The EMBO Journal vol.14 no.19 pp.4686–4694, 1995

Soluble constituents of the ER lumen are required for GPI anchoring of a model protein

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Transfer of a glycosylphosphatidylinositol (GPI) anchor to proteins carrying a C-terminal GPI-directing signal sequence occurs after protein translocation across the endoplasmic reticulum (ER). We describe the translocation and GPI modification of a model protein, preprominiPLAP, in ER microsomes depleted of lumenal content by high pH washing. In untreated microsomes preprominiPLAP was processed to prominiPLAP and GPI-anchored miniPLAP. Both products were fully translocated, since they resisted proteinase K treatment of the microsomes, and both behaved as membrane proteins by the carbonate extraction criterion. Microsomes depleted of lumenal content were able to translocate and process preprominiPLAP to give protease-protected prominiPLAP, but were unable to convert prominiPLAP to miniPLAP. Loss of GPI anchoring capacity occurred with a wash of pH >9.5. If the alkaline wash was performed after formation of prominiPLAP conversion to miniPLAP was relatively unimpaired. The results indicate that constituents of the ER lumen, possibly chaperones interacting with the proprotein and/or the GPI anchor precursor, are required in the initial steps of GPI anchoring.

Keywords: chaperone/endoplasmic reticulum/glycosylphosphatidylinositol/membrane protein/translocation

Introduction

Glycosylphosphatidylinositol (GPI) anchors are synthesized in the endoplasmic reticulum (ER) and attached to select secretory proteins bearing C-terminal GPI-directing signal sequences (reviewed most recently by Englund, 1993; McConville and Ferguson, 1993). The C-terminal signal sequence consists of a hydrophobic stretch of 10–15 amino acids and a small amino acid domain at the cleavage site (Moran *et al.*, 1991; Udenfriend *et al.*, 1991; Udenfriend and Kodukula, 1995) and in many ways resembles the N-terminal signal sequence involved in targeting ribosome-bound nascent chains of secretory proteins (including GPI-anchored proteins) to the ER. The assembly of GPI-anchored proteins requires translocation of the nascent polypeptide chain across the ER membrane, cleavage of the C-terminal signal sequence and attachment of a pre-assembled GPI moiety to the newly exposed carboxyl group. The reaction is presumed to be catalyzed by a membrane-bound transamidase. The transamidase has not been purified, but information on the mechanism of the GPI anchoring reaction has been obtained from two independent lines of investigation.

Microsomal preparations from bloodstream forms of the protozoan *Trypanosoma brucei* contain unprocessed, but appropriately situated, variant surface glycoprotein (VSG) polypeptides that are capable of being modified by *in vitro* synthesized or exogenously supplied GPIs. Transfer of radiolabeled GPIs to these endogenous VSG acceptors does not require ATP or GTP, consistent with a transamidation reaction mechanism (Mayor *et al.*, 1991). Other investigations have used a model protein substrate for the transamidase. The substrate, preprominiPLAP, was bioengineered by deleting ~60% of the internal sequence of placental alkaline phosphatase (PLAP), a GPI-anchored protein (Kodukula *et al.*, 1991; Amthauer *et al.*, 1992b). PreprominiPLAP lacks potential glycosylation sites and contains the N-terminal and C-terminal signal sequences for ER targeting and GPI attachment. Messenger RNA corresponding to preprominiPLAP can be used to program a standard *in vitro* translation–translocation system to generate preprominiPLAP, prominiPLAP and GPI-anchored miniPLAP (Kodukula *et al.*, 1991, 1992a,b). MiniPLAP formation lags behind prominiPLAP (Kodukula *et al.*, 1991), providing an opportunity to load microsomes with prominiPLAP and study C-terminal processing separately from protein synthesis and early translocation events, such as N-terminal signal sequence cleavage. Analyses of prominiPLAP-loaded microsomes (re-isolated to remove the translation mix) showed that conversion to GPI-anchored miniPLAP required ATP and/or GTP, suggesting that in a translation-independent system C-terminal processing is an energy-dependent process (Amthauer *et al.*, 1992a). These data were taken to indicate that energy-dependent steps were involved prior to processing of prominiPLAP to miniPLAP (Amthauer *et al.*, 1992a). It was proposed that ATP was involved in relieving a chaperone–prominiPLAP interaction (Amthauer *et al.*, 1992a) and support for this hypothesis was obtained from *in vivo* studies that demonstrated the association of BiP/GRP78 with proproteins (Amthauer *et al.*, 1993). It was suggested that BiP may facilitate proper folding of the translocated proprotein or stabilize a particular conformation until recognition by the GPI transamidase occurred (Amthauer *et al.*, 1993).

Several lines of evidence now indicate that lumenal ER proteins, in particular BiP/GRP78, not only play a role in protein folding (Gething and Sambrook, 1992, Helenius *et al.*, 1992), but are also required for translocation of the full range of secretory and membrane proteins (Vogel *et al.*, 1990; Sanders *et al.*, 1992; Nicchitta and Blobel, 1993). It has been shown that lumen-depleted canine pancreas microsomes are defective in transfer of soluble nascent chains into the ER lumen, but are not impaired

in signal sequence cleavage (Nicchitta and Blobel, 1993). The exact function of BiP in this process is unclear, but it has been proposed that BiP and other chaperones act to bias or drive the movement of the translocating chain, resulting in net transfer into the ER lumen (Simon *et al.*, 1992; Schekman, 1994; Glick, 1995).

In this report we show that constituents of the ER lumen are necessary for the post-translocational modification of prominiPLAP by a GPI anchor. We use a mild alkaline extraction procedure to deplete thymoma cell ER-derived microsomal vesicles of lumenal content (Bulleid and Freedman, 1988; Paver *et al.*, 1989; Nicchitta and Blobel, 1993) and use these lumen-depleted vesicles to study the translocation and processing of preprominiPLAP. The results show that miniPLAP production is abolished in lumen-depleted microsomes; we speculate that the necessary lumenal constituents may be 'chaperones' that interact with the proprotein as well as the GPI anchor precursor.

