Role of calnexin in the glycan-independent quality control of proteolipid protein

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The endoplasmic (ER) quality control apparatus ensures that misfolded or unassembled proteins are not deployed within the cell, but are retained in the ER and degraded. A glycoprotein-specific system involving the ER lectins calnexin and calreticulin is well documented, but very little is known about mechanisms that may operate for non-glycosylated proteins. We have used a folding mutant of a non-glycosylated membrane protein, proteolipid protein (PLP), to examine the quality control of this class of polypeptide. We find that calnexin associates with newly synthesized PLP molecules, binding stably to misfolded PLP. Calnexin also binds stably to an isolated transmembrane domain of PLP, suggesting that this chaperone is able to monitor the folding and assembly of domains within the ER membrane. Notably, this glycan-independent interaction with calnexin significantly retards the degradation of misfolded PLP. We propose that calnexin contributes to the quality control of non-glycosylated polytopic membrane proteins by binding to misfolded or unassembled transmembrane domains, and discuss our findings in relation to the role of calnexin in the degradation of misfolded proteins.

Keywords: endoplasmic reticulum/ER-associated degradation/membrane proteins/polytopic/transmembrane domain

Introduction

The endoplasmic reticulum (ER) is a major site for the synthesis of membrane and secretory proteins. The lumen of the ER contains a variety of molecular chaperones and folding factors which assist the folding and oligomeric assembly of newly synthesized proteins. These include the Hsp 70 family member BiP, the ER-resident lectins calnexin and calreticulin, and the oxidoreductases PDI and ERp 57. In order to restrict the deployment of potentially toxic misfolded or unassembled proteins within the cell, the ER has developed stringent quality control systems to ensure that only correctly folded and assembled proteins are allowed to exit and proceed along the secretory pathway (Hurtley and Helenius, 1989). Most proteins that fail this quality control system are ultimately

degraded by the cytosolic proteasome through a process termed ER-associated degradation (ERAD) (Wiertz *et al.*, 1996).

A common post-translational modification that occurs in the ER is the addition of N-linked glycans, and the mechanisms of glycoprotein-specific quality control have been particularly well studied (Ellgaard and Helenius, 2001). Trimming of the triply glucosylated glycan that is added to the growing polypeptide produces a monoglucosylated form which allows the glycoprotein to interact with calnexin and calreticulin in combination with ERp 57 (High *et al.*, 2000). Removal of the final glucose from the N-linked glycan releases the glycoprotein from calnexin/calreticulin, and if the glycoprotein has attained its native conformation it will exit the ER. If not, reglucosylation of the glycan by a glucosyl transferase that specifically recognizes misfolded proteins causes the glycoprotein to rebind calnexin/calreticulin, thereby retaining it in the ER and providing further time for folding. Terminally misfolded glycoproteins are released from this cycle of calnexin/calreticulin binding by trimming of mannose residues in the glycan. This inhibits reglucosylation, and thus the interaction with calnexin/calreticulin, and targets the protein for degradation (Braakman, 2001; Cabral *et al.*, 2001).

In contrast, very little is known about the quality control of non-glycosylated proteins, although a system is presumed to exist. The growing list of diseases that are associated with the ER retention of membrane and secretory proteins (not all of which are glycosylated) highlights the importance of understanding the molecular mechanisms that underlie the quality control of non-glycosylated as well as glycosylated proteins (Brooks, 1997).

We have used proteolipid protein (PLP) as a model for non-glycosylated polytopic membrane proteins to examine the quality control systems that exist for this class of polypeptides. PLP is expressed primarily by oligodendrocytes and Schwann cells, and is the major membrane protein of central nervous system myelin. The structure of PLP is relatively simple compared with other polytopic proteins; it has four transmembrane (TM) domains, a large extracellular/ER luminal domain between the third and fourth TM domains, and short cytosolic N- and C-terminal domains (Figure 1A). PLP has two disulfide bonds within its large extracellular domain and up to six sites for palmitoylation. PLP interacts with lipid rafts during its transit from the ER to the cell surface (Simons *et al.*, 2000), where it is incorporated into the myelin membrane. Two features make PLP a particularly useful model for the study of ER protein quality control. First, it has no consensus sites for N-glycosylation and therefore will interact with components of the putative glycan-independent quality control system. Secondly, a large number of

Fig. 1. Structure and subcellular distribution of wt and *msd* PLP. (A) Cartoon depicting the topology of PLP. Palmitoylation sites are shown by squiggles, disulfide bonds are shown by lines, the position of the *msd* mutation in TM4 is shown by an asterisk and the position of the artificial glycosylation sites (CHO1 and CHO2) are shown by arrowheads. (B) COS-7 cells were transiently transfected with wt (left and center column) or *msd* (right column) PLPmh, fixed with 2% formaldehyde and 0.2% glutaraldehyde, permeabilized and stained for myc (top row; green), PMCA, Lamp 2 or calnexin (cnx) (center row; red). Merged images are shown in the bottom row. Bar, 20 μm. (C) COS-7 cells were transfected with PLPmh (lanes 1–2, 7–8), PLPmh containing artificial glycosylation site CHO1 (lanes 3–4, 9–10) or CHO2 (lanes 5–6, 11–12), labeled with [³⁵S]methionine/cysteine for 2 h, solubilized and immunoprecipitated with anti-ha and left untreated (–) or treated with EndoH (+). PLPmh (– CHO) and artificially glycosylated PLPmh (+ CHO) are shown by arrows.

naturally occurring point mutations in the sequence of PLP are associated with diseases of the central nervous system, including Pelizaeus–Merzbacher disease. Most of these mutations prevent PLP from reaching the cell surface, and the mutant protein appears to be misfolded and retained in the ER (Gow and Lazzarini, 1996), indicating that it is recognized by an ER quality control system.

