Bip/GRP78 but not calnexin associates with a precursor of glycosylphosphatidylinositol-anchored protein

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When fused in-frame with a C-terminal propeptide of placental alkaline phosphatase (PLAP), rat α_{2u} -globulin (α GL), a non-glycosylated secretory protein, was expressed on the cell surface as a glycosylphosphatidylinositol (GPI)-linked chimaeric protein (α GL-PLAP). In contrast with the wild-type α GL-PLAP, a mutant, in which Asp at the cleavage/attachment site of GPI was replaced by Trp, failed to become a GPI-linked mature form and was retained as a precursor form within the cell [Oda, Cheng, Saku, Takami, Sohda, Misumi, Ikehara and Millán (1994) *Biochem. J.* **301**, 577–583]. To elucidate the molecular interactions involved in the retention of the proform within the cell, we examined the association of the proform with molecular chaperones in the endoplasmic reticulum (ER). Antibody against the ER retrieval motif KDEL coimmunoprecipitated a 25 kDa proform, but not a 22 kDa GPI-linked mature form. Pulse-chase experiments showed that the wild-type α GL-PLAP with a cleavable propeptide was converted into the mature form, whereas the mutant α GL-PLAP with an uncleavable propeptide

remained associated with ER-resident proteins with a KDEL motif and underwent rapid degradation in a pre-Golgi compartment. Chemical cross-linking studies showed that, of the several ER-resident proteins immunoreactive with the anti-KDEL antibody, a 78 kDa protein was the only protein associated with the proform. Furthermore this 78 kDa protein was dissociated from the precursor molecule on incubation with ATP, allowing us tentatively to assign it as Bip/GRP78. Anti-calnexin antibody, however, failed to coprecipitate any form of the chimaeric protein. Immunoelectron microscopy showed that the proform with the uncleavable propeptide was localized in the ER, but not detected in the Golgi apparatus or plasma membranes. Taken together, these results suggest that Bip/GRP78 is associated with pro α GL-PLAP and retains it within the ER until pro α GL-PLAP is either modified by GPI or degraded, thereby participating in the quality control of this GPI-linked chimaeric protein.

INTRODUCTION

An increasing number of membrane proteins [1–3] have been reported to be anchored on the cell surface via glycosylphosphatidylinositol (GPI). Placental alkaline phosphatase (PLAP) is one of the best characterized mammalian GPI-linked proteins [4–8]. PLAP is initially synthesized as a preproenzyme with N- and C-terminal extensions. The N-terminal peptide is cleaved off as a typical signal sequence during the translocation of newly synthesized PLAP across the endoplasmic reticulum (ER) membrane, yielding proPLAP. The C-terminal extension (propeptide) contains a stretch of 23 predominantly hydrophobic amino acids in a 29-residue sequence [9]. Newly formed proPLAP is converted into a GPI-linked form with a half-life of approx. 5 min [10]. The preassembled GPI is attached to an aspartic acid (Asp⁴⁸⁴) at a cleavage/attachment site of GPI through an amido linkage catalysed by a transamidase [4–7]. The propeptide serves as a GPI-anchor-attachment signal, since, when fused in-frame to the C-terminus of otherwise soluble secretory proteins, the propeptide is capable of rendering them GPI-anchored membrane proteins [11,12]. The importance of the hydrophobicity of the propeptide as a GPI-anchor signal has been extensively studied [11,13]. The propeptide might transiently hold proPLAP on the ER membrane in such a way that the sequence around Asp⁴⁸⁴ is presented to the catalytic domain of the putative transamidase [8].

When a chimaeric protein consisting of rat α_{2u} -globulin (α GL) and the C-terminal 33 amino acids of PLAP (α GL-PLAP) was

expressed in the COS-1 cell, a GPI-linked chimaeric protein was found on the cell surface, whereas the proform (pro α GL-PLAP), still retaining a cleavable GPI-anchor signal, did not appear on the cell surface [12]. Likewise, a mutant α GL-PLAP with an uncleavable GPI-anchor signal failed to reach the cell surface. As judged by immunofluorescence microscopy [12], pro α GL-PLAP molecules were largely localized in a fine reticular network and nuclear envelope in the cell expressing the mutant chimaeric protein, suggesting their ER localization. These findings prompted us to identify the molecular interactions responsible for the retention of the precursor molecules of α GL-PLAP within the ER.