Results

ER translocation and processing of preprominiPLAP in a cell-free system

PreprominiPLAP (Figure 1A) can be sequentially processed by ER enzymes (the signal peptidase complex and GPI transamidase) to generate prominiPLAP and miniPLAP (Figure 1A; Kodukula *et al.*, 1991; Udenfriend *et al.*, 1991; Amthauer *et al.*, 1992b). The processing reaction is readily observed using standard *in vitro* translation–translocation systems (a time course is shown in Figure 1B). Although there is no direct proof that miniPLAP contains a GPI moiety, a number of observations indicate that this is almost certainly the case. Evidence for the structure of miniPLAP as a GPI-modified protein includes its molecular weight, lack of reactivity to antibodies directed against the C-terminal signal peptide, characteristic partitioning between Triton X-114 and water before and after treatment with phospholipases and, importantly, the observation that microsomes isolated from cells defective in GPI assembly are unable to synthesize miniPLAP from prominiPLAP unless supplemented with lipid extracts from wild-type cells (Kodukula *et al.*, 1992a,b).

Both prominiPLAP and miniPLAP remained in the membrane fraction following carbonate (pH 11.5) extraction of microsomes after a 120 min translation–translocation reaction (Figure 1C). The resistance to carbonate extraction suggests that both prominiPLAP and miniPLAP are firmly associated with the membrane bilayer and behave as integral membrane proteins. Figure 1C also shows that preprominiPLAP is mainly associated with the pellet fraction after carbonate treatment. In samples prepared from shorter translation–translocation reactions (30 min) preprominiPLAP distributes roughly equally between the carbonate-resistant pellet fraction and the supernatant. These data, and the observation that preprominiPLAP is completely protease sensitive in intact microsomes and therefore is not fully translocated, suggest that its carbonate inextractability is due to signal sequence-mediated membrane association that is somehow more effective in translocation assays incubated for long (2 h) rather than short times (30 min).

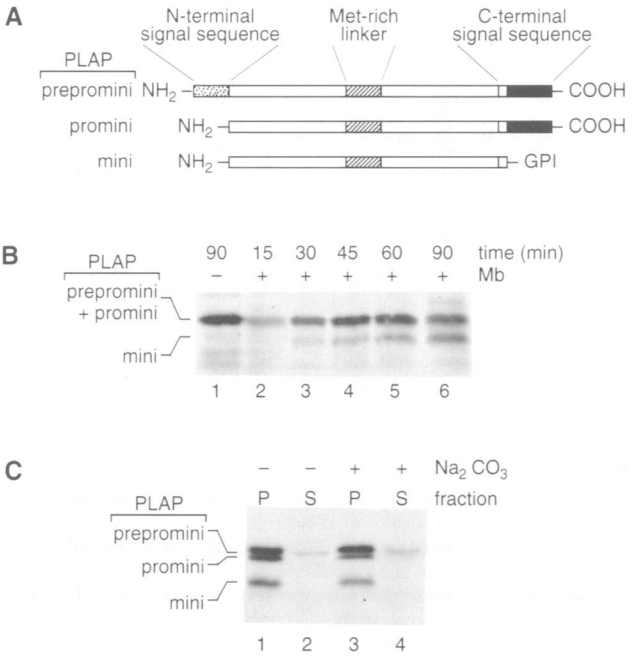

Fig. 1. Cell-free processing of preprominiPLAP to miniPLAP. (**A**) Structures of preprominiPLAP, prominiPLAP and miniPLAP. The schematic shows the main features of preprominiPLAP and the two products (prominiPLAP and miniPLAP) obtained by microsomal processing. PreprominiPLAP (Kodukula *et al.*, 1991) was constructed by replacing ~60% of the internal sequence of placental alkaline phosphatase (PLAP) with a methionine (Met)-rich linker. (**B**) Kinetics of conversion of preprominiPLAP to miniPLAP. PreprominiPLAP mRNA was translated in the absence (lane 1) or presence (lanes 2–6) of ER-derived microsomes (Mb). At the indicated time points the samples were placed on ice and analyzed by SDS–PAGE (15% gels) and fluorography. PreprominiPLAP and prominiPLAP were not resolved in the particular analysis shown. (**C**) Carbonate extraction of microsomal samples containing preprominiPLAP and derivatives. PreprominiPLAP mRNA was translated in the presence of ER microsomes for 120 min. The samples were then placed on ice, exposed to sodium carbonate as described in Materials and methods and centrifuged to generate membrane (pellet, P) and soluble (supernatant, S) fractions. The fractions were analyzed by SDS–PAGE and fluorography. Analysis of control samples (not treated with carbonate) is shown in lanes 1 and 2; carbonate-treated samples are shown in lanes 3 and 4.

In accordance with data reported in the original description of preprominiPLAP processing (Kodukula *et al.*, 1991), analysis of the kinetics of miniPLAP formation *in vitro* showed that miniPLAP could only be detected 15–30 min after initiation of the translation–translocation reaction, well after the initial appearance of prominiPLAP (Figure 1B). This lag period was exploited by Amthauer *et al.* (1992a) to obtain a preparation of microsomes loaded with prominiPLAP and devoid of soluble components of the translation system. Incubation of these prominiPLAP-loaded microsomes in the presence of ATP and/or GTP resulted in the conversion of prominiPLAP to miniPLAP (Amthauer *et al.*, 1992a), suggesting that protein modification by GPI could occur in the absence of ongoing translation. A similar analysis is shown in Figure 2. ProminiPLAP-loaded microsomes were incubated in the absence or presence of ATP, GTP or ATP + GTP and miniPLAP yield was monitored. Although some miniPLAP was formed in the absence of nucleotides, the yield was improved in the presence of nucleotides (GTP or ATP), with a combination of ATP and GTP being most effective.