Here, we have used a well-characterized folding mutant of PLP to study the glycan-independent quality control pathways operating in the ER. We find that calnexin binds to newly synthesized PLP, and show that it contributes directly to the ER quality control of PLP. Notably, calnexin interacts with the fourth TM domain of PLP, binding stably to it when it is misfolded or unassembled. We provide evidence that this interaction with calnexin inhibits the degradation of misfolded PLP, thereby

exacerbating the potentially toxic accumulation of an ER-retained protein.

Results

Subcellular localization of wild-type and misfolded PLP

In order to facilitate subsequent analysis, we added either a myc/His₆ (mh) or ha epitope tag to the C-terminus of PLP (Figure 1A). This region of PLP is the most variable between species and contains few disease-associated mutations, suggesting that by locating tags here we were unlikely to interfere with correct folding of the protein. A well studied mutation, the *msd* allele, that causes complete ER retention of the mutant protein and results in severe disease in transgenic mice is the substitution of a valine for

an alanine at position 242 in the fourth TM domain of PLP (Figure 1A, asterisk). We have used this as a natural folding mutant with which to examine the quality control of a non-glycosylated membrane protein.

The subcellular distribution of wt and *msd* PLP in COS-7 cells was examined by indirect immunofluorescence microscopy (Figure 1B). The majority of wt PLPmh was transported to the cell surface and colocalized with the plasma membrane ATPase PMCA, with a variable amount seen in intracellular structures that colocalized with the lysosomal marker Lamp 2, as has been seen previously in cultured cells (Gow and Lazzarini, 1996; Simons *et al.*, 2002). In contrast, staining of *msd* PLPmh was restricted to a reticular pattern typical of the ER (Figure 1B). This was confirmed by costaining with the ER marker calnexin, which colocalized with the *msd* form of PLPmh. Although the misfolded *msd* PLP appeared to concentrate in a perinuclear region, we found no evidence of aggresome formation even in the presence of proteasome inhibitors, and no rearrangement of vimentin filaments characteristic of aggresomes (Johnston *et al.*, 1998) occurred under these conditions (data not shown). Similar results were obtained with untagged wt and *msd* PLP and wt and *msd* PLPha (data not shown). These results demonstrate that the intracellular trafficking of the epitope-tagged PLPs is similar to that of the untagged proteins in COS-7 cells. More strikingly, it is clear that the *msd* form of PLP is recognized as misfolded and retained in the ER by quality control machinery that acts independently of N-glycosylation.

In order to demonstrate that epitope-tagged PLP has the orientation shown in Figure 1A, we introduced artificial glycosylation sites into the first (CHO1, Asn 47) and second (CHO2, Asn 222) luminal loops of wt and *msd* PLPha. As shown in Figure 1C, both sites were glycosylated, resulting in an Endo H-sensitive shift in the molecular weight of the protein. This confirms that these regions are located within the ER lumen and that the *msd* mutation does not alter the predicted orientation of the protein, as previously shown by Gow *et al.* (1997). Similar results were obtained when the protein was synthesized in a cell-free system supplemented with semipermeabilized mammalian cells (Figure 2A), confirming that the topology of PLP is maintained in this *in vitro* system.

***In vitro* analysis of PLP folding; the role of glycan-independent chaperones**

In order to establish whether any previously identified 'glycan-independent' chaperones are involved in the folding of PLP, we synthesized the polypeptide in a cell-free system supplemented with semipermeabilized mammalian cells and co-immunoprecipitated the radiolabeled PLP with various anti-chaperone antibodies (Figure 2B). Some newly synthesized PLP was co-immunoprecipitated with cytosolic Hsc 70 and its cofactor Hsp 40, and a smaller amount of PLP co-immunoprecipitated with PDI. The strongest association that we could detect was with BiP (Figure 2B, lane 4), a well characterized chaperone that is regulated by ATP hydrolysis (Munro and Pelham, 1986). The authenticity of the interaction between newly synthesized PLP and BiP was confirmed by showing that the majority of PLP could be released by the addition of ATP (Figure 2B, compare lanes 4 and 5), and by mixing

Fig. 2. Interaction of newly synthesized PLP with glycan-independent chaperones. (A) [35 S]PLPha (lanes 1–2, 7–8), PLPha containing artificial glycosylation site CHO1 (lanes 3–4, 9–10) or CHO2 (lanes 5–6, 11–12) was translated *in vitro* for 1 h, immunoprecipitated with anti-ha and left untreated (–) or treated with EndoH (+). PLPha (– CHO) and artificially glycosylated PLPha (+ CHO) are shown by arrows. (B) [35 S]-labeled wt PLP was translated *in vitro* for 15 min, incubated at 30°C for 45 min and co-immunoprecipitated with anti-chaperone or control (ctl) antibodies as indicated. Lane 1 shows 25% of the total amount of PLP used for each immunoprecipitation, and 5 mM ATP was added to the immunoprecipitation buffer where indicated. (C) [35 S]-labeled wt or *msd* PLP was translated *in vitro* for 15 min and incubated at 30°C for the time indicated. Then, the samples were split in two, depleted of ATP and immunoprecipitated with anti-BiP or anti-calnexin (Figure 4B). Co-immunoprecipitated PLP (top panel) was quantified and expressed as a percentage of the total input. The graph (bottom panel) shows the mean $\pm$ SEM of three independent experiments.

experiments which show that BiP does not bind to PLP following cell lysis (see Supplementary figure 2 available at *The EMBO Journal* Online). A strong interaction of PLP with BiP is consistent with studies showing that nascent proteins preferentially bind to calnexin and calreticulin if glycosylated within 50 residues of the N-terminus, but are likely to associate with BiP in the absence of glycosylation (Molinari and Helenius, 2000). We next examined the

kinetics of BiP binding to newly synthesized wt and *msd* PLP *in vitro* by co-immunoprecipitation of radiolabeled PLP with anti-BiP at different times following the termination of translation (Figure 2C). A transient interaction of the wt protein with BiP was observed, which peaked at 30–60 min and decreased to background levels by 4 h after synthesis. In contrast, BiP bound stably to *msd* PLP over the time course of the experiment. These results show that BiP is able to distinguish between the wt and misfolded *msd* PLP, forming a stable complex with the misfolded protein. Such a complex would be retained in the ER by virtue of BiP's KDEL retrieval motif, and therefore could play a key role in preventing misfolded PLP leaving the ER.

