Export of a misprocessed GPI-anchored protein from the endoplasmic reticulum in vitro in an ATP- and cytosol-dependent manner

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Abstract Strict quality control mechanisms within the mammalian endoplasmic reticulum act to prevent misfolded and unprocessed proteins from entering post-endoplasmic reticulum (ER) compartments. Following translocation into the ER lumen via the Sec61p translocon, nascent polypeptide chains fold and are modified in an environment that contains numerous chaperones and other folding mediators. Recently it has emerged that polypeptides failing to acquire the native state are re-exported from the ER to the cytosol for ultimate degradation by the proteasome ubiquitin system, apparently mediated again via Sec61p. Substrates for this degradation pathway include proteins destined to become glycosyl phosphatidylinositol (GPI)-anchored, but which fail to be processed and retain the C-terminal GPI signal peptide. In order to characterise this process we have used a model GPI-anchored mutant protein, prepro mini human placental alkaline phosphatase (PLAP) W179, which cannot be processed efficiently on account of being a poor substrate for the transamidase which cleaves the GPI signal peptide and adds the GPI anchor in a coupled reaction. In vitro transcription, translation and translocation into canine pancreatic microsomes resulted in ER-targeting signal sequence cleavage and formation of prominiPLAP in the ER lumen. We were able to show that prominiPLAPW179 could be exported from the microsomes in a time-dependent manner and that release requires both ATP and cytosol. Export was not supported by GTP, indicating a biochemical distinction from glycopeptide export which we showed recently requires GTP hydrolysis. The process was not affected by redox, unlike several other GPI-anchored model proteins. These data demonstrate that misprocessed proteins can be exported in vitro from mammalian microsomes, facilitating identification of factors involved in this process. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Misfolded protein; Glycopeptide; Glycosyl phosphatidylinositol; Endoplasmic reticulum; Endoplasmic reticulum-associated degradation; Transport

1. Introduction

In eukaryotic cells, endoplasmic reticulum (ER)-targeted proteins are translocated into the ER lumen cotranslationally or posttranslationally via the Sec61p translocon complex. During or shortly after translocation various processes, including removal of signal peptide, disulfide bond formation, addition of a glycosyl phosphatidylinositol (GPI) anchor and N-glycosylation, are initiated and the protein enters into one of various folding pathways [1,2]. A stringent quality control machinery at the ER level ensures that misfolded and unassembled proteins are retained in the ER lumen by molecular chaperones and then exported back to the cytosol where they are degraded by the ubiquitin/proteasome systems [1,3–8]. Interestingly, these proteins are exported via the same channel through which they were originally imported into the ER, namely the Sec61p translocon [9,10]. Allowing misfolded proteins and folding intermediates into the Golgi complex or other post-ER secretory pathway compartments can lead to competition with true secretory cargo for the various modification and trafficking processes with potential deleterious consequences. Recent data suggest that the calnexin component of the quality control system is able to sense subtle differences between conformation of folding proteins [11].

The GPI anchor, a complex glycolipid, is the sole mechanism of cell membrane attachment for many proteins in most if not all eukaryotic cells [12,13]. GPI attachment requires translocation of the nascent polypeptide chain across the ER membrane followed by cleavage of a C-terminal signal between two small side chain amino acids 10–12 residues N-terminal of the hydrophobic domain and its replacement, in a coupled process, by a preformed GPI moiety [13]. When GPI anchor addition is prevented by mutation of the cleavage site, instead of rapid export of the protein to the cell surface the protein fails to be included into ER transport vesicles [14], is retained and in common with other misfolded proteins is degraded by the ubiquitin/proteasome system [8]. GPI anchoring has been efficiently reproduced in an in vitro system [13] using an engineered model protein miniPLAP (mPLAP) based on the human placental alkaline phosphatase (PLAP). Prepro-mPLAP is devoid of N-glycosylation sites, the catalytic site and most cysteine residues [13]. A mutant form of prepro-mPLAP (S179W substitution) was in vitro translated and translocated into the ER, its N-terminal signal sequence was cleaved off to give promPLAPW179 but it was not further processed by the transamidase and did not receive a GPI anchor [15]. We used this system to study the quality control of GPI-anchored proteins in canine pancreatic microsomes. We show that mPLAPW179 indeed enters the ER but does not become GPI-anchored. Most significantly, we find that promPLAPW179 is exported out of the ER in an ATP-, cytosol- and time-dependent manner.

