The Endoplasmic Reticulum (ER) Translocon Can Differentiate between Hydrophobic Sequences Allowing Signals for Glycosylphosphatidylinositol Anchor Addition to Be Fully Translocated into the ER Lumen*

Received for publication, April 16, 2003, and in revised form, September 26, 2003 Published, JBC Papers in Press, October 6, 2003 DOI 10.1074/jbc.M303978200

Jane A. Dalley and Neil J. Bulleid‡
From the School of Biological Sciences, 2.205 Stopford Building, University of Manchester, Manchester M13 9PT, United Kingdom

The signal sequence within polypeptide chains that designates whether a protein is to be anchored to the membrane by a glycosylphosphatidylinositol (GPI) anchor is characterized by a carboxyl-terminal hydrophobic domain preceded by a short hydrophilic spacer linked to the GPI anchor attachment (o) site. The hydrophobic domain within the GPI anchor signal sequence is very similar to a transmembrane domain within a stop transfer sequence. To investigate whether the GPI anchor signal sequence is translocated across or integrated into the endoplasmic reticulum membrane we studied the translocation, GPI anchor addition, and glycosylation of different variants of a model GPI-anchored protein. Our results unequivocally demonstrated that the hydrophobic domain within a GPI signal cannot act as a transmembrane domain. The domain is fully translocated even when followed by an authentic charged cytosolic tail sequence. However, a single amino acid change within the hydrophobic domain of the GPI-signal converts it into a transmembrane domain that is fully integrated into the endoplasmic reticulum membrane. These results demonstrated that the translocation machinery can recognize and differentiate subtle changes in hydrophobic sequence allowing either full translocation or membrane integration.

Glycosylphosphatidylinositol (GPI) is a complex glycolipid that is covalently attached to the carboxyl terminus of many eukaryotic cell surface proteins (1). It provides an alternative mechanism to a hydrophobic transmembrane domain for membrane attachment and enables stable but reversible anchoring of a protein to the lipid bilayer (2). GPI anchor addition occurs during the initial stages of nascent protein entry into the secretory pathway, and by its very nature must occur post-translationally. The preformed GPI moiety is transferred en bloc to the carboxyl terminus of a precursor protein very soon after translocation of the polypeptide chain across the ER membrane (3, 4). The precursor protein contains two signal sequences to ensure GPI anchor addition, one at the amino terminus targeting the protein to the endoplasmic reticulum and one at the carboxyl terminus for recognition by the GPI-anchoring machinery (5). The signal for GPI anchor addition is characterized by a carboxyl-terminal hydrophobic domain preceded by a short hydrophilic spacer linked to the GPI anchor attachment (o) site (6). The carboxyl-terminal hydrophobic domain shares many characteristics with a classical transmembrane domain, which has led to the suggestion that this region could partition into the lipid bilayer either during or soon after protein translocation (7). Irrespective of this partitioning event the recognition of the carboxyl-terminal signal sequence by the enzyme catalyzing GPI anchor addition occurs rapidly following completion of protein synthesis (8) giving rise to the possibility that protein translocation and GPI anchor addition are coordinated events.

The enzyme responsible for GPI anchor addition has been termed a transamidase as it catalyzes the cleavage of the carboxyl-terminal signal sequence and the concomitant addition of a preformed GPI anchor (9). The GPI transamidase is a multisubunit complex containing at least five polypeptide chains. These have been identified both by genetic (10, 11) and biochemical approaches (12–14) and are termed Gpi8p, Gaa1p, PIG-S (Gpi17p), PIG-T (Gpi16p), and PIG-U (Cdc91p). Gpi8p belongs to a novel family of cysteine proteases and has been shown both to bind to the polypeptide substrate (15, 16) and to contain the catalytic active site (13, 17). The other components of the complex are less well characterized, but it is clear that the presence of each polypeptide is essential for activity of the complex (14). A non-functional protein complex will form in the absence of Gpi8p, Gaa1p, PIG-S, or PIG-U but not in the absence of PIG-T, leading to the suggestion that PIG-T functions to assemble the complex (14). The exact function of each of the components of the complex along with their functional interaction with each other, the precursor protein, and the GPI anchor remains to be fully elucidated.

The close similarity between the hydrophobic region of the GPI anchor signal sequence and a hydrophobic transmembrane domain raises the question of whether the translocon can distinguish between the two and whether they share common mechanisms of membrane insertion. Transmembrane domains are usually flanked by charged residues carboxyl-terminal to the hydrophobic domain that act as stop transfer signals preventing full translocation through the translocon pore (18). The transmembrane domains are then thought to partition into the lipid bilayer by lateral gating of the subunits of the translocon (19). It is not clear whether a similar mechanism of membrane insertion occurs during GPI signal sequence translocation or whether the GPI anchor signal is fully translocated into the

* This work was supported by Wellcome Trust Grant 66190. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed. Tel.: 44-161-275-5103; Fax: 44-161-275-5082; E-mail: neil.bulleid@man.ac.uk.
1 The abbreviations used are: GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum; PLAP, placental alkaline phosphatase; FL, full-length; VSVG, vesicular stomatitis virus G; TM, transmembrane; SP, semipermeabilized; DTT, dithiothreitol.
lumen of the ER prior to recognition by the transamidase. Uncleaved precursors of GPI-anchored proteins are not membrane-associated suggesting that the hydrophobic sequence does not partition into the lipid bilayer (20). Modification of the hydrophobic domain that results in a lack of translocation across the ER membrane also prevents GPI anchor addition, indirectly suggesting that full translocation of the signal across the lipid bilayer is required for recognition by the transamidase and GPI anchor addition (21). These results would suggest that the signal is fully translocated into the ER lumen, although such a mechanism has not been proven directly and seems counterintuitive given the hydrophobic nature of the GPI anchor signal sequence.