In vivo analysis

In order to examine the role of BiP in the quality control of misfolded PLP *in vivo*, we generated stable HeLa cell lines expressing wt or *msd* PLPmh under the control of a tetracyclin inducible promoter. The distribution of wt and *msd* PLPmh in these cell lines (Figure 3A) was essentially the same as seen in transiently transfected COS-7 cells (cf. Figure 1B). Western blotting of cell lysates confirmed that, following induction with doxycycline, both cell lines synthesized a myc-tagged protein with a molecular weight corresponding to that of PLP (Figure 3B, arrowhead). A higher molecular weight myc-reactive product was also induced in cells expressing *msd* PLPmh (Figure 3B, asterisk). This form is also observed when *msd* PLPmh or ha are expressed transiently and most likely represents an SDS-resistant dimer of *msd* PLP.

When PLP was immuno-isolated via its myc tag, we found that BiP could be co-immunoprecipitated with *msd* but not wt PLPmh (Figure 3C, compare lanes 1 and 5), demonstrating that BiP binds stably to misfolded PLP *in vivo*. Inclusion of ATP in the lysis buffer abolished co-immunoprecipitation of BiP with *msd* PLPmh, and no co-immunoprecipitation was seen in the absence of induction or with a control antibody (Figure 3C, lanes 3 and 6). Very little BiP co-immunoprecipitated with wt PLPmh *in vivo*, reflecting the transient nature of the chaperone–wt protein interaction. The failure to co-immunoprecipitate BiP with wt PLPmh was not due to lower levels of PLP expression, since similar levels of wt and *msd* PLPmh were found in the total cell lysates (Figure 3D).

BiP is not solely responsible for the ER retention of misfolded PLP

We reasoned that if the retention of misfolded PLP is mediated primarily through binding to BiP, overexpression of a secreted form of BiP in which the KDEL sequence was replaced with an myc tag (hereafter referred to as BiP_S) would be expected to cause at least some *msd* PLP to leave the ER and move with BiP_S to the cell surface. A similar approach has been used to demonstrate that PDI is responsible for the retention of unassembled procollagen C-propeptides (Bottomley *et al.*, 2001). Although the ratio of BiP_S to endogenous BiP was approximately 10:1 (Supplementary figure 3) and the majority of BiP_S exits the ER and is secreted into the medium (Figure 3E), we saw no staining of *msd* PLPha at the cell surface or in Lamp 2-positive structures in cells that co-expressed BiP_S (Figure 3F). This indicated that the

Fig. 3. *In vivo* analysis of PLP folding. (A) Cell lines expressing wt (left) or *msd* (right) PLPmh were induced overnight, fixed with methanol and stained with anti-myc followed by FITC-conjugated secondary antibody. (B) Lysates of cells induced to express wt or *msd* PLP for the time indicated were western blotted with anti-myc followed by an HRP-conjugated secondary antibody. PLPmh is shown by an arrowhead and a putative PLP dimer by an asterisk. The anti-myc cross-reactive band just above the asterisk is present in all samples even without induction. (C) Stable cell lines were induced to express wt or *msd* PLPmh overnight, solubilized and immunoprecipitated with anti-myc or control (ctl) antibody. Co-immunoprecipitated material was western blotted with anti-BiP. Immunoprecipitates from uninduced *msd* PLPmh cells (*msd* unind), and from cells solubilized with buffer containing 5mM ATP are shown where indicated. An eighth of the total lysate used for immunoprecipitation is shown in lane 9. The heavy chain of the immunoprecipitation antibody is just visible at the bottom of the panel. (D) An eighth of the total lysates used for immunoprecipitation in (C) were western blotted with anti-myc. Each lane was exposed to film for the same time to allow comparison of the amount of PLPmh in each sample. (E) COS-7 cells were transfected with BiP_Smyc or BiP_{KDEL}myc. After 24 h, the cells and medium were separated and immunoprecipitated with mouse anti-myc; the immunoprecipitated material was western blotted with rabbit anti-myc. (F) COS-7 cells were cotransfected with BiP_Smyc and *msd* PLPha, fixed with methanol and stained with anti-myc (left) and anti-ha (right) followed by FITC or Texas Red conjugated secondary antibodies, respectively. Images also show nuclei stained with DAPI. Bar, 20 μ m.

misfolded PLPha was unable to leave the ER when co-expressed with BiP_S. Hence, although misfolded PLP interacts strongly with BiP, this cannot be the only mechanism by which it is retained in the ER and additional pathways must exist for the quality control of non-glycosylated membrane proteins.