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2. Materials and methods

2.1. Materials

Nuclease-treated rabbit reticulocyte lysate, amino acid mixture minus methionine, canine microsomal membranes, signal sequence control (prolactin) mRNA, RNasin ribonuclease inhibitor and nuclease-free water were all supplied by Promega. mPLAP pGEM plasmids were the kind gift of Dr E. Medof (University of Cleveland, OH, USA). Radiolabelled compounds were from ICN Pharmaceuticals. Restriction enzymes were from Stratagene and other reagents were either from Sigma or from other suppliers and of the highest available grade.

2.2. Preparation of mPLAP mRNA

pGEM mPLAP plasmids (200 μg) were digested with HindIII. Digestions were shown to be complete by analysis of an aliquot by agarose gel electrophoresis. Plasmid DNA preparations were rendered RNase-free by ethanol precipitation following phenol extraction (twice) and resuspended in diethyl pyrocarbonate-treated water. mRNA was synthesised using a RiboMax kit (Promega). A typical 250 μl reaction mixture contained 50 μl of SP6 transcription 5×buffer, 50 μl of a mixture of rNTPs (25 μM ATP, CTP, GTP, UTP), 125 μl linear DNA template (50–100 μg total) plus nuclease-free water and 25 μl of enzyme mix (SP6). The reaction mix was incubated at 37°C for 4 h. Following in vitro transcription, the RNA was DNase-treated and RNA extracted by the addition of one volume of TE-saturated (pH 4.5) phenol:chloroform (25:24). The solution was vortexed for 1 min and then spun in a microfuge for 2 min. The upper aqueous phase was transferred to a fresh tube and one volume of chloroform was added. This was vortexed and spun as before. The resulting aqueous phase was again transferred to a fresh tube and the RNA ethanol precipitated. The RNA samples were stored in aliquots at −85°C until subsequent use.

2.3. Translation/translocation/processing of mPLAP

Cell-free translation reactions were conducted using the rabbit reticulocyte lysate as described by Pelham and Jackson [16] with the addition of canine pancreatic microsomal membranes for processing. Typically a 25 μl reaction mixture contained, in the following order, 1.5 μl of nuclease-free water, 3 μl of mRNA (either mPLAPS179 or mPLAPW179 mRNA), 1 μl of RNasin ribonuclease inhibitor, 12.5 μl of the nuclease-treated lysate, 1 μl of a mixture of amino acids minus methionine (1 mM), 2.5 μl of canine microsomes and 4 μl of [^{35}S]Trans-Label (ICN). In order to compare the unprocessed form of the protein with the processed form, control translation reactions were carried out in the absence of microsomes. The mRNA was denatured at 65°C for 3 min in nuclease-free water and immediately cooled on ice prior to the addition of the other components. Reaction mixtures were then incubated at 30°C for 90 min. Prior to analysis by SDS–PAGE or further assays, the samples were typically centrifuged at 20 000×g at 4°C for 10 min in order to pellet the membranes. The membranes were then resuspended in 25–70 μl of B88 reaction buffer (20 mM HEPES, pH 7.4, 150 mM potassium acetate, 250 mM sorbitol, 5 mM magnesium acetate), and 5 μl aliquots of this were taken for assay or analysis.

2.4. Protease protection assay

Proteinase K from a stock solution was added to a final concentration of 100 μg/ml and incubated on ice for 30 min. The digestions were quenched by the addition of phenylmethylsulphonyl fluoride (PMSF) to a final concentration of 4 mM. The treated samples were immediately heated in SDS–PAGE sample buffer at 95°C and analysed by SDS–PAGE.

