Release of calreticulin from neutrophils may alter C1q-mediated immune functions

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Calreticulin is an abundant intracellular protein which is involved in a number of cellular functions. During cytomegalovirus infection, as well as inflammatory episodes in autoimmune disease, calreticulin can be released from cells and detected in the circulation, where it may act as an immunodominant autoantigen in diseases such as systemic lupus erythematosus. Calreticulin is known to bind to the molecules of innate immunity, such as C1q, the first subcomponent of complement. However, the functional implications of C1q–calreticulin interactions are unknown. In the present study we sought to investigate, in greater detail, the interaction between these two proteins following the release of calreticulin from neutrophils upon stimulation. In order to pinpoint the regions of interaction, recombinant calreticulin and its discrete domains (N-, P- and C-domains) were produced in *Escherichia coli*. Both the N- and P-domains of calreticulin were shown to bind to the globular head regions of C1q. Calreticulin also appeared to alter C1q-mediated immune functions. Binding of calreticulin to C1q inhibited haemolysis of IgM-sensitized erythrocytes. Both the N- and P-domains of calreticulin were found to contain sites involved in the inhibition of C1q-induced haemolysis. Full-length calreticulin, and its N- and P-domains, were also able to reduce the C1q-dependent binding of immune complexes to neutrophils. We conclude that calreticulin, once released from neutrophils during inflammation, may not only induce an antigenic reaction, but, under defined conditions, may also interfere with C1q-mediated inflammatory processes.

INTRODUCTION

Calreticulin is an autoantigen of clinical interest, because antibodies against it are found in many patients suffering from lupus disorders and Sjogren's syndrome [1,2]. Human calreticulin has been cloned and found to be 417 amino acids in length, with a calculated molecular mass of 60 kDa [3]. Calreticulin has been found in nearly all eukaryotic cell types with the exception of erythrocytes, at concentrations ranging from 20 to 500 μg/g of tissue. The precise biological roles of calreticulin are still under discussion, but the protein, by virtue of its C-terminal KDEL sequence, is known to be found associated with the lumen of the endoplasmic reticulum (ER), where it is believed to function as a high-capacity Ca^{2+} storage and regulatory protein [4]. In leucocytes, the ER is very poorly defined, and 'calreticulin-like' proteins are present in discrete subcellular compartments other than the ER. For example, HL-60 cells possess a 60 kDa Ca^{2+}-binding protein in 'calciosomes' with an N-terminal sequence that shares 93 % identity with calreticulin over the first 15 amino acid residues [5], and calreticulin has also been identified in discrete secretory granules of cytolytic T-lymphocytes [6]. Calreticulin has also been detected at low levels in the plasma of normal individuals, probably originating from cells [7]. It is also overexpressed on the surface of lung fibroblasts in response to cytomegalovirus infection [8].

Several studies have indicated that calreticulin shares 50–60 % amino acid identity with proteins found in the human parasites *Onchocerca volvulus* [9] and *Schistosoma mansoni* [10], which may help to explain why calreticulin is a target for autoimmunity. What is less clear is whether, on its release from cells, calreticulin has a physiological/pathological role. In a previous study we showed that C1q, a subunit of the first component of complement (C1), which provides the initial trigger for the activation of the classical complement cascade, binds to calreticulin [11]. Potentially, the association of calreticulin with C1q may interfere with complement activation. In classical pathway complement activation, the C-terminal globular head region of C1q binds to the CH_2 domains of immune complex (IC)-fixed immunoglobulin [12]. When C1q–IC interactions are formed *in vivo*, the subcomponents C1r and C1s become activated and initiate complement activity. The cascade is regulated in part by C1 inhibitor, which binds to activated C1r and C1s, thereby inactivating these enzymes, which become dissociated from C1q–IC. In this process, complement is consumed during IC formation, which is reflected in patients with systemic lupus erythematosus (SLE), who have decreased complement levels [13]. Complement activation is an inflammatory process, but it is also involved in the prevention of formation of large insoluble ICs, which might cause tissue injury [14]. In SLE, it appears that both the prevention of antigen–antibody complexes and the consequent clearance of ICs at sites of deposition are impaired.

Leucocytes contain a large intracellular store of calreticulin. In the present study we show that calreticulin is released from activated neutrophils, and thus has the potential to associate with serum proteins. Employing native and recombinant calreticulin, and the individual N-, P- and C-domains of this protein, we have attempted to locate the binding domain for C1q–calreticulin interactions and examine whether calreticulin

Abbreviations used: C1qR, collectin receptor; $DGVB^{++}$, isotonic Veronal buffered saline containing 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1 % (w/v) gelatin and 1 % (w/v) glucose; EA, sensitized sheep erythrocytes; ER endoplasmic reticulum; FMLP, formylmethionyl-leucyl-phenylalanine; HAGG, heat-aggregated human IgG; IC, immune complex; MBP, maltose-binding protein; PMA, phorbol 12-myristate 13-acetate; SLE, systemic lupus erythematosus.

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interferes with classical complement activation, which is important for IC solubilization and clearance. In particular, the N-terminal portion of calreticulin was found to (a) reduce C1q-dependent IC binding to neutrophils, and (b) interfere with C1q-dependent haemolysis of sensitized erythrocytes in a dose-dependent manner. These results suggest that the release of calreticulin from leucocytes during inflammation may impede some of the functions of the classical complement pathway that are necessary for IC clearance.