We approached the question of GPI anchor signal sequence translocation and the requirements for membrane integration by translation of a number of variants of the model GPI-anchored substrate prepmimi-PLAP (22) in a cell-free system (16). By the addition of N-glycosylation sites close to and within the GPI anchor signal sequence we probed the accessibility of the signal to the oligosaccharyltransferase and thereby determined whether the signal is fully translocated into the ER lumen. We showed conclusively that the signal is indeed fully translocated and that the hydrophobic domain of a GPI anchor signal sequence cannot function as a transmembrane domain even when flanked by charged residues. Interestingly we found that a single amino acid change can convert the hydrophobic domain within the GPI signal to an authentic transmembrane domain that becomes fully integrated into the ER membrane. These results highlight the crucial role played by hydrophobic domains of transmembrane proteins that prevent full translocation thereby allowing membrane integration via lateral gating of the translocon.

MATERIALS AND METHODS

Plasmid Construction—The cDNA encoding mini-PLAP was a gift from Dr. M. E. Medof (Case Western Reserve University, Cleveland, OH). Mini-membrane PLAP was constructed by extension PCR of full-length (FL)-PLAP (16) using the following primers: 5'-CGCGGGCTTCTTCCTCTTCGTG-3', which was complementary to a sequence upstream of a StuI restriction enzyme site in FL-PLAP, and 3'-primer, 5'-CGCCATCGGCTCAGTTAAGATTTGCGCCTGGGAGCAGTGGCCGTCTCCAG-3'. The resulting construct contained additional residues at the carboxyl terminus (Arg-Arg-Lys-Ser) and was called FL-membrane PLAP. FL-membrane PLAP was digested with StuI and HindIII, and the resulting 390-bp StuI/HindIII fragment was subcloned into a StuI/HindIII-digested and purified pGem3Z/mini-PLAP, creating the plasmid pGem3Z/mini-membrane.

TM-VSVG was created using a previously constructed FL-PLAP TM-VSVG construct. FL-PLAP TM-VSVG was created by extension PCR from FL-PLAP template using the following primers: 5'-oligonucleotide primer 5'-CGCGGGCTTCTTCCTCTTCGTG-3' was complementary to a sequence upstream of a StuI restriction enzyme site in FL-PLAP; 3'-primer, for signal VSVG, 5'-CTGCTTTGTTTGTTTTAATTTAATGCAAAGATGGATACCAACTCGGGGAGCAGTGGCCGTCTCCAG-3', and for signal VSVG-G, 5'-CTGCTTTGTTTGTTTTAATTTAATGCAAAGATGGATACCAACTCGGGGAGCAGTGGCCGTCTCCAG-3', and contained a stop codon and a HindIII restriction enzyme site. The PCR products were gel-purified and digested with StuI and HindIII and subcloned into a StuI/HindIII-digested and purified pGem3Z/mini-PLAP.

TM-VSVG was created using a previously constructed FL-PLAP TM-VSVG construct. FL-PLAP TM-VSVG was created by extension

![Diagram of translocation of signal for GPI attachment](https://example.com/diagram.png)
PCR with the following primers: 5’ oligonucleotide primer 5’-CGCGGCTTCTCCTCTCTCTGC-3’ was complementary to a sequence upstream of a StuI restriction enzyme site in FL-PLAP and the overlapping 3’ primers 5’-TAGTCGATTAAACCTATGAAAGAAAAAGGCG-CATAGACCGCCCCGGGTGCGCGGCGTC-3’, 5’-CGCGGACAGGACGTTATCTCACCCTATGATAAAGAAAAAAGGCAATAGACCGCCCCGGGTGCG-3’ and the overlapping 3’ primers 5’-CGCGGACAGGACGTTATCTCACCCTATGATAAAGAAAAAAGGCAATAGACCGCCCCGGGTGCG-3’ and 5’-CGCGGACAGGACGTTATCTCACCCTATGATAAAGAAAAAAGGCAATAGACCGCCCCGGGTGCG-3’ were gel-purified and digested with Stul and KpnI and subcloned into a Stul/KpnI-digested and purified pGem3Z-mini-PLAP. 

TM minus tail was created using a previously constructed FL-PLAP TM minus tail construct. This was created by extension PCR with the following primers: 5’-CGCGGCTTCTCCTCTCTCTGC-3’ and the overlapping 3’ primers 5’-TAGTCGATTAAACCTATGAAAGAAAAAGGCG-CATAGACCGCCCCGGGTGCGCGGCGTC-3’, and 5’-CGCGGACAGGACGTTATCTCACCCTATGATAAAGAAAAAAGGCAATAGACCGCCCCGGGTGCG-3’ and 5’-CGCGGACAGGACGTTATCTCACCCTATGATAAAGAAAAAAGGCAATAGACCGCCCCGGGTGCG-3’. The resulting construct was digested with XhoI and KpnI and subcloned into a XhoI/KpnI-digested and purified pGem3Z2-mini-PLAP.

Introduction of glycosylation sequons within mini-PLAP and mini-membrane was achieved by site-directed mutagenesis using the following sense primers: G1 glycosylation mutant A175N, 5’-CGCGGCGTGGTCACTCGA-GTGCTGCTGACGGCCACTGCTCCC-3’ and 5’-CGCGGCGTGGTCACTCGA-GTGCTGCTGACGGCCACTGCTCCC-3’ depicts a change from threonine to asparagine.

