# Evidence That C1q Binds Specifically to $C_H2$-like Immunoglobulin $\gamma$ Motifs Present in the Autoantigen Calreticulin and Interferes with Complement Activation†

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ABSTRACT: Calreticulin (CRT) is located predominantly in the endoplasmic reticulum (ER) of cells, where it functions as a quality control controller of protein folding. However, CRT is also a prevalent autoantigen in patients with systemic lupus erythematosus (SLE), where its release from the cell may arise as a results of dysfunctional apoptosis and inefficient removal of ER vesicles, which are an abundant source of CRT and other autoantigens. Indicative of this is the presence of autoantibodies against CRT in the sera of 40−60% of all SLE patients. Once released into the circulation, CRT might bind directly to C1q and we have suggested that this association may result in a defect in C1q-mediated clearance of antigen−antibody complexes. It has been previously shown that CRT under physiological salt conditions binds to the globular head of C1q. It is known that the globular head region of C1q binds to the $C_H2$ domain in the Fc portion of immunoglobulin $\gamma$ (IgG). The N-terminal half of CRT contains a number of short regions of 7−10 amino acids that show sequence similarity to the putative C1q binding region in the $C_H2$ domain of IgG. By use of a series of 92 overlapping CRT synthetic peptides, a number of C1q binding sites on the CRT molecule have been identified, including several containing a $C_H2$ -like motif similar to the ExKxKx C1q binding motif found in the $C_H2$ domain of IgG. A number of these peptides were shown to inhibit binding of C1q to IgG and reduce binding of native CRT to C1q. Moreover, several of the peptides were capable of inhibiting the classical pathway of complement activation. These studies have identified specific binding sites on the CRT molecule for C1q and lend support to the hypothesis that interaction of CRT with C1q may interfere with the ability of C1q to associate with immune complexes in autoimmune-related disorders.

Calreticulin (CRT)[1] is a calcium binding protein found in all eukaryotic cells, except erythrocytes. It is localized mainly in the endoplasmic reticulum, but it is also found in other cellular compartments and several biological functions have been proposed (*1*). Calreticulin has occasionally been found extracellularly, and evidence to explain how CRT may be released from cells during infection, stress, and cell death has recently been reviewed (*2*). Once in the extracellular environment, CRT is of clinical relevance as it is an autoantigen prevalent in systemic lupus erythematosus (SLE) disease (*3*). Calreticulin (*4*), together with C1qRp (*5*) and CR1 (*6*), belongs to a group of proteins that have recently been identified as binding to C1q, a subcomponent of the first component of complement. Initially all three C1q binding proteins were thought to act as receptors for C1q. However, recent evidence has revealed that CRT is an intracellular protein that, upon excretion from cells, may interfere with the activation of the complement cascade at sites of inflammation by binding to C1q, possibly interfering with C1q interaction with antibody-associated immune complexes (IC). Alternatively, the targeting of CRT by C1q may be a protective innate immune response by the host to remove the autoantigen from the circulation, leaving C1q unavailable for interaction with immune complexes.

It has been proposed that CRT can be divided into three structurally distinct domains (*7*, *8*), termed the amino-terminal (N-domain), proline-rich (P-domain), and carboxy-terminal (C-domain). Under various ionic conditions, both the collagen-like region and globular heads of C1q have been shown to bind to the N-terminal half of CRT containing the N- and P-domains in vitro (*9*, *10*). While the CRT binding site on the collagen-like region of C1q remains to be identified, the globular head, and in particular the B-chain, of C1q appears to contain a CRT binding site (*10*). Moreover, in the same study, full-length CRT, the N-domain, and also the P-domain to a lesser extent are potent inhibitors of C1q hemolytic activity. The fact that CRT competes with antibody for binding to C1q implies that CRT and immunoglobulins may share similar binding sites recognized by C1q. Further,

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[1] Abbreviations: CRT, calreticulin; $DGVB^{2+}$, 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; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FL, full length; IgG, immunoglobulin $\gamma$; ghC1q, globular heads of C1q; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; SLE, systemic lupus erythematosus disease; PBS, phosphate-buffered saline.