MATERIALS AND METHODS

Antibodies and reagents

Affinity-purified rabbit antibodies against recombinant human calreticulin expressed in the baculovirus system [15] and against synthetic peptides corresponding to N-terminal residues 7–28 and the final C-terminal residues (399–417) of human calreticulin were prepared as previously described [3]. Murine anti-(human C1q) was purchased from Quidel®, San Diego, CA, U.S.A. FITC-labelled rabbit and mouse anti-human IgG were obtained from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A.). Dulbecco's PBS (with and without calcium and magnesium), formylmethionyl-leucyl-phenylalanine (FMLP) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The expression vector pMal-c2, amylose resin and Factor Xa protease were obtained from New England Biolabs (Beverley, MA, U.S.A.).

Serum samples

Blood samples were drawn by venepuncture from healthy consenting adults in accordance with the Declaration of Helsinki of the World Medical Association. Serum was isolated from the blood by centrifugation.

Isolation and preparation of neutrophils

Human neutrophils were isolated from whole blood taken from normal donors by a one-step isolation procedure using Polymorphprep™ (Nycomed), as previously described [16]. The isolated cells (> 95% pure) were then suspended in PBS, pH 7.4, without calcium or magnesium, and maintained at 4 °C. For the experiments requiring stimulated neutrophils, cells were prewarmed to 37 °C for 5 min before stimulation with either FMLP (0.1 μM) or PMA (100 ng/ml) for 15 min. The cells were then washed and resuspended in the appropriate buffer. Neutrophils, before and after stimulation, were stained with Trypan Blue for 10 min and then examined by light microscopy to monitor the integrity of the plasma membrane.

Purification of native C1q and calreticulin

Haemolytically active C1q was isolated from human serum as described by Reid [17] and its purity assessed by SDS/PAGE on a 5–20% (w/v) polyacrylamide gel under reducing conditions. C1q was subjected to linear sucrose gradient centrifugation (5–31%), which confirmed the presence of a single non-aggregated protein. As a precaution, C1q was centrifuged at 4 °C at 14000 ***g*** for 15 min immediately prior to use, to eliminate any aggregates which may have formed during storage. Globular heads of C1q were prepared by digestion of C1q with collagenase purified from *Achromobacter iophagus* [18]. Similarly, collagen tails of C1q were prepared by digestion of C1q with pepsin [19]. The recombinant globular head of the B chain of C1q was produced as described [20]. Native calreticulin was purified using the published method [21].

Construction of plasmids expressing different domains of calreticulin

For the expression and isolation of recombinant proteins, a maltose-binding protein (MBP) fusion system was used consisting of the pMal-c2 plasmid [22]. This expression vector encodes *E. coli* MBP followed by the Factor Xa cleavage site, a unique blunt-end *Xmn*I cloning site at the 5′ end and an *Eco*RI site at the 3′ end. For the purposes of this study, we expressed the protein in three domains: the N-terminal domain (N-domain; amino acids 1–181), the proline-rich domain (P-domain; amino acids 182–292) and the C-terminal domain (C-domain; amino acids 293–417). The putative domain organization was based on the known intron/exon structure [4]. PCR was used to amplify open reading frames encoding full-length calreticulin and the N-, P- and C-domains. A 1.9 kb *Sac*I fragment containing cDNA for human calreticulin was used as a template. The following six terminal primers were designed: FP-ND, 5′ GCCGTCGCCGTCGCCCGGGGAGCCCGCCGTCTAC 3′ (34-mer); RP-ND, 5′ CCCCCCAATTCCTATTCCAAGGAGCCGGA 3′ (30-mer); FP-PD, 5′ GGCTCCTTGGAACCCGGGGACGATTGG 3′ (27-mer); RP-PD, 5′ CCCCCCGAATTCCTAGGCATAGATACTGGG 3′ (30-mer); FP-CD, 5′ CCCAGTATCTATGCCCGGGTATGATAAC 3′ (27-mer); RP-CD, 5′ CCCCCCAGGAATTCTCTACAGCTCGTCCTTGGG 3′ (33-mer). The PCR products corresponding to full-length calreticulin and the N-, P- and C-domains (ND, PD and CD refer to N-, P- and C-domains respectively) were cleaved with *Sma*I and *Eco*RI and subcloned into the pMal-c2 vector restriction-digested with *Xmn*I and *Eco*RI. The recombinant plasmids containing the N-, P- and C-domains of human calreticulin were designated pN-CRT, pP-CRT and pC-CRT respectively.

Expression and purification of recombinant proteins

E. coli BL21 harbouring various constructs were grown in Luria broth containing ampicillin (50 μg/ml) at 37 °C with vigorous aeration to an A_{600} of 0.6. Isopropyl β-D-thiogalactoside (NOVA Biochem) was added to a final concentration of 0.4 mM to induce the P_{tac} promoter in the expression vector. The cells were shaken under these conditions for 3 h and harvested. Cells from 1 litre of culture (3 g of cell pellet) were suspended in 50 ml of lysis buffer [20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 0.25% (v/v) Tween 20, 1 mM EGTA, 1 mM EDTA and 5% (v/v) glycerol]. Lysozyme and PMSF (Sigma) were added to final concentrations of 100 μg/ml and 0.1 mM respectively. All subsequent steps of purification were carried out at 4 °C. The cell suspension was incubated with lysis buffer over ice for 30 min and the lysate was then sonicated at 60 Hz for 30 s bursts with intervals of 1 min (15 cycles) to shear the bacterial chromosomal DNA. After centrifugation at 14000 ***g*** for 30 min at 4 °C, the supernatant was collected and diluted 5-fold with column buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.25% (v/v) Tween 20, 1 mM EDTA and 5% (v/v) glycerol], and loaded on to an amylose resin column (50 ml bed volume; New England Biolabs) equilibrated with the same column buffer. The column was washed successively with 3 bed volumes of column buffer, 5 bed volumes of column buffer without Tween 20 and 5 bed volumes of Factor Xa buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$ and 5% (v/v) glycerol]. The fusion protein, in each case, was eluted with 100 ml of Factor Xa buffer containing 10 mM maltose. The peak fractions were pooled and Factor Xa

(1 unit/μl; New England Biolabs) was added (1 unit of Factor Xa per 100 μg of fusion protein) to release the domains from the fusion partner, wherever required.