2.5. Export assay

Reaction mixtures were prepared for export assays in a total of 25 μl and contained 12.5 μl B88 buffer, 5 ml of resuspended membranes, 5 μl cytosol and finally 2.5 μl of 10×ATP regenerating mix (10 mM ATP, 400 mM creatine phosphate, 2 mg/ml creatine kinase, 500 μM GDP-mannose [17]). Controls included reactions without ATP mix, without cytosol and full reaction incubated on ice for the duration of the assay. For some reactions GTP was added to a concentration of 10 μM. Reactions were incubated at 32°C for a specified period of time after which samples were centrifuged for 10 min at 20 000×g at 2°C for 10 min. The supernatants were removed, representing the

cytosolic fractions, and placed in fresh tubes. The pellets (membrane fractions) were resuspended in 20 μl of phosphate-buffered saline. Equivalent aliquots of the samples were analysed by SDS–PAGE.

2.6. Gel electrophoresis and autoradiography

The denatured proteins were fractionated on 15% SDS polyacrylamide mini-gels according to Laemmli [18]. After SDS–PAGE, the gels were fixed and stained with Coomassie blue (0.1% brilliant blue, 50% methanol, 10% acetic acid and 40% distilled water) for 1 h then destained. Destained gels were then soaked with En^{3}Hance (NEN Life Science Products). Following impregnation with En^{3}Hance, the gels were dried and exposed to film (Kodak Biomax film) for 1–4 days at −85°C. Autoradiographs were digitised using a Scanmaker II scanner and analysed using NIH-Image.

2.7. Preparation of rat liver cytosol

Cytosol used was prepared from rat liver as previously described [19,20]. Briefly, rat livers obtained from Harlan Sera-Lab Ltd. (Loughborough, UK) were homogenised in B88 buffer (20 mM HEPES, pH 7.4, 150 mM potassium acetate, 250 mM sorbitol, 5 mM magnesium acetate) containing 1 mM PMSF and 1 mM dithiothreitol (DTT). The homogenate was cleared of large cell debris by centrifugation at 8000×g for 10 min and the supernatant was subjected to a 100 000×g centrifugation for 40 min. The supernatant was gel-filtered through a Sephadex G-10 column into B88 buffer containing 1 mM DTT. The cytosol was stored at −85°C in 100 μl aliquots at 35 mg/ml protein. A fresh aliquot was used for each experiment.