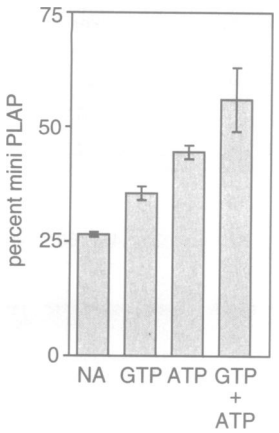

Fig. 2. Effect of nucleoside triphosphates on the post-translocational conversion of prominiPLAP to miniPLAP. PreprominiPLAP mRNA was translated in the presence of ER-derived microsomes for 20 min. The samples were placed on ice, diluted into HEPES buffer and centrifuged as described in Material and methods to re-isolate the microsomes. The microsomal pellet was resuspended in 30 µl buffer C and supplemented as shown in the figure: NA (no addition) or GTP or ATP or ATP + GTP as described in Materials and methods. The samples were incubated at 30°C for 2 h to assess the GPI anchoring reaction, i.e. the production of miniPLAP; an additional sample prepared without any additions was kept on ice as a control. At the end of the incubation the samples were treated with proteinase K (100 µg/ml final concentration), quenched with phenylmethylsulfonyl fluoride, boiled in SDS sample buffer and analyzed by SDS–PAGE. Quantitative estimates of band intensities were obtained from densitometric analysis of duplicate samples and are displayed as percent miniPLAP, i.e. miniPLAP/(miniPLAP + prominiPLAP); the bar at the top of each shaded column indicates the range of the duplicate measurements. The unsupplemented sample maintained on ice contained ~5% miniPLAP.

Loss of ER lumenal content affects the GPI anchoring reaction

The ATP requirement in prominiPLAP processing (Amthauer *et al.*, 1992a) was attributed to a post-translocational preparatory reaction, such as chaperone-mediated maturation of prominiPLAP to a conformation suitable for recognition by the GPI transamidase. Co-immunoprecipitation of the ER lumenal chaperone BiP/GRP78 with a number of proproteins *in vivo* (Amthauer *et al.*, 1993) supported this hypothesis and suggested that BiP was the chaperone in question. We decided to investigate this phenomenon further by analyzing the GPI anchoring reaction in microsomes depleted of their lumenal content by a mild alkaline pH extraction procedure.

It has been previously shown that canine pancreas microsomes can be depleted of lumenal content by a protocol that involves dilution in a high pH buffer, incubation on ice, then re-isolation of the vesicles by centrifugation through a sucrose cushion at pH 7.5. The microsomes are recovered as sealed, right-side-out vesicles completely depleted of major lumenal proteins (Nicchitta and Blobel, 1993). For the experiments described here thymoma cell ER microsomes were diluted 10-fold in HEPES/CAPS buffers prepared in the pH range 7.5–10.5. After incubation on ice the membranes were resealed by centrifugation through a sucrose cushion. Aliquots of the pH-washed membranes were taken for SDS–PAGE and Western blot analysis to determine the remaining levels of the representative ER lumenal proteins protein disulfide isomerase (PDI) and BiP. As shown in Figure 3A and C,

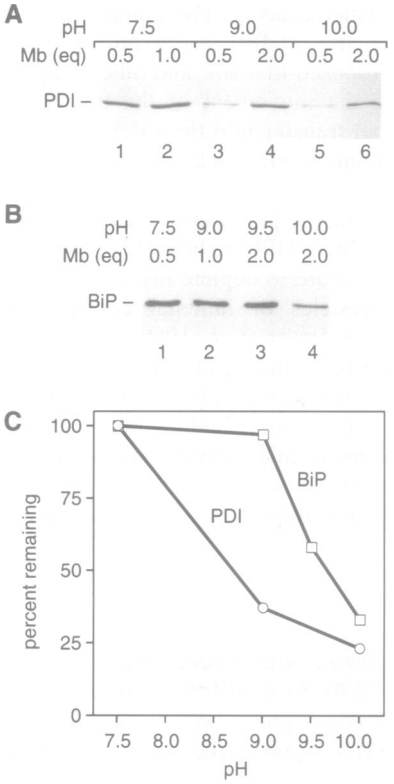

Fig. 3. Depletion of lumenal ER proteins upon exposure of microsomes to alkaline pH. Microsomes were diluted 1:10 in 50 mM HEPES, 50 mM CAPS buffer at the indicated pH values and incubated on ice for 30 min. After incubation the membranes were collected by centrifugation through a sucrose cushion, resuspended in sample buffer and analyzed by SDS–PAGE and Western blotting using antibodies to BiP/GRP78 (**A**) and PDI (**B**). The amount of sample (membrane equivalents) loaded in each gel lane is indicated. (**C**) Densitometric quantitation of the Western blots (A and B) indicating recovery of BiP and PDI in the membrane pellet as a function of the wash pH.

~63% of PDI was depleted from the microsomes after a pH 9.0 wash and ~77% was lost after a pH 10.0 wash. BiP was more resistant to alkaline washes and was only effectively depleted with a wash of pH >9.5 (Figure 3B and C; note that at pH 9.0 no BiP was lost and significant loss (~67%) occurred only when the wash pH was raised to 10.0).

To determine whether depletion of lumenal ER content affected the GPI anchoring reaction, alkaline pH-treated membranes were tested for their ability to process *in vitro* translated prepromini PLAP to GPI-anchored miniPLAP. Exposure of microsomes to alkaline buffers up to pH 9.0 had no effect on the formation of miniPLAP (Figure 4 and data not shown). When membranes were pre-treated with buffers of pH >9.0 a dramatic decrease in GPI anchoring activity was observed. As shown in Figure 4, the yield of GPI-anchored miniPLAP was ~50% lower in pH 9.5-washed membranes compared with pH 7.5-washed membranes. Further increase in the wash pH to 10.0 resulted in 90–95% inhibition of miniPLAP synthesis.

Proteins such as PDI are lost when the microsomes are subjected to a wash of pH 9.0 and it would be expected that most of the smaller components of the microsomal lumen, such as low molecular weight factors and metal cations, would be lost at this pH as well. However,

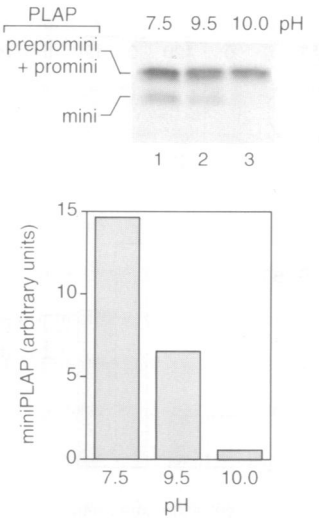

Fig. 4. GPI anchoring of preprominiPLAP is inhibited upon membrane exposure to alkaline pH. ER-derived microsomes were washed in buffers of different pH (in the range 7.5–10.0) and re-isolated as described in the legend to Figure 2. The washed microsomes were assayed for preprominiPLAP translocation and conversion to GPI-anchored miniPLAP as described in Materials and methods. Quantitative estimates of data presented were obtained via phosphorImager analysis.