Indirect immunofluorescence

Neutrophils were incubated for 30 min with 0.1 μM FMLP or 100 ng/ml PMA in PBS. Then 1×10^4 cells in 10 μl aliquots were allowed to adhere to glass slides for 30 min at 37 °C in a humidified atmosphere. Slides were washed extensively with PBS and fixed in 2% (v/v) paraformaldehyde for 1 h at 4 °C. Cells were washed and then incubated in a 1:20 dilution of preimmune serum or anti-calreticulin antibodies for 1 h. The secondary antibody (1:50 dilution) was added [anti-rabbit IgG $(Fab)'_2$ conjugated to rhodamine; Tago Immunologicals, Burlingame, CA, U.S.A.]. Following a 1 h incubation, the slides were mounted in Cytoseal 60 mounting medium (Stephens Scientific, Riverdale, NJ, U.S.A.). The cells were examined using a Nikon inverted microscope Diaphot TMP employing epifluorescence microscopy under oil immersion at 1000× magnification. A Nikon N6000 camera containing Kodak Tri-X pan 400 ASA black-and-white film was used to photograph the rhodamine-stained cells.

Measurement of calreticulin levels by ELISA

Cell suspension media from control and stimulated cells were collected by gently pelleting the cells at 325 ***g*** for 1 min. The supernatant was spun at 16000 ***g*** for 20 min to remove any particulate matter, and then coated on to microtitre plate wells. BSA and calreticulin purified from Wil-2 cells (150 ng/well) were used as negative and positive control proteins respectively. All samples were prepared in sodium carbonate buffer (pH 9.6) at the required concentration and allowed to bind to the wells for 14 h at 4 °C. Preimmune rabbit IgG acted as a non-specific immunoglobulin. Plates were washed in PBS/Tween 20 and non-specific binding sites were blocked with 5% (w/v) fat-free milk/100 mM glycine. Immunoaffinity-purified anti-calreticulin antibody (1:200 dilution) was added to each well for 1 h at 37 °C, followed by horseradish peroxidase-conjugated anti-rabbit polyclonal IgG (1:1000 dilution). 3,3′,5,5′-Tetramethyl benzidine-peroxidase substrate was added to each well and the colour was allowed to develop for 20 min. The reaction was terminated by adding 150 μl of 2 M H_2SO_4 and the A_{450} of the plate was read.

Assay of C1q-dependent haemolysis

C1q-deficient serum (Sigma) was diluted 1:40 times in $DGVB^{++}$ [isotonic Veronal buffered saline containing 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% (w/v) gelatin and 1% (w/v) glucose], and various concentrations of purified C1q were added in a final volume of 100 μl prior to incubation at 37 °C for 1 h. C1q haemolytic assays were performed by adding 100 μl of sensitized sheep erythrocytes (EA) (10^7 cells) in $DGVB^{++}$ and incubating for a further 1 h. The reaction was stopped by transferring the tubes to an ice bath and adding 0.6 ml of ice-cold $DGVB^{++}$. The unlysed cells were pelleted by centrifugation and the A_{412} values of 100 μl aliquots of supernatant were read to monitor haemoglobin release. The amount of C1q required to cause 75–85% haemolysis was determined.

Various samples for assay of C1q-dependent haemolysis were prepared as follows. Highly purified C1q (3 μg) was added to various concentrations of native or recombinant calreticulin or its domains (0–4 μg) in a final volume of 100 μl of $DGVB^{++}$ and incubated at 37 °C for 60 min. To each sample was added 100 μl of EA (10^7 cells in total), followed by incubation at 37 °C for an additional 1 h. The unlysed cells were pelleted and the amount of haemoglobin released was determined spectrophotometrically at 412 nm. Total haemolysis was the amount of haemoglobin released upon cell lysis with water. C1q-dependent haemolytic activity was expressed as a percentage of total haemolysis. *E. coli* MBP was used as a control protein.

Binding of heat-aggregated human IgG (HAGG) to neutrophils in the presence of C1q and calreticulin

IgG was isolated from human serum as described previously [23]. Purified IgG (18–20 mg/ml) in water was heated to 63 °C for 30 min. The immunoglobulin was centrifuged at 14000 ***g*** for 20 min to remove insoluble precipitates. The HAGG was then adjusted to a concentration of 10 mg/ml and stored at 4 °C. C1q (30 μg/ml) was incubated with and without calreticulin (5 μg) or its domains for 1 h at 37 °C, and then incubated again with HAGG at a final concentration of 50 μg/ml for an additional 1 h at 37 °C. Aliquots (100 μl) of the various preparations were then incubated with 5×10^5 neutrophils in PBS for 30 min at 4 °C. After washing, the binding of HAGG, HAGG–C1q and HAGG–C1q–calreticulin preparations to neutrophils was determined by incubating the cells with rabbit anti-human IgG (1:50 dilution) followed by goat anti-rabbit FITC-conjugated antibody (1:50 dilution). Analysis was performed on 5000 cells by flow cytometry employing Lysis II software version 1.1 (Becton Dickinson) and the mean fluorescence intensity was presented.