Calnexin, but not calreticulin, binds misfolded PLP

In order to identify other ER factors that might contribute to the quality control of *msd* PLP, we immunoprecipitated wt and *msd* PLPmh from induced cells under non-

denaturing conditions and screened the products with a panel of antibodies to known ER chaperones. Given that PLP lacks N-linked glycans, we were surprised to observe a particularly strong association of *msd*, but not wt PLPmh, with the ER lectin calnexin (Figure 4A, lanes 1 and 3). We examined the kinetics of calnexin binding to newly synthesized PLP *in vitro* as described above for BiP. As shown in Figure 4B, calnexin interacts transiently with wt PLP, but binds stably to the misfolded protein. These results demonstrate that calnexin, like BiP, is able to distinguish between wt and misfolded *msd* PLP, forming a stable complex with the misfolded protein. Since calnexin can bind to wt PLP, we established whether the stable association with calnexin we detected *in vivo* was specific for misfolded PLP or whether it was due to the ER localization of *msd* but not wt PLPmh. To do this, we artificially retained wt PLPmh in the ER by adding Brefeldin A (BFA) to cells when they were induced with doxycyclin. Whilst this treatment caused all the wt PLPmh to be retained in the ER (data not shown), it had no significant effect upon the amount of wt PLPmh that we found associated with calnexin (Figure 4C, compare lanes 2 and 4). In contrast, a significant amount of calnexin was bound to the *msd* PLPmh in both the absence and presence of BFA (Figure 4C, compare lanes 6 and 8). Hence, we conclude that, like BiP, calnexin is able to monitor the folding of a non-glycosylated membrane protein and bind stably to a misfolded version. The association of *msd* PLPmh with calnexin was not inhibited by prolonged treatment of the cells with tunicamycin or castanospermine (Figure 4D, lanes 1–6), both of which considerably reduce the glycan-mediated association of calnexin with a control glycoprotein opsin (Figure 4D, lanes 10–15). These results confirm that the interaction between calnexin and PLP is not dependent on glycosylation or glucose trimming, and rule out the unlikely possibility that calnexin binding to PLP occurs via a nascent intermediate factor that is itself N-glycosylated.

Since calnexin is a relatively abundant integral membrane protein of the ER, we further investigated the specificity of its interaction with *msd* PLPmh by analyzing immuno-isolated PLPmh for other abundant ER membrane proteins (Figure 4E). We found no evidence for the association of wt or *msd* PLPmh with subunits of the Sec61 translocon or the SPC complex. Therefore we conclude that the association of *msd* PLPmh with calnexin reflects a specific interaction and is not due to the immunoprecipitation of protein aggregates, patches of ER membrane or similar phenomena (Cannon *et al.*, 1996).

We were unable to detect any association between *msd* PLPmh and calreticulin, the soluble equivalent of calnexin (Figure 4F). These two chaperones possess similar substrate specificities (Danilczyk *et al.*, 2000) and have closely related structures apart from the TM domain which is present only in calnexin. This led us to suspect that calnexin may recognize and bind to the TM domains of misfolded PLP.

Calnexin binds to the fourth transmembrane domain of PLP

The *msd* mutation lies within TM4 of PLP and, although this mutation clearly affects the folding of the luminal and cytosolic domains (as evidenced by the association of BiP

Fig. 4. Calnexin binds stably to misfolded PLP. (A) Stable cell lines were induced to express wt or *msd* PLPmh overnight, solubilized and immunoprecipitated with anti-myc or control (ctl) antibodies. Co-immunoprecipitated material was western blotted with anti-calnexin (cnx). An eighth of the total lysates used for immunoprecipitation is shown in lanes 5 and 6. (B) 35 S-labeled wt or *msd* PLP was translated *in vitro* for 15 min and incubated at 30°C for the time indicated. Then the samples were split in two, depleted of ATP and immunoprecipitated with anti-BiP (Figure 2C) or anti-calnexin. Co-immunoprecipitated PLP was quantified and expressed as a percentage of the total input. The graph shows the mean $\pm$ SEM of three independent experiments. (C) Stable cell lines were induced to express wt or *msd* PLPmh overnight in the presence or absence of 5 μ g/ml BFA and then analysed as in (A). An eighth of the total lysate from induced wt and *msd* cells used for immunoprecipitation is shown in lanes 9 and 10. (D) Stable cell lines were induced to express *msd* PLPmh overnight (lanes 1–9) and HeLa cells transfected with opsin (lanes 10–15) were incubated with 10 μ g/ml tunicamycin (Tun) or 0.5 mM castanospermine (Cas) for 6 h, and then analysed as in (A). An eighth of the total lysates used for immunoprecipitation are shown in lanes 7–9. (E) Stable cell lines were induced and immunoprecipitated as in (A), and then western blotted with the antibodies indicated. An eighth of the total lysates used for immunoprecipitation are shown in lanes 5 and 6. (F) Stable cell lines were left uninduced (–) or induced (+) and immunoprecipitated as in (A), and were then western blotted with anti-calreticulin (crt). An eighth of the total lysates used for immunoprecipitation are shown in lanes 5–8.

Fig. 5. Calnexin binds the fourth TM domain of PLP. (A) Cartoon depicting the TM4ha construct. The preprolactin signal sequence is shown in gray, TM4ha is shown in black and the position of the *msd* mutation is indicated by an asterisk. (B) COS-7 cells were transiently transfected with wt (left) or *msd* (right) TM4ha, fixed with methanol and stained with anti-ha followed by a FITC-conjugated secondary antibody. Bar, 20 μm. (C) COS-7 cells were transiently transfected with *msd* TM4ha, fixed with 2% formaldehyde and 0.2% glutaraldehyde, permeabilized with 0.05% SDS (left column) or 40 μg/ml digitonin (right column) and then stained with anti-ha (top row), anti-KDEL antibody 1D3 (center row) and DAPI (bottom row). Bar, 20 μm. (D) Lysates of COS-7 cells transiently expressing wt or *msd* PLPha and wt or *msd* TM4ha were western blotted with anti-ha. TM4 with (ssTM4ha) and without (TM4ha) the signal sequence present are indicated. (E) COS-7 cells transiently expressing wt or *msd* PLPha and wt or *msd* TM4ha were solubilized and immunoprecipitated with anti-ha or control (ctl) antibodies. Co-immunoprecipitated material was western blotted with anti-calnexin (top panel) or anti-BiP (bottom panel). (F) COS-7 cells transiently expressing *msd* TM4ha were solubilized and immunoprecipitated with anti-calnexin (cnx) or control (ctl) antibodies, and immunoprecipitated was material western blotted with anti-ha. The ratio of TM4ha to ssTM4ha in calnexin immunoprecipitates and total lysates was approximately 3.5:1 and 4.5:1, respectively.