3. Results and discussion

3.1. MiniPLAPW179 mutant protein is imported in vitro into canine pancreatic microsomes but not GPI-anchored

We first wished to ensure that the mPLAPW179 did not become GPI-anchored by our canine microsome system. To achieve this we compared the translation products of mPLAPW179 and mPLAPS179 in vitro transcribed mRNAs. The mPLAPS179 variant is a good substrate for transamidase and is GPI-anchored by the in vitro system (Fig. 1A) [13]. We performed translation reactions in the presence or absence of microsomes and the resulting proteins were analysed by SDS–PAGE and autoradiography. In the absence of microsomes prepromPLAPW179 was the sole translation product detected from the corresponding mRNA (M_r ∼28 kDa) (Fig. 1B) indicating translation but no further processing. However, in the presence of microsomes this species was converted quantitatively to the pro form indicating N-terminal signal sequence cleavage (Fig. 1B; M_r ∼27 kDa). Significantly, no GPI-anchored form of mPLAPW179 can be seen which is in agreement with previous work [15]. As a control, β-lactamase mRNA was translated under the same conditions and shown to be translocated into microsomes and signal peptide processed efficiently (Fig. 1B). A small amount of a lower molecular weight species was also detected which was processed in the presence of microsomes suggesting that it is a form derived from prematurely terminated translation. When mPLAPS179 was similarly translated in the presence of microsomes, it was quantitatively N-terminally processed and partially GPI-anchored (not shown and Fig. 1C). To demonstrate that the S and W mPLAP species had been translocated to the interior of pancreatic microsomes, we performed a protease protection assay following translocation. Translation reactions (with or without microsomes) were treated with proteinase K at 100 μg/ml on ice for 30 min [21] and products then analysed by SDS–PAGE. In the absence of microsomes both mPLAPS179 and mPLAPW179 were fully susceptible to proteinase K treatment (Fig. 1C) whereas in the presence of mi-
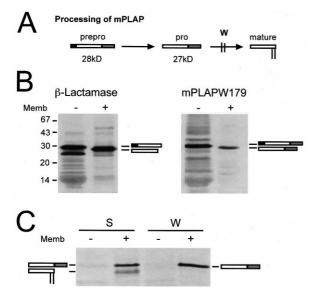
Fig. 1. Processing of mPLAP by canine pancreatic microsomes. A: Schematic presentation of processing of mPLAP. PrepromPLAP (28 kDa) has both an N-terminal signal peptide for directing insertion into the ER and a C-terminal signal peptide for transamidase cleavage and addition of the GPI anchor. The N-terminal signal is removed cotranslationally by signal peptidase to give the pro form (27 kDa) which for mPLAPSer179 is processed by the transamidase to the mature GPI-anchored form. mPLAPW179 is not processed beyond the pro form and therefore is expected to be handled by the ERAD system. B and C: Cotranslational processing and translocation of mPLAP and β-lactamase into canine pancreatic microsomes. B: mPLAPW179 and β-lactamase mRNA were translated with a rabbit reticulocyte lysate system in the presence or absence of canine pancreatic microsomes. In the absence of microsomes prepromPLAP ($M_r \sim 28$ kDa) accumulated whereas in the presence of microsomes it was quantitatively processed by the signal peptidase to promPLAP. β-Lactamase also shows the signal peptide cleavage of the prepro to the pro form. mPLAPS179 mRNA gave similar results to mPLAPW179 except that an additional band with smaller M_r (~ 24 kDa) is present when microsomes were added indicating transamidase processing and GPI anchoring (C). C: mPLAP translated in the presence of microsomes is protected from proteinase K treatment. After translation in the presence or absence of microsomes, samples were proteinase K-treated. Proteinase K was quenched by the addition of PMSF and reactions were boiled immediately in sample buffer and analysed by SDS–PAGE and autoradiography as described in Section 2. Microsomes resulted in the protection of the signal peptide processed mPLAP (S and W) and the formation of a GPI-anchored species in the case of mPLAPS179. S = mPLAPS179; W = mPLAPW179; Icons correspond to various processing forms described in A. Molecular weight standards are indicated in kDa.

crosomes the translocated chains were protected; these correspond to both the pro and mature forms of mPLAPS179 and the pro form of mPLAPW179. This confirms that the signal peptide-processed mPLAP polypeptides are indeed within the microsome lumen and that the W179 mutant behaves as predicted and is not processed by GPI addition.

3.2. Reconstitution of export of mPLAPW179

mPLAPS179 was only partially GPI-anchored in canine pancreatic microsomes with the consequence that the majority of this translation product could potentially enter the re-export system. This mixed population of species is unsuitable for further analysis as a control construct. Hence, as we were interested in the fate of the mutant mPLAPW179 due to lack of a GPI anchor, we focused our studies exclusively on this protein. Previous in vivo studies on a mutant GPI-an-

chored protein derived from human growth hormone (hD28) implicated the ER-associated degradation (ERAD) system for its disposal as the protein half-life was increased by proteasome inhibitors and was decreased by ubiquitination [8]. Studies on in vitro systems in yeast have demonstrated that ERAD requires ATP and cytosol for reconstitution [6]. Addition of proteasome inhibitors to cells expressing ERAD substrates results in accumulation of mutant protein in the cytosol [22], and it has recently been shown that ERAD substrates are re-exported to the cytosol via the Sec61p pore complex [9,10]. Using the above information, assays were constructed in order to reconstitute the process of ERAD in this in vitro system using mPLAPW179 as substrate. We used the release of mPLAPW179 polypeptide into soluble form as a readout for export. Once the translation/processing reactions had been completed, the pelleted microsomal membranes were resuspended in B88 buffer, and aliquots incubated under various conditions (see Section 2 for details). After incubation for 90 min at 32°C, the samples were again centrifuged at $20\,000 \times g$ for 10 min. The supernatant (cytosolic fraction)
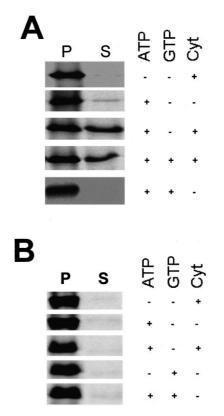