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the C1q binding of the full-length CRT and its N-domain is enhanced at half ionic strength of the buffer compared to physiological salt concentration (*10*). This indicates that the interactions between CRT and C1q are mainly of a polar or electrostatic type.

In the initial step of classical complement activation, C1q interacts with immunoglobulins associated with immune complexes (ICs). It is known that C1q binds to the $C_H2$ domain in the Fc portion of mouse IgG2b and, more specifically, to a C-terminal sequence with the motif ExKxK (positions 318, 320, and 322) (*11*). Further determinants for complement activation on the hinge-region of human IgG have also been identified (*12*). Duncan and Winter (*11*) showed also that, in the ExKxK motif, the glutamine can be replaced by a threonine or asparagine and the lysines by arginines without impairing the lytic propensity of IgG2b. A mutant with the middle lysine replaced by a glutamine had similar affinity for C1q as the wild type but was nonlytic. There are other three variations of the ExKxK motif present in the sequence of $C_H2$ region of IgG2b. These are KxKxT (positions 246, 248, and 250), NxKxK (positions 286, 288, and 290) and KxRxE (positions 290, 292, and 294). Examination of the three-dimensional crystal structure of $C_H2$ of IgG2b (*13*) shows, however, that none of these three short fragments align the charged/polar residues in a linear fashion. This is in contrast to the correct binding site, where the charged residues are arranged on a $\beta$-strand with the side chains pointing out into solution. Hence a linear arrangement appears to be mandatory for C1q binding.

The aim of the present study was to identify potential C1q-binding sites in the CRT sequence encompassing the N- and P-domains of the protein. Initial examining of the primary sequence revealed that the C1q-binding motif of IgG, that is, ExKxK, is present in the sequence comprising CRT N- and P-domains. Peptide fragments containing the potential interaction sites were produced synthetically and comprised the main focus of this study. A peptide with the sequence AEAKAKA mimicking the binding site of IgG was also synthesized and used as a positive control in analogy with earlier studies (*11*). In order that other potential C1q binding sites on CRT molecule unrelated to $C_H2$-like motifs were identified, a series of overlapping peptides spanning the whole N- and P-domains were prepared. The assumption that a flexible peptide fragment, which interconverts rapidly between random conformations in solution, mimics the behavior of the binding site in the context of a complete folded protein is justified in this particular case. First, the interaction appears to be of strong but fairly nonspecific character, as it is governed by electrostatic interactions. Second, an unstructured, flexible peptide in dynamic conformational equilibrium in solution readily adopts the required linear arrangement. The present investigation enabled us to identify possible interaction sites, but as long as the three-dimensional architecture of the complete folded protein is not known, it is not known for certain whether all the C1q binding peptides identified would be accessible on the surface of CRT in a linear orientation. The biological activity of the peptide fragments thus recognized is verified through their propensity to inhibit hemolysis of IgG-sensitized erythrocytes. Furthermore, the ability of C1q and the globular heads of C1q to bind to the peptides and the propensity of the peptides to competitively inhibit C1q binding to aggregated IgG was determined.

## EXPERIMENTAL PROCEDURES

*Multiple Sequence Alignments and Secondary Structure Prediction.* Initially a multiple sequence alignment was performed on residues 18−308 from the human CRT, comprising the N- and P-domains while excluding the 17-residue signal sequence. Predictions of secondary structure and solvent accessibility were carried out on the basis of the alignment. The automatic PredictProtein www-server of Rost (*14*) was employed to scan the sequence library of the SWISSPROT data bank. The secondary structure prediction was repeated by using the automatic www-server of Geourjon and Deleage (*15*), which provides predictions accomplished by four different prediction algorithms and their consensus. The various methods yielded virtually identical results.