In this study, we examined the association of pro α GL-PLAP with two ER-resident chaperones, Bip/GRP78 [14,15] and calnexin [16], by coimmunoprecipitation assays. We show that, of several ER-resident proteins with the KDEL motif, only Bip/GRP78 is responsible for retaining the precursor within the ER. In contrast, pro α GL-PLAP was not coprecipitated by anti-calnexin antibody, indicating that calnexin is not involved in the retention of pro α GL-PLAP in the ER.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: [³⁵S]methionine from Dupont–New England Nuclear (Boston, MA, U.S.A.); ¹⁴C-

Abbreviations used: α GL, α_{2u} -globulin; Bip, binding protein; DMEM, Dulbecco's modified Eagle's medium; DSP, dithiobis(succinimidyl propionate); ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; GRP78, glucose-regulated 78 kDa protein; KDEL, lysine-aspartate-glutamate-leucine; MEM, minimum essential medium; PLAP, placental alkaline phosphatase; PI-PLC, phosphatidylinositol-specific phospholipase C.

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methyated proteins from Amersham Corp. (Arlington Heights, IL, U.S.A.); Protein A–Sepharose CL-4B from Pharmacia–LKB Biotechnology (Uppsala, Sweden); lipofectin reagent from Gibco–BRL (Gaithersburg, MD, U.S.A.); anti-(78 kDa glucose-regulated protein) monoclonal antibody (directed against a synthetic heptapeptide KSEKDEL) from StressGen Biotechnologies Corp. (Victoria, Canada); aprotinin, apyrase (type I) and dithiobis(succinimidyl propionate) (DSP) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); pansorbin from Calbiochem–Novabiochem. (La Jolla, CA, U.S.A.); brefeldin A from Wako Chemical Co. (Tokyo, Japan); antipain, chymostatin, elastatinal, leupeptin and pepstatin from Protein Research Foundation (Osaka, Japan); phosphatidylinositol-specific phospholipase C (PI-PLC) from Funakoshi, Co. (Tokyo, Japan); yeast hexokinase from Oriental Yeast Co. (Osaka, Japan); CHAPS from Dojin Chemical Co. (Kumamoto, Japan). Nigericin was a gift from Lilly Laboratories. Antiserum against rat α GL was raised in rabbits as described previously [17]. Antiserum (anti-C-peptide) against the decapeptide (Cys-Leu-Leu-Leu-Glu-Thr-Ala-Thr-Ala-Pro) corresponding to the C-terminal nonapeptide of proPLAP was prepared and purified as described previously [10]. Antiserum against calnexin was raised in rabbits against a synthetic peptide (Lys⁴⁸⁷–Cys⁵⁰⁵) of calnexin as described previously [18]. COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum as described previously [12].

Plasmids and transfection

A full-length cDNA for rat α GL was isolated and subcloned into a unique *EcoRI* site of the mammalian expression vector pSG5 (Stratagene, San Diego, CA, U.S.A.) as described previously [12]. pSG5- α GL-PLAP encoding a wild-type chimaeric protein and pSG5- α GL-PLAP encoding a mutant chimaeric protein, in which Asp¹⁵⁹ at a cleavage/attachment site of GPI was replaced with tryptophan, was constructed as described previously [12]. Each purified plasmid (4–5 μ g) in 50 μ l of PBS was mixed with 4–5 μ g of lipofectin reagent in 50 μ l of PBS and the mixture was incubated at room temperature for 15 min. COS-1 cells, which had been inoculated (10⁵ cells/35 mm plastic dish) 24 h before transfection, were washed three times with DMEM and incubated with the plasmid–lipofectin complex in 1 ml of DMEM in a humidified CO₂ incubator. After 4–6 h incubation, the medium was replaced with 2 ml of DMEM supplemented with 10% fetal calf serum and the cells were incubated for 24 h.

Metabolic labelling and immunoprecipitation

Transfected cells were metabolically labelled with 25 μ Ci of [³⁵S]methionine as described previously [12]. After removal of the medium, cells were lysed in 0.5 ml of lysis buffer A (50 mM Hepes, pH 7.5, containing 200 mM NaCl and 2% CHAPS) by the method of Hammond and Helenius [19]. Unless otherwise specified, all procedures were carried out at 4 °C. A protease inhibitor cocktail (antipain, aprotinin, chymostatin, elastatinal, leupeptin and pepstatin A) was added to cell lysates and media (10 μ g of each/ml). The lysates were centrifuged at 15000 *g* for 10 min to remove insoluble materials and incubated with 30–40 μ l of a 10% suspension of pansorbin (fixed *Staphylococcus aureus* cells) for 30 min. The precleared supernatants were incubated with an appropriate antibody for 3–16 h. Unless otherwise mentioned powdered skimmed milk was added to a final concentration of 5% (w/v) [12]. The immune complexes were collected on 30–40 μ l of Protein A–Sepharose (50% suspension in PBS) by shaking for 1 h and washed once with lysis buffer A and then three times with CHAPS wash buffer (50 mM Hepes,