Preparation of Semipermeabilized Cells—The human lymphoblastoid cell line K562 was obtained from the European collection of animal cell cultures. The cell line was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Semipermeabilized (SP) cells were prepared by treatment with digitonin as described previously (24). 

Translation in Vitro—Protein was synthesized using a rabbit reticulocyte lysate (Promega, Southampton, UK). Transcription in Vitro—Preparation reactions were carried out as described previously (25). All recombinant plasmids were linearized with HindIII and transcribed with T7 RNA polymerase (Promega, Southhampton, UK). Preparations of Semipermeabilized Cells—The human lymphoblastoid cell line K562 was obtained from the European collection of animal cell cultures. The cell line was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Semipermeabilized (SP) cells were prepared by treatment with digitonin as described previously (24).

Translation in Vitro—Protein was synthesized using a rabbit reticulocyte lysate (FlexiLyseTM, Promega). The translation reaction (25 µl) contained 16.5 µl of reticulocyte lysate, 40 µl methionine-free amino acid mixture, 45 mM KCl, 15 µCi of [35S]methionine (PerkinElmer Life Sciences), 1 µl of transcribed mRNA, and SP cell preparation (~2 × 10⁶ cells). Hydrazine (10 mM) was included in translations where indicated. Translations were incubated at 30 °C for 60 min and were terminated by centrifugation (12,000 × g for 3 min at 4 °C) to isolate SP cells. Translated products were then processed for further analysis as described below. To be noted, all translations were preformed in triplicate.

Endoglycosidase H Treatment—Following translation, isolated SP cells were solubilized in endoglycosidase H dissolution buffer (50 mM Tris-HCl, pH 8.0, 1% (w/v) SDS) for 5 min at 95 °C. Insoluble material was removed by centrifugation (15,000 × g for 10 min). An equal volume of 150 mM sodium citrate, pH 5.5, containing phenylmethylsulfonyl fluoride (0.5 mM) was added to the soluble fraction. The samples were divided into two equal aliquots and incubated for 12 h at 37 °C with either 1 unit of endoglycosidase H (Roche Applied Science) or buffer alone. Treated and untreated samples were then subjected to immunosolation with amino antibody and analysis by SDS-PAGE.

Proteinase K Digestion—After translation isolated cells were resuspended in KHM buffer (20 mM HEPES, pH 7.2, 110 mM potassium acetate, 2 mM magnesium acetate) supplemented with 10 mM CaCl₂ and were treated with proteinase K (Roche Applied Science) at a concentration of 250 µg/ml for 30 min at 0 °C either in the presence or absence of 1% (v/v) Triton X-100. Following digestion samples were treated with phenylmethylsulfonyl fluoride (1 mM) to terminate proteinase K digestion. Samples were immunocollected with amino antibody and subjected to SDS-PAGE analysis.

Immunosolation—The amino polyclonal rabbit antibody, which specifically recognizes amino-terminal signal sequence-cleaved PLAP, was produced as described previously (16). For immunosolation, SP cells were solubilized in 500 µl of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100, 0.02% (w/v) azide) and incubated with 50 µl of protein A-Sepharose (50 µl of protein A-Sepharose-bound material was centrifuged at 15,000 × g for 1 min, washed three times with immunoprecipitation buffer, and then subjected to analysis by SDS-PAGE.

Electrophoresis—Samples for SDS-PAGE were resuspended in SDS-PAGE sample buffer (0.065 x Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromphenol blue) plus 50 mM DTT and were boiled for 5 min. Proteins were resolved through 12.5% gels, 4% stacking (Zymed Laboratories Inc.) for 30 min at 4 °C. Samples were centrifuged for 1 min, washed three times with immunoprecipitation buffer, and then subjected to analysis by SDS-PAGE.

Fig. 2. Schematic diagram indicating the regions present within the various modified GPI anchor signal sequences described in the text. The regions identified include the hydrophobic region from the GPI anchor signal sequence (hatched) and the transmembrane region from VSVG (open). The hydrophilic tail from VSVG is depicted as a dotted pattern. The sites of introduced glycosylation sequons are indicated along with the amino acid changes made, e.g., T/N depicts a change from threonine to asparagine.
FIG. 3. Mini-PLAP-G1 is accessible to the oligosaccharyltransferase and is subsequently glycosylated. A, mRNA encoding mini-PLAP (lane 1) and mini-PLAP-G1 (lanes 2 and 3) were translated in a rabbit reticulocyte lysate in the presence of SP K562 cells at 30 °C for 60 min. Translated protein was post-translationally treated with (lanes 3) or without (lanes 1 and 2) endoglycosidase H (1 unit) at 37 °C for 12 h and then subjected to immunoisolation overnight at 4 °C with 2 μl of amino antibody after preclearing to remove nonspecific binding of cellular components to protein A-Sepharose. Proteins were eluted from protein A-Sepharose in SDS-PAGE sample buffer containing 20 mM DTT, resolved on a 12.5% polyacrylamide gel, and visualized by autoradiography. A star denotes the glycosylated species. B, mRNA encoding mini-PLAP (lanes 1 and 2 and mini-PLAP-G1 (lanes 3 and 4) were translated in a rabbit reticulocyte lysate system supplemented with SP K562 cells at 30 °C for 60 min in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 10 mM hydrazine. Translated protein was then immunosolated overnight at 4 °C with 2 μl of amino antibody. Proteins were eluted from protein A-Sepharose in SDS-PAGE sample buffer containing 20 mM DTT, resolved on a 12.5% polyacrylamide gel, and visualized by autoradiography. A star indicates the glycosylated species. Endo H, endoglycosidase H; HDZ, hydrazine; memb, membrane; mPLAP, mini-PLAP; mPLAPG1, mini-PLAP-G1.