RESULTS

Release of calreticulin from activated neutrophils and detection in serum

To study the release of calreticulin from activated neutrophils, cells were stimulated with 0.1 μM FMLP or 100 ng/ml PMA or left untreated for 15 min at 37 °C. Anti-calreticulin antibody against the full-length protein was used to detect extracellular release of calreticulin into the culture medium from stimulated neutrophils from seven normal subjects. ELISA revealed the presence of calreticulin in different amounts (Figure 1, part i). The concentration of calreticulin detected in the extracellular media from 1×10^5 non-stimulated and PMA-stimulated cells incubated at 37 °C ranged between 1 and 4 ng, but a 2–3-fold increase was observed after stimulation with FMLP, with values ranging between 2 and 12 ng/10^5 cells ($n = 7$, $P < 0.05$). To investigate further whether the stimulation by FMLP resulted in the release of calreticulin into the medium, immunofluorescence microscopy was used. Cells (10^7/ml) in PBS from healthy donors were stimulated under the same conditions, then probed with anti-calreticulin and goat anti-rabbit antisera conjugated to rhodamine to analyse calreticulin surface localization and expression in non-permeabilized neutrophils. As shown in Figure 1, part (ii) (panels A and C), non-stimulated and PMA-stimulated cells showed evidence of surface staining with anti-calreticulin antibodies. However, a striking difference was observed for FMLP-stimulated cells (Figure 1, part ii, panel B); the calreticulin staining on these cells, as well as being evident on or near the surface, was also present extracellularly. As a control for non-specific staining, neutrophils were stimulated in a similar fashion and probed with rabbit preimmune antiserum, which resulted in very low levels of fluorescence (results not shown).

In order to determine if significant levels of calreticulin could be detected in the sera of healthy subjects, calreticulin was measured in 23 control sera. As expected, only a small number of sera samples had measurable levels of calreticulin, with a mean value of 1.94 μg/ml, whereas in the overall majority of samples (18 out of 23), calreticulin was not detected. The C1q levels in the

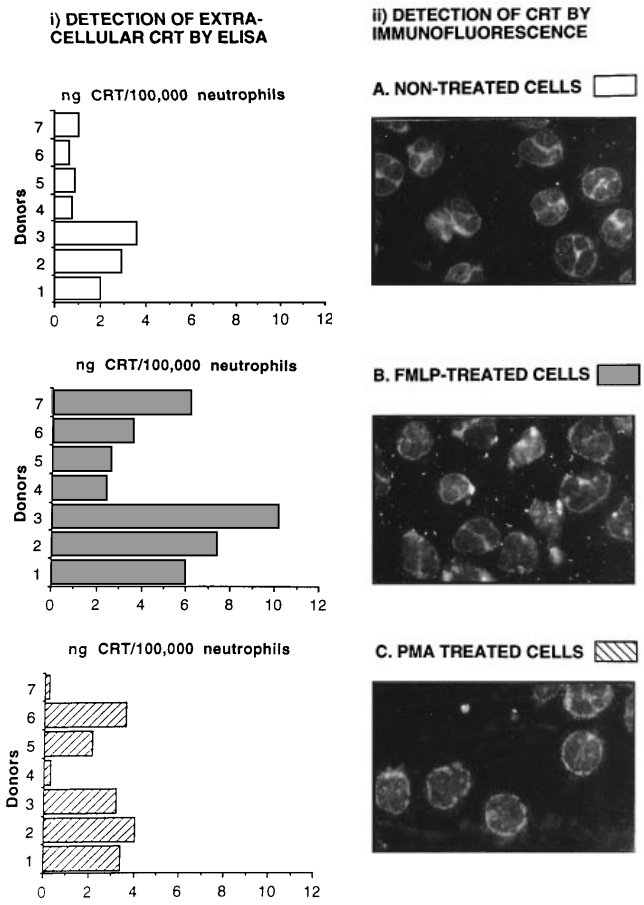

Figure 1 Release of calreticulin from stimulated neutrophils

Part (i): ELISA analysis of calreticulin released into the cell suspension buffer from non-stimulated and stimulated neutrophils from seven normal donors. Neutrophils (10^5/100 μl) were incubated with PBS, 0.1 μM FMLP or 100 ng/ml PMA for 15 min at 37 °C. The cells were pelleted at low speed and the cell suspension media were assayed for calreticulin (CRT) by a standard ELISA method. The A_{450} was read and the amount of calreticulin calculated from a standard curve obtained with purified calreticulin. Part (ii): indirect immunofluorescence of calreticulin localization in neutrophils under non-stimulated (**A**), FMLP-stimulated (**B**) and PMA-stimulated (**C**) conditions. Magnification approx. $\times$1500.

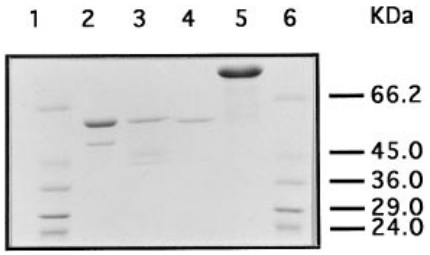

Figure 2 Purification of recombinant calreticulin and its N-, P- and C-domains

Lanes 1 and 6 contain the molecular mass standards shown (kDa). Lane 5 contains purified full-length calreticulin fused to MBP. Lanes 2–4 contain the N-, P- and C-domains respectively of calreticulin fused to MBP, after purification from an amylose resin column. All the samples were run on SDS/PAGE [10% (w/v) gel] after reduction of the disulphide bonds.

