Endoplasmic reticulum stress in the absence of calnexin

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Abstract Calnexin is a type I integral endoplasmic reticulum (ER) membrane chaperone involved in folding of newly synthesized (glycol)proteins. In this study, we used β -galactosidase reporter gene knock-in and reverse transcriptase polymerase chain reaction (RT-PCR) to investigate activation of the calnexin gene during embryonic development. We showed that the calnexin gene was activated in neuronal tissue at the early stages of embryonic development but remained low in the heart, intestine, and smooth muscle. At early stages of embryonic development, large quantities of calnexin messenger RNA (mRNA) were also found in neuronal tissue and liver. There was no detectable calnexin mRNA in the heart, lung, and intestine. The absence of calnexin had no significant effect on ER stress response (unfolded protein response, UPR) at the tissue level as tested by IRE1-dependent splicing of Xbp1 mRNA. In contrast, non-stimulated calnexin-deficient cells showed increased activation of IRE1, as measured by RT-PCR and luciferase reporter gene analysis of splicing of Xbp1 mRNA and activation of the BiP promoter. This indicates that *cnx*^{-/-} cells have increased constitutively active UPR. Importantly, *cnx*^{-/-} cells have significantly increased proteasomal activity, which may play a role in the adaptive mechanisms addressing the acute ER stress observed in the absence of calnexin.

Keywords Calnexin · Embryonic development · Endoplasmic reticulum · IRE · Unfolded protein responses · ERAD

Abbreviations

ER	endoplasmic reticulum
UPR	unfolded protein response
IRE1	inositol-requiring enzyme 1
Xbp1	X-box binding protein 1
ERAD	ER-associated degradation
PDI	protein disulfide isomerase
PBS	phosphate buffered saline
OCT	optimal cutting temperature compound

Introduction

The endoplasmic reticulum (ER) is involved in many specialized functions of the cell, including synthesis of membrane and secretory proteins, regulation of protein folding and trafficking, cellular responses to stress, and regulation of Ca²⁺ homeostasis. In the ER, there are several chaperones and folding enzymes that associate with newly synthesized proteins to assist with their correct folding and assembly (Hebert and Molinari 2007). Calnexin is a 90-kDa type I integral ER transmembrane chaperone that transiently binds newly synthesized monoglucosylated and misfolded glycoproteins, promoting their folding and oligomerization (Hebert and Molinari 2007). The protein, together with calreticulin, glucosidases, UGGT, and ERp57, is a component of a quality control of the secretory pathway (Hebert and Molinari 2007). Calnexin is ubiquitously expressed in all cells containing ER (Hebert and Molinari 2007). Surprisingly, calnexin-deficient mice survive but exhibit severe motor problems, and develop ataxic features and growth defects (Denzel et al. 2002), suggesting that expression of calnexin may play an important role during embryogenesis possibly restricted to specific organ or developmental stage.

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When protein synthesis/folding is disrupted, leading to accumulation of misfolded proteins, the cell responds by eliciting the unfolded protein response (UPR) or ER stress response (Zhang and Kaufman 2004). This is characterized by up-regulation of the synthesis of chaperone proteins [calreticulin, Grp78 (BiP), Grp94], by a decrease in general protein synthesis, and by activation of ER-associated degradation (ERAD; Zhang and Kaufman 2004; Meusser et al. 2005). The signaling proteins that mediate UPR response are ER proteins that include ATF6, PERK, and IRE1 (Zhang and Kaufman 2004). They act as proximal sensors of a disturbed ER environment. Grp78 (BiP) serves as an important UPR regulator, and under non-stress conditions, it binds to the luminal domains of ATF6, PERK, and IRE1 (Zhang and Kaufman 2004). UPR induces translocation of ATF6 to the Golgi for protease-dependent cleavage, producing a transcription factor involved in the up-regulation of expression of ER chaperones (Zhang and Kaufman 2004). Activation of PERK/PEK induces phosphorylation of the α subunit of eukaryotic initiation factor (eIF2 α) to attenuate translation (Zhang and Kaufman 2004). Finally, activation of IRE1 leads to splicing of messenger RNA (mRNA) encoding *XBPI*, producing a potent transcription factor that activates the transcription of chaperone-encoding genes (Zhang and Kaufman 2004). In addition, proteins that fail to fold correctly are translocated to the cytoplasm, ubiquitinated, and degraded by ERAD (Meusser et al. 2005).