and Hsc70 with *msd* PLP), we anticipated that the primary folding defect would be within TM4. Since calnexin, but not calreticulin, binds stably to *msd* PLPmh, we considered it likely that calnexin interacts with PLP via TM4. To test this hypothesis, we generated a truncated version of PLP encoding the fourth TM domain of PLP alone. To ensure that TM4 was properly inserted into the ER membrane, we fused the cleavable signal sequence from preprolactin to the N-terminus of TM4 and added a C-terminal ha tag to facilitate detection (Figure 5A).

When expressed in COS-7 cells, the wt TM4ha and a version containing the *msd* mutation were localized to the ER (Figure 5B). In order to establish the transmembrane orientation of TM4ha, we examined the accessibility of the C-terminal ha epitope under different conditions. We found that TM4ha could be stained with anti-ha in digitonin permeabilized cells in which the ER membrane

remains intact, as shown by the lack of staining with the luminal marker 1D3 which was clearly visible if cells were permeabilized with SDS (Figure 5C). This confirmed that the ha epitope was on the cytoplasmic face of the ER membrane. Hence TM4ha was efficiently targeted to, and correctly oriented in, the ER membrane. Consistent with this, western blotting lysates from cells transiently transfected with TM4ha showed that the signal sequence was cleaved from the majority of the expressed protein (Figure 5D, TM4ha), although a small proportion still contained the signal sequence and ran with a slightly lower mobility (Figure 5D, ssTM4ha).

We expressed *msd* TM4ha *in vivo* and found that this single transmembrane domain was able to co-immunoprecipitate calnexin with an efficiency comparable to that seen with the full-length *msd* PLPmh (Figure 5E, top panel). The luminal chaperone BiP did not co-immuno-

precipitate with wt or *msd* TM4ha (Figure 5E, bottom panel), confirming that TM4ha does not associate non-specifically with multiple ER chaperones. To confirm the interaction between TM4ha and calnexin, we performed the immunoprecipitation in reverse and showed that TM4ha was precipitated by anti-calnexin but not by a control antibody (Figure 5F). Importantly, calnexin co-immunoprecipitated the faster migrating TM4ha protein, showing that calnexin binds to the correctly oriented signal-sequence-cleaved TM4ha. The slower migrating ssTM4ha was also co-immunoprecipitated. It is likely that ssTM4 results from inefficient signal sequence cleavage, but it is formally possible that it has the opposite orientation and is also recognized by calnexin as being 'misfolded'. These results provide the first direct evidence that calnexin can bind to isolated TM domains. Calnexin might also bind to TM1, TM2 and/or TM3 of PLP, but we have not tested this here. Interestingly, calnexin associated equally well with the 'wt' TM4ha and the version containing the *msd* mutation (Figure 5E, compare lanes 6 and 8), suggesting that calnexin recognizes an 'unassembled' TM domain of a polytopic protein as being misfolded. We speculate that in full-length PLP, the *msd* mutation may prevent the proper assembly of TM4 into the folded protein, thereby causing it to be stably bound by calnexin. However, as yet we have no direct evidence that the assembly of TM domains is disrupted in the *msd* mutant and it may be some other feature of TM domains within misfolded PLP that is recognized by calnexin.

Calnexin inhibits the degradation of misfolded PLP

A key feature of PLP is the stability of the misfolded ER-retained protein relative to misfolded glycoproteins such as $\Delta F508$ CFTR which are cleared much more rapidly (data not shown). In this context, the glycan-dependent association of misfolded glycoproteins with calnexin is thought to prevent their degradation, whilst trimming of mannose residues acts as an 'off' switch to release the misfolded protein and target it for degradation. This led us to hypothesize that the glycan-independent interaction of calnexin with misfolded PLP may inhibit its degradation. We tested this directly by examining the degradation of *msd* PLPha in cells depleted of calnexin by siRNA.

Transfection of HeLa cells with either of two RNA duplexes matching nucleotide sequences within the coding sequence of calnexin resulted in loss of over 80% of cellular calnexin after 3 days of culture (Figure 6A). The depletion of calnexin had a marked effect on the rate of *msd* PLPha degradation (Figure 6B). In cells transfected with a scrambled RNA duplex, over 60% of the *msd* PLPha synthesized during a 1 h pulse remained after a 5 h chase, reflecting the stability of this misfolded protein. However, in cells treated with calnexin siRNA, the half-life of *msd* PLPha was significantly reduced to about 1.5 h. In contrast, calnexin depletion had no effect on the stability of wt PLPha (Figure 6C) or a control ER membrane protein (Supplementary figure 4). These striking results demonstrate that the glycan-independent binding of calnexin to misfolded PLP inhibits its degradation. This interaction may be particularly stable and long-lived in the absence of the 'off' switch usually provided by mannose trimming.

Fig. 6. Calnexin inhibits the degradation of misfolded PLP. (A) Lysates of cells 3 days after transfection with calnexin siRNA duplexes were western blotted with anti-calnexin (cnx) or anti-tubulin (tub). (B) Cells transfected with calnexin (cnx1 or cnx2) or scrambled (scr) siRNA duplexes and expressing *msd* PLPha were pulse labeled with [35 S]methionine/cysteine for 30 min, chased for up to 5 h and then solubilized and immunoprecipitated with anti-ha. The remaining 35 S-labeled *msd* PLPha at each time point was quantified and expressed as a percentage of the amount present after 0 h chase. The graph shows means $\pm$ SEM of seven (cnx1 and cnx2) or three (scr) independent experiments. The significance of difference between means from cells transfected with cnx1 or cnx2 and scr RNA is shown (significant at ≥ 0.5). The rate constants of degradation were 1.53 ± 0.37 and 1.40 ± 0.02 for cells transfected with cnx1 and cnx2 respectively. (C) As in (B) but the cells were transfected with wt PLPha and the graph shows means $\pm$ SEM of three independent experiments.

