Human Placental Calreticulin: Purification, Characterization and Association with other Proteins

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Calreticulin is an intracellular protein known to be involved in calcium binding, but is also known to appear as an autoantigen in certain autoimmune diseases. The cDNA sequence is known but the protein has not yet been well characterized at the amino acid level. Owing to the possible involvement of this protein in autoimmune disease and with the aim of making monoclonal antibodies for use in assay development and immunohistochemistry, we have purified calreticulin using human placental material. Amino acid analysis of the purified protein confirmed the cDNA-derived composition, and only one discrepancy between the cDNA-predicted sequence and the amino acid sequence was found by peptide mapping and microsequencing. The protein contains one disulfide bridge and has one free SH group and the protein is neither glycosylated nor phosphorylated. Affinity chromatography of a placental protein extract on a column with immobilized calreticulin showed the existence of at least six proteins interacting with calreticulin. Using the purified calreticulin in Western blots, two out of eight patients with autoimmune disease diagnosed as having anti DNA antibodies in their serum were found also to contain autoantibodies to calreticulin in their serum.

Materials and methods

Chemicals. NaCl, Na₂HPO₄, NaH₂PO₄, sodium acetate (NaAc), ammonium persulfate CH₃COOH, MeOH, glycercol, 30% H₂O₂, CH₃CN, N,N-dimethylformamide (DMF) and Brij-35 were from Merck (Darmstadt, Germany). Aminohexyl (AH) Sepharose, Sephadex G-100, Mono Q columns and low molecular weight electrophoresis standards were from Pharmacia (Uppsala, Sweden). Diethylaminoethyl (DEAE) cellulose was from Whatman (Maidstone, UK). Acrylamide, bisacrylamide and N,N,N′,N′-tetramethylethylendiamine were from Bio-Rad (Richmond, USA). Triton X-114, 3-amin-9-ethylcarbazole, octylamine, 3-cyclohexylamino-1-propanesulfonic acid (CAPS), Coomassie Brilliant Blue R-250, tris(hydroxymethyl)aminomethane (TRIS), sodium dodecyl sulfate (SDS), dithioerythritol (DTE), ICH₂COOH, bromophenol blue, bovine serum albumin (BSA), trypsin and pepsin were from Sigma (St. Louis, USA). Trifluoracetic acid (TFA) and amino acid sequenator reagents were from Applied Biosystems (Foster City, USA). S. aureus V8 protease was from ICI (Meckenheim, Germany). Poly(vinyl difluoride) (PVDF) membranes were from Millipore (Boston, USA). Peroxidase conjugated avidin and peroxidase conjugated swine anti rabbit antisera were from DAKO (Glostrup, Denmark). Skimmed milk powder was from Chr. Hansen’s Lab. (Copenhagen, Denmark).
Denmark). Biotinylated lectins (Pea lectin, *Lens culinaris* lectin, peanut agglutinin, soy bean agglutinin, *Solanus tuberosum* agglutinin, *Griffonia simplicifolia* lectin 1 and 2) were from Kem-En-Tek (Copenhagen, Denmark).

**Placentas and sera.** Placentas were obtained from Rigs-hospitalet, Copenhagen, Denmark. The placentas were frozen immediately after delivery and were kept frozen at -20°C until required for use. Sera from patients with systemic lupus erythematosus (SLE) were obtained from Statens Serum Institut, Department of Autoimmunity.

**Extraction of placentas.** Using a Polytron PT 3000 homogenizer (Kinematica, Littau, Switzerland) each placenta was homogenized sequentially with 1 L 0.1 M phosphate buffer, pH 7.2 (PB), 1 L PB, 1% Triton X-114 or Brij-35 and finally 1 L PB, 1% Triton X-114 or Brij-35, 1 M NaCl. After each extraction the extract was clarified by centrifugation and extraction was continued with the precipitate.

**Chromatography on AH-Sepharose.** The column (1 cm × 20 cm or 1 cm × 100 cm) was preequilibrated with 5 mM PB pH 7.2 and the extracted sample was dialysed against 5 mM PB pH 7.2. The sample (100–1000 ml) was pumped through the column which was then washed with 5 mM PB until A_{280} was less than 0.05. The column was then eluted with a linear gradient of increasing PB concentration until a final concentration of 0.5 M was reached. Finally the column was eluted with 0.5 M PB, 20 mM octylamine. Flow: 1 ml min⁻¹.