FIG. 4. Mini-membrane is glycosylated and processed to give rise to a hydrazide product. A, mini-PLAP and mini-membrane mRNA were translated at 30 °C for 1 h in a rabbit reticulocyte lysate in the presence or absence (lanes 1 and 3) of 10 mM hydrazine. Translated protein was precleared for 30 min in the presence of protein A-Sepharose at 4 °C and subsequently subjected to immunoisolation with 2 μl of amino antibody overnight at 4 °C. Immunoisolated proteins were resolved on a 10% SDS-polyacrylamide gel and visualized by autoradiography. The putative hydrazide product is indicated. The experiment was carried out three times with similar results. B, mRNA encoding mini-PLAP (lane 1) and mini-membrane G2 (lanes 2 and 3) were translated as above. Translated protein was post-translationally treated with (lane 3) or without (lanes 1 and 2) endoglycosidase H (1 unit) at 37 °C for 12 h and then subjected to immunoisolation overnight at 4 °C with 2 μl of amino antibody. Immunoisolated proteins were resolved on a 10% SDS-polyacrylamide gel and visualized by autoradiography. A star indicates the glycosylated species. Endo H, endoglycosidase H; HDZ, hydrazine; memb, membrane; mPLAP, mini-PLAP.

fixed in 10% (v/v) acetic acid, 10% (v/v) methanol, and dried, and labeled products were visualized by autoradiography or imaged using a Fuji BAS 2000 phosphorimaging system.

RESULTS

We have previously shown that the initial stages of GPI anchor addition can be faithfully reconstituted by translating a model GPI-anchored protein in the presence of SP cells derived from cells grown in culture (16). Here we used this experimental approach to investigate the ability of the ER translocon to fully translocate the hydrophobic region of the GPI anchor signal sequence. Recognition of the GPI anchor signal by the transamidase could occur following complete translocation of the polypeptide chain across the ER membrane requiring a rapid recognition of the signal by the transamidase in the aqueous environment of the ER lumen (Fig. 1A). Alternatively the translocon could recognize and bind to the hydrophobic sequence preventing full translocation and allowing integration into the lipid bilayer by lateral gating of the translocon prior to interaction with the transamidase (Fig. 1B).
To investigate these two alternative models we constructed variants of the GPI-anchored protein prepromini-PLAP, creating a potential glycosylation site either just amino-terminal to the cleavage site or within the hydrophobic portion of the GPI anchor signal sequence (Fig. 2). Prepromini-PLAP is not normally glycosylated so the addition of an oligosaccharide side chain would indicate that the glycosylation site is accessible to the machinery for N-linked glycosylation. We also created variants that contain additional sequence at the carboxyl terminus of the GPI anchor signal. These additional sequences include either a series of charged residues that would be predicted to act as a stop transfer signal or a naturally occurring cytosolic domain from VSVG protein. Further variants were constructed whereby the hydrophobic region of the signal for GPI anchor addition was either modified, by changing a single charged residue to a more hydrophobic residue, or replaced with the transmembrane region from VSVG protein with or without the cytosolic tail. To simplify the interpretation of the results, all products of translation from the various constructs were subjected to immunoisolation and processed to give rise to a hydrazide product.

Previously been demonstrated that these products represent promini-PLAP and GPI-anchored mini-PLAP, respectively (22). Translation of the glycosylation variant prepromini-PLAP-G1 in the presence of SP cells yielded three major immunoprecipitated products with relative molecular masses of 33, 29, and 25 kDa (Fig. 3A, lane 2). The 33-kDa product was sensitive to digestion with endoglycosidase H (Fig. 3A, lane 3), demonstrating that this glycosylation site was accessible to the oligosaccharyltransferase and that this product is glycosylated promini-PLAP-G1.

To further identify the products of translation of prepromini-PLAP-G1, we carried out translations in the presence of hydrazine (Fig. 3B). Hydrazine can act as an alternative nucleophile during the transamidation reaction resulting in the formation of a hydrazide product, which has a greater electrophoretic mobility than the GPI-anchored form (9). This difference in mobility is clearly demonstrated when prepromini-PLAP was translated in the presence of SP cells and hydrazine (Fig. 3B, lane 2). The 25-kDa GPI-anchored mini-PLAP now migrated with a relative molecular mass of 23 kDa. When prepromini-PLAP-G1 was translated in the presence of SP cells and hydrazine an additional immunoprecipitated product with relative molecular mass of 27 kDa was formed (Fig. 3B, lane 4). This result demonstrates that the 29-kDa product synthesized in the absence of hydrazine contains both unglycosylated promini-PLAP and glycosylated mini-PLAP. The fact that the G1 glycosylation site is utilized and that the glycosylated product can be GPI-anchored demonstrates that the addition of an oligosaccharide side chain close to the cleavage site does not inhibit the transamidase or that the glycosylation site is still accessible to the transferase following GPI anchor addition.

Is the Signal for GPI Anchor Addition Accessible to the Glycosylation Machinery?—Our initial approach to address the question of whether the signal for GPI anchor addition is fully translocated into the lumen of the ER was to determine whether a glycosylation site positioned four amino acids from the cleavage site could be accessible to the glycosylation machinery. The glycosylation sequon would only be glycosylated if it is accessible to the oligosaccharyltransferase and therefore in the lumen of the ER. When prepromini-PLAP was translated in the presence of SP cells and the products were immunoisolated, two major translation products were identified with relative molecular masses of 29 and 25 kDa (Fig. 3A, lane 1). It has
samples were then immunoisolated overnight at 4°C. The stop transfer sequence, a region of 20 hydrophobic amino acids followed by charged residues (18), is strongly predicted to behave as a transmembrane domain based upon hydrophobicity (predictions carried out using software such as TMbase (25)), therefore flanking the cytoplasmic domain with charged residues could potentially convert the signal to a stop transfer sequence with the consequence of integrating the polypeptide chain into the lipid bilayer.

