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Cation-Dependent Interactions of Calreticulin with Denatured and Native Proteins

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Calreticulin has sequence homology with the molecular chaperone calnexin, which is known to control folding and assembly of nascent proteins in the endoplasmic reticulum in a calcium-dependent manner. We have investigated the interaction between human placental calreticulin and denatured placental and serum proteins under various incubation conditions. The interactions with denatured proteins differed significantly from the interactions with native proteins. The interactions were highly dependent on divalent metal ions or polyamines, but were not influenced by detergent and sulfhydryl agents. Our results indicate that calreticulin might have a similar role in protein folding as the chaperone calnexin.

Calreticulin is a highly conserved 46 kDa Ca^{2+}-binding protein localized mainly to the endoplasmic reticulum (ER) in non-muscle cells.[1,2] It has a KDEL ER retention signal in the *C*-terminus,[1] and a nuclear localization signal from residues 187 to 195;[2] further, it has also been detected in the cytoplasm.[3] Calreticulin is an acidic protein with an isoelectric point of 4.7, and is composed of 417 aminoacids which are divided into three domains called *N*-, *P*- and *C*-domains.[4] The *N*-domain is believed to be globular and made of eight anti-parallel β-sheets with a helix–turn–helix motif at the *N*-terminus.[1,2] The *P*-domain is rich in proline and serine residues, followed by the highly acidic *C*-terminal domain, containing high capacity, low-affinity Ca^{2+}-binding sites, which are responsible for the bulk binding of Ca^{2+} ions.[5] Furthermore the *P*-domain contains a low-capacity, high-affinity Ca^{2+}-binding site,[5] and the putative nuclear localization signal.[2] A glycosylation site has been found at residue 326, but human calreticulin does not seem to be glycosylated under most conditions.[6]

The storage of Ca^{2+} ions in intracellular compartments such as the ER plays an important role in regulating the cytoplasmic concentration of Ca^{2+} ions. Ligand binding to cell membrane receptors triggers a release of the membrane derived second messenger IP3 (inositol 1,4,5-triphosphate), which interacts with receptors in the ER membrane to release Ca^{2+} into the cytoplasm, where it regulates a wide spectrum of biological functions.[7] The Ca^{2+} ions are pumped back into the ER by Ca^{2+}-ATPase, and when the Ca^{2+} ions are bound to a storage protein like calreticulin, the concentration gradient of free Ca^{2+} ions, which the ATPases have to pump against, is reduced.[6]

Calreticulin binds to a conserved sequence KXGFFKR on the cytoplasmic domain of the α-subunits of integrins, which locks the extracelluar part of the α-subunit in a high-affinity state, thereby increasing binding of ligands to the cell surface receptor.[7] The same conserved motif has been found in the DNA-binding domain of nuclear hormone receptors, and binding of calreticulin to both glucocorticoid, androgen and retinoic acid receptors inhibits the interaction of these nuclear receptors with their respective response elements.[8,9] This indicates a role for calreticulin in transducing signals from the extracellular matrix to the the nucleus.[10]

Comparison of the cDNA-derived amino-acid sequence of calreticulin with other proteins shows a homology to the C1q receptor (C1qR).[11] Antibodies raised against the C1qR cross-react with calreticulin, and calreticulin exhibits some of the binding properties of C1qR such as binding to the serum lectins, mannan binding protein and C1q.[12] It has been proposed that calreticulin, C1qR and an *Onchocerca volvulus* antigen (RAL1) belong to the same conserved protein family and share some structural and functional properties.[13]

Another Ca^{2+}-binding ER protein with homology to calreticulin, calnexin, is known to function as a chaperone in the folding of newly synthezised glycoproteins in the ER.[14,15] Calreticulin has been eluted with ATP, stimulated by Ca^{2+} from denatured protein columns under similar conditions as those required for elution of the ER chaperones GRP78 and GRP94.[16] In addition calreticulin interacts with the myeloperoxidase precursor apopro-MPO in a human myeloid cell line PLB 985.[17]

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We present data demonstrating that calreticulin binds to denatured serum and placental proteins in a cation- or polyamine-dependent manner, which supports the theory of calreticulin being a molecular chaperone.