In this study, we investigated the activation of the calnexin gene and the expression of calnexin during embryonic development, as well as the impact of calnexin deficiency on UPR. During embryonic development, the calnexin gene was activated in neuronal tissue at early stages of embryogenesis. There was no significant ER stress at the tissue level, suggesting that compensatory mechanisms may have been developed to deal with misfolded and aggregated proteins. However, UPR was activated in cultured non-stimulated calnexin-deficient cells. Importantly, *cnx*^{-/-} cells had increased proteasomal activity, potentially involved in adapting to the acute ER stress observed in the absence of the chaperone.

Experimental procedures

Transgenic mice

Embryonic stem (ES) cells with a deletion of one allele of the calnexin gene were created by a gene-trap approach, interrupting a coding region of the gene with a complementary DNA (cDNA) encoding β -galactosidase (Leighton et al. 2001; Mitchell et al. 2001). These cells (designated KST286) were created at BayGenomics (<http://www.baygenomics.ucsf.edu/>) and used to generate *cnx*^{+/-} mice.

The KST286 cell line was generated from 129P2 (formerly 129/Ola) ES cell lines. Parental cell lines (CGR8 and E14Tg2A) were established from delayed blastocysts on gelatinized tissue culture dishes in ES cell medium containing LIF. The KST286 cells were transfected with the gene trap vector pGT1TMpfs (<http://www.baygenomics.ucsf.edu/>) containing β -galactosidase-neomycin genes to interrupt the calnexin gene. ES cells were microinjected into 3.5-day-old C57BL/6J blastocysts to generate chimeric mice. Chimeric males were analyzed for germline transmission by mating with C57BL/6J females, and the progeny were analyzed by polymerase chain reaction (PCR), β -galactosidase staining, and Western blot analyses. Rabbit anti-calnexin antibody was used at dilution of 1:1,000. *cnx*^{+/-} mice used in this study were indistinguishable from wild-type littermates.

Staining of embryos for β -galactosidase activity

For histological analysis, wild-type and *cnx*^{+/-} embryos were harvested, washed in phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde in PBS, pH 7.2 for 3 h at 4°C. The embryos were washed in PBS and saturated with 15% sucrose in PBS overnight at 4°C. The embryos were then incubated with 25% optimal cutting temperature compound (OCT, Tissue-Tek)/15% sucrose in PBS for 20–60 min, in 50% OCT/25% sucrose in PBS for 20–60 min, and then in 75% OCT/15% sucrose in PBS for 20–60 min. The embryos were frozen in OCT by submersion into methyl butane chilled in liquid nitrogen and cryosectioned. Slides were dried overnight, washed in PBS to remove the OCT and incubated in PBS X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside) staining solution containing 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% Nonidet-P 40, 20 mM Tris, pH 8.0, 5 mM potassium ferricyanide, 1 mg/ml X-gal in *N,N*-dimethylformamide for 1–5 h at 37°C. The slides were washed in PBS, counterstained with eosin (0.1% eosin in 0.1% acetic acid), washed in 95% ethanol, 100% ethanol, and mounted with Cytoseal (Richard-Allen Scientific).

Cell culture

Mouse embryonic fibroblasts were isolated from day-12 calnexin-deficient and wild-type embryos. Cells were immortalized as described previously (Mesaeli et al. 1999) and maintained in Dulbeccos modified Eagles medium with 10% fetal bovine serum, 1% Penicillin/Streptomycin, and 2.5 ng/ml of Plasmocin. Cells were treated with 2 μ g/ml of tunicamycin or 1 μ M of thapsigargin for 16 h. Cells were lysed in a buffer containing 50 mM Tris at pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol *bis*(2-aminoethyl ether)-*N,N,N,N'*-

tetraacetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamide, 0.05 µg/ml aproprotin, 0.025 µg/ml phosphoridone, 0.05 µg/ml TLCK, 0.05 µg/ml amidinophenylmethylsulfonyl fluoride, 0.05 µg/ml E-64, 0.025 µg/ml leupeptin, and 0.01 µg/ml pepstatin; Milner et al. 1991), incubated on ice for 10 min and spun down at 12,100×g for 10 min. Supernatant containing cellular proteins was collected and proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Thirty micrograms of protein was separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose (Nakamura et al. 2000). The nitrocellulose membrane was blocked with 5% milk powder and 0.1% Tween 20 in PBS or Odyssey Blocking Buffer followed by probing with specific antibodies. Antibodies used were rabbit-anti-calnexin at dilution of 1:1,000, rabbit-anti-protein disulfide isomerase (PDI) at dilution of 1:1,000, goat-anti-calreticulin at dilution of 1:500, rabbit-anti-ERp57 at a dilution of 1:300, rabbit-anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at a dilution of 1:1,000 (Abcam), mouse-anti-IRE1 at a dilution of 1:500 (a generous gift from Dr. R. Kaufman), rabbit-anti-Grp94 at dilution of 1:1,000 (StressGen), or rabbit-anti-Grp78 at dilution of 1:1,000 (StressGen). Secondary antibodies used were goat anti-rabbit (1:10,000), rabbit-anti mouse (1:10,000) and rabbit anti-goat (1:10,000). Blots were analyzed using the Odyssey Infrared Imaging System (Li-Cor) or by electrogenerated chemiluminescence reaction (Nakamura et al. 2000).

