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Purification and characterization of a soluble calnexin from human placenta ☆

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ABSTRACT

Calreticulin (Crt) and calnexin (Cnx) are homologous endoplasmic reticulum (ER) chaperones involved in protein folding and quality control. Crt is a soluble ER luminal Mr 46 kDa protein and Cnx is a Mr 67 kDa ER membrane protein. During purification of Crt from human placenta a soluble form of Cnx (sCnx) was consistently identified in a separate ion exchange chromatography peak. The sCnx was further purified and characterised. This showed that the protein had been cleaved after residue 472 (between Gln and Met), thus liberating it from the transmembrane and cytoplasmic parts of Cnx. The extraction and initial purification steps were carried out in the presence of protease inhibitors, thus ruling out that the cleavage was an artefact of the isolation procedure. This indicates that sCnx may have a physiological chaperone function similar to that of Crt.

Introduction

Calnexin (Cnx)[1] and calreticulin (Crt) are important chaperones in the endoplasmic reticulum (ER) and participate in the folding and quality control of many client proteins [1–3]. The two proteins are homologous with 45% sequence identity (based on Crt) in their globular and P domains (Fig. 1; Suppl. Fig. 1). However, they differ with respect to several features: Cnx is a transmembrane protein and has a short cytoplasmic C-terminus and ER luminal N and P domains, whereas Crt is a soluble ER luminal protein with a large capacity for Ca^{2+} binding in the C-terminus. Despite their similar structures and overlapping properties they seem to complement each other in the folding of other proteins, in particular the folding of MHC I [4–7]. Moreover, they seem to be able to substitute for each other in many respects [8–9]. Therefore, differences in some of their cellular functions may stem from one being a membrane protein and the other a soluble protein. However, some differences may also be related to the small differences in structure: Cnx has a longer P domain "arm" and Crt has a longer C-terminus (Fig. 1), otherwise their structures in the globular domains are essentially similar.

Previously, Crt has been isolated from human placenta and characterized extensively [10–16]. Here, the isolation and characterization of a soluble Cnx (sCnx) variant from human placenta is reported.

Materials and methods

Chemicals

Carbonate buffer pH 9.6 (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.001% (w/v) phenolred), TTN buffer, pH 7.5 (0.025 M Tris, 0.5% (v/v) Tween 20, 0.15 M NaCl), alkaline phosphatase (AP) substrate buffer, phosphate-buffered saline (PBS) (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.3, 0.15 M NaCl) were made in-house. Pyronin G, Tween 20, NaH_2PO_4, Na_2HPO_4, NaCl, CH_3COOH, $Na_2S_2O_3$, $CaCl_2$, $MgCl_2$, $(NH_4)_2SO_4$ and dimethylformamide (DMF) were from Merck (Darmstadt, Germany). Tris, BisTris, ammonium sulfate, *para*-nitrophenylphosphate (*p*NPP) substrate tablets, 5-bromo-4-chloro-3-indolylphosphate (BCIP), nitroblue tetrazolium (NBT), phenol red, Triton X-114, $NaHCO_3$, Na_2CO_3, NH_4HCO_3, glycine, urea, thiourea, guanidine (Gu) hydrochloride, trifluoroacetic acid (TFA), CH_3CN, glycerol, HCHO (37% (w/v), diethanolamine, AP-conjugated rabbit immunoglobulins (Igs) against mouse IgG, AP-conjugated goat Igs against rabbit IgG and endoprotease AspN (E.C. 3.4.24.33) were purchased from Sigma Aldrich (St. Louis, MO, USA). HCOOH and bovine serum albumin (BSA) were from Fluka (St. Louis, MO, USA). Protein purification equipment (Äkta Prime™), Q-Sepharose Fast Flow™, Phenyl-Sepharose Fast Flow™ and Sephacryl S-100

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[1] *Abbreviations used:* Crt, calreticulin; Cnx, calnexin; ER, endoplasmic reticulum; MHC, major histocompatibility complex; PPI, protein prolyl isomerase.

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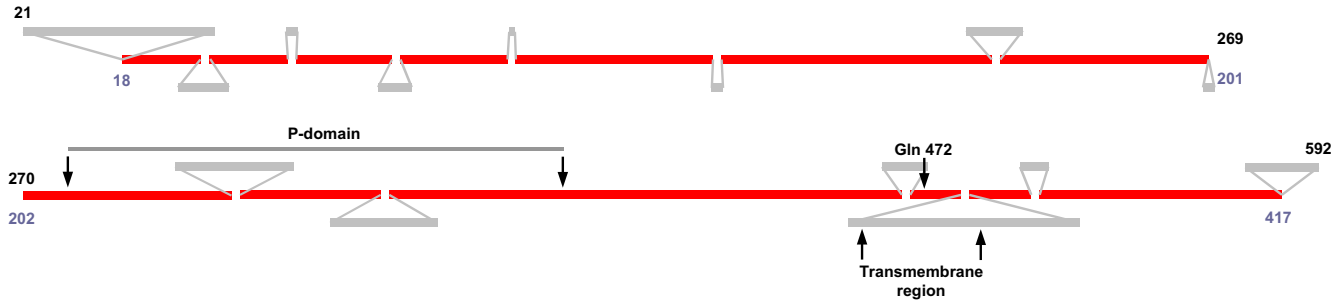

Fig. 1. Comparison of calnexin and calreticulin. The red line indicates the sequence of Crt and Cnx that aligns according to the alignment in Suppl. Fig. 1 with the inserts into the Crt sequence shown as grey bars. The location of the P-domain, the transmembrane region and the terminal Gln 472 of sCnx are indicated. Numbering is according to the sequence including signal sequence. Numbers above the line are for Cnx and below the line for Crt.