Discussion

To date, studies have focused on dissecting the ER quality control pathways that operate for glycoproteins. In order to characterize mechanisms that exist for non-glycosylated membrane proteins, we have used a disease-associated mutant of PLP as a model for this process. Strikingly, we find that the ER lectin calnexin binds stably to misfolded PLP in a glycan-independent fashion, and we show that this interaction protects misfolded PLP from degradation.

The precise way in which calnexin and calreticulin contribute to protein folding and quality control is a matter of considerable debate. On the one hand, these chaperones are known lectins that show a marked preference for

binding to proteins containing N-linked glycans and are components of a glycan-based system that specifically monitors the folding of glycoproteins (High *et al.*, 2000; Ellgaard and Helenius, 2001). On the other, it has been shown that calnexin and calreticulin can bind and promote the folding of non-glycosylated proteins, presumably via protein-protein interactions. Most of the evidence for this mode of calnexin/calreticulin function has come from *in vitro* studies using cytosolic substrate proteins that would not normally encounter ER chaperones (Ihara *et al.*, 1999; Saito *et al.*, 1999), or using drug treatments/mutagenesis to inhibit glycosylation artificially (Danilczyk and Williams, 2001, and references cited therein) making it possible to argue that these chaperones recognize some other motif common to glycoproteins. Our findings that calnexin, but not calreticulin, associates with newly synthesized PLP and binds stably to misfolded PLP under robust solubilization conditions (1% Triton X-100) provide strong evidence that this chaperone does indeed function in the folding and quality control of naturally occurring non-glycosylated proteins *in vivo* and suggest a role for the TM domain of calnexin in these processes.

Calnexin binds to misfolded transmembrane domains of polytopic membrane proteins

The TM domain of calnexin has been implicated in determining the specificity of its interactions since calnexin and calreticulin associate with different substrate glycoproteins despite having similar luminal domains. The function of this TM domain in substrate selection is unclear: it could promote association with membrane proteins by localizing calnexin to the ER membrane, or more controversially it could directly bind the TM domains of target proteins. Interestingly, a recent study has shown that calnexin interacts with a truncated ER-retained form of CD82 even though the luminal domain is correctly folded (Cannon and Cresswell, 2001), suggesting that the TM domains of this molecule are misfolded and may be recognized by calnexin. In this study we find that calnexin can discriminate between two versions of PLP with a single amino acid change in TM4, and show that calnexin can interact stably with an isolated TM domain of PLP. These data provide the first direct evidence that calnexin is able to bind misfolded and/or unassembled TM domains.

It is unclear what constitutes a 'misfolded' TM domain since these are often relatively simple helical structures. However, there must be some feature or motif that causes this single TM domain to be recognized by calnexin. Polar residues are rare in TM domains of single-spanning membrane proteins but are common in polytopic proteins, where they may help pack the TM domains together (Reggiori and Pelham, 2002). TM4 of PLP contains a number of polar residues and it is tempting to speculate that calnexin recognizes exposed polar residues in misfolded/unassembled TM domains within the hydrophobic environment of the lipid bilayer, as has been proposed for Rer1p (Sato *et al.*, 2001) and Tul1p (Reggiori and Pelham, 2002). Such a mechanism would be analogous to the binding of BiP to hydrophobic patches exposed in the aqueous environment of the ER lumen. Since calnexin forms part of a network of chaperones in the ER (Tatu and Helenius, 1997), some of which are associated

with the translocon, it is possible that calnexin samples TM domains as they are integrated into the ER membrane, binding stably to those that fail to fold or assemble correctly.

Role of calnexin in the degradation of non-glycosylated membrane proteins

Terminally misfolded proteins synthesized at the ER are normally degraded by the cytosolic proteasome, and a number of observations indicate that glycan-dependent association with calnexin/calreticulin can protect proteins from degradation (Cabral *et al.*, 2001). The trimming of mannose residues is thought to act as a 'timer' to release terminally misfolded glycoproteins from calnexin/calreticulin, thereby promoting degradation, and may also produce a specific 'disposal' signal (Jakob *et al.*, 1998; Braakman, 2001). Thus the presence of N-linked glycans provides a means of targeting terminally misfolded glycoproteins for degradation. Clearly such mechanisms cannot operate for non-glycosylated proteins like PLP, which may therefore be protected from degradation by a stable interaction with calnexin. In fact, we find that a key feature of PLP is the relative stability of the misfolded ER-retained protein compared with misfolded glycoproteins such as CFTR $\Delta F508$. Furthermore, the depletion of cellular calnexin dramatically enhances the rate of misfolded PLP degradation. Calnexin depletion does not induce the unfolded protein response, since BiP levels do not increase (data not shown), and therefore it is unlikely that the enhanced degradation we observe is due to increased expression of the ERAD machinery. Indeed, our results are consistent with a recent finding that deletion of the TM domain of a misfolded single-spanning glycoprotein (BACE457) reduced its interaction with calnexin and enhanced its rate of degradation (Molinari *et al.*, 2002). We propose that the glycan-independent binding of calnexin to the TM domain(s) of misfolded PLP inhibits degradation, and that this interaction is stable and long lived in the absence of the 'off' switch usually provided by mannose trimming. Therefore the misfolding of non-glycosylated membrane proteins may represent a particular threat to the normal function of the ER quality control machinery.