**Chromatography on DEAE cellulose.** The sample was mixed with an equal volume of 50 mM TRIS pH 8.5 (A buffer) and pH adjusted to 8.5 with 1 M NaOH. The sample was pumped through the column (1 cm × 20 cm) which was washed with A buffer until A_{280} was less than 0.05, and then eluted with a linear gradient of NaCl in A buffer. Flow: 1 ml min⁻¹.

**Gel filtration.** The sample was chromatographed on a 4 cm × 100 cm G-100 fine column which was equilibrated and run in 0.1 M PB, pH 7.2. Flow: 1 ml min⁻¹.

**Affinity chromatography on calreticulin–divinyl sulfone–agarose.** Two ml divinyl sulfone-agarose was washed in 0.1 M carbonate buffer, pH 9.0, and allowed to react overnight at 4°C with 0.4 mg purified calreticulin. The matrix was then washed for 2 h in 1 M TRIS, 0.4 M glycine, pH 9.0, and finally washed extensively in 0.1 M phosphate buffer, pH 7.2. Placental protein extract (100 ml) in 0.1 M PB, 1.0% Brij-35 was passed through the column which was then washed extensively with 0.1 M PB, pH 7.2, 0.1% Brij-35, until A_{280} was less than 0.05. Elution of bound proteins was done with 1 M acetic acid and 1 ml fractions were collected during elution.

**Phase separation experiments.** Samples with 1% Triton X-114 were incubated at 37°C for 1 h and centrifuged briefly.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).** Polyacrylamide gels (12.5%) were run according to Laemmli. Gels were cast and run using a Mighty Small apparatus (Hoefer, San Francisco, USA). Gels were stained in 0.1% Coomassie Brilliant Blue in 40% MeOH, 10% CH₃COOH and destained in 10% CH₃COOH.

**Electroblotting.** Gels were electroblotted onto PVDF membranes using a semi-dry Electroblotter (JKA Biotech, Copenhagen, Denmark). The PVDF membrane was placed on top of three pieces of Whatman No. 3 and three pieces of Whatman No. 1 paper. The gel was placed on top of the PVDF membrane and covered with three pieces of Whatman No. 1 and three pieces of Whatman No. 3 paper. The paper pieces were wetted with 10 mM CAPS, pH 11 and the PVDF membrane was wetted with MeOH and then with CAPS buffer. The current density was 0.1 mA cm⁻² and transfer time was 16–24 h. Membranes were stained in 0.1% Coomassie Brilliant Blue in 40% MeOH and destained in 40% MeOH.

**Immunodetection of proteins.** The Coomassie Brilliant Blue stained PVDF membranes were destained completely in MeOH after cutting out appropriate lanes. The strips were then blocked in 10% skimmed milk powder in PB or in commercial skimmed milk for 1–2 h. Strips were then incubated with primary antibodies diluted 1:200 or 1:500 in 10% skimmed milk in PB. Incubation was done overnight at 5°C and then 1 h at room temperature. Strips were then rinsed three times with 10% skimmed milk in PB and incubated for 1 h with secondary antibody (peroxidase conjugated swine anti rabbit Ig or rabbit anti human Ig) diluted 1:500 in 10% skimmed milk. After three washes in PB and one in 50 mM acetate buffer pH 5, bound antibodies were visualized with 3-aminio-9ethylcarbazole (50 mg dissolved in 2 ml DMF to which 50 ml 50 mM NaAc pH 5.0 had been slowly added) and H₂O₂ (0.0015%). Strips were then rinsed in water and dried.

**Reduction and alkylation.** Samples were alkylated directly with 50 mM ICH₃COOH or treated first with 40 mM DTE and then alkylated. Samples were then passed through a G-10 gel filtration column to remove low molecular weight compounds.

**Peptide mapping.** Samples were incubated with enzyme (1:1000, w/w) at 37°C and then analysed by SDS-PAGE or high performance liquid chromatography (HPLC). For analysis by SDS-PAGE samples were boiled with sample buffer 1:1 and then 10 µl were loaded per lane. For analysis by HPLC the digestions were stopped by addition of TFA to a final conc. of 1%, and the sample
Fig. 1. Western blot analysis of placental extracts using antiserum against purified calreticulin as primary antibody and peroxidase conjugated swine anti rabbit Ig as secondary antibody. Samples were boiled 1:1 with sample buffer and run on a 12% SDS-PAGE gel. After electrottransfer to PVDF membrane, the blot was incubated with antisera and finally developed using 3-amino-9-ethylcarbazole and H$_2$O$_2$: A, PB extract; B, PB+1% Triton X-114 extract; C, PB+1% Triton X-114+1 M NaCl extract. Indicated molecular weight markers were phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin.