Immunohistochemistry

For immunocytochemistry, calnexin-deficient or wild-type cells were grown on glass coverslips. Cells were fixed with 3.7% formaldehyde in PBS for 15 min, permeabilized for 5 min in PBS containing 1% saponin and blocked for 1 h in PBS/1% saponin containing 1% milk powder. Primary antibodies used were rabbit anti-PDI (1:50), rabbit anti-Grp78 (1:50), and rabbit anti-Grp94 (1:50). Secondary antibody was anti-rabbit Alexa Fluor 546 (1:100). The cells were also stained with fluorescein isothiocyanate (FITC)-Concanavalin A (Sigma). The cells were then mounted onto glass slides and fluorescent signals visualized using an inverted fluorescent microscope.

Reverse transcriptase polymerase chain reaction

Calnexin-deficient and wild-type cells were treated with thapsigargin (1 µM), tunicamycin (2 µg/µl), brefeldin-A (2.5 µM), or castanospermine (5 µg/ml) for 16 h, and total RNA was isolated using TRIzol Reagent (Invitrogen Life Technologies). cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and subsequently ampli-

fied with Taq polymerase (Sigma) with specific primers as follows: for Xbp1, forward primer 5'- CCTGTGGTT GAGAACCAGG-3' and reverse primer 5'-CTAGA GGCTTGGTGTATAC-3'; for calnexin, forward primer 5'-CATGATGGACATGATGATGACAC-3' and reverse primer 5'-GGTCTTCAGACTTGCATCTGGC-3'; for Grp78, forward primer 5'-TGGTATTCTCCGAGTGACAGC-3' and reverse primer 5'-AGTCTTCAATGTCCGCATCC-3'; for GAPDH, forward 5'-AACTTTGGCATTGTGGAAGG-3' and reverse primer 5'-ACACATTGGGGGTAGGAACA-3'; for tubulin, forward primer 5'-CCGGACAGTGTGGCAA CCAGATCGG-3' and reverse primer 5'-TGGCCAAAA GGACCTGAGCGAACGG-3'. PCR products were separated on acrylamide gels containing a 7.5% acrylamide (for Xbp1 PCR products) or 1% acrylamide (for calnexin, Grp78, GAPDH, and tubulin PCR products). For quantitative analysis, three independent experiments were carried out, and intensities of DNA bands were quantified using ImageJ Software.

Cell transfection and luciferase assay

Wild-type and calnexin-deficient cells were transfected with pRL-XFL vector encoding Renilla luciferase and firefly luciferase reporter genes as described previously (Back et al. 2006). The cells were co-transfected with pGL3-Grp78-luciferase (firefly luciferase reporter driven by a 311-bp fragment (−304 to +4) of the Grp78 promoter (Yoshida et al. 1998) and with Renilla luciferase reported plasmid pRL-CMV (Promega) at 1:50 ratio, respectively, using Effectene (Qiagen) with 0.2 µg total DNA. Cells were treated with 1 µM thapsigargin for 16 h, lysed, diluted 1:10 in passive lysis buffer (Promega) and then assayed with the dual-luciferase assay kit (Promega) using a luminometer (Berthold-Lumat LB 9501). Relative light units (RLUs) were normalized to internal control (pRL-XF) or to Renilla luciferase reporter gene under control of the CMV promoter (pRL-CMV or pGL3-Xbp1) (Back et al. 2006). Average values ± standard deviation of three independent experiments ($n=3$) are reported.

Proteasome activity assay

Proteasome activity of cell extracts was measured using 20S proteasome activity assay kit from Chemicon International. Cells were washed twice with PBS and lysed (1×10^6 cells per sample) for 30 min on ice in a buffer containing 50 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) at pH 7.5, 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100. Lysates were centrifuged at 14,000 rpm for 15 min at 4°C followed by measurement of fluorescence using 380 nm excitation and 460 nm emission filters in a fluorometer (Spectramax Gemini XPS).