*Preparation of the Peptides.* A set of overlapping peptides, 15 amino acid residues long, spanning the whole N- and P-domains of CRT were synthesized by Fmoc-based solid-phase peptide synthesis with a BT7400 manual peptide synthesizer (Biotech Instruments Ltd., Kimpton, Hertfordshire, U.K.). The peptides were made as peptide amides using a Rink Amide MBHA acid-labile linker on a polystyrene resin support and were cleaved from the support with 90% trifluoroacetic acid containing phenol and triisopropylsilane as scavengers. Following lyophilization, peptides were analyzed by reverse-phase HPLC [Gilson (Anachem, Luton, U.K.)] using an analytical C18 column [Vydac (Anachem, Luton, U.K.)]. Additional sets of shorter $C_H2$-like IgG motif peptides (see Table 2) were obtained from the Oxford Centre for Molecular Sciences (OCMS) Peptide Synthesis Facility. Peptides were synthesized by standard Fmoc chemistry on an Applied Biosystems synthesizer. Preparative reverse-phase high-performance liquid chromatography (HPLC) was used to purify the peptides and they were lyophilized prior to resuspension in the appropriate buffer. The purity of the peptides was confirmed by electrospray mass spectroscopy and analytical HPLC.

*Purification of Full-Length C1q and Globular Heads of C1q.* Hemolytically active C1q was isolated from 100 mL of human serum on the basis of a modified method of Reid's (*16*). Briefly, serum was dialyzed against 5 L of water overnight at 4 °C, and the resulting precipitate was harvested by centrifugation at 10000*g* and solubilized in 30 mL of 500 mM NaCl, 20 mM Tris-HCl, and 10 mM EDTA, pH 7.4. The C1q-containing solution was further diluted five times with Tris−EDTA buffer (TEB; 20 mM Tris-HCl and 5 mM EDTA, pH 7.4) and passed through a 40 mL FPLC Q-Sepherose column (Pharmacia) to retain C1r/s and IgM. The C1q-enriched flowthrough was then applied to a SP−Sepharose column (Pharmacia). IgG was eluted first by extensive washing of the column with 150 mM NaCl in TEB and finally the C1q was eluted with a salt gradient over the concentration range 150−500 mM NaCl in TEB. The resulting C1q was concentrated by ultrafiltration and separated from any remaining immunoglobulins on a 100 mL Superose 6 size-exclusion column (Pharmacia). The purity of C1q was assessed by SDS−PAGE on a 5−20% (w/v) polyacrylamide gel under reducing conditions where it appeared as three bands, corresponding to the a, b, and c chains of 34, 32, and 27 kDa, respectively. As a precaution, immediately prior to use, C1q was centrifuged at 4 °C at 14000*g* for 15 min to eliminate any aggregates that may have formed during storage. Globular heads of C1q (ghC1q) were

prepared by digestion of C1q with collagenase purified from *Achromobacter iophagus* (*17*), resulting in an equimolar mixture of the A, B, and C heads. Briefly, 1−5 mg of C1q was dialyzed overnight with dialysis buffer (50 mM Tris, 150 mM NaCl and 10 mM $CaCl_2$, pH 7.2) and concentrated by ultrafiltration to a volume of 1−5 mL. Then between 1 and 5 mg of C1q was incubated with 1 mg of collagenase (Sigma Chemical Co.) overnight at 37 °C. Finally the ghC1q are separated from collagenase and undigested C1q by size-exclusion chromatography on a Superose 12 column. The purity of both C1q and the globular heads of C1q used in study was assessed by SDS−PAGE.

*Biotinylation of C1q and Calreticulin.* The biotinylation procedure was identical to the method published previously, which provides biotinylated C1q in a functional state as assessed by hemolytic activity (*18*).