To ascertain whether the translocon could recognize a GPI anchor signal as an authentic stop transfer sequence, we added the amino acids -RRKS to the carboxy-terminal end of the hydrophobic signal. The resulting construct termed mini-membrane was translated in the presence of SP cells in the absence and presence of hydrazine to determine whether the modified signal was still a substrate for the transamidase. The main product of translation for mini-membrane migrated with a slightly slower mobility than promini-PLAP (Fig. 4A, compare lanes 1 and 3). This product is “promini-membrane” since these translation products have been immunoisolated with an antibody recognizing the amino-terminal signal sequence-cleaved translation products. In the presence of hydrazine an additional product was observed that had an identical mobility to the hydrazide product formed when mini-PLAP was translated in the presence of hydrazine (Fig. 4A, compare lanes 2 and 4).

Although this is a minor product it was reproducibly seen when mini-membrane was translated in the presence of hydrazine. This demonstrates that the transamidase can potentially recognize and cleave mini-membrane suggesting that at least some of the translation product was fully translocated into the ER lumen despite the presence of charged residues flanking the hydrophobic GPI anchor signal sequence. The low level of transamidase activity could be a result of a low level of full translocation or due to the charged residues affecting the ability of the transamidase to recognize and cleave the GPI anchor signal sequence.

To further investigate whether mini-membrane had been fully translocated rather than integrated into the lipid bilayer we modified the hydrophobic signal to include a glycosylation site (as we did for the G2 construct) to generate mini-membrane-G2. When this construct was translated in the presence of SP cells an additional product with a slower mobility than promini-membrane was observed (Fig. 4B, lane 2). This product was sensitive to digestion with endoglycosidase H verifying that it represents glycosylated promini-membrane (Fig. 4B, lane 3). It is unlikely that translocation was due to cleavage of the additional charged amino acids as no product co-migrating with promini-PLAP can be seen after translation of mini-membrane-G2 (Fig. 4B, lane 2) even after deglycosylation (lane 3). That only a proportion of the mini-membrane-G2 translation product was glycosylated is not surprising as glycosylation sites close to the carboxyl terminus are poorly recognized by the oligosaccharyltransferase (26). The fact that the G2 site was recognized at all does demonstrates that the GPI signal was accessible to the oligosaccharyltransferase suggesting that the polypeptide chain was fully translocated into the ER lumen.

The results obtained with mini-membrane could be explained if the short stretch of amino acids added were not sufficient to convert the GPI signal into a stop transfer signal. We therefore made a further construct where we added the 29-amino acid cytosolic tail from VSVG protein to the carboxy-terminal end of the polypeptide chain (Fig. 2) either with the authentic GPI signal (signal VSVG) or with the GPI signal

---

**Fig. 6.** **A** TM-VSVG is not processed by the transamidase. A, TM-VSVG-G (lanes 1–4) and TM-VSVG (lanes 5 and 6) mRNA were translated individually in a rabbit reticulocyte lysate supplemented with SP K562 cells at 30°C for 60 min in the presence (lanes 1 and 4) or absence (lanes 2, 3, and 5) of 10 mM hydrazine. Translated TM-VSVG-G protein was post-translationally treated with lane 2 or without (lanes 1 and 3–6) endoglycosidase H (1 unit) at 37°C for 12 h. All samples were then immunoisolated overnight at 4°C with 2 μl of amino antibody. Immunoisolated protein was eluted in sample buffer containing 20 mM DTT and resolved on a 12.5% polyacrylamide gel. B, TM minus tail mRNA was translated in a rabbit reticulocyte lysate supplemented with SP K562 cells at 30°C for 60 min either in the presence (lane 2) or absence (lane 1) of 10 mM hydrazine. Samples were then subjected to immunoisolation with 2 μl of amino antibody. Immunoisolated proteins were analyzed by 12.5% SDS-PAGE and autoradiography. The experiment was carried out three times with similar results. *Endo* H, endoglycosidase H; *HDZ*, hydrazine.

To further address whether the signal sequence for GPI anchor addition is translocated prior to GPI anchor addition a glycosylation site was introduced into the hydrophobic region of the signal sequence. When prepromini-PLAP-G2 was translated in the presence of SP cells and the products of translation were immunoisolated, three major products were visualized with relative molecular masses of 33, 29, and 25 kDa (Fig. 3C, lane 1). The 29- and 25-kDa products co-migrated with the signal sequence. When prepromini-PLAP-G2 was translated in the presence of SP cells an additional product with a slower mobility than promini-membrane was observed (Fig. 4B, lane 2). This product was sensitive to digestion with endoglycosidase H verifying that it represents glycosylated promini-membrane (Fig. 4B, lane 3). It is unlikely that translocation was due to cleavage of the additional charged amino acids as no product co-migrating with promini-PLAP can be seen after translation of mini-membrane-G2 (Fig. 4B, lane 2) even after deglycosylation (lane 3). That only a proportion of the mini-membrane-G2 translation product was glycosylated is not surprising as glycosylation sites close to the carboxyl terminus are poorly recognized by the oligosaccharyltransferase (26). The fact that the G2 site was recognized at all does demonstrates that the GPI signal was accessible to the oligosaccharyltransferase suggesting that the polypeptide chain was fully translocated into the ER lumen.