Results

Developmental activation of the calnexin gene

Calnexin is an ubiquitously expressed ER protein, yet calnexin-deficient mice develop specific motor disorders and neurological problems (Denzel et al. 2002), suggesting an essential role for the protein during neuronal development. We used a transgenic mouse model to investigate the expression of calnexin during embryonic development with a special emphasis on the nervous system. First, we generated

mice expressing β -galactosidase reporter gene under control of the endogenous calnexin promoter. Second, embryos were harvested at different gestational times, fixed, frozen, and stained for β -galactosidase activity. Each embryo was tested for the presence of the β -galactosidase transgene by PCR, followed by analysis of sections prepared from transgenic and wild-type littermates. Figure 1 shows sagittal sections of embryos at different stages of development. At embryonic day 11 (E11.5), there was high activity of the calnexin promoter and relatively uniform β -galactosidase staining throughout the embryo (Fig. 1a). Interestingly, there was low

Fig. 1 Activation of the calnexin gene during embryogenesis. Histological analysis and detection of the β -galactosidase reporter gene activity of *cnx*^{+/+} embryos were carried out as described under “Experimental procedures.” **a** A ubiquitous activation of the calnexin promoter in E11.5 embryos. As early as E12.5 (**b**) and E13.5 (**c**), there was activation of the calnexin gene in the brain and cartilage but not in the liver. **d** In the E15.5 embryo, the strongest β -galactosidase signal was observed in the brain and liver but not in the heart. Activation of the calnexin promoter was also seen in the lungs. At embryonic stages, E17.5 (**e**) and E18.5 (**f**) activation of calnexin promoter was most apparent in the brain, lungs, kidney, and cartilage tissues. No significant staining was detected in the heart

activity of the calnexin promoter in the E11.5 embryonic liver (Fig. 1a). As the embryo aged, activation of the calnexin gene became more localized to specific regions. Already at day 12, specific activity of the β -galactosidase reporter was highest in the neuronal tissue and cartilage, while the signal in liver tissue remained distinctively low (Fig. 1b, E12.5). This pattern became more apparent in later stages of embryogenesis. Figure 1c shows that the calnexin gene was highly activated in neuronal tissue, lungs, and cartilage. β -galactosidase activity remained low in the liver, heart, arteries, and ganglions (Fig. 1c, E13.5). At day 15 (Fig. 1d, E15.5), the calnexin promoter remains highly active in neuronal (brain, spine) and in cartilage tissues. At this stage of embryonic development, no significant staining for β -galactosidase was detected in the heart, liver, and smooth muscle (Fig. 1d). A high level of activation of the calnexin promoter continued in the neuronal tissue and cartilage in the 17- and 18-day-old embryos (Fig. 1e, f). In the 17-day-old embryo, there was also activation of the calnexin gene in the lungs, kidney, but very little in the liver (Fig. 1e, E17). At day 18 of embryonic development, the calnexin gene remained highly active in the brain, spine, cartilage, and lungs (Fig. 1f, E18.5). At this stage of embryonic development, there was significant activation of the calnexin gene in the liver (Fig. 1f, E18.5), while the activity remained below detection in the heart, intestine, and smooth muscle (Fig. 1f, E18.5).

Next, we isolated RNA from a variety of mouse tissues at different stages of embryonic development as well as from newborn animals, followed by reverse transcriptase PCR (RT-PCR) analysis of the expression of calnexin mRNA (Fig. 2). In agreement with observed β -galactosidase signal, calnexin was highly expressed in the brain at all stages of embryonic and postnatal development (Fig. 2). At early stages of embryonic development, large quantities of calnexin mRNA were found in the liver, and there was no detectable mRNA in the heart, lung, and intestine (Fig. 2, E13.5). In contrast to the RT-PCR analysis of calnexin mRNA in embryonic liver (Fig. 2, E13.5), there was a relatively low activity of the β -galactosidase reporter gene in the liver of E13.5 embryos (Fig. 1c). This indicates that despite a low transcriptional activity of the calnexin gene, there was a significant accumulation level of calnexin mRNA in the embryonic liver. Expression of calnexin mRNA increased at later stages of development in the lung, intestine, and kidney (Fig. 2). In the E17.5 embryos, expression of calnexin mRNA in the brain remained high, and there was no detectable mRNA signal in the heart, intestine, spleen, kidney, and skeletal muscle (Fig. 2). After birth, a significant level of calnexin mRNA was also detected in the heart and other tested tissues (Fig. 2). In conclusion, both β -galactosidase reporter gene studies (Fig. 1) and RT-PCR analysis (Fig. 2) indicated that throughout embryonic

Fig. 2 RT-PCR analysis of calnexin mRNA in mouse embryonic tissues. RT-PCR analysis was carried out as described under “Experimental procedures.” Relatively high level of calnexin mRNA was observed in the brain, lungs, and liver at all stages of development tested

development, the calnexin gene is highly activated in the nervous system, especially in the brain.

