is that the ~70-kDa protein (the crosslinking partner leading to the ~100-kDa adduct) and Gpi8p recognize proproteins sequentially, with the ~70-kDa protein handing off proproteins to Gpi8p for conversion to GPtdIns-anchored proteins. As p1 cannot be GPtdIns-anchored it accumulates at Gpi8p and back up to the ~70-kDa protein accounting for crosslinks to both proteins. If the ~70-kDa protein is indeed Gaa1p, this suggests a functional role for Gaa1p in the transamidase complex.

**Photocrosslinking of miniPLAP translocation intermediates (p5, p6, p7) to ER proteins**

We next used photocrosslinking to investigate the near-neighbor-protein environment of the translocation intermediates p5, p6, and p7 (Fig. 1B and C), in comparison with that of the full-length protein p2 (Fig. 4). These experiments were carried out to provide additional specificity controls for our crosslinking procedure and also to test whether the functional and nonfunctional GPtdIns signals contained in p5 and p7, respectively, were to be found in the same ER protein environment. For example, proteins such as p6 that are truncated N-terminal to the GPtdIns signal sequence (Fig. 1B and C) are expected to be bound to the ribosome and in contact with the translocon component Sec61α. Similarly, p5 and p7 (Fig. 1B and C) are ribosome-bound and expected to be retained in the translocon, although p5 is clearly also recognized by the GPtdIns transamidase as it can be processed to p5/GPtdIns (Fig. 2B).

Fig. 4 (panel A, lanes 7–8 and panel B, lane 1) re-establishes that proteins of ~40 kDa and ~120 kDa can be photocrosslinked to p2, yielding adducts of ~66 kDa and ~145 kDa similar to those seen in Fig. 3. The ~145-kDa adduct is seen only faintly in Fig. 4B, lane 1, although the ~66-kDa adduct is quite intense; in contrast, the intensity of both adducts is comparable in Fig. 4A, lane 7. We note these experiment-to-experiment variations in the yield of particular photoadducts and, as a result, we concentrate here on the adducts that are seen most consistently between experiments. Fig. 4 also shows that samples containing the translocation intermediates p5, p6, or p7 yield photoadducts running in the general vicinity of the 66-kDa molecular mass marker (panel A, lanes 9–12, panel B, lane 2). None of these adducts were seen in samples that were not exposed to UV light (Fig. 4A, lanes 1–6). Many of the adducts seen with the translocation intermediates were visualized as smears on the fluorogram; the reason for this is not clear. Taking the maximum molecular mass indicated by the smears, Fig. 4 shows that adducts of ~70 kDa, ~60 kDa, and ~70 kDa are formed with p5, p6, and p7, respectively, indicating crosslinking partners of ~40 kDa (Table 1). In addition, an adduct of ~85 kDa was detected with p5 (Fig. 4B, lane 2).

In order to investigate the nature of these photoadducts further, we tested whether the adducts could be immuno-precipitated with antibodies raised against the translocon component Sec61α. Fig. 5 shows that while all constructs tested (p3, p6, p5, p7) yielded photoadducts of size close to the 66-kDa molecular mass marker (Fig. 5, lanes 1–4), only the polypeptides p6 (truncated N-terminal to the GPtdIns signal sequence) and p7 (bearing a nonfunctional GPtdIns signal sequence) generated products that could be
clearly immunoprecipitated with anti-Sec61α Ig (Fig. 4, lanes 5–8). The result with p6 is expected [33] and confirms our experimental procedures. The crosslink between p7 and Sec61α indicates that the ribosome-bound p7 polypeptide is retained in the translocon. No ~70-kDa photoadduct was seen if p7 was translated in the presence of preradiated εANB-Lys-tRNA (data not shown). The molecular mass of Sec61α deduced from these studies is consistent with that reported by others (37–40 kDa [21,34]).

Photoadducts obtained with p3 and p5 were not detected in the immunoprecipitation lanes (Fig. 5, lanes 5 and 7), although a faint, immunoprecipitated band of ~66 kDa could be seen in the p5 sample with longer exposure. The p3 polypeptide is a full-length (nonribosome-bound) chain, and its interaction with translocon components is most likely transient and therefore undetectable under our experimental conditions, i.e. p3 will have left the translocon before the sample is photolyzed. The p5 polypeptide chain, despite being bound to the ribosome like p7, appears not to be efficiently crosslinked to Sec61α. This may simply be because the two photoactive lysine residues in K176, K184) are not appropriately situated with respect to Sec61α, whereas the additional photoactive lysine in p7 (K194) contacts the translocon permitting formation of a p7-Sec61α photoadduct (the efficiency of crosslinking between translocation intermediates and Sec61α is known to vary depending on the position of the photoreactive lysine [21]). In support of this idea we note that p6 and p7 have a photoactive lysine residue 55 (K114 in p6) or 56 (K194 in p7) amino acids distant from the ribosome-attached C-terminus; this is likely to be the lysine residue involved in efficient photocrosslinking to Sec61α.

Another possibility to explain the inefficient crosslinking of p5 to Sec61α is that processing of p5 to p5/GPtdIns results in escape of the protein from the translocon, out of crosslinking range; we view this as unlikely as identical results were obtained when photolysis was carried out after a 20-min incubation when conversion to GPtdIns/p5 would be minimal (data not shown). Similarly, the proteolysis model (discussed above in the context of Fig. 2) in which p5 is released from the ribosome and so escapes the translocon does not provide an adequate explanation, as proteolysis would apply equally to both p5 and p7.