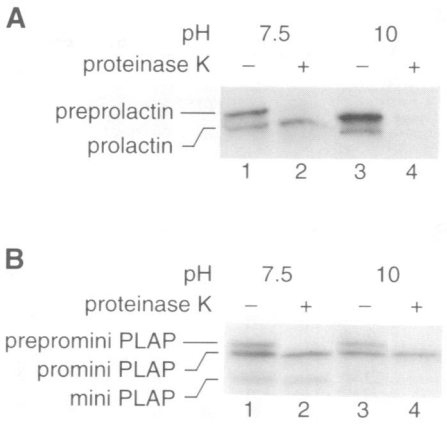

Fig. 5. ProminiPLAP is translocated across and retained by reticuloplasm-depleted microsomes. Protection of translocated prolactin (**A**) or prominiPLAP (**B**) from exogeneously added proteinase K upon membrane exposure to alkali pH. Following 30 min translocation reaction the samples were diluted to 50 µl in 150 mM KOAc, 50 mM HEPES–NaOH, pH 7.5, 2.5 mM $Mg(OAc)_2$, 1 mM DTT and treated with proteinase K at a final concentration of 100 µg/ml. Proteolysis was stopped by adding 3 mM phenylmethylsulfonyl fluoride. The samples were diluted with 2 vol. saturated ammonium sulfate and incubated on ice for 30 min. Following centrifugation for 20 min at 12 000 *g* the pellet was washed with 7% trichloracetic acid and analyzed by SDS–PAGE.

reduction in the yield of miniPLAP only occurred when microsomes were washed at pH >9.5, the pH range required to remove BiP (Figure 3B and C). The coincident loss of prominiPLAP processing and BiP supports the hypothesis that BiP (or other components of the ER lumen that are removed only at pH >9.5) is required at some stage in the processing of prominiPLAP to miniPLAP. Alternative explanations, such as the inability of the lumen-depleted microsomes to retain prominiPLAP or loss of GPI transamidase activity on high pH treatment, are ruled out by the data presented below. Also, it is unlikely that the transamidase is a lumenal protein (or complex of proteins) that can be removed by high pH washing.

Alkali-washed microsomes are able to translocate preprominiPLAP

It has recently been shown that lumenal constituents of the ER are necessary for the translocation of soluble proteins such as prolactin (Nicchitta and Blobel, 1993). Lumen-depleted canine pancreas microsomes are able to translocate preprolactin to the extent that it is acted on by signal peptidase, but they are unable to retain the soluble product prolactin, which is found mainly in the extramicrosomal space (Nicchitta and Blobel, 1993). These and other data suggested that ER lumenal chaperones are required to bias transport of the nascent chain and achieve net transfer into the ER lumen (Nicchitta and Blobel, 1993). We were able to reproduce these results for prolactin using pH 10.0-washed thymoma cell ER microsomes that were largely (~70%), but not completely, depleted of BiP. Figure 5A shows that both pH 7.5- and pH 10.0-washed microsomes are able to process preprolactin to prolactin (lanes 1 and 3), but prolactin generated in pH 10.0-washed microsomes is not protected from proteinase K digestion (compare lanes 2 and 4). A similar experiment was performed with preprominiPLAP. As shown in Figure 5B, the amount of translocated and proteinase K-protected prominiPLAP was essentially the same in mock-treated membranes (pre-washed at pH 7.5) and in high pH-washed membranes (pre-washed at pH 10.0). These results indicate that unlike prolactin, prominiPLAP is retained by microsomes that are comparatively depleted of lumenal constituents. Furthermore, the results indicate that the lack of conversion of prominiPLAP to miniPLAP in high pH-washed microsomes cannot be due to non-translocation of prominiPLAP. The ability of the lumen-depleted microsomes to retain prominiPLAP but not prolactin is most likely because prominiPLAP is a membrane protein (Figure 1C), whereas prolactin is soluble. Thus there appears to be a differential requirement for constituents of the ER lumen in the translocation of soluble proteins versus membrane proteins.

High pH treatment does not affect the GPI transamidase

Little is known about the mechanism of protein modification by GPI and it is unclear whether the high pH wash employed to deplete microsomal lumenal content has a damaging effect on GPI transamidase activity. To examine this possibility pelleted microsomes were resuspended in unbuffered sucrose and exposed to pH 10.0 buffer at dilutions of 1:1, 1: 5 and 1:10. After 30 min incubation on ice the membranes were re-isolated by centrifugation through a sucrose cushion and tested for GPI anchoring activity. As shown in Figure 6A and B, membranes washed by diluting 1:1 in pH 10.0 buffer were able to synthesize miniPLAP almost (~90%) as efficiently as mock-treated membranes (diluted 1:1 in pH 7.5 buffer), suggesting that the GPI anchoring machinery is not intrinsically sensitive to high pH. Wash dilutions of 1:5 or 1:10 in pH 10.0 buffer were required to see a significant effect on the yield of miniPLAP. As shown in Figure 6C, a large wash