pH 7.5, containing 200 mM NaCl and 0.5% CHAPS) [19]. The immune complexes were boiled in Laemmli's sample buffer [20] and analysed by SDS/PAGE (12.5% polyacrylamide gel). Gels were fixed and equilibrated in 1 M sodium salicylate before fluorography as described by Chamberlain [21]. In some experiments the cell lysates were subjected to a first immunoprecipitation with anti-KDEL antibody. Washed immune complexes–Protein A beads were then boiled in 0.5% SDS in PBS for 3 min to disrupt the immune complexes. After the samples had been centrifuged at 15000 *g* for 1 min, the supernatants were diluted 10-fold with 1% Nonidet P40 in PBS and were subjected to a second round of immunoprecipitation with anti- α GL or anti-(C-peptide) IgG.

For PI-PLC digestion, metabolically labelled cells were subsequently incubated with 0.1–0.2 unit of PI-PLC in 0.7 ml of minimum essential medium (MEM) for 1 h in a CO₂ incubator as described previously [10,12]. Media were removed and centrifuged at 5000 *g* for 5 min to remove detached cells. The resultant supernatants were subjected to immunoprecipitation.

Chemical cross-linking

Chemical cross-linking experiments were performed essentially as described by Margolese et al. [22]. After being metabolically labelled, cells were lysed in 0.5 ml of lysis buffer B (50 mM Bicine/NaOH, pH 8.0, containing 150 mM NaCl, 1.0% Nonidet P40 and 5 mM iodoacetamide and 0.5 mM PMSF) in the absence or presence of the thiol-cleavable amino-reactive cross-linking reagent DSP at a final concentration of 0.2 mM. After vigorous vortexing, the cell lysates were stood on ice for 30 min. Cross-linking reactions were stopped by the addition of 50 μ l of 50 mM glycine in distilled H₂O and the cell lysates were placed on ice for 10 min. After centrifugation at 15000 *g* for 10 min, the resultant supernatants were subjected to immunoprecipitation as described above. The immune complexes–Protein A beads were washed once with lysis buffer B, twice with a high-salt washing buffer (10 mM Tris/HCl, pH 7.5, containing 500 mM NaCl, 0.5% Nonidet P40 and 1 mM EDTA), and twice with a low-salt washing buffer (10 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 0.5% Nonidet P40 and 1 mM EDTA).

ATP depletion of cell lysates and dissociation of Bip/GRP78 by ATP

In order to deplete cellular ATP, cells were lysed in 0.5 ml of lysis buffer C (50 mM Tris/HCl, pH 7.5, containing 1% Nonidet P40, 150 mM NaCl and 1 mM PMSF) containing either 100 units/ml apyrase or 25 units/ml apyrase, 11 mM glucose and 8 units/ml hexokinase as described by Lamandé et al. [23]. After 30 min on ice, iodoacetamide (5 mM final concn.) and the protease inhibitor cocktail were added to cell lysates, which were then centrifuged to remove insoluble material. The resultant supernatants were incubated with pansorbin. Precleared supernatants were divided into two equal parts and incubated with anti- α GL or anti-KDEL antibody for 3 h. Skimmed milk powder was omitted from an immunoprecipitation procedure. The immune complexes were recovered on Protein A–Sepharose beads, washed twice with lysis buffer C and twice with a washing buffer (50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 0.1% Nonidet P40 and 1 mM EDTA). For ATP-dependent dissociation of Bip/GRP78, immune complex–Protein A–Sepharose beads were suspended in 200 μ l of the washing buffer containing 10 mM MgCl₂ and incubated in the absence or presence of 4 mM ATP at 23 °C for 30 min. The beads were then washed once with the washing buffer and boiled in Laemmli's sample buffer for SDS/PAGE [23].

Immunoelectron microscopy

Immunoelectron microscopy was performed as described previously [24,25]. Transfected COS-1 cells were fixed for 2 h at room temperature with the periodate/lysine/paraformaldehyde fixative and washed. After being permeabilized with 0.1% saponin, cells were incubated with anti- α GL for 1 h and then with peroxidase-conjugated goat Fab to anti-rabbit IgG for 1 h. The peroxidase reaction was carried out at room temperature for 15 min in a substrate medium containing diaminobenzidine and H_2O_2 .