Chromatographed on a model 130A HPLC system (Applied Biosystems) using a 0.4 cm ¥ 20 cm reversed-phase C-18 column (5 μm particle size). The column was eluted with a linear gradient of CH$_3$CN in 0.1% TFA. Flow: 275 μl min$^{-1}$.

Amino acid analysis (AAA). Samples were hydrolysed in 6 M HCl, 0.5% phenol, 0.05% dithiodipropionic acid for 24 h at 110°C, and analysed as described by Barkholt and Jensen.12

Amino acid sequence analysis. Samples were analysed on a model 477 A sequenator (Applied Biosystems) using chemicals and software supplied by the manufacturer.

Results
As a result of investigating several matrices in affinity chromatography of human proteins, a human placental protein was obtained by affinity chromatography of a placental extract on AH-Sepharose, from which the protein could be affinity eluted using 10 mM octylamine, and subsequently purified by ion-exchange chromatography on DEAE-cellulose and Mono-Q columns (not shown). The purified protein had an apparent $M_r$ of 60000 and an

Fig. 2. Purification of human calreticulin from placenta: A, ion-exchange chromatography on DEAE-cellulose of a PB+1% Triton X-114 extract; fractions were tested for the presence of calreticulin by Western blotting; B, Sephadex G-100 gel filtration chromatography of fractions from A containing calreticulin; fractions were tested for calreticulin by Western blotting and the column was calibrated using the indicated marker proteins; C, ion-exchange chromatography of fractions from B containing calreticulin; 1 ml aliquots were chromatographed separately on a Mono Q column; D, SDS-PAGE analysis of purified calreticulin from C. The sample was boiled 1:1 with electrophoresis sample buffer and run on a 12% SDS-PAGE gel.
N-terminal sequence homologous to murine calreticulin, and was subsequently identified as human calreticulin by further peptide mapping and amino acid sequencing studies (see later). The purified protein was used for raising antibodies, which were used for designing an optimal extraction and purification scheme. Sequential extraction of placenta with 0.1 M PB, pH 7.2, PB + 1% Triton X-114 and PB + 1% Triton X-114 + 1 M NaCl showed that calreticulin was mainly located in the PB + 1% Triton X-114 extract and PB + 1% Triton X-114 + 1 M NaCl extract (Fig. 1). Temperature-induced phase separation with this extract showed, however, that calreticulin remained in the water phase, making it likely that calreticulin is a soluble protein located mainly in an intracellular membrane-bound compartment, a prediction also made by the presence of a C-terminal KDEL sequence in the cDNA-derived sequence. Calreticulin was purified to homogeneity from the PB + Triton X-114 extract as shown in Fig. 2. After chromatography on DEAE cellulose, fractions with calreticulin were subjected to gel filtration on Sephadex G 100, where calreticulin eluted with a $M_r$ of 60000. Individual fractions from gel filtration were chromatographed separately by ion-exchange chromatography on Mono Q columns to yield pure protein. The purified protein was occasionally found to form dimers (Fig. 3b) and treatment with iodoacetic acid before analysis by SDS-PAGE resulted in the appearance of a new additional band with slightly higher $M_r$ (Fig. 3a), as would be expected for a protein in equilibrium with a disulfide bonded homodimer. This shows that the purified protein has a free SH group and can dimerize by formation of a disulfide bond. Furthermore, this notion is supported by the presence of three cysteines in the cDNA-derived sequence of calreticulin.  

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<th>Table 1. Amino acid analysis of human calreticulin.</th>
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* Derived from cDNA sequence. n.d. = not determined.

![Fig. 3. SDS-PAGE analysis of purified calreticulin and reaction with DTE and iodoacetic acid. Samples were boiled with electrophoresis sample buffer with DTE (a) or without DTE (b) and run on a 12% gel. In (a) the sample had been pretreated by incubation in 40 mM iodoacetic acid in PB pH 8 for 1 h in the dark at room temperature and then gel-filtered on a PD-10 column to remove the excess of iodoacetic acid.](image)