The results obtained with mini-membrane could be explained if the short stretch of amino acids added were not sufficient to convert the GPI signal into a stop transfer signal. We therefore made a further construct where we added the 29-amino acid cytosolic tail from VSVG protein to the carboxy-terminal end of the polypeptide chain (Fig. 2) either with the authentic GPI signal (signal VSVG) or with the GPI signal
containing a potential glycosylation site (signal VSVG-G). When the signal VSVG-G construct was translated in the presence of SP cells a translation product was synthesized that had a slower mobility than the signal VSVG translation product (Fig. 5A, compare lanes 3 and 5). This translation product was sensitive to digestion with endoglycosidase H (Fig. 5B, lane 2) verifying that the glycosylation site within the GPI signal was utilized. Additionally when translated in the presence of hydrazine both the signal VSVG and the signal VSVG-G were converted to the mini-PLAP hydrazide albeit rather inefficiently (Fig. 5A, lanes 4 and 6). Again these results suggest that both these constructs are translocated into the ER lumen where they are accessible to both the glycosylation machinery and the transamidase. Therefore, the presence of the VSVG cytosolic tail does not seem to convert the GPI signal into a stop transfer sequence and prevent translocation.

As the levels of glycosylation and transamidase activity were low our results might be explained by the SP cells allowing a minor level of translocation even of authentic transmembrane proteins. To investigate this point we made a construct where we replaced the hydrophobic region of the GPI signal with the stop transfer sequence from VSVG (called TM-VSVG) and a construct that contains a potential glycosylation site within the hydrophilic region (termed TM-VSVG-G) (Fig. 2). We then ascertained whether the hydrophobic region within TM-VSVG was at any stage accessible to the transamidase or the glycosylation machinery. When TM-VSVG-G was translated in the presence of SP cells a single translation product was observed that was not sensitive to digestion with endoglycosidase H (Fig. 6A, lanes 1 and 2). Additionally no hydrazide product was seen when TM-VSVG-G or TM-VSVG was translated in the presence of hydrazine (Fig. 6A, lanes 4 and 6).

To demonstrate that the hydrophobic domain from the VSVG stop anchor sequence could be recognized by the transamidase we prepared a construct that was identical to TM-VSVG but without the VSVG cytosolic tail (TM minus tail). Translation of

| A | signal VSVG | signal VSVG-G | TM-VSVG |
|---|---|---|---|
| PK | - | + | - |
| TX100 | 1 | 2 | 3 |
| Lane | | 4 | 5 | 6 | 7 | 8 | 9 |

| B | signal VSVG | signal VSVG-E/L |
|---|---|---|
| PK | - | + | - |
| TX100 | 1 | 2 | 3 |
| Lane | | 4 |

| C | signal VSVG-G | signal VSVG-G-E/L |
|---|---|---|
| Endo H | - | + | - |
| Lane | 1 | 2 | 3 |

| D | Mini-PLAP | signal VSVG E/L |
|---|---|---|
| HDZ | + | - | - |
| Lane | 1 | 2 | 3 | 4 |

Fig. 7. Signal VSVG is fully translocated across the ER membrane. A, signal VSVG (lanes 1–3), signal VSVG-G (lanes 4–6), and TM-VSVG (lanes 7–9) mRNAs were translated individually in rabbit reticulocyte lysate in the presence of SP K562 cells for 1 h at 30 °C. Isolated cells were treated with proteinase K (250 μg/ml) (lanes 2, 3, 5, 6, 8, and 9) with the addition of 1% (w/v) Triton X-100 as indicated (lanes 3, 6, and 9). After 30 min at 0 °C the protease was inhibited by addition of phenylmethylsulfonyl fluoride (1 mM). Samples were then subjected to immunoisolation with 2 μl of amino antibody. Immunoisolated proteins were analyzed by 12.5% SDS-PAGE and autoradiography. B, signal VSVG-E/L mRNA was translated as above. Isolated cells were treated with proteinase K (250 μg/ml) (lanes 2 and 3) with the addition of 1% (w/v) Triton X-100 (lanes 3). After 30 min at 0 °C the protease was inhibited by addition of phenylmethylsulfonyl fluoride (1 mM). Samples were then subjected to immunoisolation with 2 μl of amino antibody. Immunoisolated proteins were analyzed by 12.5% SDS-PAGE and autoradiography. C, signal VSVG-G (lanes 1 and 2) and signal VSVG-G-E/L (lanes 3 and 4) mRNA were translated individually in a rabbit reticulocyte lysate in the presence of SP K562 cells for 1 h at 30 °C. Isolated cells were incubated with (lanes 2 and 4) or without (lanes 1 and 3) endoglycosidase H (1 unit) at 37 °C for 12 h. Samples were then subjected to immunoisolation with 2 μl of amino antibody overnight at 4 °C. Immunoisolated protein was solubilized in SDS-PAGE sample buffer containing 20 mM DTT, resolved by 12.5% SDS-PAGE, and then visualized by autoradiography. D, mini-PLAP (lanes 1 and 2) and signal VSVG-E/L (lanes 3 and 4) mRNA was translated individually in a rabbit reticulocyte lysate supplemented with SP K562 cells at 30 °C for 60 min in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 10 mM hydrazine. All samples were then immunosolated overnight at 4 °C with 2 μl of amino antibody. Immunoisolated protein was eluted in sample buffer containing 20 mM DTT and resolved on a 12.5% polyacrylamide gel. Endo H, endoglycosidase H; HDZ, hydrazine; PK, proteinase K.
TM minus tail in the presence of SP cells resulted in the appearance of a single translation product (Fig. 6B, lane 1). When hydrazine was included in the translation an additional product was seen (Fig. 6B, lane 2). Hence the TM minus tail was recognized by the transamidase and cleaved to form a hydrazide product. Taken together these results demonstrate that the low efficiency of glycosylation and transamidase activity cannot be simply explained by a low level of translocation as no glycosylation or transamidase processing of TM-VSVG or TM-VSVG-G occurred.