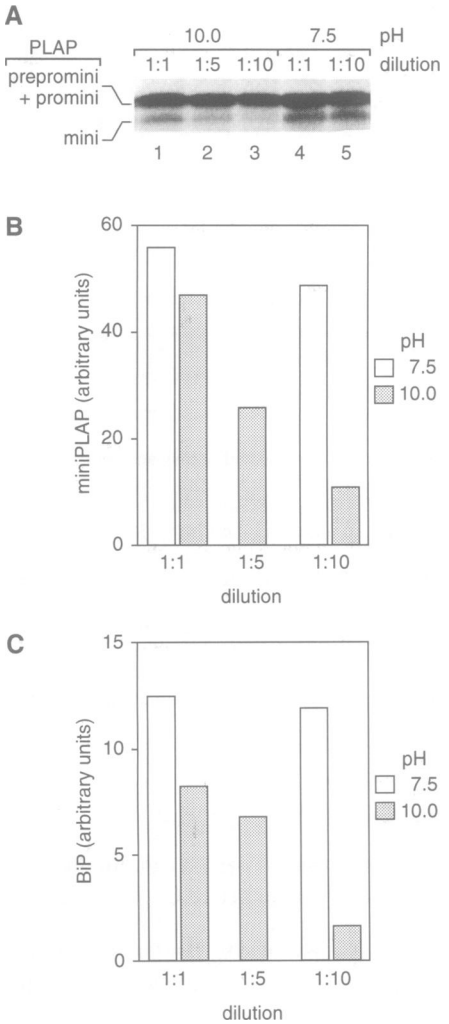

Fig. 6. Reduction in miniPLAP yield in high pH-washed microsomes depends on the dilution factor used for the alkaline pH wash. Microsomes were pelleted through a sucrose cushion and resuspended in unbuffered sucrose. Aliquots were diluted 1:1, 1:5 or 1:10 with 50 mM HEPES, 50 mM CAPS buffer at pH 7.5 or 10.0 as indicated. After 30 min incubation on ice membranes were re-isolated by centrifugation through a sucrose cushion, resuspended and assayed for their ability to convert preprominiPLAP to miniPLAP. Aliquots (2 equivalents) were also analyzed for BiP content by Western blotting and densitometric analysis, as in Figure 3. (**A** and **B**) MiniPLAP synthesis in membranes washed at pH 7.5 or 10.0 using different dilutions. (B) Quantitative estimates of the data shown in (A). (**C**) Amount of BiP recovered in membranes washed at pH 7.5 or 10.0 using different dilutions.

dilution is also required for the efficient removal of lumenal constituents. The correlation between the amount of microsomal lumenal content (exemplified by BiP; Figure 6C) and microsomal ability to form mature GPI-anchored miniPLAP is consistent with the interpretation that the decrease in GPI anchoring activity occurs through the loss of lumenal content and not through direct loss of activity on exposure of the transamidase to high pH.

Post-translocational depletion of lumenal content has little effect on conversion of prominiPLAP to miniPLAP

In order to analyze further the requirement for ER lumen constituents in GPI anchoring the high pH lumen-depletion

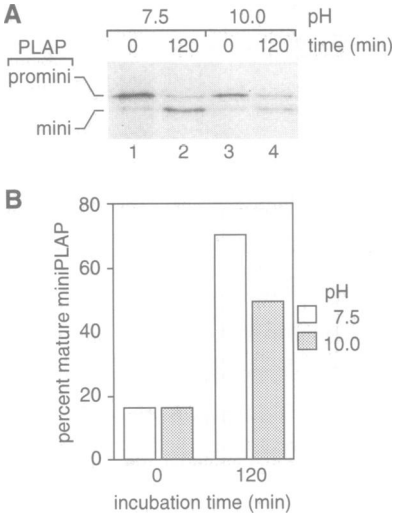

Fig. 7. Conversion of translocated prominiPLAP to mature miniPLAP upon membrane exposure to alkali pH. (**A**) Membranes were pre-loaded with prominiPLAP as described in Material and methods, then diluted 1:10 with 50 mM HEPES, 50 mM CAPS buffer at pH 7.5 or 10.0, incubated on ice for 30 min and re-isolated by centrifugation through a sucrose cushion. The membrane pellets were resuspended in buffer C and assayed for formation of miniPLAP as described in Materials and methods. (**B**) Quantitation of data presented in (A).

wash was carried out after pre-loading the microsomes with prominiPLAP as follows. PreprominiPLAP mRNA was translated in the presence of microsomes for 20 min, after which the sample was chilled on ice to stop further reaction. The microsomes (containing mainly promini-PLAP; see Figure 1B) were pelletted by centrifugation, resuspended and diluted 10-fold with 50 mM HEPES, 50 mM CAPS buffer at either pH 7.5 or pH 10.0. After 30 min incubation on ice the membranes were re-isolated by centrifugation and resuspended in buffer at pH 7.5 supplemented with GTP and an ATP regenerating system. Half of each sample was reserved for analysis, while the remainder was incubated for 2 h at 30°C to permit conversion (if any) of prominiPLAP to miniPLAP. All samples were then treated with proteinase K and analyzed by SDS–PAGE and fluorography. The results are shown in Figure 7. At the 0 time point, i.e. after the wash in pH 7.5 or 10.0 buffer but without further incubation at 30°C, the microsomal samples contain mainly (>80%) prominiPLAP, with only a small amount of miniPLAP (Figure 7A, lanes 1 and 2). The amount of prominiPLAP recovered in the microsomal sample washed at pH 10.0 was less than in the pH 7.5-washed sample, possibly because of membrane losses during the pH 10.0 wash and centrifugation steps. After 2 h incubation at 30°C the pH 7.5-washed membranes showed a decrease in the intensity of the prominiPLAP band and a corresponding increase in miniPLAP (the miniPLAP yield increased from <20% of the labeled bands to >70%; Figure 7B, white bars). Interestingly, miniPLAP was also formed when the pH 10.0-washed membranes were incubated for 2 h at 30°C (Figure 7A, lanes 3 and 4). The yield of miniPLAP in this case was somewhat lower than in the pH 7.5-washed sample, but considerable nevertheless (the miniPLAP yield increased from <20% of the labeled bands to ~50%; Figure 7B, shaded bars). The results indicate that lumenal constituents of the ER play a role

in the GPI anchoring reaction after translocation of the proprotein, but before the proprotein is recognized and processed by the transamidase.