Experimental

Chemicals. Na_2HPO_4, NaH_2PO_4, H_3PO_4, NaCl, $(NH_4)_2SO_4$, Na_2CO_3, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, KCl, calcium lactate and 5,5-diethylbarbituric acid were from Merck (Darmstadt, Germany), and Triton X-114 was from Merck-Schuchardt (Schuchardt, Germany). *N*-Hydroxysuccinimidobiotin, trizma base, ammonium sulfate, putrescine, spermine, spermidine, Ponceau S and glycerol were from Sigma (St. Louis, MI). Coomassie Brilliant Blue G-250 was from Serva (Heidelberg, Germany). Phosphate buffered saline (PBS), pH 7.38, made of 7.6 g NaCl, 1.42 g Na_2HPO_4 and 0.27 g KH_2PO_4 in 1 L Milli-Q water, and PBS, 0.05% Tween 20, were from Statens Seruminstitut (Copenhagen, Denmark). Ammonium persulfate, SDS-molecular weight standards (low range), gel filtration standard, acrylamide and bisacrylamide were from BIO-RAD (Hercules, CA). Ethanol was from Danisco (Aalborg, Denmark). CH_3COOH was from Bie & Berntsen (Rødovre, Denmark). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Reduced glutathione was from Boehringer Mannheim GmbH (Germany). HRP-streptavidin was from Zymed (San Francisco, CA). Enhanced chemiluminescence (ECL) Western blotting detection reagents and Hyperfilm-ECL were from Amersham (Buckinghamshire, UK). X-Ray developer LX24 and X-ray fixer AL4 were from Kodak (New Haven, CT). Agarose HSA 1000 was from FMC Litex A/S (Vallensbaek, Denmark). S-200 Sephacryl and Mono Q columns were from Pharmacia (Uppsala, Sweden). Water filtration equipment was from Millipore Corp. (Bedford, MA).

Placentas and sera. Human placentas were obtained from Rigshospitalet maternity ward (Copenhagen, Denmark). The placentas were frozen at −20 °C immediately after delivery. Human sera were obtained from Statens Seruminstitut, Department of Autoimmunology (Copenhagen, Denmark).

Extraction of placentas. One placenta, frozen immediately after delivery, was cut into pieces and further homogenized in three sequential steps. In the first step the tissue was homogenized in 0.5 L of 0.1 M phosphate buffer (PB), pH 7.2, followed by 0.5 L of 0.1 M of PB, 1% Triton X-114 and finally 0.5 L of 0.1 M PB, 1% Triton X-114, 1 M NaCl. After each step the homogenate was centrifuged for 30 min at 16 300 *g*, and the pellet was transferred to the next extraction buffer.

Purification of placental calreticulin. One placenta, frozen immediately after delivery, was homogenized in 0.5 L of PB, pH 7.2 and centrifuged 30 min at 16 300 *g*. The pellet was homogenized in 0.5 L of PB, 1% Triton X-114, pH 7.2 and centrifuged. An equal volume of 0.1 M PB, 1% Triton X-114 was added to the supernatant, and phase separation was performed at 37 °C overnight. To the water phase ammonium sulfate was added until a final concentration of 337 g/L was reached and the solution was stirred at 4 °C overnight. A second round of ammonium sulfate (150 g/L) precipitation was performed at pH 4.7 (adjusted by addition of H_3PO_4). The pellet from the second precipitation was dissolved in 250 mL of 10 mM PB, pH 7.5 and dialyzed against the same buffer. Further purification was obtained by ion-exchange chromatography on a Mono-Q column. 2 mL dialysate were pumped on the column, which was then eluted with a linear gradient of increasing salt concentration until a final concentration of 1.0 M NaCl was obtained.

Biotinylation of calreticulin. To 1 mL of purified calreticulin, dissolved in 0.1 M Na_2CO_3, pH 9, 0.4 mg of *N*-hydroxysuccinimidobiotin in 10 µL *N,N*-dimethylformamide was added, and the solution was mixed end-over-end for 2 h at room temperature. The mixture was dialyzed against 0.1 M PBS, pH 7.4 and diluted twice with glycerol.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gels (12.5%) were run according to the method of Laemmli[21] using a vertical slab gel unit from Hoefer Scientific Instruments (Minnesota). Gels were stained in 0.25 w/v% Coomassie Brilliant Blue G 250 in 50% ethanol, 7.5% CH_3COOH and destained in 35% ethanol, 10% CH_3COOH, 1% glycerol.

Western blots. The polyacrylamide gels were electroblotted to 0.45 µm nitrocellulose membranes using a semi-dry electroblotter (Ancos, Denmark) and 10 times diluted SDS-running buffer as transfer buffer. Membranes were stained in Ponceau S to visualize the lanes for cutting. After the individual lanes had been cut out, the membrane strips were blocked in 0.5% Tween 20 for 30 min, a treatment which also removed all Ponceau S from the proteins. The strips were incubated with biotinylated calreticulin (diluted 1 : 500) in 10 mM tris, 0.05% Tween 20 pH 8.5, together with one of the following agents; $MgCl_2$, $CaCl_2$, NaCl, KCl, reduced glutathione, Tween 20 (0.5%), putrescine, spermine or spermidine. The strips were washed three times in the same buffer in which the incubation had been performed, and were afterwards incubated with horse radish peroxidase-conjugated streptavidin, diluted 1 : 5000, washed three times as above and rinsed in water. Bands were detected with 2 mL mixture of ECL detection reagent 1 and 2 (mixed in equal volumes), using a Hyperfilm-ECL in 10–60 s.