To demonstrate directly that the signal for GPI anchor addition is translocated fully and efficiently across the ER membrane we carried out a protease protection experiment. The products of translation from signal VSVG and signal VSVG-G synthesized in the presence of SP cells were subjected to digestion with proteinase K either in the presence or absence of Triton X-100. For both constructs the translation products were protected from proteolysis in the absence of added detergent but were degraded after the membranes were solubilized (Fig. 7A, lanes 2, 3, 5, and 6). This result demonstrates that the translation products were completely sequestered within the lumen of the ER with no region of the translation product being accessible to the protease. If the modified GPI signal behaved as a stop transfer sequence then the integration of the polypeptide chain into the lipid bilayer would result in the cytosolic tail being exposed to and cleaved by the protease. To demonstrate this effect we translated TM-VSVG in the presence of SP cells. A single translation product was observed (Fig. 7A, lane 7) that was sensitive to proteolysis after treatment with proteinase K (Fig. 7A, lane 8). The appearance of a slightly faster migrating translation product indicates that the polypeptide chain was integrated into the lipid bilayer but that the cytosolic tail was still accessible to and degraded by the protease. The translation products were degraded completely when the SP cells were solubilized with detergent prior to proteolysis (Fig. 7A, lane 9). Taken together these results prove that the hydrophobic region from the GPI signal cannot act as a stop transfer sequence even when an authentic cytosolic tail is added to the carboxyl terminus. Both the glycosylated and the unglycosylated form of signal VSVG-G was protected from proteolysis showing that the low level of glycosylation is not due to a lack of translocation (Fig. 7A, lanes 4 and 5).

These data suggest that the hydrophobic domain from the GPI anchor signal sequence contains some characteristic that prevents normal recognition by the translocon to allow integration into the ER membrane. The sequence (VPAPPPLLAGTLLLLLETATAP) contains one charged residue and two internal proline residues that could influence either overall hydrophobicity or helical content. To determine whether these residues have an influence over the ability of this sequence to act as a transmembrane domain we initially changed the charged residue (Glu) to a hydrophobic residue (Leu) to created signal VSVG-E/L. When this construct was translated in the presence of SP cells a single translation product was observed (Fig. 7B, lane 1) that was sensitive to proteolysis after treatment with proteinase K (Fig. 7B, lane 2). The appearance of a slightly faster migrating translation product indicates that the polypeptide chain was integrated into the lipid bilayer. The translation products were degraded completely when the SP cells were solubilized with detergent prior to proteolysis (Fig. 7B, lane 3). We also introduced this mutation into the signal VSVG-G construct (signal VSVG-G-E/L) and found that when this construct was translated in the presence of SP cells the synthesized polypeptide was refractory to glycosylation (Fig. 7C, lanes 3 and 4). Additionally no hydrazide product was seen when signal VSVG-E/L was translated in the presence of hydrazine (Fig. 7D, lane 3). These results clearly demonstrate that a single amino acid change converts the GPI anchor signal sequence into a transmembrane domain.

**DISCUSSION**

The initial stage in the attachment of a GPI anchor to the carboxyl-terminal end of a polypeptide chain involves recognition of the GPI anchor signal sequence by the transamidase complex. The results presented here demonstrate conclusively that this recognition event occurs following full translocation of the chain across the ER membrane. The consequence of this observation is that the process of protein translocation and GPI anchor addition are segregated and that the hydrophobic GPI anchor signal sequence will be transiently exposed to the lumen of the ER. However, it is highly likely that protein translocation and GPI anchor addition are tightly coordinated in a similar fashion to N-linked glycosylation (27). Following translocation the hydrophobic GPI anchor signal sequence would need to be rapidly recognized by the transamidase as any prolonged exposure to the aqueous environment may lead to nonspecific association or sequestration away from the protein surface. Hence it would seem reasonable to suggest that the transamidase, like the oligosaccharyltransferase, is associated with the translocon ready to recognize and interact with newly translocated proteins. The transamidase itself is a multisubunit complex with a clearly defined function for only one of the five subunits (17). One function of the complex could be to interact with a ribosome-associated translocon to allow rapid and efficient recognition and processing of GPI anchor signal sequences.

The results presented here also clearly demonstrate that the hydrophobic region within the GPI signal cannot act as a transmembrane domain even when followed by an authentic cytosolic domain. This would suggest that there is some characteristic of the hydrophobic domain from a GPI anchor signal sequence that allows the ribosome or translocon to distinguish it from a
Translocation of Signal for GPI Attachment

The GPI anchor signal sequence reveals the presence of a single charged residue that facilitates recognition as a potential transmembrane domain. However, the GPI signal is unlikely to be due to overall hydrophobicity as algorithms designed to detect transmembrane domains predict that the hydrophobic region of a GPI signal should potentially act as a transmembrane domain. This is likely due to the presence of a single charged residue within the sequence that could influence conformation. Recent work has demonstrated that a disproportionate number of proline residues are found in transmembrane domains. The presence of proline actually increases the α-helical content by thermodynamically disfavoring the formation of β-sheet conformations within detergent micelles. Hence the presence of proline does not preclude the possibility that this domain can adopt an α-helical conformation. Exchanging single charged residues within GPI anchor signal sequences can, however, render the sequence able to act as a transmembrane domain. Our results demonstrate that the presence of proline did not prevent membrane integration; rather the presence of the charged residue was responsible for the lack of recognition of this domain by the translocon. Whether the single amino acid change results in a significant change of conformation or a change in helical surface leading to a change in recognition by the translocon is not clear and will be the subject of future work.