![Fig. 4. Peptide maps of purified calreticulin subjected to cleavage with trypsin (A), S. aureus V8 protease (B) and pepsin (C). Individual peaks were collected from the HPLC for subsequent sequence analysis.](image)
The purified calreticulin was used for amino acid analysis (Table 1), N-terminal sequence analysis and for peptide mapping studies using trypsin, *S. aureus* V8 protease and pepsin (Fig. 4). The amino acid analysis confirms the acidic nature of the protein, predicted by the cDNA-derived protein sequence, and in accordance with this more peaks were seen in the *S. aureus* V8 protease digests than in the trypsin and pepsin digests. Individual peaks from HPLC analysis of the proteolytic digests were used for obtaining sequence information. The sequences obtained from the enzymatic digests were aligned with the cDNA-derived calreticulin sequence (Fig. 5) and only one discrepancy was found with the cDNA sequence: a valine at position 349 instead of a tyrosine. Further support for valine at this position can be found by comparing published sequences for calreticulin from various species, which shows that this valine occurs in a highly conserved region of calreticulin, with valine at this position in all calreticulins. In addition this comparison reveals a very high degree of similarity between published calreticulin sequences, showing that this protein is highly conserved. When the protein had been alkylated with iodoacetic acid before digestion one peak gave the sequence Asp-Ile-Arg-Cys (cm)-Lys, showing that the cysteine in this position has a free SH group, whereas one fragment isolated was found by N-terminal sequencing to cover the whole N-terminal region, and was found by amino acid analysis to contain two cysteines not derivatized by iodoacetic acid and one cysteine derivatized to form carbamomethyleysteine. Thus the cysteines at positions 105 and 137 most likely form a disulfide bridge. No evidence for glycosylation in the form of glucosamine or galactosamine was found, either by AAA in short or longer hydrolysis.

**Fig. 5.** Comparison of amino acid sequences with the cDNA-derived human calreticulin sequence (10). Capital letters indicate positively assigned residues, while lower letters indicate tentatively assigned residues. * indicates that no residue was assigned. A single discrepancy with the cDNA-derived sequence was found at position 349. The cysteine at position 163 was identified as carboxymethylcysteine.

**Fig. 6.** Western blots of purified calreticulin incubated with sera from patients with diagnosed anti DNA autoimmune disease. The purified calreticulin was run on a 12% SDS-PAGE gel and electrotransferred to a PVDF membrane. The blot was stained with Coomassie blue and individual lanes were cut out and destained completely with MeOH for subsequent incubation with sera. Lane I and St. = standard proteins were not destained after Coomassie staining. Lanes A–H were incubated with sera from individual patients diluted 1:1000 in skimmed milk. After incubation the eight strips were washed and incubated with peroxidase conjugated rabbit anti human IgG in skimmed milk. Bound antibodies were visualized by incubation with 3-amino-9-ethylcarbazole and H$_2$O$_2$. 

- 97 000
- 68 000
- 45 000
- 30 000
- 20 000
experiments, or on Western blots using a panel of biotinylated lectins (results not shown), and no signs of glycosylation were found by sequencing across the potential glycosylation site. No signs of phosphotyrosine, phosphothreonine or phosphoserine were found by AAA using short or longer hydrolysis times and no sign of phosphotyrosine was found on Western blots using a monoclonal antibody against phosphotyrosine (not shown). Thus, even if calreticulin contains one potential glycosylation site and many potential phosphorylation sites, these sites are not post-translationally modified in the placentally derived protein.

UV spectra (not shown) of the purified calreticulin showed that the protein had a high content of nucleic acids, most likely RNA, in accordance with previous results showing that Ro proteins, and also calreticulin, bind to small RNAs (hY RNA).\textsuperscript{9,13,14}

Previously it has been shown that calreticulin can be precipitated by immunoglobulins from patients with systemic lupus erythematosus (SLE). To investigate whether it is calreticulin itself or the associated RNA that is the autoantigen, Western blots with the purified protein incubated with sera from patients with autoimmune disease were performed. Experiments with sera from SLE patients with diagnosed anti DNA autoantibodies, showed that two out of eight patients had antibodies to calreticulin in their serum (Fig. 6).

Since calreticulin most likely interacts with other endoplasmic reticulum (ER) proteins and possibly also nuclear proteins, an attempt to characterize some of these proteins after affinity chromatography on a calreticulin column was made. This could also help in the elucidation of the function of calreticulin. Since calreticulin resides in an intracellular membrane-bound compartment these experiments were performed using the Triton X-114 extracts, also used for purifying calreticulin itself. Chromatography of this extract on a column with calreticulin immobilised on a divinyl sulfonylglarose column identified six major bands, as revealed by SDS-PAGE of proteins bound to the column and eluted with 1 M acetic acid (Fig. 7). The relative molecular weights of these proteins were 100 000, 85 000, 58 000, 47 000, 28 000 and 15 000, respectively. Since these proteins give rise to rather diffuse bands in SDS-PAGE they might be membrane-associated proteins retaining a high proportion of detergent in affinity chromatography. Further characterization of these proteins should help in the elucidation of the function of calreticulin.