Calnexin-deficient mouse fibroblasts

Calnexin is an important component of the quality control of the secretory pathway involved in folding of newly synthesized (glyco)proteins (Williams 2006). It is expected that in the absence of calnexin, there might be a buildup of misfolded proteins in the ER and, consequently, activation of the UPR. To test if the absence of calnexin affects UPR, we isolated mouse fibroblasts from wild-type and *cnx*^{-/-} mice. As expected, Western blot analysis revealed that wild-type cells contained immunoreactive calnexin whereas calnexin-deficient cells did not (Fig. 3a). There was approximately a twofold increase in the level of Grp94 and ERp57 in *cnx*^{-/-} cells and a slight (0.5-fold) increase in Grp78 (Fig. 3a). We found no significant difference in the level of expression of calreticulin and PDI in the absence of calnexin (Fig. 3a). Thus, the lack of calnexin in these cells did not appear to significantly impact expression of other ER-folding proteins. Moreover, the morphological appearance of the wild-type and calnexin-deficient cells was indistinguishable (Fig. 3b, c). The cells attached firmly to plastic and showed typical, ER-like staining with FITC-ConA, anti-PDI, anti-Grp78, and anti-Grp94 antibodies (Fig. 3b, c).

Fig. 3 Chaperone expression in calnexin-deficient cells. **a** Western blot analysis of ER folding factors in wild-type (*wt*) and calnexin-deficient (*cnx*^{-/-}) mouse embryonic fibroblasts. Wild-type and calnexin-deficient cells were lysed, and proteins were separated by

SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies as indicated in the figure. **b** and **c** Immunostaining of wild-type and calnexin-deficient cells, respectively, with anti-Grp78, anti-Grp94, anti-PDI antibodies, and FITC-ConA

ER stress in calnexin-deficient mouse embryonic fibroblasts

Next, we tested if calnexin deficiency led to activation of UPR in cultured cells. In the present study, we examined the expression of ER-resident stress-sensitive proteins (Fig. 4a), splicing of Xbp1 (Fig. 4b and c, Fig. 5a and b), and activation of the BiP promoter (Fig. 5a and b; Zhang and Kaufman 2004). Upon the accumulation of misfolded proteins, IRE1, an ER, type 1 transmembrane protein, homodimerizes and undergoes trans-autophosphorylation, leading to activation of its cytoplasmic endoribonuclease function (Back et al. 2005). The target of this endoribonuclease is mRNA encoding Xbp1 (Back et al. 2005; Zhang and Kaufman 2004). We used two ER stress inducers to test for the UPR-dependent expression of ER-resident proteins in the absence of calnexin: tunicamycin (*Tn*), an inhibitor of N-glycosylation, and thapsigargin (*Tg*), an inhibitor of sarco/endoplasmic reticulum Ca^{2+} -ATPase, an ER localized Ca^{2+} -ATPase. Figure 4a shows that both thapsigargin and tunicamycin induced expression of Grp78 (BiP), Grp94, and IRE1 in wild-type and calnexin-deficient cells. Grp78 expression was induced twofold or 1.5-fold in the presence of thapsigargin or tunicamycin, respectively, in the wild-type and calnexin-deficient cells (Fig. 4a). Expression of Grp94 was induced threefold or sixfold by thapsigargin in wild-type and calnexin-deficient cells, respectively. Tunicamycin induced expression of Grp94 by 2.3- and 2.6-folds in wild-type and calnexin-deficient cells, respectively (Fig. 4a). Although the same level of ER stress has been detected in non-stimulated, untreated calnexin-deficient cells as indicated by a slight up-regulation of the Grp78 and Grp94 genes, there was still a significant, tunicamycin- or thapsigargin-dependent up-regulation of UPR in the absence of calnexin (Fig. 4a).