Fig. 2. mPLAPW179 is specifically exported from canine microsomes in an ATP- and cytosol-dependent manner. After translation/translocation of mPLAPW179 (A) or β-lactamase (B) mRNA, microsomes were reisolated and used for export. After 90 min at 32°C, the supernatant (S) was separated from the microsomes (P) by centrifugation and equivalent amounts analysed by SDS–PAGE. ATP or cytosol alone had no effect on the distribution of the mPLAPW179 (A, upper two rows) whereas when added together, a significant amount of protein was recovered in the cytosolic fraction (S in third row from top). GTP at 10 μM has no additional effect on export when added with ATP and cytosol (bottom second row) and did not stimulate export when added with ATP alone (bottom row). Experiments were performed twice and done in duplicate. In contrast, β-lactamase localisation (B) was not affected by incubating the microsomes under any conditions which allowed mPLAPW179 release (compare third rows of A and B).

was separated from the pellet (membranes) and analysed by SDS–PAGE (Fig. 2A). Incubating the loaded microsomes with cytosol at 32°C had no effect on the partitioning of mPLAPW179 as the vast majority of the protein was recovered in the microsomal fraction (Fig. 2A, top row). Incubation with an ATP regenerating system (see Section 2) also had very little effect on the localisation of the mutant protein (which remained within the microsomes). These assays also indicate that handling of the microsomes did not cause non-specific leakage of contents. By contrast, when both ATP and cytosol were present in the incubation reactions, a significant amount of mPLAP (∼20%) was released into the medium indicating export from ER to cytosol (Fig. 2A, 3rd row). Addition of GTP at 10 μM to cytosol (not shown) or to cytosol plus ATP had no additional effect on the proportion of exported mPLAPW179 (Fig. 2A, 4th row). Significantly, GTP did not bypass the requirement for cytosol in the presence of an ATP regenerating system for mPLAPW179 export (Fig. 2A, bottom row) which is in contrast to glycopeptide export [23]. Microsome latency was not compromised under the export conditions described here as evidenced by the retention of translocated β-lactamase in the microsomal fraction

Fig. 4. Redox conditions have no significant effect on export of mPLAPW179 from microsomes. 5 mM DTT was added to the export reaction mix as indicated on the graph and reactions were then incubated at 32°C for 90 min. Reactions were split into pellet and supernatant and analysed as in Fig. 3. Only reactions containing both ATP and cytosol showed significant amounts of mPLAPW179 release into the supernatant. Controls (cytosol, ATP and GTP alone) represent single data points, for full reactions error bars are indicated ($n = 2$).

after incubation with ATP, cytosol, GTP or these components in combination (Fig. 2B).

Hence, export of mPLAPW179 from the ER can be reconstituted in vitro and requires the presence of both cytosol and ATP, similar to observations for in vitro ER export of misfolded proteins in *Saccharomyces cerevisiae* [6]. Significantly, the failure of GTP to replace cytosol is in contrast to our recent data which demonstrate that glycopeptide export may be reconstituted with ATP and GTP alone [23]. These observations thus indicate that glycopeptide export and misfolded protein export are biochemically distinct and that misfolded protein export requires additional cytosolic factors.

3.3. Export of mPLAPW179 is time-dependent

In order to confirm that mPLAPW179 export was physiologically relevant, we studied the microsome export kinetics in the presence of ATP and cytosol. Fig. 3 shows that export was time-dependent with about 20% of the protein exported after 120 min. Export is linear with time and requires both ATP and cytosol. ATP or cytosol alone control incubations showed no significant export. These data demonstrate that mPLAPW179 is exported in a time-dependent fashion and also that membrane integrity was not compromised over the 120 min, confirming that a bona fide specific export reaction was reconstituted. No further export was observed after 2 h and reached a maximum of 20%. This low efficiency could be due to a tight binding of the mPLAPW179 to molecular chaperones like BiP in the ER [24].

