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In vitro mapping of calnexin interaction with ribosomes

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Abstract

Calnexin is an endoplasmic reticulum (ER) resident type I integral membrane phosphoprotein. This protein is actively involved in the ER glycoprotein quality control through its luminal domain. In addition, although calnexin also interacts with membrane-bound ribosomes, the nature of this interaction remains poorly characterized. Herein, using in vitro approaches, we demonstrate that calnexin cytosolic domain directly interacts with, at least 5 ribosomal proteins. Furthermore, we characterize more specifically its interaction with the ribosomal protein L4 and that L4 binds to the 19 carboxy terminal amino acids of calnexin. We suggest that the direct interaction of calnexin with membrane-bound ribosomes may represent a regulatory mechanism for its lectin-like chaperone function.

Keywords: Endoplasmic reticulum; Ribosome; Translocon; Calnexin; Ribosomal protein L4

Glycoproteins that traverse the eukaryotic secretory pathway are targeted to the ER where they are co-translationally inserted through the membrane by the translocon [1]. Maturation and processing of the polypeptide chain, including signal sequence cleavage, transfer, trimming of N-linked glycans, chaperone binding, disulfide bond formation, and folding, occur when proteins emerge in the ER lumen [2]. The initial folding of glycoproteins synthesized in the ER is taking place in the aqueous protein-lined channel connected to the exit tunnel of the large ribosomal subunit. The translocon assists this maturation process by providing a protective environment that minimizes the opportunity for protein aggregation [3]. Historically, the translocon has been characterized as a molecular complex co-sedimenting with ribosomes [4]. Further studies demonstrated that the aqueous pore was formed by the Sec61 protein and by several other accessory proteins (TRAM, signal peptidase, oligosaccharyl transferase, and others) which showed significant binding to ribosomes. Recently, a direct interaction between ribosomes and Sec61 was observed using both biochemical and structural approaches [5]. To allow a tightly regulated junction between the ribosome and the translocation channel, interactions between ribosomal proteins and ER resident integral membrane proteins need to be established.

Calnexin is an endoplasmic reticulum (ER) type I integral membrane chaperone which transiently binds to newly synthesized mono-glucosylated glycoproteins, thereby promoting their folding and oligomerization [6,7]. This chaperone also displays a prolonged interaction with misfolded proteins, leading to their retention in the ER [8]. Calnexin is part of a molecular machinery, including calreticulin, UGGT, and Erp57, which "proofreads" the protein folding process in the ER [7] and named ER glycoprotein quality control [9].

In the present study, we confirm the previously described interaction of calnexin with membrane-bound ribosomes [10], using in vitro interaction systems with an *Escherichia coli* recombinant cytosolic domain of calnexin, and further we identify one of the major ribosomal calnexin interacting proteins as the ribosomal protein L4.

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Materials and methods

Materials. HepG2 cells were cultivated as previously reported [11]. Rabbit anti-sera were raised against a synthetic peptide (C4) corresponding to amino acids 555–573 of canine calnexin (anti-CNX) [12]. Human L4 cDNA was purchased from the IMAGE consortium and sequence verified. The C4 peptide and its scrambled counterpart were synthesized at the Sheldon Biotechnology Centre (McGill University). All other reagents were of the highest grade commercially available and purchased from Sigma unless indicated.

Bacterial expression, purification, and coupling of calnexin cytosolic domain. GST-calnexin cytosolic domain (GST-CCD) was previously described [10]. The GST-CCD was digested with thrombin for 4 h on ice and the supernatant was collected and incubated with benzamidine-Sepharose for 45 min at 4 °C. Beads were then centrifuged and the protein content in the supernatant was quantified and qualitatively analyzed by SDS–PAGE followed by Coomassie blue staining. One milligram of purified calnexin cytosolic domain (CCD) was then covalently coupled to CH4-B-activated Sepharose according to the manufacturer's recommendations. Similarly, 1 mg BSA was coupled to CH4-B Sepharose as above.