Discussion

GPI-anchored proteins are synthesized in the ER from precursor polypeptides (preproproteins) containing an N-terminal ER-targeting signal sequence and a C-terminal GPI-directing sequence. The proteins are translocated across the ER membrane and in almost all cases the N-terminal signal sequence is removed by the signal peptidase complex to yield the proprotein. Since the C-terminal signal sequence is typically 18–32 amino acids long and the ribosomal cleft protects ~40 amino acids, translation must be complete before GPI addition can occur in order to release the nascent chain from the ribosome and expose the GPI modification site. In this paper we use a model protein, preprominiPLAP (derived from placental alkaline phosphatase; PLAP) to study conversion of proproteins to the GPI-anchored form. We show that conversion of prominiPLAP to the GPI-anchored form (miniPLAP) is dependent on components of the ER lumen; these may be molecular chaperones, such as BiP/GRP78, or hitherto unidentified protein(s) interacting with the GPI anchor precursor. We also show that the requirement for ER lumenal components in protein translocation (Nicchitta and Blobel, 1993) is more stringent for translocation of water soluble polypeptides than for translocation of membrane proteins, such as the proprotein precursors of GPI-anchored proteins.

Synthesis of GPI-anchored miniPLAP is abolished in ER microsomes depleted of lumenal content

In order to explore prominiPLAP conversion to GPI-anchored miniPLAP we prepared ER-derived thymoma cell microsomes that were largely depleted of lumenal components such as BiP/GRP78 and PDI. Lumen depletion was achieved by diluting the microsomes in high pH buffers and re-isolating the microsomes by centrifugation through a sucrose cushion at pH 7.5. Exposure of thymoma cell ER vesicles to pH 10.0 buffer resulted in ~67% loss of BiP, somewhat lower than that reported by Nicchitta and Blobel (1993) for canine pancreas microsomes, where high pH caused the loss of almost all BiP/GRP78. However, as shown previously, the release of lumenal proteins varies depending on the source of membranes (Paver *et al.*, 1989). Also, the incomplete release of proteins like BiP can be explained by the observation that a large fraction of BiP appears to be immobilized in a calcium stabilized matrix in the ER lumen (Booth and Koch, 1989). This last point is consistent with our observations that indicate that substantial amounts (~67%) of PDI, and presumably other lumenal constituents (proteins and small molecules), are lost with a pH 9.0 wash, while removal of BiP requires a wash pH >9.0.

Processing of prominiPLAP to GPI-anchored miniPLAP was abolished in microsomes washed at pH >9.0. Control experiments showed that this observation could not be attributed to a direct effect of pH on the GPI transamidase nor could it be assigned to a translocation defect, as prominiPLAP translocation was unaffected in lumen-depleted microsomes. The latter observation is discussed more fully below. The loss of prominiPLAP processing occurred only when microsomes were washed in buffers of pH high enough to remove BiP; no effect on GPI anchoring was seen when washes at pH <9.0 were used, although at this pH PDI, and presumably other lumen constituents (proteins and small molecules), are largely removed. This observation is in good agreement with previous results showing that proproteins *in vivo* are found in a complex with BiP (Amthauer *et al.*, 1993). The cumulative data are consistent with an essential role for a lumenal protein such as BiP/GRP78 (or other lumenal constituents that are removed only by washes of pH >9.0; see below) in the conversion of prominiPLAP to miniPLAP. We did not attempt to verify the specific participation of lumenal proteins, particularly BiP, in the GPI anchoring reaction by re-introducing the protein(s) into lumen-depleted microsomal vesicles.

Role of ER lumenal content in protein translocation

Our analyses show that lumen-depleted microsomes are fully active in translocation and N-terminal signal cleavage of preprominiPLAP and the signal-cleaved product, prominiPLAP, is protected from exogenously added proteinase K (Figure 5). These data suggest that, unlike the situation with prolactin (Nicchitta and Blobel, 1993; see also Figure 5A for data on prolactin), a comparative absence of lumenal proteins does not affect translocation of prominiPLAP. It has been reported previously that various protein modifications can prevent reversible transport of translocated polypeptide chains. For example, *cis*-side release of signal sequence-cleaved bactericidal/permeability-increasing protein could be stopped by either addition of a stop codon, further extension of the chain or addition of a glycosylation site (Ooi and Weiss, 1992). The most likely explanation for the difference between prolactin and prominiPLAP translocation (Figure 5) in microsomes with a reduced concentration of lumenal ER proteins is that while prolactin is a soluble protein, prominiPLAP contains a hydrophobic C-terminal signal sequence which functions as a membrane anchor (Figure 1C). In this context it is worth mentioning that although there is no primary sequence homology between the C-terminal sequences of the proprotein precursors of GPI-anchored proteins, the general features of the GPI signal sequence, such as critical length (10–15 amino acid residues) of the hydrophobic domain and the requirement for a small amino acid at the cleavage site, are preserved in various species (Udenfriend and Kodukula, 1995). This suggests that the C-terminal hydrophobic signal sequence might be important both for anchoring the translocated proprotein in the membrane bilayer and for recognition by the GPI transamidase.

Potential roles for ER lumenal proteins in GPI anchoring

Complexity in the processing of proproteins to GPI-anchored forms was originally identified by Amthauer *et al.* (1992a), who exploited the observation that in cell-free assays GPI modification (i.e. appearance of miniPLAP) lags behind synthesis of prominiPLAP by ~15 min (see also Figure 1B). Microsomes isolated by centrifugation at this point in the assay contain promini-

PLAP and very little GPI-anchored miniPLAP. Incubation of these pre-loaded microsomes at 30°C resulted in some conversion of prominiPLAP to miniPLAP, but efficient conversion required that the assay mixture be supplemented with ATP and/or GTP (Figure 2). Non-hydrolyzable forms of the nucleotides were ineffective (Amthauer *et al.*, 1992a). Amthauer *et al.* (1992a) suggested that ATP participates in prominiPLAP processing via an ATP-dependent, chaperone-mediated reaction in which prominiPLAP undergoes some form of conformational maturation that permits the GPI signal sequence to be recognized by the transamidase. In separate experiments Amthauer *et al.* (1993) garnered support for this hypothesis by showing that BiP/GRP78 co-immunoprecipitates with a number of proproteins.