The sequence identity between p5 and p7 (except for the leucine to lysine substitution at position 194), together with the observation that p7 can be crosslinked to Sec61α, indicates that both proteins represent genuine translocation intermediates. We suggest that a p5-Sec61α photoadduct is not clearly detected because, unlike p7, p5 does not contain an appropriately situated photoactive lysine residue in contact with Sec61α. As we show below, the photoadduct smear of ~66–72 kDa seen with p5 (Fig. 4B, lane 2, Fig. 5, lane 3) most likely represents a crosslink between p5 and the ~40-kDa transamidase subunit Gpi8p.

**Photocrosslinking studies with p2 and p5 using microsomes containing FLAG-tagged Gaa1p and Gpi8p**

In order to interpret the photocrosslinking results further and to test the possibility that the ~40-kDa target protein (found in photoadducts involving p2, p3, and p5, but not p6 and p7) corresponds to the transamidase subunit Gpi8p, we used ER microsomes prepared from HeLa cells expressing FLAG-tagged Gaa1p and/or Gpi8p. The use of epitope-tagged proteins was necessary because we have had little success with antibodies raised against transamidase components. The FLAG-protein-expressing HeLa microsomes were able to convert both p2 and p5 to GPtdIns-anchored forms, indicating that expression of one or both FLAG-tagged proteins had no adverse effect on production of p2/GPtdIns and p5/GPtdIns; indeed, expression of FLAG-tagged Gpi8p correlated with an increased yield of p2/GPtdIns and p5/GPtdIns (Fig. 6A, compare lane 1 with lanes 3 and 5, and lane 2 with lanes 4 and 6).

We next used the FLAG-Gpi8p- and FLAG-Gaa1p-containing microsomes in photocrosslinking experiments similar to those shown in Figs 3–5. To examine if photocrosslinking experiments contained the FLAG-tagged proteins, the photolyzed reaction mixtures were subjected to immunoprecipitation with anti-FLAG Ig. The data are shown in Fig. 6. Direct analysis of the photolyzed reaction mixtures showed photoadducts of ~66 kDa, ~85 kDa, and ~145 kDa with p2, and a smeared band of ~66–74 kDa with p5. All these products could be immunoprecipitated with anti-FLAG Ig (Fig. 6, lanes 3, 4), whereas no photoadducts were immunoprecipitated with anti-FLAG Ig when microsomes derived from nontransfected HeLa cells were used. (data not...
The immunoprecipitation data imply that FLAG-proteins are either components of the photoadducts, or noncovalently associated with the adducts so as to permit coimmunoprecipitation. Thus, given the expected molecular mass of FLAG-Gpi8p, it appears reasonable to propose that the ~66-kDa adduct seen with p2 represents p2+FLAG-Gpi8p. Similarly, the smeared band of 66–74 kDa seen with p5 likely represents a p5-FLAG-Gpi8p adduct. However, the ~85-kDa and ~145-kDa adducts seen with p2 and immunoprecipitated with anti-FLAG Ig suggest that p2 can be crosslinked to ER proteins of molecular mass ~60 kDa and ~120 kDa, respectively, that are noncovalently associated with one or both of the FLAG-tagged transamidase subunits. This presumed association would have to be strong enough to resist detergent extraction and sample workup prior to SDS/PAGE analysis. We conclude that miniPLAP variants with functional GPtdIns signal sequences are found in the immediate vicinity of a set of ER proteins that includes the genetically defined transamidase subunits Gpi8p and Gaa1p. We further conclude that proteins of molecular mass ~60 kDa and ~115–120 kDa are likely to represent additional subunits of the GPtdIns transamidase complex.

CONCLUSIONS

The data presented in this paper represent an initial exploration of the protein-biochemical environment encountered by promiPLAP during its conversion to GPtdIns-anchored miniPLAP. The detailed results of the photocrosslinking studies are summarized in Table 1. We consistently observed crosslinks to proteins of molecular mass ~40 kDa that corresponded to Gpi8p or Sec61α, depending on the reporter construct used. When ribosome-bound translocation intermediates such as p6 and p7 were tested, crosslinks to Sec61α were detected (neither p6 nor p7 goes on to acquire a GPtdIns anchor). With a number of other reporters, crosslinks to Gpi8p were identified; these reporters included p1, p2, p3 (of which only p2 and p3 could be GPtdIns-anchored), and p5. The p1 protein clearly associates with Gpi8p despite the inability of the transamidase to process it to GPtdIns-anchored p1. The p5 protein similarly associates with Gpi8p despite being ribosome-bound. Other crosslinked adducts were also clearly detected, albeit less consistently, possibly because they are formed as a result of a transient interaction between an ER protein and the miniPLAP reporter used. An example is the ~145-kDa adduct seen with p2 and p3, that is detected only in a certain time-window during the translation-translocation reaction (Fig. 3F). Another example is the ~60-kDa protein that can be crosslinked to p2 or p5 in some experiments and not others. More detailed analyses need to be performed before the nature of these presumably transient interactions can be fully evaluated.
Our principal conclusions are that (a) proproteins can be crosslinked to the transamidase subunit Gpi8p, (b) a pro-protein that cannot be GPtdIns-anchored is in close proximity to a ~70-kDa protein that may correspond to Gaa1p, (c) a ~120-kDa protein is transiently associated with proproteins in the course of GPtdIns-anchoring, (d) the ~120-kDa protein as well as another protein of ~60 kDa are likely to represent additional subunits of the transamidase complex, and (e) it is possible for ribosome-bound, translocon-associated proproteins to be converted to GPtdIns-anchored proteins. In the absence of a biochemical assay for the transaminidation reaction using purified components, these data provide the first direct evidence that the genetically identified transaminidase subunit Gpi8p is in close proximity to a proprotein as it undergoes conversion to a GPtdIns-anchored protein. The data also implicate other protein components of the ER membrane that may participate in the anchoring reaction, and demonstrate the potential of photocrosslinking methodology to study the GPtdIns-anchoring reaction.

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