Calnexin-Sepharose purification. HepG2 cell lysate was performed as described previously [10]. Lysates (2 mg protein) were first incubated with albumin coupled CH4-B Sepharose beads (100 μl) for 2 h at 4 °C. Beads were pelleted and the supernatant was then incubated with 50 μl CCD-Sepharose beads for 2 h at 4 °C. Beads were then pelleted and washed 4 times with lysis buffer containing 0.5% Chaps. Beads were then resuspended in Laemmli sample buffer.

Ribosome pull-down. Ribosomes were purified as previously reported [10]. Increasing amounts of GST-CCD (0–2 μg) were incubated with 2.5 μg of purified ribosomes for 30 min on ice and spun through a 1.5 M sucrose cushion for 3 h at 95,000 rpm in a Beckman TLA100 rotor. Similar experiments were performed with a constant amount of 0.5 μg GST-CCD and increasing amounts of purified ribosomes (0.1–5 μg). Pellets were then re-suspended in Laemmli sample buffer, resolved by SDS–PAGE, transferred onto nitrocellulose membrane, and immunoblotted with anti-GST antibodies. Experiments were repeated at least three times and the affinity binding was determined as previously described [13]. Similar experiments were also carried out in the presence of 0, 10 or 50 μg of either rabbit pre-immune serum or anti-calnexin (C4) anti-serum.

Far-Western blot. Ribosomal proteins (150 μg) were resolved by SDS–PAGE and transferred onto nitrocellulose membranes. Proteins were renatured as previously described [14]. Membranes were then blocked using PBS, 0.1% Tween 20, and 3% BSA for 45 min at 4 °C. Membranes were then incubated with 50 μg/ml GST or GST-CCD or 10 mM C4 peptide or its scrambled counterpart overnight at 4 °C in PBS containing 0.1% Tween 20 and 0.3% BSA. After 5 washes with the same buffer, membranes were incubated either with anti-GST or anti-C4 antibodies for 2 h at room temperature followed by 3 washes with the same buffer and incubation for 45 min with Protein-A-HRP. Immunoblots were revealed using enhanced chemiluminescence.

In vitro transcription translation. The hL4 cDNA in pBluescript pSKII was linearized in the polylinker region downstream of the stop codon (*Xba*I). A *Xba*I linearized empty vector was used as negative control. Plasmids DNA were then incubated with the T7 TNT rabbit reticulocyte kit (Promega) in the presence of ^{35}S-Met following the manufacturer's recommendations. Radiolabelled newly translated products were then resuspended in PBS containing 1.5% Chaps and protease inhibitors. After 30 min incubation, lysates were centrifuged at 4 °C in a benchtop centrifuge at top speed for 30 min. Clarified lysates were then normalized for protein concentration (1 mg) and incubated with glutathione (GSH)–Sepharose beads (100 μl) for 45 min at 4 °C. Beads were pelleted and the supernatants were then incubated with GST-CCD bound to GSH–Sepharose beads for 2 h at 4 °C. After 5 washes with PBS and 0.5% Chaps, beads were resuspended in Laemmli sample buffer. The purified radiolabelled material was then resolved by SDS–PAGE and visualized by fluorography.

Results

In vitro, direct, and specific cytosolic domain of calnexin binding to ribosomes

We have previously demonstrated that calnexin associates to membrane-bound ribosomes either directly or indirectly but independently of the nascent chains [10]. In addition, this ribosome-binding ability is enhanced by phosphorylation of the cytosolic domain of calnexin (CCD) by ERK-1 [10]. To confirm that the interaction of calnexin with ribosomes occurred through its cytosolic domain, recombinant canine CCD was covalently conjugated to CH4-B-activated Sepharose beads. HepG2 lysate was first depleted using albumin-conjugated CH4-B Sepharose beads and the resulting protein extract chromatographed onto CCD-Sepharose matrix. After extensive washing (50 beads volumes), beads were then resuspended in Laemmli buffer. HepG2 lysate and CCD-bound proteins were resolved by SDS–PAGE and visualized either by Coomassie blue staining (Fig. 1, CB left panel) or silver staining (Fig. 1, SS right panel), respectively. Ten silver-stained bands corresponding to CCD-binding complexes were analyzed by N-terminal Edman degradation microsequencing. The name of the identified proteins is indicated by an arrow