The ability of GTP to substitute for ATP in supporting prominiPLAP processing is less easily explained, as GTP, unlike ATP, is poorly transported across microsomal membranes (Clairmont *et al.*, 1992) and must therefore act on the external surface of the microsomal vesicles. GTP is required for protein translocation (Gilmore, 1993) and is known to stimulate GPI biosynthesis (Stevens, 1993). Since protein modification by GPI is post-translocational, GTP is unlikely to exert an effect via components of the translocation machinery. However, although the assay system is not responsive to supplementation with sugar nucleotides [UDP–GlcNAc required for the first reaction in GPI assembly (Doering *et al.*, 1989) and GDP–mannose, required indirectly for the mannose components (Menon *et al.*, 1990)], it is conceivable that GTP stimulates GPI synthesis from endogenous pools of partially assembled GPI structures and endogenous donors of anchor components [mannosylphosphoryldolichol (Menon *et al.*, 1990) and phosphatidylethanolamine (Menon *et al.*, 1993)]. Since GPIs are essential for C-terminal processing of prominiPLAP (Kodukula *et al.*, 1992b), GTP may act by promoting GPI synthesis to generate the amount of anchor precursor needed to function in the transamidase reaction.

In the framework of these ideas it may be appropriate to postulate the existence of other lumenal proteins that participate in GPI anchoring by interacting with the GPI structure rather than with the pro-protein. Evidence from mammalian and protozoal systems suggests that GPIs are biosynthesized on the cytoplasmic face of the ER and that the GPI anchor precursor is flipped into the lumenal leaflet for transfer to protein (Vidugiriene and Menon, 1993, 1994; Mensa-Wilmot, 1994). We speculate that the ER lumen may contain a protein(s) that serves to retain newly flipped anchor precursors in the lumenal leaflet of the ER, and 'guide' the precursors to the transamidase for transfer to protein. Such a protein may well be a GPI binding lectin which, like BiP, is removed by washing microsomes only at high dilution in buffers of pH >9.0. The recent identification of a number of lectins, or proteins with proposed lectin-like properties, in the ER, the ER–Golgi intermediate compartment and other organelles in the eukaryotic secretory pathway (Fiedler *et al.*, 1994; Helenius, 1994, Arar *et al.*, 1995) suggest that carbohydrate recognition events may serve a general function in the modification and transport of secretory proteins.

In summary, ER lumenal proteins may be involved in the GPI anchoring reaction by 'preparing' both substrates, the proprotein and the GPI anchor precursor, for recognition/processing by the transamidase. Reaction progress from either direction, i.e. ATP-dependent, chaperone (BiP/GRP78?)-mediated maturation of the proprotein or GTP-stimulated GPI biosynthesis followed by flipping and recognition by a lumenal lectin, would appear to be sufficient to drive processing of prominiPLAP in the post-translocational, translation-independent GPI anchoring assay.

Materials and methods

Materials

Translation grade [^{35}S]methionine was obtained from DuPont NEN (Wilmington, DE). SP6 RNA polymerase and *Hin*dIII were purchased from New England Biolabs. Rabbit reticulocyte lysate and a mixture of amino acids (minus methionine) were obtained from Promega (Madison, WI) and creatine phosphate, creatine kinase and *Staphylococcus aureus* nuclease were from Boehringer Mannheim Biochemicals. Alkaline phosphatase-conjugated rabbit anti-chicken/turkey IgG was purchased from Zymed Immunochemicals (San Francisco, CA). Horseradish peroxidase-linked anti-rabbit IgG and the ECL Western blotting kit were from Amersham Corporation. Plasmid pGEM-4Z/miniPLAPωSer was a gift from Dr Sidney Udenfriend (Roche Institute of Molecular Biology, Nutley, NJ) and preprolactin mRNA was provided by Dr Chris Nicchitta (Duke University Medical Center). Rabbit anti-BiP antibodies were generously provided by Dr Nathan Brot (Roche Institute of Molecular Biology, Nutley, NJ) and chicken antibodies against PDI were provided by Dr Ronald Raines (University of Wisconsin–Madison).

Cell culture

The mouse thymoma cell line BW5147.3 was maintained in suspension culture in DME supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37°C.

Microsome preparation

Rough microsomes were prepared as described previously (Vidugiriene and Menon, 1993) with minor procedural modifications. BW5147.3 thymoma cells ($2–5\times10^8$) were collected by centrifugation, washed with phosphate-buffered saline (PBS), resuspended in buffer A [0.25 M sucrose, 50 mM HEPES–NaOH, pH 7.5, 1 mM dithiothreitol (DTT)] supplemented with 1 μg/ml leupeptin and 0.1 mM TLCK and disrupted by nitrogen cavitation, followed by three strokes of a tight pestle dounce homogenizer. The resulting lysate was treated with *S.aureus* nuclease (1.5 U/ml) for 20 min on ice, then clarified by centrifugation at 10 000 *g* for 15 min at 4°C. The supernatant was layered on a cushion of 1.3 M sucrose in 50 mM HEPES–NaOH, pH 7.5, and centrifuged in a Beckman 70Ti rotor (Beckman Instruments Inc., Fullerton, CA) at 43 000 r.p.m. for 3 h at 4°C. The membrane pellet was resuspended in buffer A at a concentration of 50 A_{280} U/ml (determined in 1% SDS), diluted with an equal volume of buffer B (1 M KOAc, 50 mM HEPES–NaOH, pH 7.5, 10 mM EDTA, 1 mM DTT) and incubated for 20 min on ice. The resulting salt-washed (ribosome-free) membranes were collected by centrifugation through a cushion of 0.5 M sucrose, 0.5 M KOAc, 50 mM HEPES–NaOH, pH 7.5 (Beckman TLA 100.3 rotor, 75 000 r.p.m., 45 min, 4°C). The pellet was resuspended to the original starting volume in buffer A and aliquots were rapidly frozen and stored at –70°C.

Depletion of microsomal lumenal content

For depletion of lumenal content microsomes (typically 25 μl) were diluted 10-fold in 50 mM HEPES, 50 mM CAPS at the indicated pH. Following 30 min incubation on ice the membranes were pelleted through a 50 μl cushion of 0.5 M sucrose in 50 mM HEPES–NaOH, pH 7.5 (Beckman TLA 100.1 rotor, 45 min, 90 000 r.p.m., 4°C). The pellet was resuspended in the original starting volume in buffer A. In some experiments microsomes were re-isolated by centrifugation through a cushion of unbuffered 0.5 M sucrose before being diluted into high pH buffer (different fold dilutions in the range 1:1–1:10 were used as specified).