HR™ were from Pharmacia (Uppsala, Sweden). Sequencing grade trypsin (porcine) (E.C. 3.4.21.4) was from Promega (Madison, WI, USA). Trifluoroacetic acid (TFA) and bicinchoninic acid (BCA) protein assay kit were from Thermo Fisher Scientific (Waltham, MA, USA). MaxiSorp™ microtitre plates were from Nunc (Roskilde, Denmark). Complete protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany). Rabbit antibodies recognizing the N-terminus or C-terminus of Crt were produced as described [17]. Rabbit antibodies to ERp57 were a generous gift from Lars Ellgaard (Department of Biology, University of Copenhagen, Denmark). Rabbit antibodies recognizing the N-terminus or C-terminus of Cnx were from Calbiochem (La Jolla, CA, USA). Rabbit antibodies to ERp72 were from Abcam (Cambridge, UK). Rabbit antibodies to PDI were from Stressgen (Victoria, British Columbia, Canada). Precast Tris–glycine gels (4–20%) were from NOVEX (San Diego, CA, USA). GelCode Blue Stain Reagent and bis(succinimidyl)suberate (BS3) were from Pierce (Rockford, IL, USA). Prestained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) molecular weight standards were from BioRad (Hercules, CA, USA). SDS and $AgNO_3$ were from BDH Chemicals (Poole, Dorset, UK). EtOH was from Kemetyl (Køge, Denmark). Poros oligo R2 microcolumns were from Applied Biosystems (Foster City, CA, USA). Ultradiafilters were from Millipore (Billerica, MA, USA). ReproSil Pur C18 AQ 3 um was from Dr. Maisch GMBH (Ammerbuch-Entringen, Germany).

SDS–PAGE

SDS–PAGE was carried out according to Laemmli [18] as described by Studier [19] using precast 4–20% gels. Samples were boiled 1:2 with sample buffer (70 mM SDS, 100 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.05 M Tris pH 6.8, 0.06% (w/v) pyronin G), 15 μL was added/well and electrophoresis was carried out, using 0.024 M Tris, 0.192 M glycine, 0.1% SDS (w/v) pH 8.8 as running buffer, at 125 mA/gel. Gels were rinsed 2×10 min in water, stained with Coomassie Brilliant Blue (GelCode Blue) for 1 h and then destained in water. Silver staining of gels was done as follows: first, gels were fixed in 50% (v/v) EtOH, 12% (v/v) CH_3COOH for 1 h, then they were rinsed in 30% (v/v) EtOH for 3×20 min, treated with 0.02% (w/v) $Na_2S_2O_3$ for 1 min, rinsed in water 3×20 s, stained with 0.02% (w/v) $AgNO_3$, 0.03% (w/v) HCHO for 20 min, rinsed in water 2×20 s and developed in 6% (w/v) Na_2CO_3, 0.4×10^{-3}% (w/v) $Na_2S_2O_3$, 0.02% (w/v) HCHO for 2–10 min. The staining was stopped with 50% (v/v) EtOH, 12% (v/v) CH_3COOH for 15 min and gels finally rinsed with water.

Immunoblotting

SDS–PAGE gels were sandwiched between 12 sheets of Whatman paper no 1 equilibrated in 10 times diluted electrophoresis buffer and subjected to electroblotting to nitrocellulose membranes overnight at 0.1 mA/cm^2 using a semi-dry electroblotting apparatus (JKA Biotech, Copenhagen, Denmark). The membranes were blocked for 1 h in 50 mM Tris, 1% (v/v) Tween 20, 0.3 M NaCl, pH 7.5 (TTN), washed three times in TTN, and then incubated with antibodies diluted 1:1000 in TTN. After 1 h incubation the membranes were washed three times in TTN, followed by incubation for 1 h with AP-conjugated rabbit Igs against mouse IgG or AP-conjugated goat Igs against rabbit IgG diluted 1:1000 in TTN. After another three washes, bound antibodies were detected by incubation with staining solution (12.5 mg 5-bromo-4-chloro-3-indolylphosphate in 0.5 ml DMF and 25 mg nitroblue tetrazolium in 0.5 ml 70% (v/v) DMF added to 75 ml 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

Cnx and Crt purification

Crt was purified from human placenta as previously described [11] with minor modifications (Fig. 2). A placenta was first homogenized twice with 20 mM BisTris, 1 mM $CaCl_2$, pH 7.5 in the presence of protease inhibitors (Complete tablets) followed by two rounds of homogenization with 0.5 L 20 mM BisTris, 1 mM $CaCl_2$, pH 7.5, 1% (v/v) Triton X-114. Between homogenizations, the precipitate was separated from the supernatant by 20 min of centrifu-

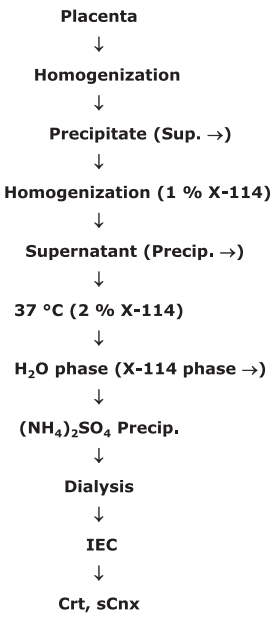

Fig. 2. Purification scheme for human placenta Crt. The soluble Cnx was obtained during the Q Sepharose ion exchange chromatography (IEC).