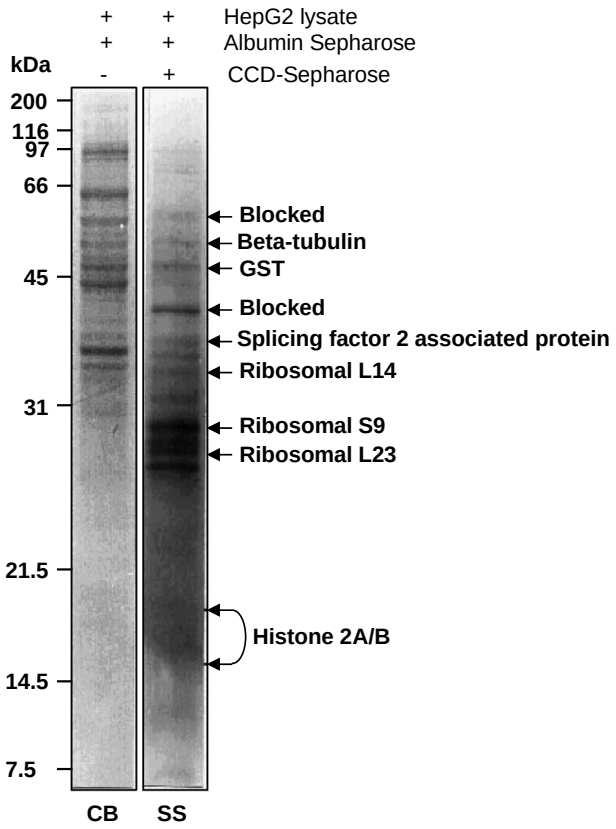

Fig. 1. Evidence of in vitro binding of calnexin cytosolic domain (CCD) to ribosomes. HepG2 lysates were incubated (+) or not (−) with CCD-Sepharose beads. The proteins were then resolved by SDS–PAGE and stained with Coomassie blue (CB) or silver (SS).

pointing to the corresponding silver-stained band. Affinity chromatography of HepG2 lysate on CCD-Sepharose matrix led to the identification of three major ribosomal proteins: L14, S9, and L23, thus suggesting that the CCD may directly interact with entire ribosomes.

To evaluate the affinity of the CCD for ribosomes, we established an in vitro assay in which the cytosolic domain of calnexin fused to GST (GST-CCD) was tested for its capacity to directly bind to purified ribosomes (Fig. 2A) as described in Materials and methods. Fig. 2B shows that the CCD fused to GST was able to bind directly to ribosomes. Indeed, the association of increasing amounts of GST-CCD or ribosomes with a constant amount of their respective binding partners, ribosomes and GST-CCD, led to saturable binding curve (Fig. 2C). Scatchard analysis of the corresponding values revealed a dissociation constant K_D of 1.7×10^{-6} M for the binding of GST-CCD to ribosomes (Fig. 2C, insert), reflecting a relatively low affinity of calnexin for ribosomes.

