The Use of Calnexin and Calreticulin by Cellular and Viral Glycoproteins*

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Calnexin and calreticulin are homologous lectin chaperones that assist maturation of cellular and viral glycoproteins in the mammalian endoplasmic reticulum. Calnexin and calreticulin share the same specificity for monoglucosylated protein-bound N-glycans but associate with a distinct set of newly synthesized polypeptides. We report here that most calnexin substrates do not associate with calreticulin even upon selective calnexin inactivation, while BiP associates more abundantly with nascent polypeptides under these conditions. Calreticulin associated more abundantly with orphan calnexin substrates only in infected cells and preferentially with polypeptides of viral origin, showing stronger dependence of model viral glycoproteins on endoplasmic reticulum lectins. This may explain why inactivation of the calnexin cycle affects viral replication and infectivity but not viability of mammalian cells.

About 20% of mammalian proteins are predicted to be secretory proteins (1). Most of them are N-glycosylated during transit through the endoplasmic reticulum (ER) translocon. The preassembled glycan added to nascent chains is composed of 2 N-acetyl glucosamines, 9 mannoses, and 3 terminal glucose residues. Two glucose residues are rapidly trimmed by ER-resident glucosidases, and the generated monoglucosylated N-glycan directs association of the nascent polypeptide with the lectin chaperones calnexin (Cnx) and calreticulin (Crt) and with the glycoprotein-specific oxidoreductase ERp57 (2–6). For most glycoproteins, transient association with Cnx, Crt, and ERp57 is not essential, but contributes to the efficiency of the maturation process. Although Cnx and Crt share the same ligand specificity, they bind a distinct set of polypeptides, possibly because the first is membrane-bound whereas the latter is soluble in the ER lumen. It has been shown in fact that Cnx binds to membrane-proximal glycans whereas Crt binds to glycans that emerge deeper into the lumen (7–15).

Recently, Cnx-knockout mice were established (16). An unexpected recombination event resulted in the generation of a second mouse strain expressing a shorter version of Cnx (sCnx). Cnx-deficient mice and mice expressing sCnx were phenotypically identical. Directly after birth, their size, respiration, and feeding were normal. At 8–10 days after birth, they were one-third lighter than their littermates, and they progressively developed severe motor abnormalities. Most of the mice either died or had to be sacrificed within 4 weeks (16), showing the incapacity of Crt to fully replace Cnx activity. Because of severe breeding difficulties of the original Cnx-deficient strain, only the strain expressing sCnx was continued (16). Here we made use of embryonic fibroblasts derived from these mice to better characterize the function of Cnx and Crt during protein folding.

We first established that sCnx originated from an alternative splicing of the Cnx gene that removed several functional residues, thus selectively depleting cells of Cnx activity. No evidence of compensatory unfolded protein response (UPR) was found, indicating the lack of substantial protein-folding defects at the cellular level upon eradication of Cnx activity. Despite the fact that Crt and Cnx share the same specificity for monoglucosylated N-glycans, Crt did not normally associate with orphan Cnx substrates upon selective depletion of Cnx activity. Rather, enhanced association of nascent chains with BiP was observed.

Three cellular Cnx substrates, the folding-competent BACE501 and the folding-defective BACE457 and BACE457Δ were expressed in cells selectively depleted of Cnx activity to determine whether the membrane-bound versus soluble status, the number of protein-bound N-glycans, or the substrate folding competence affected the capacity of Crt to associate with orphan Cnx substrates. None of these specific Cnx substrates associated with Crt upon eradication of Cnx activity. Crt did associate with orphan Cnx substrates only in virus-infected cells and, preferentially, with orphan Cnx substrates of viral origin. These results showed an intriguing diversity among cellular protein and viral proteins expressed in infected cells in the use of and in the dependence on the Cnx/Crt chaperone system.

EXPERIMENTAL PROCEDURES

Cell Lines, Transient Transfections, and Viral Infections—wt and S-Cnx mouse embryonic fibroblasts (MEF) were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum/penicillin/streptomycin (Invitrogen, Life Technologies, Inc.).