It would appear that subtle differences in the hydrophobic region of GPI anchor signal sequences prevent the recognition of the region by the ribosome or translocon and allow full translocation across the ER membrane. Hence an essential feature of a GPI signal is that it contains not just a hydrophobic domain but a hydrophobic domain that has some characteristic, such as the positioning of charged residues, that prevents recognition as a potential transmembrane domain. Conversely overall hydrophobicity cannot guarantee that a sequence will behave as a transmembrane domain; a positive recognition by the ribosome or translocon must occur to ensure the pathway of membrane integration rather than full translocation occurs.

Acknowledgments—We thank Professor Stephen High for many fruitful discussions during the course of this work and Dr. Martin Poole for critical reading of the manuscript.

REFERENCES

1. Ferguson, M. A. (1999) *J. Cell Sci.* **112**, 2799–2809
2. McConvilie, M. J., and Ferguson, M. A. (1993) *Biochem. J.* **294**, 305–324
3. Bangs, J. D., Hereld, D., Krakow, J. L., Hart, G. W., and Englund, P. T. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3297–3311
4. Tartakoff, A. M., and Singh, N. (1992) *Trends Biochem. Sci.* **17**, 470–473
5. Udenfriend, S., Micanovic, R., and Kodukula, K. (1991) *Cell Biol. Int. Rep.* **15**, 729–759
6. Udenfriend, S., and Kodukula, K. (1995) *Annu. Rev. Biochem.* **64**, 563–591
7. Berger, J., Micanovic, R., Greenspan, R. J., and Udenfriend, S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 1457–1460
8. Kodukula, K., Maxwell, S. E., and Udenfriend, S. (1995) *Methods Enzymol.* **250**, 536–547
9. Maxwell, S. E., Ramalingam, S., Gerber, L. D., Brink, L., and Udenfriend, S. (1995) *J. Biol. Chem.* **270**, 19576–19582
10. Hamburger, D., Egerton, M., and Riezman, H. (1995) *J. Cell Biol.* **129**, 629–639
11. Benghezal, M., Benachour, A., Rusconi, S., Aebi, M., and Conzelmann, A. (1996) *EMBO J.* **15**, 6575–6583
12. Fraering, P., Imhof, I., Meyer, U., Strub, J. M., van Dorselaar, A., Vionnet, C., and Conzelmann, A. (2001) *Mol. Biol. Cell** **12**, 3295–3306
13. Ohishi, K., Inoue, N., Maeda, Y., Takeda, J., Riezman, H., and Kinoshita, T. (2000) *Mol. Biol. Cell* **11**, 1523–1533
14. Kodukula, K., Inoue, N., and Kinoshita, T. (2001) *EMBO J.* **20**, 4088–4098
15. Vidugiriene, J., Vainauskas, S., Johnson, A. E., and Menon, A. K. (2001) *Eur. J. Biochem.* **268**, 2290–2300
16. Spurway, T. D., Dalley, J. A., High, S., and Bulleid, N. J. (2001) *J. Biol. Chem.* **276**, 15975–15982
17. Meyer, U., Benghezal, M., Imhof, I., and Conzelmann, A. (2000) *Biochemistry* **39**, 3461–3471
18. High, S., and Dobberstein, B. (1992) *Curr. Opin. Cell Biol.* **4**, 581–586
19. Martgolia, B., Hofmann, M. W., Brunner, J., and Dobberstein, B. (1995) *Cell* **81**, 207–214
20. Delahunty, M. D., Stafford, F. J., Yuan, L. C., Shaz, D., and Benifacino, J. S. (1993) *J. Biol. Chem.* **268**, 12017–12037
21. Wang, J., Maziarz, K., and Ratnam, M. (1995) *J. Mol. Biol.* **256**, 1305–1310
22. Kodukula, K., Micanovic, R., Gerber, L., Tamburrini, M., Brink, L., and Udenfriend, S. (1991) *J. Biol. Chem.* **266**, 4446–4470
23. Gurevich, V. V., Pokrovskaya, I. D., Obukhova, T. A., and Zozulya, S. A. (1991) *Anal. Biochem.* **195**, 207–213
24. Wilson, R., Allen, A. J., Oliver, J., Brookman, J. L., High, S., and Bulleid, N. J. (1995) *Biochem. J.* **307**, 679–687
25. Hofmann, K., and Stoffel, W. (1993) *Biochem. J. Hoppe-Seyler* **374**, 166
26. Nilsson, I., and von Heijne, G. (2000) *J. Biol. Chem.* **275**, 17338–17344
27. Whitley, P., Nilsson, I. M., and von Heijne, G. (1996) *J. Biol. Chem.* **271**, 6241–6244
28. Liao, S., Lin, J., Do, H., and Johnson, A. E. (1997) *Cell* **90**, 31–41
29. McCormick, P., Miao, Y., Shao, Y., Lin, J., and Johnson, A. E. (2003) *Cell* **12**, 329–341
30. Wilkinson, B. M., Critchley, A. J., and Stirling, C. J. (1996) *J. Biol. Chem.* **271**, 25590–25597
31. Wigley, W. C., Corboy, M. J., Cutler, T. D., Thibodeau, P. H., Olden, J., Lee, M. G., Rizo, J., Hunt, J. F., and Thomas, P. J. (2002) *Nat. Struct. Biol.* **9**, 381–388