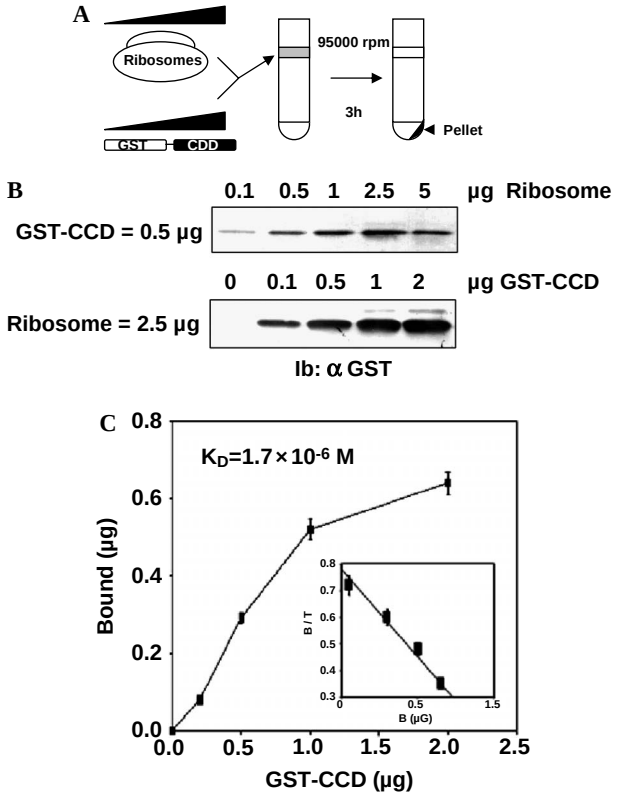

Fig. 2. Calnexin cytosolic domain (CCD) binds specifically to ribosomal proteins. (A) Increasing amounts of GST-CCD were incubated with a purified ribosome fraction for 30 min on ice and spun through a 1.5 M sucrose cushion for 3 h at 95,000 rpm. Similar experiments were performed with a constant amount of GST-CCD and increasing amounts of purified ribosomes. Ribosomal pellets were then collected. (B) Pull-down performed after in vitro association of either increasing amounts of purified ribosomes (0.1–5 µg) with 0.5 µg of GST-calnexin cytosolic domain (CCD; top panel) or increasing amounts of GST-CCD (from 0 to 2 µg) with a constant amount of purified ribosomes (2.5 µg, bottom panel). Ribosomal pellets were then resolved by SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-GST antibody. (C) Scatchard plot of data from Fig. 2B. The dissociation constant (K_D) was then determined from the slope of the obtained curve.

Characterization of the interaction between CCD and ribosomal proteins

To characterize the ribosomal proteins binding directly to the CCD, we examined these interactions using a Far-Western blot approach (Fig. 3A). Purified ribosomes were resolved by SDS–PAGE and transferred to nitrocellulose membranes. After protein renaturation, membranes were then blocked and incubated with either purified GST (Fig. 3B, left panel) or purified GST-CCD (Fig. 3B, right panel) followed by immunoblotting using anti-GST antibody. We found that 5 potential ribosomal proteins (50, 48, 35, 33, and 28 kDa, respectively) directly associate to GST-CCD (Fig. 3B, right panel). As expected, no interaction with ribosomal proteins was detected with GST alone (Fig. 3B, left panel).

To further dissect out these interactions, we selected a specific region of the CCD expected to participate in ribosome binding. Indeed, since phosphorylation of the CCD by ERK1 at Ser563 had previously been shown to enhance calnexin-ribosome association [10], we tested whether the corresponding region of the CCD (containing Ser563) directly interacted with ribosomal proteins. To this end, we used the peptide corresponding to the amino acids 555–573 (C4 peptide) of the canine calnexin protein sequence and its scrambled counterpart. These peptides were used in our Far-Western blot approach (Fig. 3A). The resulting membrane was then immunoblotted using anti-calnexin-C4 antibodies (Fig. 3C). Our data demonstrate that the C4 peptide interacts specifically with one ribosomal protein of approximately 50 kDa (Fig. 3C, right panel) whereas no corresponding signal was detected with the scrambled peptide (Fig. 3C, left panel). Interestingly, this band corresponds to one of the most intense bands revealed using GST-CCD as a probe (Fig. 3B). Two parallel membranes were available, the band corresponding to C4-binding protein visualized by amido black staining, then analyzed by N-terminal Edman degradation microsequencing and identified as the ribosomal protein L4, a component of 60S ribosome large subunit. No other surrounding bands were visible using this staining approach thus suggesting that only one ribosomal protein displayed this apparent molecular weight which was identified as L4.