For infections, cells were plated in 6-cm Petri dishes to 70–80% confluence and incubated for 30–60 min at room temperature with the selected virus diluted to 30 plaque-forming units per cell in RPMI/0.2% bovine serum albumin, pH 6.8. The inoculum was replaced with cell culture medium, and incubation proceeded for 4 h. Cells were then metabolically labeled. For transfections, cells plated at 90% confluence were transfected with BACE expression plasmids using Lipofectamine...
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2000 (Invitrogen, Life Technologies, Inc.) according to the manufacturer’s instruction for adherent cells and as described in Ref. 17.

**Metabolic Labeling, Preparation of Cell Extracts, Immunoprecipitation, and EndoH Treatments—Virus-infected (4 h after infection) or transiently transfected cells (17 h after transfection) were starved for 20 min in Met/Cys-free medium, pulsed for 10 min with 150 μCi of [35S]Met/Cys in 1 ml of starvation medium/dish and chased for the times indicated in the figures with Dulbecco’s modified Eagle’s medium supplemented with 5 ml cold Met/Cys. Postnuclear supernatants were prepared by solubilization of cells in 800 μl/dish of ice-cold 2% CHAPS in Hepes buffer saline, pH 6.8, containing 20 μM ice-cold N-ethylmaleimide, protease inhibitors (HBS), and 10 units of apyrase/dish (Sigma) to rapidly deplete cellular ATP for anti-BiP immunoprecipitations. Cell extracts were prepared by 10 min of centrifugation at 10,000 × g and analyzed by reducing SDS-PAGE.

Immunoprecipitations were performed by adding protein A beads (Sigma, 1:10 w/v swollen in HBSS) and the selected antibody to the cell extracts. Incubations were for 1–4 h in a cold room.

The immunoprecipitates were washed three times with HBSS/0.5% CHAPS and resuspended in sample buffer for SDS-PAGE. Gels were exposed to BioMax (Kodak) films and scanned with an Agfa scanner.

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**RESULTS**

**Characterization of sCnx**—We first assessed intracellular expression and localization of sCnx by indirect immunofluorescence of semipermeabilized cells. sCnx conserved the N-terminal ER-targeting signal sequence and the C-terminal ER retention motif of full-length Cnx, because it co-localized with the ER marker BiP (Fig. 1A). Replacement of Cnx with sCnx did not affect cell viability and did not trigger a compensatory up-regulation of other ER-resident proteins such as BiP, PDI, ERp57, or Crt (Fig. 1B) showing that MEF expressing sCnx (S-CNX MEF) were not under debilitating ER stress.

Analysis of sCnx transcripts (see “Experimental Procedures”) revealed a deletion of 417 base pairs (Fig. 1C), from base 307 to 725, because of excision of the Cnx exons four, five, and six (Fig. 1D). The gene product was characterized by the substitution G103V, and by the deletion of 139 residues (Lys104–Leu242) (Fig. 1E) resulting in faster electrophoretic mobility (Fig. 1B). Among the residues deleted are those involved in glucose binding that mediates association of Cnx with substrates (Tyr165, Lys167, Tyr186, Met189, and Asp217) (18), two cysteines involved in a surface-exposed disulfide bond required for substrate binding (Cys183 and Cys196), and the conserved Asp118 involved in the coordination of a calcium ion. Thus, deletion of the Cnx glycan binding site and of other residues possibly involved in maintenance of the native Cnx architecture did not affect cell viability, ER morphology, and did not trigger a UPR in cultured cells (Fig. 1). However, it did cause the same phenotypic alteration at the organism level as deletion of Cnx (18).

sCnx Does Not Associate with Newly Synthesized Glycoproteins—We next determined whether sCnx binds to newly synthesized glycoproteins in the ER. wt and S-CNX cells were pulsed with 10 min of [35S]methionine and [35S]cysteine and chased for 0–60 min with unlabeled amino acids. Detergent extracts were immunoprecipitated with antibody raised to the cytosolic tail of Cnx and of sCnx (Fig. 2A).

Several labeled cellular proteins coprecipitated with Cnx. Most of these proteins were chased away in 60 min, indicating that they were Cnx substrates that associated transiently with the ER lectin (Fig. 2A, wt). Deletion of the 139 residues upon alternative splicing of the Cnx gene abolished association of labeled cellular polypeptides with sCnx (Fig. 2A, S-CNX).