The efficiency of the high pH wash in depleting microsomal lumenal content was assessed by determining the amount of BiP and PDI remaining in the membrane fraction. Analysis of proteins was carried out by SDS–PAGE and Western blotting, typically loading 0.5–3 membrane

equivalents/gel lane. After probing with primary antibodies (chicken anti-PDI or rabbit anti-BiP) blots were developed using peroxidase-conjugated second antibodies and the ECL method (BiP) or alkaline phosphatase-conjugated second antibodies and a colorimetric method (PDI). Band intensities were determined by densitometry.

Translation–translocation assays

PreprominiPLAP mRNA was prepared from plasmid pGEM-4Z/mini-PLAPωSer (Kodukula *et al.*, 1991; Amthauer *et al.*, 1992) by *Hin*dIII linearization and SP6 RNA polymerase-mediated transcription. *In vitro* translation reactions were performed in nuclease-treated rabbit reticulocyte lysate as previously described (Kodukula *et al.*, 1991), using [^{35}S]methionine to tag the translation product. For translocation of preprominiPLAP and formation of mature GPI-anchored miniPLAP membranes were included in the translation reaction mixture at a final concentration of 1 equivalent/11 μl (Walter and Blobel, 1981). The reaction was typically allowed to proceed for 2 h at 30°C, after which the products were analyzed by SDS–PAGE (15% gels) and fluorography. A maximum of 10 μl/gel lane of the reaction mixture was loaded to obtain adequate separation of radioactive bands. Bands were quantitated by phosphorImager analysis (Molecular Dynamics).

Protein translocation was assessed by protease protection assays. After the translation–translocation reaction samples were placed on ice, diluted to 50 μl with 150 mM KOAc, 50 mM HEPES–NaOH, pH 7.5, 2.5 mM $Mg(OAc)_2$, 1 mM DTT and proteinase K was added to a final concentration of 100 μg/ml. The samples were incubated for 30 min on ice before adding phenylmethylsulfonyl fluoride (4 mM final concentration, added from a 150 mM stock in ethanol) to stop proteolysis. The samples were diluted with 2 vol. saturated ammonium sulfate. Following 30 min incubation on ice, products were precipitated by centrifugation for 20 min at 12 000 *g*. The pellet was washed with 7% trichloracetic acid and analyzed by SDS–PAGE as described above.

Translation-independent translocation of preprominiPLAP

Translation-dependent translocation of preprominiPLAP was carried out for 20 min at 30°C as described above, with the exception that 3–5 reaction mixtures were combined. The translocation reaction was stopped by placing samples on ice and diluting 5-fold with 50 mM HEPES–NaOH, pH 7.5. The membranes were collected by centrifugation (Beckman TLA 100.1 rotor, 90 000 r.p.m., 45 min, 4°C) through a 50 μl cushion of 0.5 M sucrose in 50 mM HEPES–NaOH, pH 7.5. The pellet was resuspended in 30 μl buffer C [buffer A supplemented with 50 mM KOAc, 5 mM $Mg(OAc)_2$]. For standard incubations the sample was supplemented with 2.5 mM ATP, 0.5 mM GTP, 20 mM creatine phosphate and 100 μg/ml creatine kinase. For the experiments presented in Figure 2 several conditions were used: no addition was made or the reaction was supplemented with 1 mM GTP or supplemented with 1 mM ATP + 20 mM creatine phosphate + 100 μg/ml creatine kinase or supplemented with GTP + the ATP–creatine phosphate–creatine kinase mixture. For ER lumen depletion experiments the membranes were resuspended in 10 μl buffer A (instead of 30 μl buffer C) and diluted 10-fold with 50 mM HEPES, 50 mM CAPS, pH 7.5 (mock) or 10.0 (test sample) and left for 30 min on ice. The microsomes were re-isolated by centrifugation through a sucrose cushion and the membrane pellets were resuspended in buffer C. All samples were then processed as follows. Half of the sample was kept on ice, while the other was incubated at 30°C for 2 h to induce the GPI anchoring reaction. At the end of the reaction the samples were placed on ice, treated with proteinase K and analyzed on SDS–PAGE as described above.

Carbonate extraction

Membranes were carbonate extracted according to previously described procedures (Nicchitta and Blobel, 1993). Briefly, translocation reactions were diluted 5-fold with ice-cold 0.1 M Na_2CO_3, pH 11.5 or with a buffer consisting of 150 mM potassium acetate, 50 mM HEPES–NaOH, pH 7.5, 2.5 mM $MgCl_2$. Following 20 min incubation on ice, samples were layered onto a 50 μl cushion of 0.5 M sucrose, 0.1 M Na_2CO_3 or 0.5 M sucrose, 50 mM HEPES–NaOH, pH 7.5, respectively. The membranes were recovered by centrifugation at 4°C in a Beckman TL100.1 rotor (Beckman Instruments Inc., Palo Alto, CA) for 45 min at 90 000 r.p.m. The pelleted microsomes were resuspended in SDS sample buffer and analyzed by electrophoresis using 15% SDS–polyacrylamide gels. The supernatant and cushion fractions were combined and adjusted to pH 7.5 with glacial acetic acid in the case of carbonate-treated samples. The samples were processed by adding 2 vol. saturated ammonium sulfate and incubating on ice for 20 min to precipitate proteins. The precipitate was recovered by centrifugation for 20 min at 12 000 *g*, washed with 5% trichloracetic acid and analyzed by SDS–PAGE using 15% gels.

Acknowledgements

This work was supported by NIH grant AI28858 and the University of Wisconsin. The authors are grateful to Drs Nathan Brot, Chris Nicchitta, Ron Raines and Sidney Udenfriend for generously providing reagents and Laura van der Ploeg and Adam Steinberg for preparing the figures. A.K.M. would like to acknowledge Bob Dylan, Ethan Allen Hawley, Horace Rumpole, the Rosemount Estate, I.Menon and K.M.M. for stimulation and K.M.M. for help with manuscript preparation.

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Received on March 17, 1995; revised on May 30, 1995