### *Solid-Phase Enzyme-Linked Immunosorbent Assays*

*Epitope Mapping of C1q Binding Sites in the N−P Region of Calreticulin.* Synthetic peptides of 15 amino acids in length and overlapping by 12 peptides were coated on Falcon microtest flexible 96 well plates overnight. After extensive washing, unoccupied absorption sites were blocked with 3% BSA and washed, and human C1q (500 ng/well) was added to each peptide-coated well overnight at 4 °C. After further washing, C1q bound to the peptides was detected rabbit anti-human $(Fab)'_2$ fragment antiserum against C1q (1:2000 dilution), followed by anti-rabbit antiserum conjugated to peroxidase (1:5000 dilution). After washing, bound peroxidase was detected using 3,3′,5,5′-tetramethylbenzidine (TMB) as substrate and absorbance results read at 450 nm.

*Binding of Globular Heads of C1q to the IgG $C_H2$-Domain-like Control Peptide and Selected Peptides of Calreticulin.* The globular head region of C1q contains the binding region that recognizes the $C_H2$ domain of IgG. An ELISA was used to detect ghC1q binding to selected synthetic peptides representing the IgG $C_H2$ domain motif (AEAKAKA, peptide 1) and CRT homologue peptides listed in Table 2. The peptides at a concentration of $2.5 \times 10^{-4}$ M were bound on Polysorb ELISA plates in triplicate overnight at 4 °C in sodium carbonate buffer (0.015 M $Na_2CO_3$ and 0.035 M $NaHCO_3$, pH 9.6). Next, the wells were washed three times with PBS-T and unoccupied absorption sites in the wells were blocked by 1 h incubation with 3% (w/v) BSA, and the plates were then washed three more times with PBS-T. The bound peptides were then incubated with 1000 ng of globular heads of C1q (70 nM) in 100 $\mu$L of PBS, overnight at 4 °C. Non-peptide-coated wells were also loaded with globular heads of C1q to assess nonspecific binding of C1q to the wells. To detect globular heads, the wells were probed with rabbit anti-(human C1q) $(Fab)'_2$ antibody or rabbit preimmune serum (both 1:2000 dilution) followed by anti-rabbit $(Fab)'_2$ antiserum conjugated to horseradish peroxidase (1:4000 dilution). The use of preimmune serum acted as a control, to assess nonspecific binding of the antibodies in rabbit serum that react with human ghC1q bound to the solid-phase peptides.

*Competitive Inhibition of Biotin-Labeled C1q Binding to IgG by Unlabeled C1q.* The detection of whole C1q binding to solid-phase IgG by indirect antibody detection methods often results in high background due to nonspecific binding of immunoglobulin to C1q complexed with IgG. To assess binding of C1q to IgG directly, purified C1q was biotinylated and competed with unlabeled C1q for binding to IgG. Biotinylation was performed by the method of Ghebrehiwet et al. (*18*). Competitive inhibition of binding of biotin−C1q was performed at subsaturation binding conditions with 5.45 $\mu$M (250 ng/100 $\mu$L) C1q per 200 ng of IgG, for 4 h. This concentration of biotin−C1q was kept constant and combined with increasing concentrations (2.18−218 $\mu$M; 100−10000 ng/100 $\mu$L) of unlabeled C1q and the mixture was added to the IgG-coated microtiter wells. Bound biotin-labeled C1q was determined by probing with 100 $\mu$L of strepavidin−peroxidase conjugate [1:1000 dilution in PBS-Tween 20 with 0.5% (w/v) BSA], for 1 h. After washing, bound peroxidase was detected with TMB as substrate. In control assays, strepavidin−peroxidase alone was added to IgG-coated wells to ensure that the conjugate was not binding directly to immobilized IgG. Egg lysozyme (2−200 $\mu$M) was used as a negative control to compete for C1q binding to IgG. Results are expressed as percentage of biotin−C1q binding in the presence of unlabeled proteins compared to the value of biotin-labeled protein alone (100%).