Calreticulin Does Not Normally Associate with Orphan Calnexin Substrates—Cnx and Crt associate with a wide array of substrates because they bind monoglucosylated N-glycans, which are transiently exposed by all glycosylated polypeptides expressed in the ER (19–24). Unexpectedly, however, they were found to associate with a distinct set of polypeptides in living cells (7–14). To verify if Cnx inactivation resulted in changes in Crt specificity, acquiring novel substrates, the de-
polypeptides. The black lines in Fig. 2 show a selection of 14 glycoproteins that differentially labeled wt and S-CNX MEF chased for 0–60 min. No Cnx substrate does co-precipitate with sCnx, confirming that sCnx is not functional as a lectin chaperone. B, same as described in A, for Crt. C, samples immunoprecipitated with Cnx (X) or Crt (R) antibody have been loaded side-by-side to compare co-precipitating-labeled cellular polypeptides. Chase time is 3 min. Asterisks show Cnx in lanes 1 and 5, Crt in lanes 2, 3, and 6, and sCnx in lane 4. D, same as described in A, for BiP. Comparison of labeled polypeptides co-precipitating with BiP after a 0- and a 60-min chase allows identification of cross-reacting, unspecific bands (+). In all gels, + shows labeled bands that cross-react with protein A beads.

To better analyze this, wt and S-CNX MEF were pulse-labeled, chased for 3 min, detergent-solubilized, and Cnx and Crt immunoprecipitates were analyzed in the same gel (Fig. 2C). In wt cells, the pattern of cellular proteins associating with Cnx (Fig. 2C, lane 1) and Crt (lane 2) was only partially overlapping. Cnx associates with newly synthesized proteins of all sizes, whereas Crt associates preferentially with larger polypeptides. The black lines in Fig. 2C show a selection of 14 labeled polypeptides of molecular masses between 30 and 100 kDa that associate with Cnx but not with Crt in wt cells. None of these Cnx substrates were found to associate with Crt upon Cnx inactivation (lane 3). Thus, most cellular Cnx substrates remain inaccessible to Crt upon selective eradiation of Cnx activity.

Lanes 4–6 in Fig. 2C are controls showing that deletion of Cnx activity prevents substrate association (lane 4) and that cell incubation with the glucosidase inhibitor castanospermine, which prevents trimming of N-glycans to the monoglycosylated form, substantially inhibits association of newly synthesized polypeptides with Cnx and Crt (lanes 5 and 6, respectively). Association of newly synthesized polypeptides with Crt was unaffected by Cnx inactivation. However, this was not true for substrate association with the other abundant ER chaperone BiP/GRP78. This was significantly increased, especially at early chase times (0 and 5 min of chase, Fig. 2D), indicating increased association of BiP with nascent chains upon Cnx inactivation. Overall, substrate association with BiP and with Crt remained transient in S-CNX cells. Together with the findings reported above (depletion of Cnx did not trigger UPR and viability of S-CNX cells was normal), this was a further indication that (most) newly synthesized cellular proteins remained folding-competent upon selective deletion of Cnx activity.

Association with ER Chaperones and Maturation of β-Secretase in wt Cells and in Cells Expressing sCnx—Unbiased analysis of labeled cellular proteins (Fig. 2) did not show the dramatic increase of Crt-associated radioactivity that would be expected if Crt replaces Cnx and would associate with orphan Cnx substrates upon Cnx inactivation. We next specifically tested the competence of Crt to associate with selected cellular Cnx substrates and analyzed whether membrane-bound versus soluble status or extent of N-glycosylation made a difference. To this end, three variants of human BACE were individually transfected in the two cell lines. BACE457a is a soluble, di-glycosylated protein, and BACE457 and BACE501 are type I membrane proteins with 2 and 4 N-glycans, respectively. BACE457Δ and BACE457 are folding-incompetent and are eventually degraded from the ER (25–27), whereas BACE501 folding is productive, and the native glycoprotein is transported along the secretory pathway to the plasma membrane (28, 29).