Mapping of the L4 region which interacts with CCD

We have demonstrated a direct interaction of calnexin cytosolic domain with ribosomes most likely (and at least) through the binding of the extreme carboxy-terminal region of calnexin to the ribosomal protein L4. In an attempt to confirm the involvement of this domain of calnexin in the binding to ribosome, an assay similar to that described in Fig. 2 was carried out except that experiments were performed in the presence of increasing amounts of

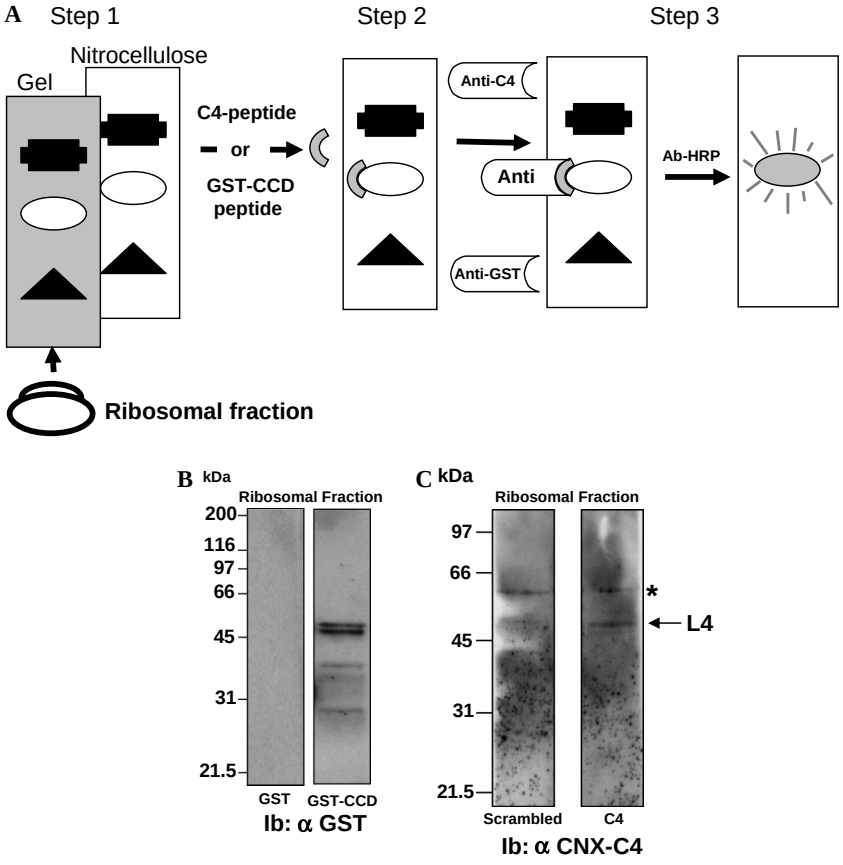

Fig. 3. Calnexin cytosolic domain (CCD) interacts directly with the ribosomal protein L4. (A) Schematic representation of our Far-Western blot approach. Step 1: After ribosome purification, the ribosomal proteins were resolved by SDS–PAGE and then transferred onto nitrocellulose membrane; Step 2: peptide (C4 or GST-CCD) was used to probe the ''prey'' ribosomal proteins on the membrane; Step 3: Immunoblotting with anti-peptide antibodies to detect ribosomal proteins bound to peptide. (B) Far-Western blot in which GST (left panel) or GST-CCD (right panel) was used to probe ribosomal fraction immobilized on a membrane after SDS–PAGE and then immunoblotted with anti-GST antibody. (C) Far-Western blot in which the C4 peptide (right panel) and its scrambled (left panel) counterpart were used to probe ribosomal fraction as described in Fig. 3A (asterisk, nonspecific band; arrow, L4).