*Competitive Inhibition of Biotin-Labeled C1q Binding to IgG by Unlabeled IgG $C_H2$-Domain-like Control and Calreticulin Peptides*. Purified biotin-labeled C1q was incubated with and without unlabeled peptides before the ability of C1q to bind to IgG-coated microtiter wells was assessed. A subsaturation concentration of labeled C1q (5.45 nM; 250 ng/100 $\mu$L) was combined with increasing concentrations (100−250 000 nM) of unlabeled peptides and the mixtures were added to the IgG-coated microtiter wells (200 ng added/well). Bound biotin-labeled C1q was determined by assessing strepavidin−peroxidase binding followed by TMB substrate to detect the peroxidase conjugate bound. Unlabeled C1q was used to detect strepavidin−peroxidase and TMB substrate nonspecific binding. Results were read on a microtiter ELISA plate reader at 450 nm and compared to the value of biotin-labeled C1q and ghC1q alone.

*Competitive Inhibition of Biotin-Labeled CRT Binding to C1q by Unlabeled IgG $C_H2$-Domain-like Control and Calreticulin Peptides*. Purified biotin-labeled CRT (molar ratio 50:1) was incubated with and without unlabeled peptides. A subsaturation concentration of labeled CRT (0.5 nM) was combined with increasing concentrations (16.6−255 $\mu$M) of unlabeled peptides and the mixtures were added to the C1q-coated microtiter wells (2 $\mu$g added/well). Bound biotin-labeled CRT was detected upon addition of 50 $\mu$L of strepavidin−peroxidase (1:5000 dilution), followed by TMB substrate to detect the peroxidase conjugate bound. Unlabeled CRT was used to detect strepavidin−peroxidase and TMB substrate nonspecific binding. Results were read on a microtiter ELISA plate reader at 450 nm and compared to the value of biotin-labeled CRT alone.

*Assay of Whole Complement and C1q-Dependent Hemolysis*. To determine if the IgG $C_H2$-domain-like peptides could block complement activation, freshly prepared serum was diluted 1:50 times in $DGVB^{2+}$ and preincubated with various concentrations [$(0-5) \times 10^{-4}$ M] of synthetic CRT and [$(0-7.5) \times 10^{-4}$ M] IgG $C_H2$-domain-like peptide purified by reverse-phase HPLC for 1 h at 37 °C. Next, to each sample, was added 100 $\mu$L of EA cells ($10^7$ in total), followed by

Table 1: Details of Synthetic Peptides Used

| molecule | peptide no. | residue | amino acid sequence[a] | mol wt |
|---|---|---|---|---|
| IgG | $CH_2$-Domain-like | | | |
| | 1 | 318−322 | A**E**A**K**A**K**A | 670 |
| Calreticulin | N-Domain | | | |
| | 2 | 21−29 | I**E**S**K**H**K**S**D**F | 1016 |
| | 3 | 42−48 | D**E**E**K**D**K**G | 799 |
| | 4 | 131−137 | F**N**Y**K**G**K**N | 853 |
| | 5 | 142−149 | **KD**I**R**C**K**D**D** | 973 |
| Calreticulin | P-domain | | | |
| | 6 | 202−209 | W**D**E**R**A**K**I**D** | 1012 |
| | 7 | 256−262 | G**E**W**K**P**R**Q | 876 |
| Arbitrary Control Peptides | | | | |
| | 8 | N/A | KKISPP**T**P**K**P**R**PPR | 1548 |
| | 9 | N/A | PPRPTPVAPGSSKT | 1344 |
| | 10 | N/A | N**N**Y**E**P**R**S | |

[a] Amino acids shown in boldface type represent charged or polar residues participating in the proposed binding motif.

incubation at 37 °C for an additional 30 min. The unlysed cells were pelleted and the amount of hemoglobin released was determined spectrophotometrically at 412 nm. Total hemolysis was assessed as the amount of hemoglobin released upon cell lysis with water. The complement hemolytic activity was expressed as a percentage of total hemolysis.