Table 1 C1q binds predominantly to the N-terminal half of calreticulin

Microtitre wells were coated with 0.25, 1.0 or 4.0 μg of full-length calreticulin or its N-, P- or C-domains. MBP and BSA were used as control proteins. After washing three times and blocking with milk/PBS, 125 ng of C1q (50 μl) was added to each well in the presence of normal or half physiological salt concentrations for 2 h at 37 °C. After additional washing, binding of C1q to each protein was detected by probing with rabbit anti-(human C1q) (1:5000 dilution), followed by anti-rabbit IgG (1:4000 dilution) conjugated to horseradish peroxidase, each for 1 h at 37 °C. The ELISA plates were then developed and A_{450} readings taken. Results are means $\pm$ S.D. of three experiments.

Amount of calreticulin or domain added/well (μg)	Binding (A_{450} units)			
	Native CRT	N-domain	P-domain	C-domain
Half physiological salt concentration				
4.0	0.89 $\pm$ 0.23	0.95 $\pm$ 0.22	0.43 $\pm$ 0.16	0.26 $\pm$ 0.12
1.0	0.78 $\pm$ 0.36	0.44 $\pm$ 0.09	0.16 $\pm$ 0.03	0.12 $\pm$ 0.09
0.25	0.43 $\pm$ 0.20	0.24 $\pm$ 0.24	0.15 $\pm$ 0.18	0.19 $\pm$ 0.22
Physiological salt concentration				
4.0	0.23 $\pm$ 0.14	0.37 $\pm$ 0.14	0.07 $\pm$ 0.03	0.04 $\pm$ 0.04
1.0	0.07 $\pm$ 0.06	0.17 $\pm$ 0.06	0.03 $\pm$ 0.05	0.02 $\pm$ 0.06
0.25	0.07 $\pm$ 0.06	0.05 $\pm$ 0.06	0.01 $\pm$ 0.06	0.02 $\pm$ 0.09

same subjects were also measured and were found to be normal (56.4 $\pm$ 14.9 μg/ml; mean $\pm$ S.D.).

Recombinant production of human calreticulin and its various domains in *E. coli*

Human calreticulin was expressed as three distinct domains. The expression and purification of each fusion protein containing *E. coli* MBP was performed as described previously [24]. Calreticulin migrated with an apparent molecular mass of 60 kDa on SDS/PAGE, although the predicted molecular mass is 46 kDa. The MBP–calreticulin fusion protein also migrated with the same anomalous mobility as a major band of 97 kDa (Figure 2, lane 5). The three domains of calreticulin, N-, P- and C- (Figure 2, lanes 2–4 respectively), of predicted molecular masses of 20, 16 and 16 kDa respectively, migrated as bands of approx. 60 kDa when fused to MBP (42 kDa). The full-length recombinant protein and the three domains reacted with polyclonal rabbit anti-(human calreticulin) antiserum in Western blot analysis (results not shown). MBP alone did not react with anti-calreticulin antiserum.

Identification of the region of calreticulin that binds to C1q

As calreticulin is known to bind to C1q, it was felt important to localize further the region of calreticulin that binds to C1q. Therefore various amounts of the N-, P- and C-domains of calreticulin (4, 1 and 0.250 μg) were bound on ELISA plates overnight. A polyclonal antibody against calreticulin was used to confirm the binding of each recombinant domain to the wells in a dose-dependent manner. The domains were then incubated with 125 ng of native C1q in PBS or half-strength PBS for 2 h at 37 °C, followed by probing with rabbit anti-(human C1q) antibody (1:3000 dilution) and anti-rabbit antiserum conjugated to horseradish peroxidase. As shown in Table 1, C1q bound predominantly to the N-domain of calreticulin under physio-

Table 2 Calreticulin binding to different regions of C1q

Microtitre wells were coated with 0.25, 1.0 or 4.0 μg of C1q, the collagen tails of C1q, the globular heads of C1q or the recombinant globular head of the B chain of C1q (ghB). After washing three times and blocking with 1% (w/v) milk/glycine/PBS, 0.5 μg of native calreticulin was added to each well and incubated in normal and half-strength PBS for 2 h at 37 °C. After additional washing, calreticulin binding was determined by probing with rabbit anti-(human calreticulin) (1:4000 dilution, 2 h, 37 °C) and then analysed by ELISA and A_{450} values measured. Results are means ± S.D. of triplicate experiments. MBP acted as a negative control protein.

Amount of C1q or fragments added/well (μg)	Binding (A_{450} units)				
	Native C1q	C1q tails	C1q heads	ghB	MBP
Half physiological salt concentration					
4.0	1.84 ± 0.34	0.08 ± 0.09	0.23 ± 0.30	0.48 ± 0.09	0.08 ± 0.01
1.0	1.55 ± 0.30	0.10 ± 0.03	0.20 ± 0.01	0.37 ± 0.04	0.08 ± 0.01
0.25	0.62 ± 0.02	0.06 ± 0.03	0.06 ± 0.03	0.31 ± 0.01	0.08 ± 0.02
Physiological salt concentration					
4.0	1.15 ± 0.06	0.08 ± 0.01	0.43 ± 0.16	0.26 ± 0.12	0.06 ± 0.03
1.0	0.65 ± 0.06	0.08 ± 0.01	0.16 ± 0.03	0.12 ± 0.09	0.07 ± 0.02
0.25	0.29 ± 0.09	0.07 ± 0.00	0.15 ± 0.18	0.19 ± 0.22	0.09 ± 0.08

logical salt conditions. When less stringent salt conditions were employed, C1q binding to the N-domain was enhanced, and even weak binding to the P- and C-domains was observed.