UPR activation and Xbp1 splicing in calnexin-deficient cells

Next, we examined if activation of IRE1 and splicing of Xbp1 were affected in the absence of calnexin. We isolated RNA from mouse embryonic fibroblasts followed by RT-PCR analysis of Xbp1 mRNA. In addition to tunicamycin and thapsigargin, UPR was also induced in wild-type and *cnx*^{-/-} cells with brefeldin-A (*BFA*), an ER-Golgi transport inhibitor, and castanospermine (*Cst*), an inhibitor of glucosidase I, thus inhibiting oligosaccharide processing critical for calnexin-substrate interactions (Hebert et al. 1996; Ivessa et al. 1995). Treatment of cells with these drugs leads to protein accumulation in the ER and to activation of UPR (Pahl and Baeuerle 1995). Figure 4b shows that thapsigargin (*Tg*) or tunicamycin (*Tn*) treatment of cells strongly induced a rapid and complete splicing of Xbp1 to produce a 424-bp spliced variant. Importantly, both cell lines showed similar sensitivity to thapsigargin- and tunicamycin-induced UPR. However, under the same experimental conditions, brefeldin-A and castanospermine induced only a partial splicing of Xbp1 (Fig. 4c). Most importantly, wild-type cells were more sensitive to brefeldin-A and castanospermine-induced UPR than calnexin-deficient counterparts (Fig. 4c).

To quantify Xbp1 splicing, we took advantage of a luciferase reporter system developed by Kaufman's group (Back et al. 2006). Wild-type and *cnx*^{-/-} cells were transfected with pRL-IXFL vector encoding Renilla luciferase (internal control) and firefly luciferase reporter gene, which is activated only when the 26-nt intron from Xbp1 is removed in a UPR-dependent manner (Back et al. 2006). To report activation of UPR, we also transfected cells with pGL3-Grp78-luciferase vector encoding firefly luciferase

Fig. 4 Induction of UPR in calnexin-deficient cells. **a** Western blot analysis of chaperone proteins in wild-type and calnexin-deficient cells treated with tunicamycin (*Tn*) and thapsigargin (*Tg*). Duplicate samples were analyzed by Western blotting as indicated in the figure. Densitometry was performed using Kodak Image Software and a one-tail, two-sample *t* test was done to determine significant difference from the untreated control. In wild-type cells treated with *Tg*, Grp78 increased 80% ($p=0.05$), Grp94 increased 139% ($p=0.25$), and IRE1 increased 20% ($p=0.05$). In wild-type cells treated with *Tn*, Grp78 increased 53% ($p=0.05$), Grp94 increased 153% ($p=0.25$), and IRE1 increased 7% ($p=0.05$). In calnexin-deficient cells treated with *Tg*, Grp78 increased 82% ($p=0.25$), Grp94 increased 75% ($p=0.25$), and IRE1 increased 52% ($p=0.05$). In calnexin-deficient cells treated with *Tn*, Grp78 increased 55% ($p=0.05$), Grp94 increased 34% ($p=0.05$), and IRE1 increased 3% ($p=0.05$). **b** and **c** Xbp1 splicing in wild-type and *cnx*^{-/-} cells. Wild-type (*wt*) and calnexin-deficient (*cnx*^{-/-}) cells were treated with **b** thapsigargin (*Tg*) and tunicamycin (*Tn*) or **c** brefeldin-A (*BFA*) and castanospermine (*Cst*) followed by isolation of mRNA and RT-PCR with primers specific for unspliced (*upper DNA band*) and spliced (*lower DNA band*) variant of Xbp1 mRNA. Densitometry was normalized to *wt* untreated and shown graphically below

under control of the Grp78 (BiP) promoter containing a ER stress element (Yoshida et al. 1998). Figure 5 shows that, in non-stimulated cells, there was a significant difference in UPR activation between *cnx*^{-/-} and wild-type cells as measured by splicing of Xbp1 mRNA and activation of the BiP promoter. There was a twofold and 1.7-fold higher luciferase activity in *cnx*^{-/-} cells transfected with pRL-IXFL and pGL3-Grp78-luciferase vector, respectively. Thapsigargin treatment significantly enhanced luciferase activity in wild-type and calnexin-deficient cells expressing luciferase reporter genes (Fig. 5b). Figure 5b shows that

thapsigargin treatment of wild-type and calnexin-deficient cells resulted in similar levels of activation of luciferase resulting from Xbp1 splicing. However, comparison of the means of wild-type untreated cells to wild-type thapsigargin-treated cells revealed that thapsigargin-induced luciferase activity was increased 11-fold, whereas it was induced only 6.6-fold when comparing the means of calnexin-deficient cells that are untreated to calnexin-deficient cells that are treated with thapsigargin. This was likely due to already significantly higher luciferase activity detected in non-stimulated, untreated *cnx*^{-/-} cells (Fig. 5a). Figure 5b also