Other chaperones that may contribute to the folding and quality control of PLP

Polytopic proteins present the ER with a particularly complex problem since they must achieve the correct folding of a number of luminal, cytosolic and transmembrane domains. Consistent with this, both cytosolic (Hsc/p 70, Hsp 40) and luminal chaperones (BiP, PDI) interact with newly synthesized PLP *in vitro*. A similar complexity of interactions has also been seen with the glycosylated polytopic protein CFTR (Loo *et al.*, 1998; Meacham *et al.*, 1999). One notable difference is in the selection of Hsc/p 70 cofactors; PLP appears to utilize Hsp 40 whilst the folding of CFTR is assisted by Hdj2 (Meacham *et al.*, 1999). BiP binds stably to misfolded PLP *in vivo*, suggesting that this chaperone may act in combination with calnexin to facilitate the quality control of misfolded PLP. It is also possible that an interaction with PDI contributes to the retention of misfolded PLP in the ER (compare with Bottomley *et al.*, 2001).

Implications for Perlizaeus–Merzbacher disease

Perlizaeus–Merzbacher disease (PMD) is a debilitating disease of the central nervous system caused by duplication, deletion or mutation of the PLP locus. Most clinically relevant mutations prevent PLP from reaching the cell surface and the mutant PLP accumulates in the ER (Gow and Lazzarini, 1996). In oligodendrocytes, the accumulation of misfolded protein is severe and is likely to be a direct cause of the oligodendrocyte apoptosis that is the primary clinical feature of PMD (Gow and Lazzarini, 1996; Gow *et al.*, 1998). It is noteworthy that a folding mutant of another tetraspanning myelin protein, PMP-22, has recently been shown to interact with calnexin in a glycosylation-dependent manner, recruiting the chaperone to intracellular 'myelin-like' structures (Dickson *et al.*, 2002). The authors suggest that the resulting sequestration of calnexin compromises cell function and this may be the underlying cause of diseases associated with mutations in PMP-22. Although misfolded PLP does not appear to form myelin like structures, it does accumulate in a perinuclear region which may represent a recently described 'quality control' compartment that accumulates misfolded proteins and calnexin under various conditions (Kamhi-Nesher *et al.*, 2001).

Based on our findings that calnexin interacts stably with and retards the degradation of *msd* PLP, we propose that the accumulation of misfolded PLP results from the failure of the normal ERAD machinery to dispose of this misfolded protein efficiently. Furthermore, it is possible that the sequestration of calnexin (and other chaperones) by misfolded PLP plays an additional role in the pathology of PMD. We are now in a position to test these hypotheses, and to examine the wider role for calnexin in the quality control of non-glycosylated membrane proteins.

Materials and methods

Reagents and antibodies

Cell culture reagents were from GIBCO BRL or CLONTECH Laboratories, T7 RNA polymerase and rabbit reticulocyte lysate were from Promega, Brefeldin A was from Alexis, and EasyTag L-^[35S]methionine and EXPRESS ^[35S]protein labeling mix were from NEN Dupont. All other chemicals were from Sigma or BDH/Merck. Rabbit anti-ha and mouse anti-myc (9E10) were from Sigma, mouse anti-ha from Roche, sheep anti-BiP and rabbit anti-myc from Santa Cruz, mouse anti-Hsc/p 70 and rabbit anti-Hsp 40 from Stressgen, mouse anti-Hdj-2 from Neomarkers, mouse anti-Lamp 2 from the Developmental Studies Hybridoma Bank, University of Iowa, and mouse anti-PMCA from Affinity Bioreagents. Serum from rabbits immunized with peptide AFPSKTSASIGSLC of PLP was used for detection of untagged PLP. Rabbit anti-calnexin was a gift from Professor Ari Helenius (ETHZ, Switzerland), mouse 1D3 was a gift from Dr Viki Allan (University of Manchester) and rabbit anti-PDI was a gift from Professor Neil Bulleid (University of Manchester). HRP-conjugated secondary antibodies were from DAKO.

Molecular cloning and DNA manipulations

An expressed sequence tag clone MNCb-3609 from a mouse brain library containing the 5' end of PLP was obtained from the Japanese Health Science Research Resource Bank (National Institute of Infectious Diseases, Tokyo) and sequenced to confirm that it contained the correct full-length coding sequence. The PLP cDNA was cloned using the TOPO PCR cloning system (Invitrogen) and then subcloned into the *EcoRI* site of pcDNA3 and pcDNA3.1(–)/Myc-His (Invitrogen) and the *NotI/EcoRI* sites of the tetracycline responsive expression vector PTRE2hyg (CLONTECH). QuikChange mutagenesis (Stratagene) was used to generate the Ala242Val *msd* substitution, add a C-terminal ha tag and generate artificial glycosylation sites by insertion of Asn at position 47

(CHO1) and Thr at position 224 (CHO2). TM4ha consists of the 21 amino acid signal sequence from human preprolactin fused to amino acids 232–276 of PLP (six predicted to be luminal, 27 transmembrane and 14 cytosolic) followed by an ha tag, and was constructed by PCR overlap extension. The preprolactin signal sequence and TM4ha fragments containing complementary overlaps were amplified separately and then combined and used as template to produce the final spliced product which was cloned into the *EcoRI/BamHI* sites of pcDNA3.1(–). BiP_{KDEL}myc was a gift from Professor Neil Bulleid (University of Manchester) and BiP_Smyc was generated by QuikChange mutagenesis. Mutagenized and subcloned constructs were confirmed by DNA sequencing.