Recombinant BACE457Δ and BACE457 became major Cnx (black lines in lane 3, Fig. 3, A and B, respectively) and BiP substrates in transiently transfected wt MEF (lane 7). Both glycoproteins did not associate with Crt (lane 5). BACE501 associated abundantly with Cnx and BiP (Fig. 3C, lanes 3 and 7, respectively) and weakly with Crt (Fig. 3C, lane 5).

As is shown for endogenous glycoproteins (Fig. 2), sCnx did not associate with the recombinantly expressed BACE variants (Fig. 3, A–C, lane 4) and Crt did not associate with the BACE variants even after deletion of Cnx activity (Fig. 3, A–C, compare lane 5 with 6). Instead, normalization for the slightly lower expression level of the recombinant proteins in S-CNX MEF revealed a moderate increase of BiP binding.

Altogether, the data shown in Figs. 2 and 3 confirmed the clear distinction between Cnx and Crt substrates. Furthermore, the data show that orphan Cnx substrates remain inaccessible to Crt even upon eradication of Cnx activity, no matter whether the substrate protein is membrane-bound (BACE457 and BACE501), soluble (BACE457Δ), weakly glycosylated (BACE457 and BACE457Δ) or more heavily glycosylated (BACE501), folding-competent (BACE501), or folding-incompetent (BACE457 and BACE457Δ).
We next determined whether selective depletion of Cnx activity affected maturation of BACE501. Native BACE501 is released from the ER and is subject to complex glycosylation in the Golgi that decreases electrophoretic mobility and renders BACE501 N-glycans EndoH-resistant (28, 29). Both the molecular mass increase and the acquisition of EndoH resistance, as a further estimate of the rate of BACE501 export from the ER, occurred with similar efficiency and kinetics in wt and S-CNX MEF (Fig. 3D).

SVF Glycoproteins Associate More Abundantly with Calreticulin in S-CNX Cells—Analysis of cellular polypeptides showed that the substrate specificity of Crt did not change upon Cnx depletion. We next analyzed the fate of four viral envelope glycoproteins that served as models in the past years to elucidate functioning of the ER folding machinery: the G protein of Semliki forest virus (SFV, Ref. 32), the HA of the influenza virus (20, 31), and E1 and p62 of the retrovirus: ERp57-containing mixed disulfides are shown (33).

Thus, maturation of SVF glycoproteins progressed normally without Cnx. Unlike the cellular proteins previously investigated, SVF glycoproteins did associate more abundantly with Crt in the absence of functional Cnx.

We next analyzed the behavior of a glycoprotein considered a classic Cnx substrate with undetectable association with Crt in wt cells, the VSV G protein (9, 30).

wt and S-CNX MEF were infected with VSV 4 h before pulse-chase analysis. Oxidative folding of the VSV G protein is sequentially assisted by BiP and Cnx (30). Acquisition of the native structure is monitored by following the formation of the native B2 epitope with the specific monoclonal antibody I14 (34). Here we made use of I14 to compare the kinetics of G protein maturation in the two cell lines by pulling down from detergent extracts prepared after several chase times the fraction of native G protein.

The amount of I14 reactive-labeled G protein increased at the same rate in both cell lines to about 20 min of chase, and then weakly decreased, again with similar kinetics after the 40 min chase, upon release from cells of viral particles containing labeled G protein (Fig. 5A and data not shown). Consistently, there was no difference, if any a slight acceleration in S-CNX cells, in the rate at which the G protein acquired EndoH-resistant glycans (Fig. 5B). Thus, as for the proteins tested above, maturation of VSV G protein was not affected by Cnx depletion, even though in wt cells, Cnx is the lectin chaperone of choice for the G protein. We next determined whether in the case of the G protein Crt did intervene during folding in S-CNX MEF.

Calreticulin Associates with VSV G Protein in S-CNX Cells—To identify the chaperones assisting maturation of the
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G protein, co-precipitations with BiP, Cnx, and Crt were analyzed with reducing SDS-PAGE.