Agarose gel electrophoresis. Placental extracts and human sera (diluted 1:20) were separated on a 1% agarose gel

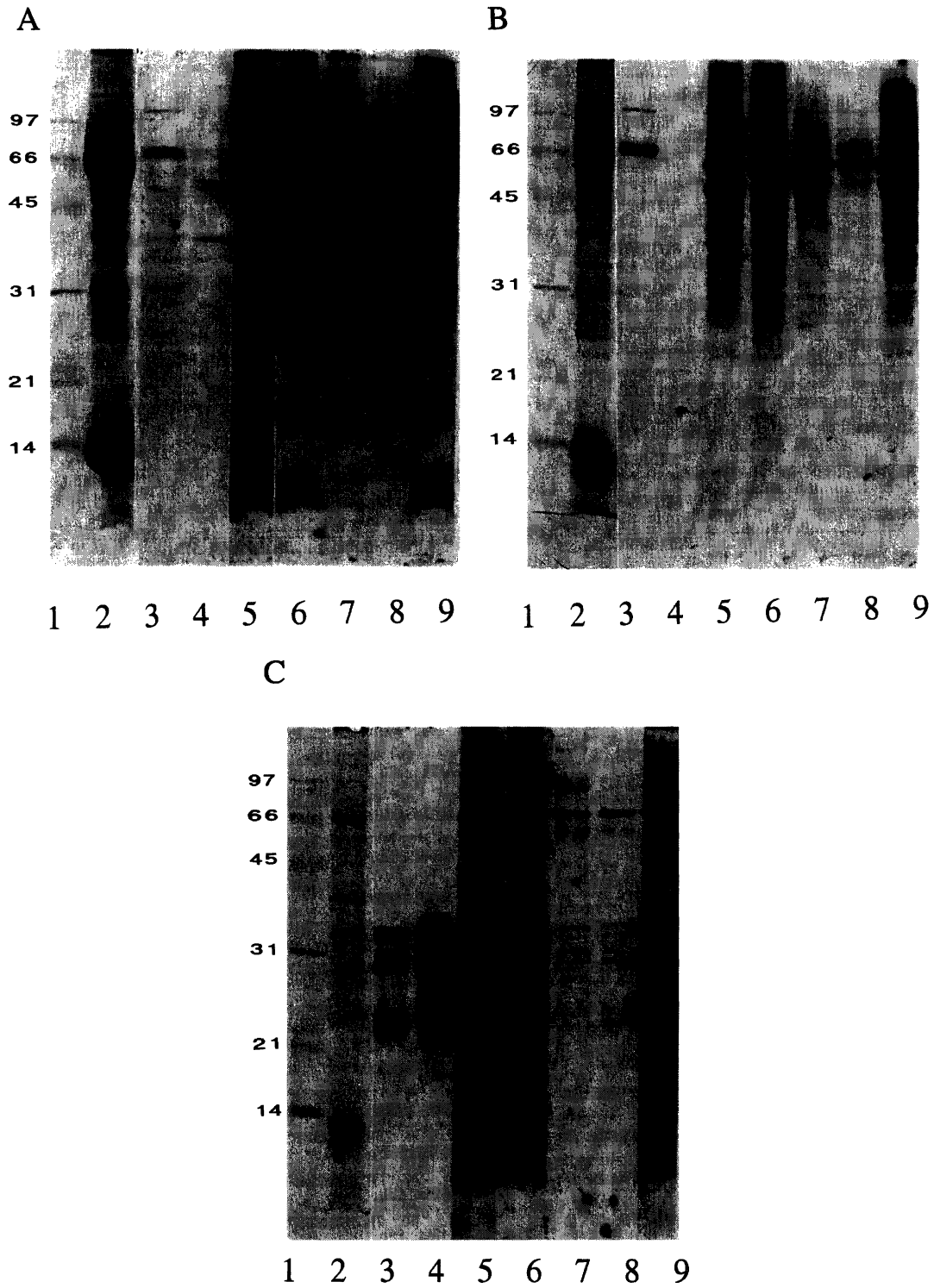


Fig. 1. Western blots showing interaction between calreticulin and denatured placental proteins. Placenta extracts were boiled 1:1 with reducing sample buffer and separated on a 12.5% SDS-gel. The proteins were electrotransferred to nitrocellulose membranes and incubated with biotinylated calreticulin in various buffers, followed by incubation with streptavidin conjugated peroxidase and finally developed by ECL. (A) (1) Molecular weight markers on SDS-PAGE; (2) PB extract on SDS-stained with Coomassie Brilliant Blue; (3–9) Western blots of PB extracts incubated without (3) or with (4–9) calreticulin in different buffers; (3) control without calreticulin in 10 mM $MgCl_2$; (4) with calreticulin in 10mM tris; (5) in 10 mM $MgCl_2$; (6) in 10 mM $MgCl_2$–1 mM glutathione; (7) in 10 mM $MgCl_2$–0.5 M NaCl; (8) in 10 mM $MgCl_2$–1.0 M NaCl; (9) in 10 mM $MgCl_2$–0.5% Tween 20. (B) (1) Molecular weight markers on SDS-PAGE; (2) PB + 1% Triton X-114 extract on SDS-PAGE; (3–9) Western blots of extracts incubated without (3) and with (4–9) biotinylated calreticulin in different buffers (see A). (C) (1) Molecular weight markers on SDS-PAGE; (2) PB + 1% Triton X-114 + 1 M NaCl extract on SDS-PAGE; (3–9) Western blots of extracts incubated without (3) and with (4–9) calreticulin in different buffers (see A).