3.4. Redox does not influence mPLAPW179 export

Previously we have shown that redox can play a critical role in the ability of ER to process misfolded protein. Specifically, the model GPI signal containing protein hD28 forms disulphide-linked homooligomers, and degradation of this protein can be accelerated by addition of DTT, which we interpreted as indicating that retranslocation to the cytosol requires monomeric forms of the hD28 [25,26]. In addition, a free cysteine in the C-terminus was found to be essential for effi-
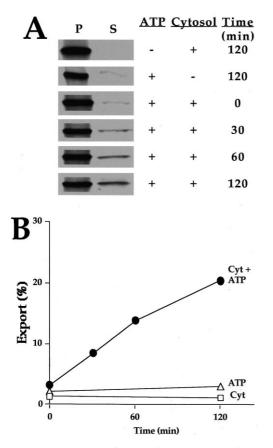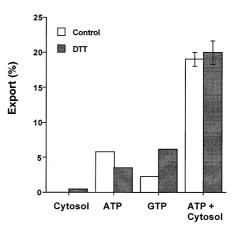

Fig. 3. mPLAPW179 export from canine pancreatic microsomes is time-dependent. Export from mPLAPW179-loaded microsomes was performed for the specified period of time. A: Autoradiograph of the protein recovered in the membrane (P) and cytosolic (S) fractions. B: Quantitation using NIH-Image analysis of this material relative to the total in both cytosol and membrane fractions. In the presence of both ATP and cytosol, a time-dependent export of the mPLAP is observed reaching about 20% after 120 min. Controls shown are ATP or cytosol only. The experiment was repeated twice with essentially identical results.

cient ER retention of hD28 [8]. We chose to investigate the influence of DTT of mPLAPW179 which has only two cysteine residues (182 and 189 of prepromPLAP) at analogous positions within the C-terminus to the critical cysteine in hD28 required for retention [15]. DTT at up to 5 mM in the export assay medium had no effect on the proportion of exported mPLAPW179 (Fig. 4). Addition of DTT with both ATP and cytosol or in the control reactions (ATP alone, cytosol alone or GTP alone) did not increase the level of export (compare white bars and grey bars in Fig. 4), hence for this substrate, redox is not an important factor for re-export.

Reconstitution of ER quality control mechanisms, and in particular the re-export pathway for malfolded proteins, has been achieved in *S. cerevisiae*, but for higher eukaryotes this has proved more problematic. Here we report the re-export of an mPLAP variant that is a model for GPI addition and quality control [13]. Translocation from the microsomal lumen requires ATP and cytosol and is time-dependent, indicating that re-export requires energy and cytosolic factors. However, the reaction is rather inefficient, with export of ~20% of mPLAP representing the maximum we have achieved with this system. This is in contrast to the high efficiency of translocation into the microsomes and also of export of simpler substrates from microsomal membranes, e.g. glycopeptides, where we routinely achieve ~80% re-export of the loaded peptide [20,23]. This low export efficiency may reflect continued engagement with chaperones, which is not expected to occur for the efficiently exported glycopeptide substrate. The differential efficiency may also reflect specific requirements for the export route; in the case of mPLAP we expect that export is via Sec61p, but for glycopeptide it is possible that alternative channels in the ER membrane are used [20,23]. Indeed, as the ER is known to be functionally composed of as yet ill defined subdomains, it is possible that much of the mPLAP translocated into the microsomal lumen is in an environment where export cannot take place as the ER fragment is derived from a region of the organelle devoid of essential export factors. Our data also indicate that the requirements for polypeptide export are more complex than for glycopeptide, as we have recently demonstrated that cytosolic proteins are not required for the latter substrate [23]. This may reflect the need to direct polypeptides efficiently into the cytosolic proteasome as they emerge from the translocon. The system described here should provide a vital tool for the identification of such factors.

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