Fig. 5 Xbp1 splicing and activation of Grp78 promoter in calnexin-deficient cells. Renilla luciferase and firefly luciferase activities were measured as described under “Experimental procedures,” and the relative ratio of firefly luciferase to Renilla luciferase activity in each cell lysates was calculated. **a** Wild-type (open bars) and *cnx*^{-/-} (black bars) cells were transfected with reporter plasmid for Xbp1 splicing or a plasmid DNA encoding luciferase reported gene under control of Grp78 promoter as described under “Experimental procedures.” Cell lysates were assayed for luciferase activity. **b** Wild-type (open bars) and calnexin-deficient (black bars) cells were transfected with reporter plasmid for Xbp1 splicing or a plasmid DNA encoding luciferase reporter gene under control of Grp78 promoter as described under “Experimental procedures.” Cells were treated with thapsigargin (Tg) followed by luciferase assay. Columns and bars represent the mean $\pm$ SD of nine measurements from three independent transfection experiments. RLU relative light units. One tail, two-sample *t* test were performed. **p* = 0.05 and ***p* = 0.025

shows that there was thapsigargin-dependent activation of the BiP promoter in wild-type and *cnx*^{-/-} cells.

Calnexin-deficient cells have increased proteasomal activity

Although calnexin-deficient cells have increased UPR, they have typical growth and survival characteristics observed under standard tissue culture conditions, suggesting that they may develop adaptive mechanisms to deal with increased stress (Rutkowski et al. 2006; Rutkowski and Kaufman 2007). One additional pathway induced by UPR is activation of ERAD and proteasomal activity, leading to the removal of misfolded proteins (Rutkowski et al. 2006; Rutkowski and Kaufman 2007; Yoshida et al. 2003). Therefore, we tested if increased proteasomal activity in the absence of calnexin compensated for constitutively active UPR in these cells. Cellular extracts from wild-type and calnexin-deficient cells were prepared and tested for

proteasomal activity based on detection of the fluorophore 7-amino-4-methylcoumarin after cleavage from the fluorescently labeled substrate LLVY (Meng et al. 1999). Figure 6 shows that proteasome activity was significantly higher in calnexin-deficient cells when compared to wild-type cells. This suggests that increased ERAD may be one mechanism of adaptation to acute ER stress seen in the absence of calnexin. In an ongoing study, we have found that expression of a myelin-specific protein P0 in calnexin-deficient cells was consistently very low. However, it can be rescued in the presence of proteasomal inhibitors, supporting and extending our conclusions that *cnx*^{-/-} cells have increased proteasomal activity (data not shown).

Neuronal tissue deficient in calnexin does not have increased ER stress

Next, we asked if there is any UPR activation in calnexin-deficient mouse tissues, as this may contribute to a neuronal phenotype of the calnexin-deficient mouse (Denzel et al. 2002). We examined splicing of Xbp1 in calnexin-deficient spine, brain, cerebellum, and liver (Fig. 7). Thapsigargin-induced UPR in wild-type cells was used as a positive control. Thapsigargin treatment of wild-type cells resulted in rapid and complete splicing of Xbp1 to produce a 424-bp spliced variant (Fig. 7, *ctrl*). Although there was a different level of expression of Xbp1 in mouse tissues tested, RT-PCR analysis of mRNA showed no detectable splicing of Xbp1 in calnexin-deficient (Fig. 7, *cnx*^{-/-}) and wild-type (Fig. 7, *wt*) tissues. Next, we carried out Western blot analysis of the level of BiP in cerebellum and brain from wild-type and calnexin-deficient animals. In agreement with Xbp1 splicing results, there was no significant increase

Fig. 6 Proteasomal activity in calnexin-deficient cells. Cellular extracts were prepared from wild-type (open bar) and *cnx*^{-/-} (closed bar) cells, and assayed for proteasome using 20S proteasome activity assay kit from Chemicon International as described under “Experimental procedures.” Fluorescence of the fluorophore 7-amino-4-methylcoumarin was measured using 380-nm excitation and 460-nm emission filters. RFU relative fluorescence units

Fig. 7 Activation of UPR in wild-type and calnexin-deficient tissues. RNA was isolated from tissues derived from wild-type (*wt*) and calnexin-deficient (*cnx*^{-/-}) 21-day-old mice followed by RT-PCR analysis for Xbp1 unspliced (450 bp) and splice (424 bp) variants.

Wild-type cells treated with thapsigargin (*Tg*) were used as a positive control (*ctrl*). RT-PCR of tubulin mRNA was used as a loading control. The arrows indicate the location of unspliced and spliced Xbp1 RT-PCR DNA fragment

in BiP expression in calnexin-deficient tissues (Fig. 8). Collectively, these results indicate that there was no significant activation of UPR in the presence and absence of calnexin in cerebellum, brain, spine, and liver.