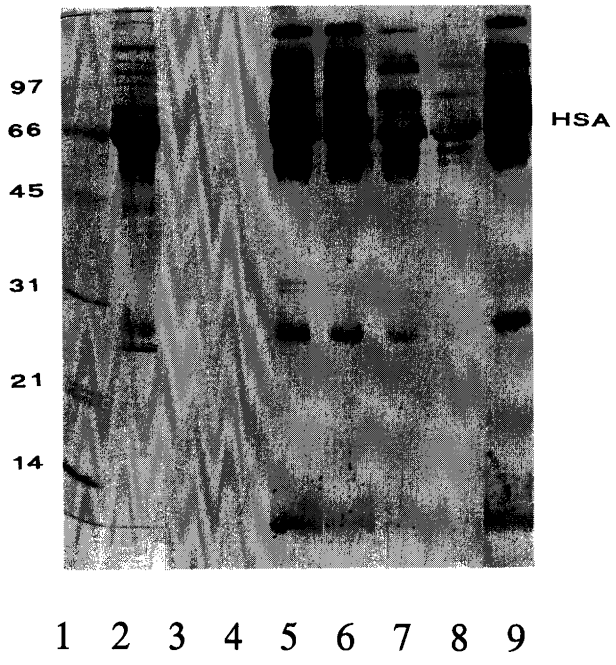


Fig. 2. Western blot showing interaction between calreticulin and denatured serum proteins (methods as described in Fig. 1). (1) Molecular weight markers on SDS-PAGE; (2) human serum diluted 1:20 on SDS-stained with Coomassie Brilliant Blue; (3–9) Western blots of serum proteins incubated without (3) or with (4–9) biotinylated calreticulin in different buffers; (3) control without calreticulin in 10 mM $MgCl_2$; (4) with calreticulin in 10 mM tris; (5) in 10 mM $MgCl_2$; (6) in 10 mM $MgCl_2$–1 mM glutathione; (7) in 10 mM $MgCl_2$–0.5 M NaCl; (8) in 10 mM $MgCl_2$–1.0 M NaCl; (9) in 10 mM $MgCl_2$–0.5% Tween 20.

with tris/veronalbuffer pH 8.5 (90 mM 5.5-diethylbarbituric acid, 0.27 M Trizma base, 1.4 mM calcium lactate) as running-buffer at 8 V cm^{-1} for 80 min.

Blotting of native proteins to nitrocellulose. The agarose gels were blotted to 0.45 μm nitrocellulose membranes under the pressure of a block of lead for 1 h, and treated like the membranes in the Western blot procedure.

Gel filtration and ion-exchange chromatography of human serum. 1.8 mL human serum was chromatographed on a 4 cm × 100 cm S-200 column, eluted with 0.1 M PBS, pH 7.4 overnight at 5 °C with a flow rate of 1 mL/min. IgG- and IgM-containing fractions were further chromatographed on an ion-exchange column (Mono Q), eluted with a gradient from 0 to 1 M NaCl in 10 mM PB, pH 7.5.

Results

Initial dot blot experiments showed that calreticulin interacts with several proteins in human placental extracts in a Mg^{2+}- and/or Ca^{2+}-dependent manner (results not shown). To investigate this further, we performed Western blots with placental proteins. Figure 1 shows interactions of calreticulin with denatured soluble (A), Triton X-114 extractable (B) and high salt extractable (C) placental proteins under different incubation conditions. As seen on the blots, calreticulin has the ability to interact with many proteins in all the extracts, showing that the interaction is not restricted to proteins derived from any particular cellular compartment. The