**Discussion**

The protein purified here has been characterized as human placental calreticulin by comparing the found amino acid sequences with published cDNA sequences. Human calreticulin is homologous to calreticulin from other species and the N-terminal sequence is identical with the N-terminal sequence of an autoantigen in human SLE\textsuperscript{9,13} and a calcium binding protein from HL 60 cells which co-purifies with an inositol 4,5-bisphosphate sensitive Ca\textsuperscript{2+} store.\textsuperscript{15} These proteins most likely represent the same protein, human calreticulin, and this suggests a role for calreticulin in inositol 4,5-bisphosphate stimulated Ca\textsuperscript{2+} release from the ER. Human calreticulin has three cysteines two of which most likely form a disulfide bridge. The three cysteines are all located in the N-terminal domain (the N-domain) and the disulfide bridge thus helps to stabilize this domain. The role of the free –SH group in dimerization or interaction with other proteins remains to be determined. No evidence either for glycosylation of the potential asparagine glycosylation site or for phosphorylation of Tyr, Ser and Thr was found. However the protein was found to be associated with RNA. Interestingly the protein could be purified on an aminohexyl-Sepharose column, from which it could be affinity eluted with octylamine. Whether this represents a true affinity for amino groups or an affinity for hydrophobic compounds remains to be determined, but the positively charged amino group may compete with Ca\textsuperscript{2+} for binding to some of the low affinity calcium sites.

Calreticulin has been assumed to function as a Ca\textsuperscript{2+} storage protein, but the finding that at least six proteins could be affinity purified on a calreticulin column indicates that calreticulin also interacts with other proteins to perform this and other specific functions, possibly some specific parts of RNA processing or regulation of Ca\textsuperscript{2+} homeostasis. In skeletal muscle the ryanodine receptor

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**Fig. 7.** SDS-PAGE analysis of proteins purified on a calreticulin column. The eluate (100 µl) was lyophilised and redissolved in 20 µl electrophoresis sample buffer. After boiling for 2 min 10 µl was analysed on a 12.5% Laemmli gel.
(M, 565000) participates in regulation of Ca$^{2+}$ homeostasis, and the C-terminal channel region of the receptor seems to form a complex with triadin (M, 94000) and caislequinin (M, 63000). Furthermore, the N-terminal region of the receptor interacts with the dihydropropyridine receptor (M, 170000, 175000, 52000 and 32000 for α1, α2, β and γ subunits). The relative molecular weights of some of the proteins in this complex correspond well to the relative molecular weights of some of the proteins found to interact with calcretulin in this work, and since calcretulin and caislequinin have similar structures and probably perform similar functions it is a likely possibility that calcretulin performs a function in coupling of the ryanodine receptor or a similar receptor to regulation of Ca$^{2+}$ levels in the ER.

Since calcretulin contains a nuclear localization signal a function as a shuttle protein between the nucleus and the ER can also be envisaged. Calcretulin has recently been shown to be capable of interaction with nuclear hormone receptors, and preliminary histochemical studies using the antibodies produced here have actually shown that the antigen is present in both the nucleus and the ER. A role for calcretulin in RNA processing and transport is compatible with its apparent involvement in several categories of autoimmunity like SLE, and addition of calcretulin to the list of autoantigens in SLE actually reinforces the possibility of a function in RNA processing, since most autoantigens in SLE have so far been found to be components of the cellular RNA processing apparatus. The finding that some SLE patients with anti DNA autoantibodies also have autoantibodies to calcretulin may help to resolve the apparent discrepancy, which seems to exist in the literature about the nature of the Ro SS-A antigen, an autoantigen in Sjögren’s syndrome (SS). The protein characterized by Deutscher et al. has M, 60000 and contains an RNA binding motif whereas the protein characterized by Lieu et al. and McCauliffe et al. does not contain a known RNA binding motif but has an Mr of 60000 and a sequence identical with that described here for human calcretulin. Since the protein characterized by Lieu et al. was purified by its ability to react with autoantibodies from a patient with SLE and since several patients with anti DNA autoantibodies were found to contain autoantibodies to the calcretulin purified here, it can be concluded that calcretulin is an autoantigen in only some cases of SLE.

References


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