In wt cells, the G protein abundantly associated with BiP and with Cnx. As previously shown in other cell lines (30, 39), BiP association (3-min chase, Fig. 5, C and F) preceded the association of G protein with Cnx (10-min chase, Fig. 5, D and F). Labeled G protein did not co-precipitate with Crt (Fig. 5E), confirming preferential association of this glycoprotein with Cnx (9).

sCNX did not associate with G protein (Fig. 5D). Lack of Cnx caused a clear change in the kinetics of G protein association with BiP (Fig. 5, C and F) and resulted in a significant transient association of G with Crt (Fig. 5, E and F). The release of G protein from Crt in S-CNXX cells ($t_{1/2} = 11$ min) was faster compared with G protein release from Cnx in wt cells ($t_{1/2} = 18$ min, Fig. 5F). This could possibly account for the slightly faster acquisition of EndoH-resistant glycans (Fig. 5B) and faster release of viral particles from S-CNXX cells (data not shown).

Thus, similar to SFV glycoproteins and unlike glycoproteins of cellular origin, G protein associated with Crt when Cnx was not available. Importantly, this was a peculiarity of the G protein expressed in infected cells. In fact if the G protein was transiently expressed upon cell transfection with a VSV G protein expression plasmid, the G protein behaved like cellular glycoprotein associated more abundantly with BiP, but not with Crt upon Cnx inactivation (Fig. 5G).

Calreticulin Retains Folding-incompetent HA in the ER—In wt MEF, maturation of HA is characterized by the stepwise conversion of the newly synthesized glycoprotein from intermediate oxidation states (IT, Ref. 35) into fully oxidized, native form (NT) that can be followed in nonreducing gels (Fig. 6A). During oxidative folding in the ER, HA transiently associates with Cnx and with Crt (Fig. 6C).

Influenza HA is strongly dependent on Cnx for maturation (14). Consistently, analysis of HA folding in S-CNXX MEF showed that about 50% of the labeled HA progressively entered disulfide-bonded (DBA) in the nonreducing gel, Fig. 6B) and in non-covalent aggregates; the latter persisting upon sample reduction (Fig. 6B). In the absence of Cnx, misfolded disulfide-bonded HA did persistently associate with Crt (Fig. 6D). Thus, folding-competent and folding-incompetent cellular Cnx substrates did not associate with Crt upon depletion of Cnx. All model viral glycoproteins tested in this study did so, in infected cells, independently of their folding competence.

DISCUSSION

We characterized and made use of embryonic fibroblasts derived from mice expressing a shorter, inactive Cnx variant to better characterize chaperone involvement during protein maturation in the mammalian ER. Cnx deletion did not result in significant protein misfolding, shown by the lack of a compensatory UPR in S-CNXX MEF and by analysis of several cellular and viral model proteins.

Unbiased analysis of labeled cellular proteins showed that despite equal specificity for monoglucosylated N-glycans expressed by nascent polypeptides, Crt and Cnx assist a different subset of proteins during maturation. Importantly, Crt remained inaccessible for most Cnx substrates even upon selective inactivation of Cnx. Rather, BiP associated more abundantly with nascent chains in the absence of functional Cnx.

Only upon viral infection, Crt associated with orphan Cnx substrates of viral origin. A saturation of the Crt binding capacity that precludes association of orphan Cnx substrates in non-infected cells is one possible explanation of our data. However, even though VSV and SFV infections shutdown host protein biosynthesis, thereby setting Crt free, influenza virus infection did not. Rather it similarly activates the “surrogate” activity of Crt.

Significantly, Crt failed to associate with orphan Cnx substrates of viral origin when these were expressed in transfected rather than in infected cells. Thus, cell infection (at least with the three viruses used in this study) activates the surrogate function of Crt, consistent with a stronger dependence of viral glycoproteins from Cnx and Crt. This is actually an Achilles heel for viruses that has been exploited for designing antiviral therapies. In fact, the Cnx/Crt cycle is required for replication and infectivity of many human pathogens (36–38), but not for viability of cultured mammalian cells. Iminosugar derivatives