Identification of the region of C1q that binds to calreticulin

To identify the region of C1q that binds to calreticulin, a range of concentrations of whole C1q, the collagen-like tail region and the globular head region of C1q, as well as the recombinant globular head B chain region of C1q (U. Kishore, P. Eggleton and K. B. M. Reid, unpublished work), were immobilized on the surface of ELISA plates. A rabbit anti-(human C1q) polyclonal antibody was used to confirm that the C1q fragments had bound to the plates in a dose-dependent manner. Calreticulin bound most strongly, and in a dose-dependent manner, to whole C1q and the globular head region of C1q, in particular the globular head B chain region (Table 2). This binding was enhanced at half ionic strength. Calreticulin bound very weakly to the collagenous tail region of C1q. C1q plays an important role in the prevention of the formation of IC and in the opsonization and clearance of any IC formed. The globular head region of C1q is important for binding to the Fc region of immunoglobulins. These results strongly suggest that calreticulin binding to C1q may interfere with C1q-mediated functions involving the globular head region.

Inhibition of complement-dependent haemolysis by calreticulin

Activation of the classical pathway of complement is brought about by the binding of the globular head regions of C1q to the F_c portions of antibody IgM or IgG in the IC. In view of the interaction between calreticulin and the C1q globular head region, a C1q-dependent haemolytic assay was used to study the effect of calreticulin on complement activation. The assay of C1q-dependent haemolysis requires C1q to be added back to C1q-deficient serum in order to reconstitute the C1 complex. In the present study, the addition of C1q (15 μg/ml) back to C1q-deficient serum was sufficient to lyse 70–80% of EA. This concentration of C1q was then chosen as the standard in a series of studies to determine whether C1q–calreticulin binding results in inhibition of C1q-dependent haemolysis. Initially, various

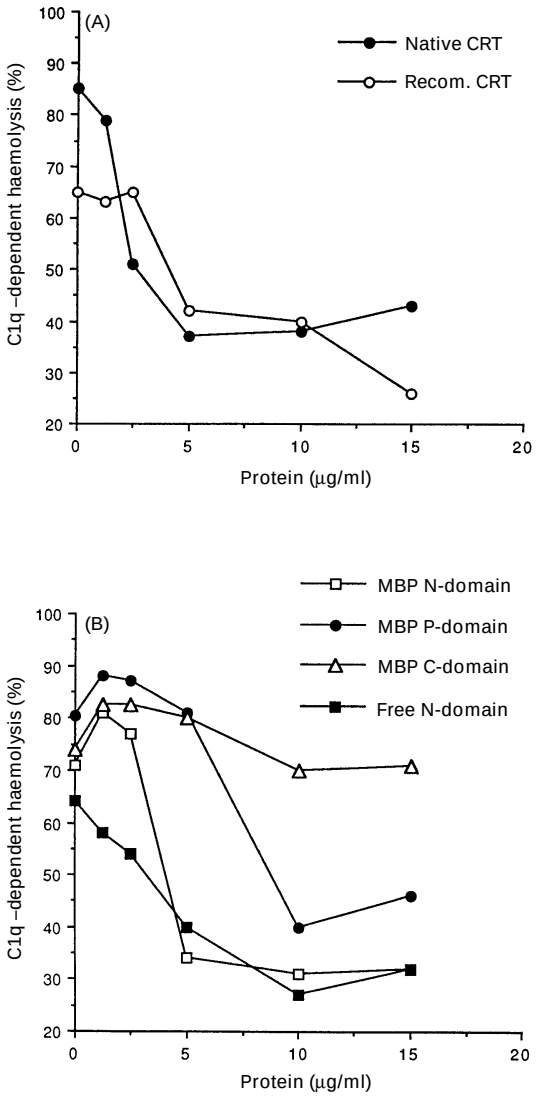

Figure 3 Inhibition by calreticulin of the C1q-dependent haemolysis of IgM-sensitized erythrocytes

(**A**) Different concentrations of native or recombinant (recom.) calreticulin (CRT) were added to a 1:40 dilution of C1q-deficient serum together with suboptimal amounts of C1q, and incubated for 1 h at 37 °C. Then 1×10^7 EA in 100 μl were added and incubated for an additional 1 h at 37 °C. The non-lysed cells were pelleted and the A_{405} values of the supernatants were measured. The percentage lysis was determined relative to complete (100%) lysis of cells. (**B**) Inhibition of the C1q-dependent lysis of EA by the N- and P-domains of calreticulin. Recombinant N-, P- and C-domains were employed in the assay described above. The means of three to six experiments are presented for each protein or domain.

concentrations of native calreticulin, as well as recombinant N-, P- and C-domains, at concentrations ranging from 0.125 to 40 μg/ml were incubated with 15 μg/ml C1q for 60 min at 37 °C before addition of the mixtures to EA. The addition of 2–5 μg/ml native, full-length recombinant calreticulin or N-domain lowered the haemolytic activity of C1q from 70–80% to below 20% (Figure 3A). A similar result was obtained when 10 μg/ml N- or P-domain was used instead of native calreticulin (Figure 3B). The C-domain of calreticulin had no effect on C1q-dependent haemolytic activity. Normal serum (diluted 1:20) and C1q-deficient serum containing no C1q or calreticulin were used as controls. The C1q-dependent haemolytic activity of normal