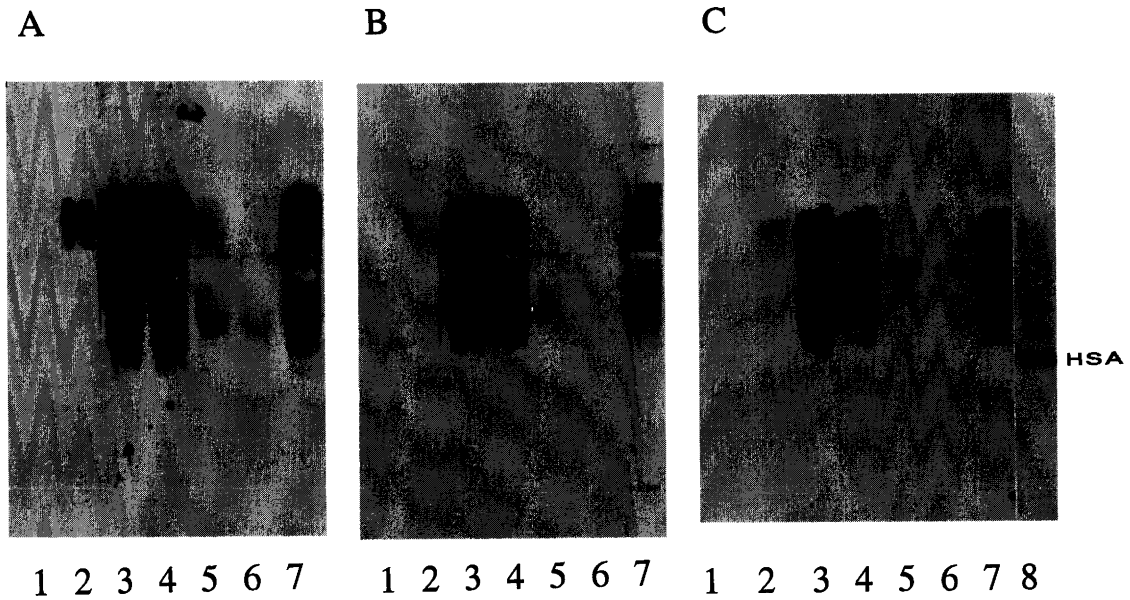


Fig. 3. Interaction between calreticulin and native serum and native placental proteins. Protein samples were separated on a 1% agarose gel under non-reducing conditions and transferred to nitrocellulose. The membranes were incubated without (1) or with (2–7) biotinylated calreticulin followed by streptavidin conjugated peroxidase and finally developed by ECL. The cathode is placed at the top. (A) PB placental extracts; (B) PB + 1% Triton X-114 placental extracts; (C) serum proteins. Incubation conditions were similar in A, B and C; (1) control without calreticulin in 10 mM $MgCl_2$; (2) calreticulin in 10 mM tris; (3) in 10 mM $MgCl_2$; (4) in 10 mM $MgCl_2$–1 mM glutathione; (5) in 10 mM $MgCl_2$–0.5 M NaCl; (6) in 10mM $MgCl_2$–1.0 M NaCl; (7) in 10 mM $MgCl_2$–0.5% Tween 20; (8) serum proteins blotted to a nitrocellulose membrane and stained with Ponceau S.