FIG. 5. Maturation of VSV G protein in wt and in S-CNXX cells. A, native form of labeled G protein is specifically pulled-down from detergent extract with the specific monoclonal antibody I14. B, release of the G protein in the Golgi has been determined by measuring acquirement of EndoH-resistant glycans. C, analysis of G protein associated with BiP. D, same as described in C, for Cnx. E, same as described in B, for Crt. F, kinetics of G protein release from Cnx and BiP in wt cells and from Crt and BiP in S-CNXX cells. G, wt (W) and S-CNXX cells (S) were transiently transfected for expression of the VSV G protein (lanes 1 and 8). Anti-Cnx (lanes 2 and 3), Crt (lanes 4 and 5), and BiP (lanes 6 and 7) are shown. The thick black lines show the position in the gel of the VSV G protein. Asterisks in C–E and G show the respective ER chaperones, arrowheads the G protein.
have proved useful in reducing the viral level in an animal model of chronic hepatitis B infection (39), of bovine diarrhea virus (40), and mortality in the case of Japanese encephalitis virus infection (41).

Is Cnx a dedicated chaperone? Cnx associates transiently with numerous glycoproteins. If Cnx assistance would be essential for their folding, dramatic consequences should emerge upon Cnx eradication or inactivation. This is obviously not the case, at least for cultured cells and during embryogenesis, and hints at a role for Cnx as an obligate chaperone only for a restricted subset of mammalian polypeptides. Several ER chaperones are dedicated to specific substrates (a comprehensive list is presented in Ref. 2). Calmegin, the testis-specific homologue of Cnx involved in spermatogenesis is an important example for this discussion. Similar to Cnx, calmegin also associates transiently with a large number of nascent, monoglycosylated glycoproteins in the sperm, but its depletion only prevents maturation of one of them, namely fertilin-β (42, 43). Consistent with the lack of effects on other glycoproteins; maturation, spernum number, viability, or motility are unaffected in calmegin-deficient mice, whereas the fertilin-β-regulated sperm’s capacity to bind to the egg zona pellucida is lost; thus, leading to male sterility (42, 44). Analogies with Cnx are striking, because Cnx is dispensable for maturation of most glycoproteins. In mice, clear signs of progressive pathology, eventually causing early postnatal death and motor disorders in the survivors (16), show however that Cnx may be required for a very restricted set of proteins, possibly expressed in tissue-specific fashion. Characterization of the physiologic Cnx substrate(s) is the next challenge.

How do we explain that deletion of Cnx, an abundant ER chaperone transiently associating with a large number of newly synthesized polypeptides, does not result in substantial protein misfolding with activation of the UPR? In contrast with cytosolic proteins, polypeptides expressed in the ER benefit from being singularly injected through individual translocans that offer a fair spacing between aggregation-prone, unstructured nascent chains, thus per se offering protection from aggregation (45). Moreover, co-translational addition of N-glycans increases the hydrophilicity of the unstructured chains and association of BiP/GRP78 with exposed hydrophobic patches (46, 47), further preventing aggregation, and may expose orphan Cnx substrates in replacing folding factors, as shown in this study by the increased association of nascent chains with this abundant luminal chaperone in S-CNX cells.

A final remark is that upon analysis of maturation, surface transport, and activity of several viral and cellular proteins (14, 48–53, and this work), influenza virus HA remains the only one substantially suffering lack of Cnx assistance. Thus, HA could represent a model for those yet-to-be-identified cellular proteins requiring Cnx for maturation. What is so special in HA? Influenza HA has a peculiar head-to-tail structure, with the N-terminal Cys14 covalently paired with the C-terminal Cys466 in S-CNX cells. D, detergent extracts of S-CNX cells were subjected to a double immunoprecipitation with anti-HA antibody.

**Fig. 6.** Maturation of influenza virus HA in wt and in S-CNX cells. A, influenza virus HA has been immunosolated from detergent extracts of metabolically labeled and chased wt MEF with specific antibody; the antibody also pulls down the viral nuclear protein (NP). B, same as described in A in S-CNX MEF. DBA are HA-containing aggregates. C, detergent extracts of wt MEF were immunoprecipitated with antibody to Cnx (left) or Crt (right), and then subjected to a second immunoprecipitation with antibody to HA. In the anti-Cnx panel, the last lane shows the lack of HA interaction with sCnx. D, detergent extracts of S-CNX cells were subjected to a double immunoprecipitation, first with anti-Crt, then with anti-HA antibody.
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