RESULTS

A precursor of GPI-linked protein associates with ER-resident proteins

Figure 1 shows structures of the wild-type and mutant chimaeric protein used in this study. The chimaeric protein (α GL-PLAP) consisted of α GL (155 amino acids) fused in-frame to a C-terminal 33-amino acid sequence of PLAP. COS-1 cells expressing the wild-type chimaeric protein were labelled with [35 S]methionine for 3 h. The chimaeric protein was immunoprecipitated from cell lysates with anti- α GL and subjected to SDS/PAGE, followed by fluorography (Figure 2). Two forms (25 kDa, 22 kDa) of the chimaeric protein were detected in the cell (Figure 2A, lane 4, and 2B, lane 1) as reported previously [12]. Anti-(C-peptide) IgG against the C-terminal nonapeptide of PLAP specifically precipitated the 25 kDa form, but not the 22 kDa form (Figure 2B, lanes 1 and 2), indicating that the 25 kDa form retained the C-terminal propeptide. The 22 kDa form was released from the cell surface on digestion with PI-PLC (Figure 2B, lane 3), demonstrating that the 22 kDa form was a mature protein modified by GPI. This is supported by a previous

finding that both [3 H]ethanolamine and [3 H]stearate, components of GPI, are incorporated into the 22 kDa form, but not the 25 kDa proform [12]. Since α GL-PLAP is not glycosylated, the difference in molecular mass (3 kDa) can be largely accounted for by the removal of the C-terminal propeptide. In some cases the 25 kDa protein migrated as a rather broad band compared with the 22 kDa protein, presumably because of co-migration of the immunoglobulin light chain (25 kDa) derived from the antibody used in the immunoprecipitation. Anti-peptide antibody (anti-KDEL) directed against the heptapeptide KSEKDEL containing the ER retrieval motif KDEL detected four major proteins including a 78 kDa protein (Figures 2 and 3). Electrophoretic patterns of the proteins precipitable with anti-KDEL antibody were somewhat different from one experiment to another, probably because we used suboptimal quantities of monoclonal antibody in each immunoprecipitation experiment. In addition to these proteins the anti-KDEL antibody co-precipitated another protein corresponding in mobility to the 25 kDa proform of α GL-PLAP on SDS/polyacrylamide gel (Figure 2A, lanes 4 and 5). To see if this protein is identical with pro α GL-PLAP, the cell lysate was first immunoprecipitated with anti-KDEL (Figure 2B, lane 5) and the immune complexes formed were then dissociated in the presence of SDS. After the addition of an excess amount of Nonidet P40, the samples were subjected to a second round of immunoprecipitation using either anti- α GL (lane 6) or anti-(C-peptide) (lane 7). Both the antibodies precipitated this 25 kDa protein, demonstrating that the 25 kDa pro α GL-PLAP, but not the 22 kDa mature form, was associated with proteins recognized by anti-KDEL. Since the proteins immunoreactive with anti-KDEL antibody associated with the newly synthesized proform *in vivo*, it is likely that these proteins represent ER-resident proteins with the KDEL retrieval signal. In contrast with the wild-type α GL-PLAP, only the 25 kDa

Figure 1 Structures of the wild-type and mutant chimaeric protein (α GL-PLAP) used in this study

(A) An amino acid sequence of the C-terminal region of pro- α GL-PLAP using a single-letter code. The amino acids derived from α GL and those from PLAP are shown by lower- and upper-case letters respectively. Position numbers of the original sequence are shown for α GL (above the sequence) and for PLAP (below the sequence). The arrowhead indicates a predicted cleavage site. Replacement of D (159) with W gives rise to the mutant α GL-PLAP. Anti-(C-peptide) antibody was raised against the C-terminal nonapeptide (thick bar) of PLAP. (B) Structures of proform and GPI-linked mature form of α GL-PLAP and their reactivities with anti- α GL and anti-(C-peptide) antibodies.

Figure 2 Association of pro-chimaeric protein with ER-resident proteins with a KDEL motif

(A) COS-1 cells expressing α GL (lanes 1–3), wild-type (lanes 4–6) or mutant (lanes 7–9) α GL-PLAP were labelled with [35 S]methionine for 3 h. The cell lysates were immunoprecipitated with anti- α GL (lanes 1, 4 and 7), anti-KDEL (lanes 2, 5 and 8) or anti-calnexin (lanes 3, 6 and 9). The immune complexes were analysed by SDS/PAGE, followed by fluorography. Left-hand lane, [14 C]-methylated proteins (from the top of the gel; 200 kDa, 97.4 kDa, 69 kDa, 46 kDa, 30 kDa and 14.3 kDa). (B) COS-1 cells expressing wild-type α GL-PLAP were labelled with [35 S]methionine for 3 h. In lanes 1–4, the cells were further incubated with PI-PLC in MEM for 1 h. The cell lysate (lanes 1 and 2) and media (lanes 3 and 4) were subjected to the immunoprecipitation with either anti- α GL (lanes 1 and 3) or anti-(C-peptide) (lanes 2 and 4) antibody. In lanes 5–7, the cells were directly lysed and subjected to a first immunoprecipitation with anti-KDEL antibody (lane 5), followed by a second round of immunoprecipitation with either anti- α GL (lane 6) or anti-(C-peptide) (lane 7) antibody. Left-hand lane, [14 C]-methylated proteins.