either pre-immune (PI) serum or anti-calnexin (anti-CNX-C4) anti-serum. Interestingly, although increasing amounts of PI led to a reduced-ribosome binding ability of GST-CCD (Fig. 4A, left panel), the presence of identical amounts of anti-CNX-C4 led to a more dramatic reduction of this association (Fig. 4A, right panel). These data indicated that masking the extreme carboxy-terminal region of the CCD using antibodies reduced the association of the CCD with ribosomes, thus confirming the direct interaction between calnexin and ribosomes through the C4 domain.

To further characterize the association of the ribosomal L4 protein with the CCD, the human L4 cDNA was cloned into pBluescript SKII vector. This vector was then linearized downstream of the hL4 STOP codon and in vitro transcribed and translated in the presence of ^{35}S-methionine using the T7 TNT kit resulting in the translation of a full-length L4 protein. Full-length radiolabelled proteins were then pulled down using GST-CCD, the purified proteins resolved by SDS–PAGE and the gels stained with Coomassie blue prior to being revealed by fluorography on X-ray films. Remarkably, the full-length radiolabelled L4 protein specifically associated with GST-CCD (Fig. 4B, middle panel) thus confirming that calnexin directly interacts with L4. When a similar experiment was carried out with GST, only the nonspecific radiolabelled band was observed as being pulled down (asterisk, Fig. 4B).

Discussion

In this study, we demonstrate the direct binding of calnexin to the ribosomal protein L4, via its cytosolic domain. This is the first report of a direct association of a ribosomal protein with the cytosolic domain of calnexin (CCD). In our study, we also observed that other putative ribosomal (or ribosome-associated) proteins may also interact directly with the CCD as revealed by Far-Western blot (Fig. 2B). The failure to detect L4 in Fig. 1 may be explained first by the fact that not all visible bands were sequenced and second that some of them displayed blocked sequence at their N-terminus.

Co-translational translocation in all organisms requires the ribosome as the major partner of the translocation