Discussion

Despite considerable understanding of the calnexin/calreticulin cycle, virtually nothing is known about the regulation of the calnexin gene and a potential role for the protein in UPR. In this paper, we report, for the first time, on the activation of the calnexin gene during embryonic development in mouse. One of the most striking and unexpected findings is that the calnexin gene is highly active in neuronal and cartilage tissues at relatively early stages of embryogenesis. In contrast, activation of the calnexin gene occurs relatively late in the liver and is undetectable in the heart. Only at the latest stages of embryo development is there activation of the calnexin gene in the liver with little to no activation of the gene in the heart. Neuronal specific

activation of the calnexin gene is of great interest because calnexin-deficient mice exhibit motor disorders (Denzel et al. 2002). In *Caenorhabditis elegans*, calnexin is also highly expressed in the dorsal and ventral nerve cord, and head and tail neurons throughout development (Lee et al. 2005). Moreover, in *Drosophila*, calnexin is required for rhodopsin maturation, with mutations in *Drosophila* calnexin leading to severe down-regulation in rhodopsin expression resulting in retinal degeneration (Rosenbaum et al. 2006). Taken together, these observations indicate that calnexin may play an important role during neuronal development. Our finding that calnexin is highly expressed in the neuronal tissue throughout embryonic development taken together with evidence that calnexin interacts with the myelin protein, PMP22 (Dickson et al. 2002), further stresses the impact calnexin may have on neurogenesis and how, in its absence, the neuroprotection function it may serve could be lost, resulting in neuropathology.

Although the chaperones calnexin and calreticulin are homologous proteins with some redundant functions, they yield very different phenotypes in their mouse deficient

Fig. 8 Expression of Grp78 protein in wild-type and *cnx*^{-/-} cerebellum and brain. Tissues were solubilized in a lysis buffer, and proteins were separated by SDS-PAGE as described under “Experimental procedures.” Increasing amount of tissue lysates were loaded into SDS-PAGE as indicated in the figure. Blots were normalized for expression of GAPDH

models. The calreticulin deficiency is embryonic lethal at day 14 of gestation due to impaired cardiac development (Mesaeli et al. 1999). This is not surprising because calreticulin is highly expressed in the heart during early stages of embryonic development (Mesaeli et al. 1999). Furthermore, this lethality is rescued by cardiac specific over-expression of calcineurin, suggesting that changes in Ca^{2+} homeostasis, in the absence of calreticulin, play an important role during cardiogenesis (Guo et al. 2002). Importantly, calnexin is not expressed in the developing heart (this study) and does not compensate for calreticulin function during cardiogenesis, further supporting the hypothesis that the Ca^{2+} homeostasis role of calreticulin is responsible for impaired cardiac development in calreticulin-deficient mice (Mesaeli et al. 1999). In sharp contrast, calnexin-deficient mice survive and exhibit severe motor problems, growth defects, and ataxic features, such as an unstable gait (Denzel et al. 2002). This is not surprising in view of our findings that the calnexin gene is highly activated in neuronal tissue at early stages of embryonic development. Importantly, the drastically different patterns of expression of calreticulin and calnexin during embryonic development, as well as phenotypes of calreticulin- and calnexin-deficient mice suggest that the functions of these proteins are not entirely redundant and that calnexin does not have a critical role in life but an important role in quality of life.

The important finding of this study is that *cnx*^{-/-} cells have constitutively active UPR. This may represent acute stress response phenomenon proposed by Kaufman's group (Rutkowski et al. 2006; Rutkowski and Kaufman 2007). Yet both wild-type and calnexin-deficient cells have a similar capacity for ER stress induction with commonly used drugs (thapsigargin and tunicamycin), but they differ in UPR levels in non-stimulated cells. This is supported by reporter gene and RT-PCR analyses, as well as by Western blot analysis of ER stress proteins including Grp78 and Grp94. Interesting *cnx*^{-/-} cells show significant up-regulation of ERp57, a protein that has not been associated with ER stress. Relatively high expression of ERp57 in these cells may represent an ER-stress-independent adaptive mechanism for cells deficient in calnexin. Increased UPR in *cnx*^{-/-} cells does not appear to have any effect on cell growth, suggesting that *cnx*^{-/-} must have developed adaptive mechanisms for dealing with an increased load of misfolded proteins. Indeed, we show here that calnexin-deficient cells have increased proteasomal activity, indicative of a high activity of ERAD. Therefore, we propose that increased ERAD may represent one mechanism of adaptation to acute ER stress in the absence of calnexin. Calnexin-deficient cells do not appear to compensate for the loss of calnexin by up-regulating expression of other chaperones. Instead, they modulate specific pathways, including ERAD, to deal with increased load of misfolded proteins.

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