Figure 3 Pulse-chase experiment of newly synthesized chimaeric proteins

In (A), COS-1 cells expressing the wild-type α GL-PLAP were pulse-labelled with [35 S]methionine for 2 h (lanes 3 and 4) and chased in MEM for 3 h (lanes 5 and 6) or 6 h (lanes 7 and 8). At each time point, cells were lysed, divided into two equal parts and subjected to immunoprecipitation with either anti- α GL (lanes 3, 5 and 7) or anti-KDEL (lanes 4, 6 and 8) antibody. In lanes 1 and 2, cells were labelled for 2 h, chased for 3 h and further incubated with PI-PLC. The cell lysate (lane 1) and medium (lane 2) were immunoprecipitated with anti- α GL. The immune complexes were analysed by SDS/PAGE, followed by fluorography. Left-hand lane, [14 C]-methylated proteins. In (B), COS-1 cells expressing the mutant α GL-PLAP were pulse-labelled with [35 S]methionine for 2 h (lanes 1 and 2) and chased in MEM for 3 h (lanes 3 and 4) or 6 h (lanes 5 and 6). At each time point, cells were lysed, divided into two equal parts and subjected to immunoprecipitation with either anti- α GL (lanes 1, 3 and 5) or anti-KDEL (lanes 2, 4 and 6) antibody. Left-hand lane, [14 C]-methylated proteins.

proform was detected in the cells expressing the mutant chimaeric protein, in which Asp¹⁵⁹ was replaced by Trp (Figure 2A, lane 7). We and other groups previously reported that Trp at a cleavage/attachment site of GPI abrogates the cleavage of propeptide and concomitant attachment of GPI *in vivo* in cultured cells, and in *in vitro* translation/translocation systems [12,26–28]. When the cells expressing the mutant α GL-PLAP were analysed, anti-KDEL antibody was capable of coprecipitating pro α GL-PLAP with the uncleavable GPI-anchor signal (Figure 2A, lane 8). Taken together, these findings indicate that

pro α GL-PLAP with either the cleavable or uncleavable propeptide associates with ER-resident proteins with the KDEL motif.

Anti-calnexin specifically precipitating a 90 kDa protein, however, failed to coprecipitate any forms of α GL-PLAP (Figure 2A, lanes 6 and 9). This result indicates that calnexin is not involved in retention of the prochimaeric protein within the ER, although the possibility is not ruled out that calnexin might be involved in a very early stage of the synthesis of α GL-PLAP. After 15 min of pulse, cell lysates were prepared and subjected to

Figure 4 Degradation of the mutant chimaeric protein in a pre-Golgi compartment

(A) COS-1 cells expressing the mutant α GL-PLAP (lanes 1–5) were pulse-labelled with [35 S]methionine for 0.5 h (lane 1) and chased in MEM for 4 h in the absence (lane 2) or presence of 5 μ g/ml brefeldin A (lane 3), 25 mM NH_4Cl (lane 4) or 5 μ M nigericin (lane 5). Cell lysates were immunoprecipitated with anti- α GL antibody. The immune complexes were analysed by SDS/PAGE followed by fluorography. Lane 6, the 25 kDa proform and 22 kDa form detected in COS-1 cells expressing wild-type α GL-PLAP. (B) The amount of α GL-PLAP was measured by densitometric analysis of a weakly exposed version of the fluorogram in (A) and expressed as a percentage of that obtained at the pulse label. BFA, brefeldin A.

immunoprecipitation with anti-calnexin. Under the conditions where several proteins were coimmunoprecipitated with calnexin, however, there was no protein corresponding to pro α GL-PLAP (results not shown). Neither anti-KDEL nor anti-calnexin coprecipitated soluble rat α GL (Figure 2A, lane 2; Figure 5, lane 1).