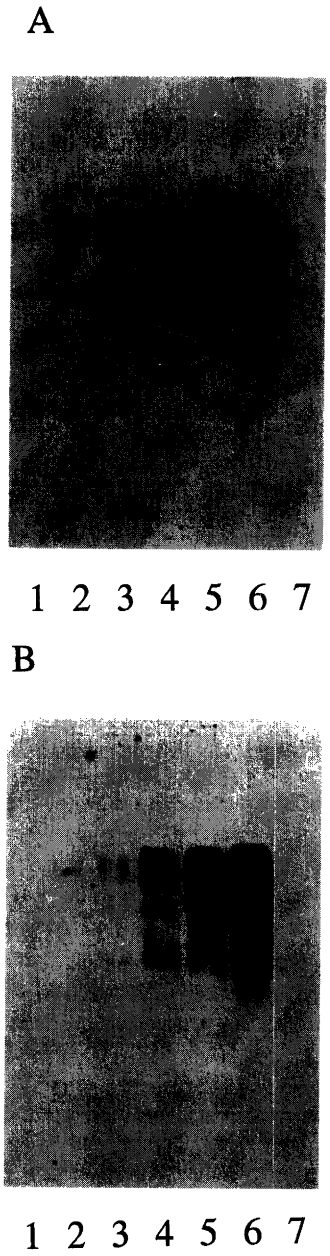


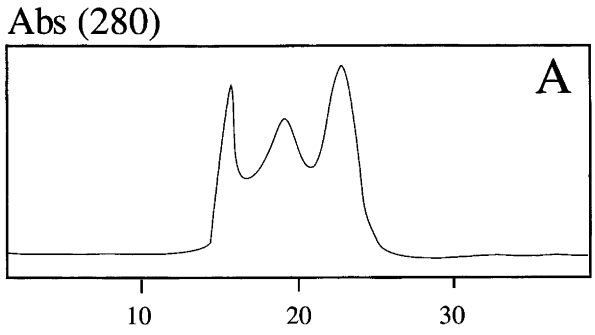
Fig. 4. Binding of calreticulin to native serum proteins and its Ca^{2+} and Mg^{2+} concentration dependency. The serum proteins were treated as in Fig. 3. (A) Serum proteins incubated with calreticulin in 10 mM tris buffer with $MgCl_2$ in different concentrations; (1) 0 mM $MgCl_2$; (2) 0.1 mM $MgCl_2$; (3) 0.5 mM $MgCl_2$; (4) 1 mM $MgCl_2$; (5) 5 mM $MgCl_2$; (6) 10 mM $MgCl_2$; (7) control without calreticulin in 10mM $MgCl_2$; (B) serum proteins incubated with calreticulin in 10 mM tris buffer with $CaCl_2$ in different concentrations; (1) 0 mM $CaCl_2$; (2) 0.1 mM $CaCl_2$; (3) 0.5 mM $CaCl_2$; (4) 1 mM $CaCl_2$; (5) 5 mM $CaCl_2$; (6) 10 mM $CaCl_2$; (7) Control without calreticulin in 10mM $CaCl_2$

interactions were strongly dependent on the presence of Mg^{2+}, but are not influenced by a sulfhydryl reagent (1 mM glutathione) or by detergent (0.5% Tween 20). On the other hand, a high salt concentration (0.5–1.0 M NaCl) diminished the interactions. To examine whether this interaction was restricted to cellular proteins, we performed a similar blot using denatured serum proteins as targets. As seen in Fig. 2 human serum contains many proteins that can interact in their denatured form with calreticulin, and the dependency on buffer composition was similar to that seen for the placental proteins. The results above indicate that calreticulin generally interacts with denatured proteins in a cation-dependent way.

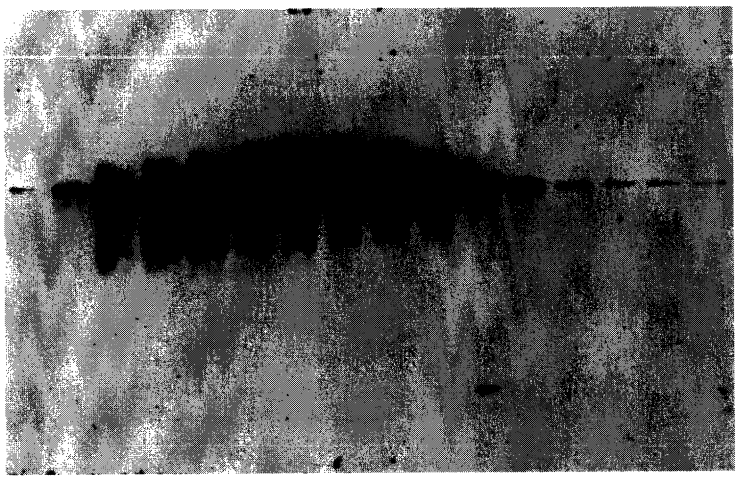
To verify that this interaction is specific for denatured proteins, we performed blots with the same protein preparations under non-denaturing conditions. As seen in Fig. 3 only a few proteins from placenta and serum interact with calreticulin under non-denaturing conditions. Native albumin does not interact with calreticulin, as it was recognized by calreticulin only under denaturing conditions. Although not clearly visible on the photographs, it can be seen on the original films that three major bands with an anodic mobility and one smaller band with a cathodic mobility do interact with calreticulin under native conditions. An investigation of the concentration dependency of the Mg^{2+}- and Ca^{2+}-stimulated interaction of calreticulin with native serum proteins showed that both ions stimulated the interaction in a concentration-dependent way (Fig. 4). Also, it was observed that Mg^{2+} was more effective than Ca^{2+} in promoting the interaction. Exactly the same concentration dependency was observed for the interaction of calreticulin with denatured proteins (not shown). The concentrations of divalent cations required for optimal binding of calreticulin to other proteins closely match the millimolar range concentration of divalent ions present in the ER.

To characterize the interacting serum proteins in more detail, we performed gel filtration and ion-exchange separation. Fractionation of human serum on a S-200 gel filtration column resulted in the emergence of three major protein peaks, containing IgM, IgG and albumin, respectively. Selected fractions representing the three major peaks were separated on an agarose gel, blotted to nitrocellulose and incubated with calreticulin under non-reducing conditions. It appeared that calreticulin interacts with native IgM and IgG, but not with native serum albumin (Fig. 5). Control experiments with purified human IgG and IgM verified that the major interacting proteins were indeed IgG and IgM (not shown). A further chromatography of IgG- and IgM-containing fractions on an ion-exchange column (Mono Q) showed that the calreticulin binding activity coeluted with IgG and IgM, respectively, and subsequent SDS-analysis (under reducing conditions) of the reactive fractions confirmed the presence of both heavy and light Ig chains (not shown).