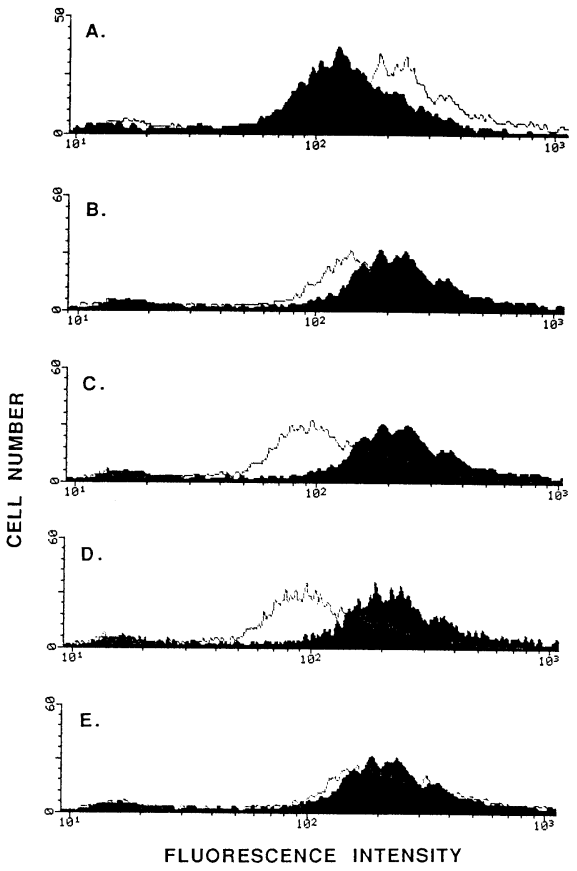

Figure 4 Flow cytometric analysis of the effects of C1q and calreticulin on binding of HAGG to neutrophils

C1q (30 μg/ml) was incubated with calreticulin or one of its three functional domains, or with MBP alone, for 1 h at 37 °C. Then 50 μg of HAGG was added followed by incubation with the various C1q–calreticulin preparations for a further 1 h at 37 °C, as described in the Materials and methods section. (**A**) HAGG binding alone (filled) or after incubation with C1q (open). (**B**)–(**E**) HAGG–C1q binding (filled) and decrease in HAGG–C1q binding in the presence of calreticulin (open): (**B**) full-length calreticulin, (**C**) N-domain, (**D**) P-domain, (**E**) C-domain. MBP alone had no effect on C1q-enhanced HAGG binding. The mean fluorescence intensities of control cells probed with primary antibody alone or with both primary and secondary antibodies were < 6 and < 60 respectively.

serum without the addition of calreticulin or additional C1q produced between 78 and 85% EA haemolysis, while C1q-deficient serum not supplemented with C1q or calreticulin resulted in only 18% haemolysis.

Effect of calreticulin on binding of HAGG to neutrophils

C1q is known to enhance IC binding and uptake by neutrophils [25]. In the present study, preincubation of 50 μg of HAGG with 30 μg of C1q led to an increase in HAGG binding to the cell surface (Figure 4A), increasing the mean fluorescence intensity from 158 to 293. As shown in Figures 4(B)–4(D), when pre-incubated with native calreticulin or its N- or P-domain, the C1q-mediated binding of HAGG to cells was impaired to various degrees, with the mean fluorescence intensity decreased from a value of 293 to values of 222, 197 and 132 respectively. The C1q-independent binding of HAGG via Fc receptors was not affected by preincubation with calreticulin or its domains. The C-domain of calreticulin (Figure 4E) did not impair the C1q-dependent enhancement of HAGG binding to neutrophils. MBP was used as a control protein and had no effect on HAGG binding.

DISCUSSION

Calreticulin was first purified over 20 years ago [26], but the precise functions of this protein are still unknown. It has been implicated as an immunodominant antigen in autoimmune disease [3]. The protein has been localized to many intracellular and extracellular sites other than the ER, including the nuclear envelope [27], cytoplasmic granules [6], cell surface [8,28,29] and bloodstream [7,30]. The N-terminal amino acid sequence of one form of C1q receptor (C1qR; collectin receptor) is identical to that of calreticulin. In addition to binding to C1q, a C1q receptor has been proposed to be involved in the clearance of ICs from the circulation [31]. To date, it has been difficult to differentiate between C1qR and calreticulin at the biochemical and molecular levels. Further evidence that calreticulin and C1qR are similar proteins comes from our previous study in which C1q was found to bind to native full-length calreticulin [11]. In the present study, we wished to further dissect the molecular interaction between C1q and calreticulin and to address some of the biological implications of such an interaction. We observed the presence of detectable levels of calreticulin in the serum of approx. 20% of healthy individuals. We also demonstrated that stimulation of neutrophils, a major source of calreticulin, with FMLP can lead to the release of calreticulin *in vitro*. Calreticulin may be released from neutrophil minor granule stores. However, attempts to isolate calreticulin from the purified major primary and secondary granules of neutrophils have not been successful [11]. Alternatively, calreticulin may be released upon perturbation of the plasma membrane during cell activation. Stendahl and co-workers [32] have shown that calreticulin and the Ca^{2+} storage marker, a Ca^{2+}-dependent ATPase, both become concentrated in the filamentous actin-rich cytoplasmic area around ingested particles during neutrophil activation. This could be a means of calreticulin release by the cells during active phagocytosis of ICs or bacteria. Alternatively, FMLP is known to activate the rapid release of Ca^{2+} from intracellular stores and to induce degranulation of a number of secretory vesicles, which may account for the release of calreticulin into the extracellular environment. Moreover, recent evidence from Rosen and co-workers [33] concerning the morphological sequence of apoptosis has shown that many autoantigen clusters originating from the ER are contained within small blebs which are translocated to the cell surface. Calreticulin is believed to be present in these blebs, together with other autoantigens including Ro, La and nucleosomal DNA [33a]. The small particulate extracellular matter observed in the FMLP-treated neutrophils in the present study stained strongly for calreticulin, consistent with this mechanism of autoantigen translocation.