Precursor with an uncleaved GPI-anchor signal undergoes rapid degradation

A precursor-product relationship between the 25 kDa proform and the 22 kDa mature form was examined as shown in Figure 3. In a previous report [12] we failed to reveal any relationship between the two forms, presumably because the abundant expression of α GL-PLAP overwhelmed the capability of the COS cell to attach a GPI tag. In this study, to attain a more moderate expression, transfected cells were cultured for 24 h instead of 48 h before being used and the cells were pulse-labelled with [35 S]methionine for 2 h, allowing ER-resident proteins with a longer half-life to be labelled. At each time point, cells were lysed and divided into two equal parts and then the cell lysates were immunoprecipitated with either anti- α GL or anti-KDEL antibody. At the beginning of the chase the 25 kDa form was the major one detected in the cell (Figure 3A, lane 3). A considerable amount of the 22 kDa form was already formed. As the chase period elapsed, the 25 kDa form decreased and reciprocally the 22 kDa form increased (lane 5), and at 6 h of chase the 22 kDa mature form was the only form detected within the cell (lane 7). Again only the 25 kDa proform was coprecipitated with anti-KDEL antibody throughout the chase period (lanes 4 and 6). When the mutant α GL-PLAP with the uncleavable GPI-anchor signal was expressed, no 22 kDa form was detected during the pulse-chase periods (Figure 3B). These results support the conclusion that the 25 kDa form with the cleavable GPI-anchor signal is a biosynthetic precursor of the 22 kDa form. Figure 3(B)

also shows that, instead of being converted into the 22 kDa mature form, the mutant α GL-PLAP with the non-functional GPI-anchor signal underwent rapid degradation (lanes 1, 3 and 5). Figure 4 illustrates that degradation of the mutant chimaeric protein occurred even under conditions in which exit from the ER was blocked by brefeldin A [25,29–32], indicating that the mutant chimaeric protein was degraded in a pre-Golgi compartment (Figure 4A, lanes 2 and 3). In support of this result, perturbants of the Golgi apparatus (nigericin) and lysosomes-endosomes (NH_4Cl) [33–35] did not inhibit the degradation of the mutant chimaeric protein (lanes 4 and 5).

Chemical cross-linking experiment

To determine which ER-resident protein specifically associates with pro α GL-PLAP, chemical cross-linking experiments were performed. After metabolic labelling the cells expressing the mutant α GL-PLAP were lysed in the absence or presence of the homobifunctional cross-linker DSP. Anti-KDEL antibody precipitated four major proteins with apparent molecular masses 94, 78, 55 and 40 kDa (Figure 5, lane 3), three of which correspond in molecular mass to GRP94, Bip/GRP78 and protein disulphide isomerase in order of decreasing molecular mass [36,37], although the possibility was not excluded that the antibody recognizes the internal KDEL motif by chance rather than the C-terminal ER retrieval motif. Only the 78 kDa protein became significantly enriched in the presence of DSP (Figure 5, lane 6).

ATP-dependent dissociation of a 78 kPa protein-pro α GL-PLAP complex

During the course of this study we noticed that the ATP content in the cell lysates markedly affects the extent to which the 78 kDa protein associates with the pro-chimaeric protein. Without ATP depletion before immunoprecipitation, we could barely detect the 78 kDa protein after immunoprecipitation with anti- α GL anti-

Figure 5 Chemical cross-linking of the 78 kDa protein to pro-chimaeric protein

COS-1 cells expressing α GL (lanes 1 and 2) or the mutant α GL-PLAP (lanes 3–6) were labelled with [35 S]methionine for 3 h. The cell lysates were immunoprecipitated with anti- α GL (lanes 2, 4 and 6), anti-KDEL (lanes 1 and 3) or anti-C-peptide (lane 5) antibody. In lane 6, the cell lysate was incubated with a homobifunctional cross-linker, DSP, before immunoprecipitation with anti- α GL antibody as described in the Materials and methods section. Left-hand lane, 14 C-methylated proteins.

Figure 6 ATP-dependent dissociation of the 78 kDa protein from pro-chimaeric protein

COS-1 cells expressing the mutant α GL-PLAP were labelled with [35 S]methionine for 3 h. After incubation with apyrase alone (lanes 1, 2, 6 and 7) or with apyrase, glucose and hexokinase (lanes 3, 4, 8 and 9) the cell lysates were subjected to immunoprecipitation with either anti- α GL (lanes 1, 3 and 6–9) or anti-KDEL (lanes 2 and 4) antibody. In lanes 6–9, immunoprecipitates were incubated in the absence (lanes 6 and 8) or presence (lanes 7 and 9) of ATP before analysis by SDS/PAGE. Lane 5, 14 C-methylated marker proteins.

body (Figures 2 and 5). The 78 kDa protein was coprecipitated by anti- α GL only after chemical cross-linking treatment (Figure 5). After ATP-depleting-treatment, which hydrolysed and/or consumed ATP in the cell lysates, however, the 78 kDa protein was reproducibly coprecipitated with pro α GL-PLAP by anti- α GL (Figure 6, lanes 1 and 3). Interestingly, anti-KDEL antibody recognized the 78 kDa protein–pro α GL-PLAP complex preferentially in the ATP-depleted cell lysates (lanes 2 and 4) compared with that of the untreated cell lysates (Figures 2, 3, 5), further supporting the proposal that, of the four proteins with the KDEL motif, this 78 kDa protein is the one that associates with pro α GL-PLAP.