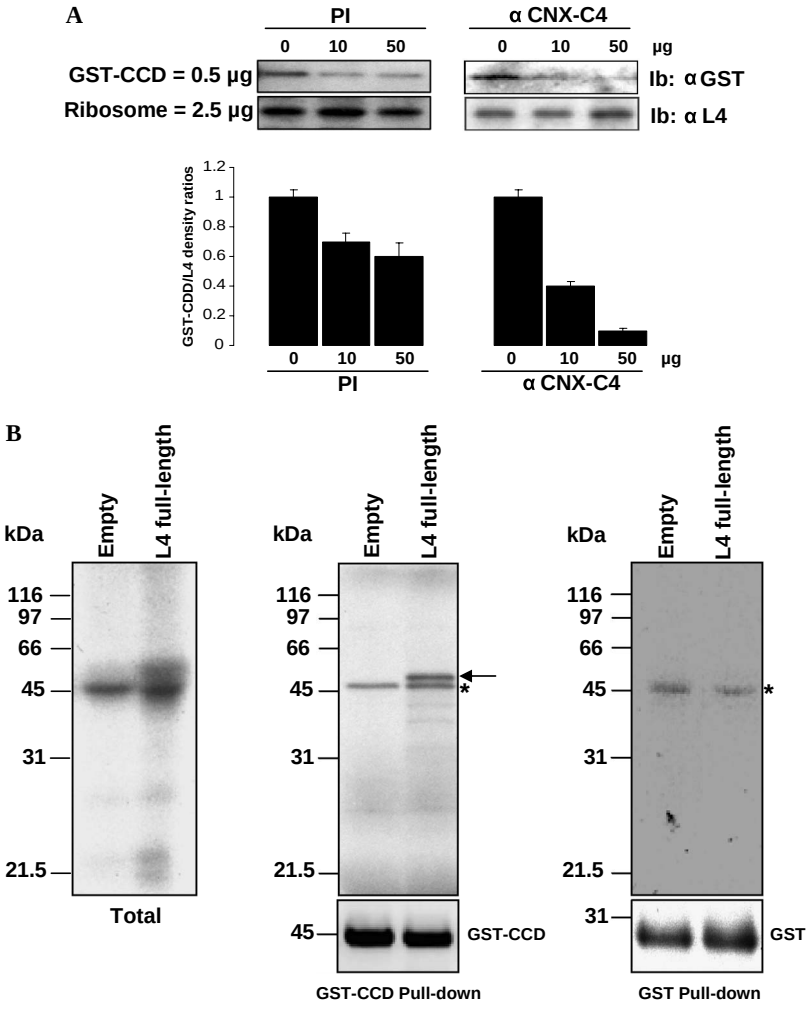

Fig. 4. Characterization of L4 binding to calnexin cytosolic domain (CCD). (A) GST-CCD pull-down experiments were carried out as previously shown in Fig. 2. 0.5 µg of GST-CCD was incubated with 2.5 µg of purified ribosomes in the presence of 0, 10, and 50 µg of either pre-immune (PI) serum or anti-CNX-C4 (α CNX-C4) antibody. The amount of GST-CCD pull-down was evaluated by immunoblot using anti-GST antibody. These amounts were normalized to the amount of ribosomal protein L4 in the ribosomal pellet. Experiments were repeated three times and quantified by scanning densitometry. The ratio GST-CCD/L4 is shown ±SD (graphs). (B) Empty pBluescript pSKII vector or the same vector containing the full-length human L4 cDNA linearized downstream of the stop codon was in vitro translated as described in Materials and methods. Radiolabelled protein products (left panel) were pulled down using 3 µg GST-CCD (middle panel) or 3 µg GST (right panel), and the purified proteins were resolved by SDS–PAGE and followed by radioautography (asterisk, nonspecific band; arrow, L4). The amount of GST and GST-CCD in the assay was visualized by Coomassie Blue staining (bottom pictures).

channel. In this context, nascent chains exit from a tunnel in the large ribosomal subunit and are directly transferred into the translocation channel. More importantly, nascent chains can be post-translationally modified as they are co-translationally translocated into the ER lumen. This is particularly true for glycoproteins which represent the largest part of secretory proteins. Indeed, coincident with the translation process, numerous glycoproteins undergo N-linked glycosylation as they are translocated [15]. Calnexin was initially identified as an abundant ER type I integral membrane lectin-like chaperone [6]. Its implication in co-translocational glycoprotein folding was extensively demonstrated [16,17], thus suggesting its presence in proximity to the translocation channel. In addition, phosphorylation of CCD by the concerted action of the protein CK2 and ERK-1 enhances calnexin targeting to membrane-bound ribosomes [10]. Our present data report that the binding of CCD to L4 occurs in a region corresponding to the most C-terminal 19 amino acids of calnexin, containing the ERK-1 phosphorylation site (Ser563).

This direct interaction between calnexin and L4 revealed the presence of calnexin in very close proximity of the translocation channel. The luminal domain of calnexin may therefore interact with the oligosaccharide chain in nascent glycoproteins and this interaction can occur while the protein is being processed by the translocation machinery. In these conditions, interaction of CCD-L4 may be required to stabilize the nascent glycoproteins ribosome-translocon junction. Moreover, a model of protein topography within 60S rat ribosomal subunit based on protein accessibility and cross-linking data suggested that L4 domains may be exposed at the surface of this ribosomal

subunit [18], thus making it accessible to CCD. As a consequence, L4 may represent an additional potential ER-ribosome contact site, but there is no structural information on this association available yet.

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