Cell culture and transfection

COS–7 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and plated out at least 24 h before transfection. CLONTECH Tet-On HeLa cells were grown in DMEM containing 10% tetracycline-tested FCS and 100 µg/ml geneticin. For biochemical studies, cells were transfected using Lipofectamine 2000 (Invitrogen) and analysed after 16–24 h. For immunofluorescence, cells were transfected using FuGene (Roche) and fixed after 16–24 h. HeLa cell lines stably expressing wt or *msd* PLP_h were generated using the CLONTECH Tet-On™ gene expression system. Stable transfectants were selected by growth in the presence of 500 µg/ml hygromycin. Clonal lines were chosen based on level of inducible expression as judged by immunofluorescence and western blotting. Expression was induced by 1 µg/ml doxycycline.

In vitro transcription, translation and immunoprecipitation

Cultured HT1080 fibroblasts were permeabilized with digitonin (Wilson *et al.*, 1995) and used as a source of ER membranes for translation. PLP was translated in a rabbit reticulocyte lysate system (Promega) for 15 min at 30°C in the presence of 0.75 µCi/µl [^{35S}]methionine and semi-permeabilized HT1080 cells. Then, 0.1 mM aurintricarboxylic acid (ACTA) was added to inhibit translation initiation and samples were incubated at 30°C for 5 min. Membrane-associated PLP was isolated by centrifugation for 5 s at 16 000 g and washed by resuspension in KHM (20 mM HEPES pH 7.2, 110 mM KOAc, 2 mM MgOAc). The resulting cell pellet was solubilized in 10 volumes of standard IP buffer [10 mM Tris–HCl pH 7.6, 140 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100] or ATP depletion buffer (10 mM Tris–HCl pH 7.6, 140 mM NaCl, 5 mM EGTA, 2 mM EDTA, 2 U/ml apyrase, 1 mM PMSF, 1% Triton X-100) for immunoprecipitation of BiP and Hsp family chaperones. Samples were incubated on ice for 1 h with intermittent vortexing and then centrifuged at 16 000 g for 10 min to remove insoluble material. Next, 2 mM methionine and 10 µl protein A or G Sepharose [50% (v/v) in IP buffer] were added to the supernatant and samples were rotated for 1 h at 4°C before centrifugation at 16 000 g for 5 min. The supernatant was rotated with the appropriate antibody for at least 4 h at 4°C and then 10 µl protein A or G Sepharose was added and the samples were rotated at 4°C for a further 3 h. Immune complexes were collected by centrifugation for 30 s at 16 000 g and washed three to five times with IP buffer. The kinetics of chaperone binding were measured by incubating samples for different times at 30°C after addition of ATCA prior to isolation of membrane-associated PLP and immunoprecipitation with anti-BiP. Samples were incubated with 25 µl sample buffer for 10 min at 70°C and then analysed by SDS–PAGE and phosphorimaging using a Fuji BAS 2000 system.

Co-immunoprecipitation from cultured cells

Cells were grown and transfected or induced in 10 cm dishes and harvested 18–24 h after transfection or induction. Cells were rinsed twice with phosphate-buffered saline (PBS), incubated with 10 mM iodoacetamide in PBS for 15 min at 4°C, rinsed twice with PBS and solubilized with IP buffer or ATP depletion buffer for co-immunoprecipitation of BiP and Hsp family chaperones. Samples were incubated on ice for 1 h with intermittent vortexing and then centrifuged at 16 000 g for 10 min to remove insoluble material. The supernatant was divided into two, incubated with anti-epitope tag antibody or species-matched control and immunoprecipitated as described above prior to SDS–PAGE and western blotting.

Indirect immunofluorescence microscopy

Cells grown on glass coverslips were fixed using methanol or using 2% formaldehyde and 0.2% glutaraldehyde, and then permeabilized with 0.1% Triton X-100 and 0.05% SDS. Secondary antibodies were from Jackson ImmunoResearch Labs. Images were obtained using an Olympus BX60 upright microscope and a MicroMax-cooled slow-scan CCD

camera (Roper Scientific) driven by Metamorph software (Universal Imaging Corporation).

RNA interference

Twenty-one-nucleotide duplexes corresponding to human calnexin coding nucleotides aagacgataccgatgataaa (cnx1) and aatgtggtggtcc-tatgtga (cnx2) with symmetric 2 nucleotide 3' (2'deoxy) thymidine overhangs were synthesized and annealed (Dharmacon Research). A scrambled siRNA (AAAACCAUCAUACCAGAGACA) was a gift from Dr Stephen Taylor (University of Manchester). HeLa cells were transfected with 6 µl of 20 µM siRNA duplex per well of a 12-well dish using Oligofectamine (Invitrogen) as described (Elbashir *et al.*, 2001). Knockdown of calnexin was apparent after 3 days.

Metabolic labeling of cells and pulse-chase analysis of PLP degradation

Cells were transfected with PLPha after 2 days of RNA interference. The next day, cells were starved for 20 min in methionine/cysteine-free MEM (Sigma) with 2 mM glutamine, and then metabolically labeled for 30 min with the same medium containing 20 µCi/ml of [³⁵S]methionine/cysteine protein labeling mix for 30 min. Cells were rinsed twice with PBS and chased in normal growth medium supplemented with 5 mM unlabeled methionine and cysteine. At the end of the chase, cells were rinsed twice with PBS and solubilized in IP buffer. Samples were denatured by heating to 70°C for 10 min and then centrifuged at 16 000 g for 10 min to remove insoluble material. Supernatants were precleared and immunoprecipitated with anti-ha as described above prior to SDS-PAGE and phosphorimaging.

Statistical analysis

Statistical analyses (one-way ANOVA and Dunnett's T3 test for significance) were carried out using SPSS 10.1 software. Averaged data were fitted to a single exponential decay ($y = y_0 + Ae^{-x/t}$) and the rate constants of degradation of msd PLPha were calculated using Microcal Origin software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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