The 78 kDa protein that specifically associates with the newly synthesized pro-chimaeric protein has the same molecular mass as Bip/GRP78, an ER-resident molecular chaperone, suggesting

Figure 7 Immunoelectron microscopy of cells expressing chimaeric proteins

COS-1 cells expressing wild-type α GL-PLAP (A) or mutant α GL-PLAP (B) were fixed, permeabilized, and incubated with anti- α GL antibody, and then stained with immunoperoxidase. GA, Golgi apparatus; M, mitochondria; N, nucleus; PM, plasma membrane.

that the 78 kDa protein is identical with Bip/GRP78. Bip/GRP78 has the C-terminal KDEL motif, which functions as an ER retrieval signal involved in the recycling of Bip/GRP78 between the ER and the Golgi apparatus [38]. Bip/GRP78, a member of the Hsp70 family, is an ATP-binding protein and dissociates from protein substrates to which it is bound in an ATP-dependent manner [39,40]. As shown in Figure 6 (lanes 6–9), on incubation with ATP the 78 kDa protein was found to be dissociated from the 78 kDa protein–pro α GL-PLAP complex. It is therefore quite likely that the 78 kDa protein with which the newly synthesized pro α GL-PLAP associates is identical with Bip/GRP78. Pro α GL-PLAP was not coprecipitated with anti-calnexin from ATP-depleted cell lysates (results not shown).

Immunoelectron microscopy

When transiently expressed in COS-1 cells α GL-PLAP with the uncleaved GPI-anchor signal did not appear on the cell surface but was confined to the nuclear envelope and reticular structures such as ER, as judged by immunofluorescence microscopy [12]. Immunoelectron microscopy confirmed that wild-type α GL-PLAP was found on the cell surface (Figure 7A), whereas mutant α GL-PLAP was absent from the Golgi apparatus and plasma membranes and accumulated in ER-like structures (Figure 7B).

DISCUSSION

Bip, originally identified as an immunoglobulin heavy-chain-binding protein [41], is identical with a glucose-regulated protein with a molecular mass of 78 kDa (GRP78) [39]. Bip/GRP78 is involved in the assembly not only of immunoglobulin, but also of many oligomeric proteins. Moreover, as an ER-resident molecular chaperone, Bip/GRP78 is thought to assist the folding processes of monomeric proteins until they assume correctly folded tertiary structures. Bip/GRP78 binds to and retains partially folded and misfolded proteins and incompletely assembled subunits within the ER, and consequently permits only correctly folded proteins and properly assembled oligomeric proteins to leave. Thus it is believed that the protein serves as a component in a quality-control mechanism [14,15]. Bip/GRP78 itself is largely localized in the ER via the KDEL retrieval motif at the C-terminus [42]. Once Bip/GRP78 leaves the ER, it binds to a cognate receptor (ERD2) in the *cis*-Golgi network and is recycled back to the ER [43]. In this study we have shown by coimmunoprecipitation assay that, when transiently expressed in the COS-1 cell, the precursor of the chimaeric protein α GL-PLAP is associated with Bip/GRP78 (Figure 2). Anti-KDEL antibody preferentially precipitated the 78 kDa protein- $\text{pro}\alpha$ GL-PLAP complex from ATP-depleted cell lysates (Figure 6). In the reciprocal experiment anti- α GL coprecipitated the 78 kDa protein (Figure 6). Furthermore this 78 kDa protein was found to be dissociated *in vitro* from the 78 kDa protein- $\text{pro}\alpha$ GL-PLAP complex on incubation with ATP (Figure 6), compatible with Bip/GRP78-protein substrate complexes breaking apart in the presence of ATP [23,39,40]. Melnick et al. [44] reported that both GRP94 and Bip/GRP78 bind to unassembled immunoglobulin chains and form ternary complexes. However, it is unlikely that other ER-resident proteins with the KDEL motif such as GRP94 (94 kDa) and protein disulphide isomerase (55 kDa) also participate in the retention of $\text{pro}\alpha$ GL-PLAP, since, of the four major proteins immunoreactive with anti-KDEL antibody, only the 78 kDa protein was significantly enriched after chemical cross-linking (Figure 5).