To elucidate further the nature of the interaction between calreticulin and denatured placental and serum proteins, Western blots were performed under varying incubation conditions (Fig. 6). The buffer composition seems to affect the interaction of both serum and placental proteins with calreticulin in the same way. Incubation with 10 mM Ca^{2+} stimulated the interaction, but to a



B



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 5. Interaction of calreticulin with serum proteins, separated by gel filtration. (A) A 0.8 mL sample of human serum was separated on a gel filtration column. (B) Selected fractions were electrophoresed on a 1% agarose gel and transferred to a nitrocellulose membrane, which was incubated with biotinylated calreticulin, followed by streptavidin conjugated peroxidase and developed by ECL: (1) gel filtration fraction 13; (2–5) gel filtration fraction nos. 14–17 containing IgM; (6–8) gel filtration fraction nos. 18–20 containing IgG; (9–13) gel filtration fraction nos. 21–25 containing serum albumin fractions; (14–16) late fractions.

remarkably lesser degree than 10 mM Mg^{2+}. A low concentration of salt (10 mM NaCl and 10 mM KCl) augmented the interaction, whereas a high concentration of NaCl was shown to inhibit the interaction (Fig. 1). The polyamine putrescine increased the specific binding of calreticulin to nearly the same level as that seen with Mg^{2+}. The other polyamines, spermine and spermidine also increased the interaction, although a high background occurred using these agents.

The binding of calreticulin to native human serum proteins in the presence of polyamines and low concentration of salt (10 mM NaCl and 10 mM KCl) was examined by a corresponding experiment using non-denaturing conditions (Fig. 7). The pattern of augmented calreticulin binding to native serum proteins (IgM and IgG) was the same as that seen under denaturing conditions.

Discussion

Newly synthezised proteins are folded and assembled in the endoplasmic reticulum under the control of molecular chaperones.[16] The amino-acid sequence of the protein is the code to the folding pattern, but many nascent proteins rely on chaperones for proper folding. The chaperones prevent unproductive intra- and intermolecular interactions under the process of folding and assembly of subunits.[15] It has been suggested that two different folding and transport pathways exist in the ER, a membrane-associated and a luminal pathway.[14] The

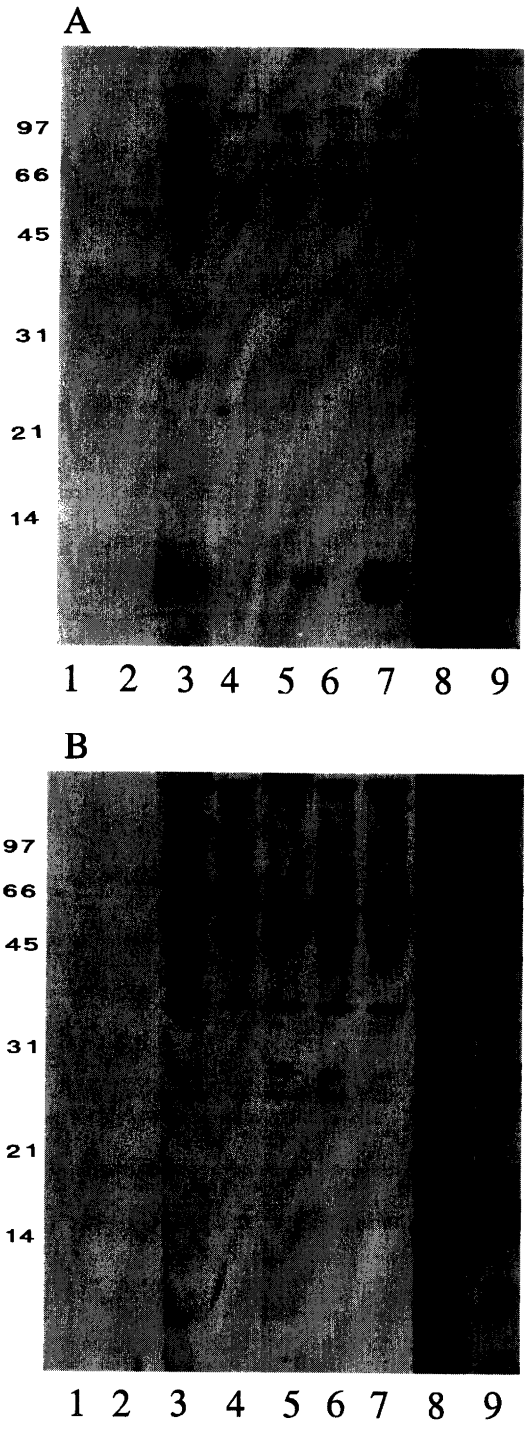


Fig. 6. The influence of various ions and polyamines on the interaction between calreticulin and denatured serum and denatured placental proteins. The procedures are as in Fig. 1. (A) Blot with serum proteins incubated with calreticulin (2–9). (B) PB extract from placenta incubated with calreticulin (2–9). Incubation conditions are similar in A and B. (1) Control without calreticulin in 10 mM $MgCl_2$; (2) 10 mM tris; (3) 10 mM $MgCl_2$; (4) 10 mM NaCl; (5) 10 mM $CaCl_2$; (6) 10 mM KCl; (7) 10 mM putrescine; (8) 10 mM spermine; (9) 10 mM spermidine.