A functional interaction between calreticulin and proteins involved in vascular homoeostasis has been examined previously. Calreticulin has been identified as an anti-thrombotic agent which binds to vitamin K-dependent coagulation factors, stimulates endothelial nitric oxide production and limits thrombosis in coronary arteries [30]. In order to study the interaction between calreticulin and the serum component C1q, the N-, P- and C-domains of calreticulin were expressed in *E. coli*. The first domain, which consists of the N-terminal half of the molecule, contains eight anti-parallel β-strands connected by protein loops. This domain is neutrally charged and has been found to contain binding sites for a number of proteins, including the DNA-binding domain of steroid receptors and the α-subunit of integrins [34,35]. The central P-domain comprises a proline-rich sequence

which, according to hypothetical modelling analysis, may keep the N- and C-terminal portions of the molecule spatially separate. The C-terminal region of the protein is highly acidic and negatively charged, and is thought to interact with blood clotting factors [30]. To test whether the binding of purified C1q to various domains of calreticulin is dependent upon ionic strength, some binding assays were performed under conditions of half-salt concentrations. Binding of C1q to the N-domain occurred under both physiological and half-salt conditions. However, binding of C1q to the P-domain only occurred under the more artificial low-ionic-strength conditions. The hypothesis that calreticulin–C1q complex-formation may be due to non-specific charge–charge interactions is unlikely, since the preponderance of negatively charged residues in the C-domain of calreticulin might have been expected to interact with charged regions of the C1q molecule; however, this is not the case. Having established the N- and P-domains of calreticulin as the sites for C1q interaction, we next examined which region of C1q binds to calreticulin. In ELISA, where native C1q, the collagen tail region, the native globular head region and the recombinant globular heads of C1q were immobilized, calreticulin bound most strongly to whole C1q and the globular heads of C1q, and insignificantly to the collagen tails.

Plasma C1q is the major molecule involved in the initiation of the complement cascade classical pathway. The globular head region of C1q binds specifically to ICs, initiating and subsequently amplifying the classical pathway of complement activation, and thereby preventing the formation of precipitating ICs in plasma. Thus the interaction of calreticulin with the globular heads of C1q probably interferes with mechanisms involving complement activation. In order to investigate this possibility further, a series of *in vitro* studies was undertaken. That calreticulin was in fact blocking the C1q–immunoglobulin interaction was confirmed when both native and recombinant full-length calreticulin were shown to be potent inhibitors of C1q-dependent haemolytic activity. The region involved could be localized to the N-terminal half (N- and P-domains) of the calreticulin molecule. Although C1q binds predominantly to the N-domain of calreticulin, which is also the most autoantigenic domain of the protein [21], incubation of C1q with the N-domain did not interfere with autoantibody binding to calreticulin (U. Kishore, K. B. M. Reid and P. Eggleton, unpublished work). This suggests that the main autoantigenic site on calreticulin and the C1q binding site are different.

The majority of circulating ICs are cleared by erythrocytes after binding to these cells via CR1 receptors. However, once ICs begin to accumulate in other tissues, leucocyte migration to these sites of IC deposition can play a role in their phagocytosis and clearance. IC binding to neutrophils is enhanced in the presence of 30 μg/ml C1q [25]. This suggests that binding of ICs to neutrophils not only is mediated through Fc receptors present on these cells, but is also enhanced by C1q-binding proteins on the cell surface, of which there are several candidates [28,36–38]. A 33 kDa C1q-binding protein, isolated from the plasma membranes of neutrophils [39], specifically binds to the globular head region of C1q and may function to enhance IC binding to neutrophils directly, or to enhance cross-linking of ICs to Fc receptors. Co-incubation of C1q with calreticulin partially prevented the enhanced binding of HAGG to neutrophils, mediated by C1q alone. These results suggest that calreticulin is capable of inhibiting the C1q-mediated binding of aggregated IgG to neutrophils, but not HAGG binding directly. Surprisingly, intact calreticulin appeared less effective than either the N- or the P-domain in inhibiting C1q-dependent binding of HAGG to cells. As the C-domain contains the majority of the acidic residues found in the protein, the improved efficiency of the N- and P-domains in inhibiting the C1q-mediated binding of HAGG to cells may be due to elimination of the negative charge contained within the C-domain of the whole calreticulin molecule. That ionic interactions may be influential for the calreticulin–C1q interaction was suggested by our observations that the binding of C1q to the full-length calreticulin as well as to the N-domain increased with decreasing ionic strength. To date, there have been no reports that calreticulin binds specifically to immunoglobulins or interferes with immunoglobulin-mediated functions.

Confirmation of calreticulin release into the serum comes from two sources: first, the direct demonstration of elevated levels of calreticulin in the sera of SLE patients and second, the presence of autoantibodies against the protein in such sera. Calreticulin appears to interfere with the early phase of complement activation by (a) decreasing IC binding to leucocytes, and (b) inhibiting complement-dependent haemolysis. Such interactions with C1q could be of pathophysiological significance, whereby inhibition of complement activation may lead to a decrease in the efficient processing of ICs or the interactions of C1q with its various candidate receptors. Moreover, at sites in other tissues where ICs have accumulated, calreticulin may impede efficient IC binding and clearance by phagocytes recruited to the sites of inflammation. This is currently under investigation.

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