When the wild-type chimaeric protein was expressed, anti-KDEL antibody coprecipitated the 25 kDa form with a propeptide. This was confirmed by a sequential immunoprecipitation procedure in which proteins recovered in the first immunoprecipitation with anti-KDEL antibody were subjected to a second immunoprecipitation using anti-(C-peptide) antibody, which specifically recognizes a C-terminal nonapeptide of PLAP (Figure 2B). Considering that Bip/GRP78 associated with neither the 22 kDa mature α GL-PLAP which lacks the propeptide or intact α GL (α GL and α GL-PLAP share the N-terminal 155 amino acids shown in Figure 1), it is likely that Bip/GRP78 binds directly to a stretch of 23 hydrophobic amino acids in the propeptide (Figures 2 and 5), to the sequence around Asp¹⁵⁹ at the cleavage/attachment site of GPI of the precursor or to a certain conformation unique to the precursor molecule encompassing the propeptide. Polypeptides that are hydrophobic in nature are favoured substrates of Bip/GRP78 *in vitro* [45,46]. By binding transiently the hydrophobic prosequence, Bip/GRP78 might block irreversible aggregation of the precursor molecule until the chimaeric protein is modified by GPI. Alternatively Bip/GRP78 may be more directly involved in the enzyme reaction catalysed by a transamidase as proposed by Amthauer et al. [47].

Pulse-chase experiments showed that the 25 kDa α GL-PLAP with the cleavable GPI-anchor signal is the precursor of the 22 kDa GPI-linked form. In contrast with wild-type α GL-PLAP, the mutant protein with the uncleavable GPI-anchor signal is

rapidly degraded via a non-lysosomal degradation pathway. This degradation of the mutant chimaeric protein probably occurs in a pre-Golgi compartment, since it proceeded even in the presence of brefeldin A, which blocks exit of proteins from the ER [24,29–32]. Compatible with this finding, both nigericin and NH_4Cl , which are known to affect acidic compartments such as lysosomes [35], endosomes [35] and Golgi apparatus [27,35,48], did not inhibit degradation of the mutant chimaeric protein. Furthermore, immunoelectron microscopic observation shows that mutant α GL-PLAP accumulates in the ER, suggesting that rapid degradation of the precursor occurs in the ER and/or its specific subcompartment. Thus, irrespective of the functional or non-functional GPI-anchor signal, $\text{pro}\alpha$ GL-PLAP is retained in the ER and/or the ER subcompartment by binding to Bip/GRP78. However, the fates of the two precursors completely diverge. After cleavage of the propeptide and attachment of GPI, which possibly trigger the release of Bip/GRP78 from wild-type α GL-PLAP, the GPI-linked mature form proceeds to the Golgi apparatus and finally reaches the cell surface. On the other hand, $\text{pro}\alpha$ GL-PLAP with uncleavable propeptide remains associated with Bip/GRP78 until the precursor is finally degraded within the ER. Thus the quality control by Bip/GRP78 and coupled selective degradation system ensure that only the GPI-linked mature form is transported out of the ER.

Under conditions in which the addition of GPI is impaired in some way, the fate of uncleaved precursors may be different from one precursor to another. Some precursors are proteolytically processed and finally secreted into the medium [10,11,49,50], whereas others are subjected to rapid degradation within the cell [51–53]. Recently Field et al. [54] showed that one (hGHDAF28) of their chimaeric proteins with a non-functional GPI-anchor signal underwent rapid degradation in a pre-Golgi compartment in transiently transfected cells. They also suggested that disulphide-linked aggregation of the precursor causes the retention of the chimaeric protein. Suzuki et al. [55] reported that association between Bip/GRP78 and a T-cell receptor α -subunit tagged with a GPI-anchor signal persists in the ER without rapid degradation, although the molecular species to which Bip/GRP78 is bound remains uncharacterized.

Calnexin (type I integral membrane protein localized in the ER) is involved in the folding and assembly of a wide range of newly synthesized proteins [16,18]. Since calnexin also retains partially folded, misfolded and unassembled proteins [56,57], it is proposed that it participates in the control of the quality of proteins in the ER, as in the case of Bip/GRP78. In some cases both calnexin and Bip/GRP78 are reported to participate sequentially in the folding of newly synthesized proteins [19,58]. However, anti-calnexin failed to coprecipitate any form of the chimaeric protein (Figure 2), indicating that calnexin is not involved in the conformational assessment of α GL-PLAP. However, this does not necessarily suggest that calnexin does not play a role in the quality control of GPI-linked proteins in general. Rather there is a possibility that it does not bind to α GL-PLAP because α GL-PLAP is not glycosylated. Calnexin is a lectin-like chaperone and preferentially binds to monoglucosylated intermediate of asparagine-linked oligosaccharide chains on newly synthesized glycoproteins [59,60], although peptide-peptide interactions also contribute to the association of calnexin with substrate proteins after initially binding through oligosaccharide portions [22,59].

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