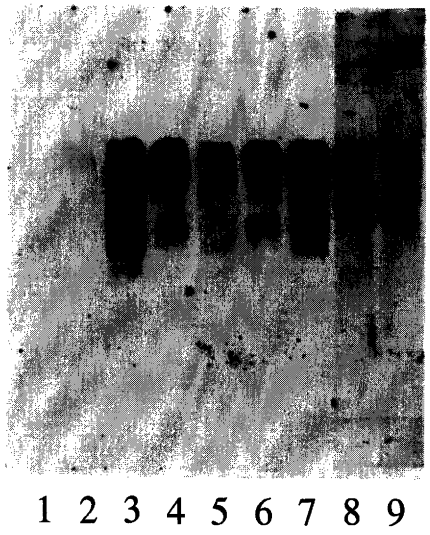


Fig. 7. The interaction between calreticulin and native human serum proteins, and its dependency of divalent ions, monovalent ions and polyamines. The procedures are as in Fig. 3. (1) Control without calreticulin in 10 mM $MgCl_2$; (2) 10 mM tris; (3) 10 mM $MgCl_2$; (4) 10 mM NaCl; (5) 10 mM $CaCl_2$; (6) 10 mM KCl; (7) 10 mM putrescine; (8) 10 mM spermine; (9) 10 mM spermidine.

transmembrane calcium-dependent chaperone calnexin selectively binds glycosylated proteins, while non-glycosylated proteins pass on to the luminal pathway.[14] Since calreticulin resides in the lumen of ER and has sequence homology with calnexin in its luminal part, we believe that calreticulin could be a calcium-dependent chaperone belonging to the luminal pathway.

In this study we have shown a cation-dependent interaction between calreticulin and denatured placental and serum proteins. With a few exceptions calreticulin could only bind such proteins in the presence of Mg^{2+} or Ca^{2+} ions, and to a lesser extent in the presence of K^+ and Na^+ ions at low concentrations (10 mM). The dependency of the binding on the presence of cations, and in particular on divalent ions, indicates that a conformational change has to occur in calreticulin before an interaction can occur. Assuming that the divalent cations bind at the low affinity sites in the *C*-terminus, it is likely that the binding of calreticulin to other proteins does not solely result from an electrostatic interaction of a negatively charged region on calreticulin and positively charged areas on other proteins. Such an interaction would clearly be inhibited by binding of cations to calreticulin. Thus it seems more likely that the binding of positively charged ions to the *C*-terminus of calreticulin induces a conformational change favouring binding of denatured proteins.

Polyamines also caused an increased interaction, which might be due to the ability of polyamines to mimic divalent ions in biological systems.[19] The detergent Tween 20 in a concentration of 0.5% had no effect on the interactions, which excludes weak hydrophobic interactions as an important prerequisite for the interaction

to take place. We also tested the effect of the sulhydryl agent glutathione to investigate whether disulfide bridges are involved in the binding, but this had no influence on the binding. On the other hand highly concentrated salt (0.5–1.0 M NaCl) inhibited the interaction, indicating that ionic interactions are involved.

As controls we performed the same binding experiments under non-denaturing conditions, which resulted in an altered pattern of binding (Figs. 3 and 7). Only a few proteins were shown to bind calreticulin under native conditions. The non-glycosylated protein albumin (M_w = 69.000) is an example of a molecule which does not bind to calreticulin in its native state but binds in its denatured form. The buffer requirement for binding of the native immunoglobulins to calreticulin was similar to that seen in the experiments performed under denaturing conditions. In this context the sequence homology of calreticulin and C1qR may indicate a functional homology of these molecules with regard to interaction with molecules of the immune system.

We conclude that calreticulin preferentially binds to denatured cellular and secreted proteins, and that the interaction is strongly dependent on the presence of a divalent cation, which supports the theory of calreticulin being a molecular chaperone. Thus, calreticulin does not only function as a Ca^{2+}-storage protein in the ER: its putative chaperone function in the ER is also dependent upon and stimulated by the cations of the ER.

When a protein has been folded and assembled in the ER it is transported to the Golgi apparatus. In HEpG2 cells the time spent by nascent proteins in the ER is the rate-limiting step of the protein secretion,[14] therefore, retention by chaperones might be the controlling link. Other experiments have shown that non-functional, mutant (class 2 mutated LDL receptor) proteins are arrested in the ER by the chaperone calnexin.[14] Calreticulin might have a similar function in the luminal pathway. We suggest that calreticulin controls folding of nascent proteins and arrests improper and mutated proteins in the ER. Moreover, a function of calreticulin in the intracellular transport of immunoglobulins may be anticipated, as these were the main native proteins of serum found to bind calreticulin in a cation-dependent way.

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