To investigate whether complement activation was inhibited during the first step in the pathway, C1q-deficient serum (Sigma Chemical Co.) was diluted 1:40 in $DGVB^{2+}$ and various concentrations of purified C1q were added back in a final volume of 100 $\mu$L prior to incubation at 37 °C for 30 min. C1q hemolytic assays were performed by adding 100 $\mu$L of EA ($10^7$ cells) in DGVB and incubating for another 30 min. The reaction was stopped by transferring the tubes to an ice bath for 5 min. The unlysed cells were pelleted by centrifugation and 150 $\mu$L aliquots of supernatant were read at 412 nm for hemoglobin release. The minimal amount of C1q required to cause 80−90% hemolysis was determined. Various samples for assay of C1q-dependent hemolysis were prepared as follows. Highly purified C1q (5 $\mu$g) was added to various concentrations of synthetic CRT peptides as described above. Freeze-dried peptides were weighed and dissolved in $DGVB^{2+}$ to a concentration of $5 \times 10^{-3}$ M, and serial dilutions were made and incubated at various concentrations at 37 °C for 30 min with 5 $\mu$g of C1q. To each sample was added 100 $\mu$L of EA cells ($10^7$ in total), followed by incubation at 37 °C for an additional 30 min. The C1q hemolytic activity was determined as above and also expressed as a percentage of total hemolysis. The IgG $C_H2$ -domain-like peptide (AEAKAKA) was used as a positive control inhibitor of complement hemolysis. Three irrelevant peptides acted as negative control peptides.

## RESULTS

*Secondary Structure Prediction of Calreticulin.* Part of the sequence alignment of the human CRT N- and P-domains with those from other species and also with calnexin sequences was examined. By use of the PredictProtein server of Rost et al. (*14*), 19 protein sequences were found having amino acid similarity >30% to the human N- and P-domains aligned simultaneously. For none of these is the three-dimensional fold known. Among these, eight are CRT homologues from different species (>59% identity) and eight are calnexins (34−48% identity), which are ER chaperones that anchor to the ER membrane through an additional membrane-spanning domain (*19*). The profile-based neural network prediction of secondary structure elements (*14*) identified nine regions in the N-domain sequence that are predicted to adopt $\beta$-conformation. An α-helical region was also located in the sequence (position 81−86), although with a low reliability index. The expected average accuracy of this secondary structure prediction method has been evaluated in cross-validation experiments to be >72% for the three states of α-helix, $\beta$-strand, and loop of water-soluble globular proteins (*14, 20*). The consensus prediction provided by Geourjon and Deleage (*15*) confirmed this prediction, localizing, in essence, the same $\beta$-regions and also the potential α-helical region. Due to the uncommonly high number of charged residues in the P-domain, no secondary structure was predicted for that part of the sequence. Several equivalents of the IgG−C1q binding motif, allowing for the residue substitutions (*11*), can be detected in the CRT sequence. Four of them are found in the N-domain and two in the P-domain. These fragments and their location within the CRT sequence are presented in Table 1. The sequence alignment showed that while these charged and polar residues spaced one amino acid apart are completely conserved among the CRTs, the same polar-charged arrangements are not conserved in the calnexins, which otherwise share reasonable similarity with CRT.

*Identification of C1q Binding Sites Present in the N- and P-Domain of C1q.* Both the N- and P-domains of CRT are effective inhibitors of C1q-mediated complement activation and have $IC_{50}$ values up to 1000-fold lower than for individual peptides alone (Table 2). This suggests either multivalent interactions at multiple $C_H2$-like sites (possible for the N-domain but not the P-domain) or binding of C1q to other sites distinct from those proposed here. Therefore, the C1q-binding activity to all sites with the N- and P-domains of CRT was evaluated under physiological salt conditions with a series of overlapping peptides. The overlapping peptides confirmed that the $C_H2$-like peptides do indeed bind to C1q (Figures 1 and 2). Interestingly, the mapping studies revealed other unique C1q binding sites in the CRT sequence of a non-$C_H2$-like nature; a site in the N-domain of CRT (peptides 35−40, Figure 1B) was observed to bind to C1q encompassing the amino acids TDMHGDSEYNIMFGPDICGPGTKKVHV (amino acids 103−129). The peptide 51 corresponding to the amino acid sequence FTHLYTLIVRPDNTY (amino acids 150−165) also demonstrated strong binding to C1q. As shown in

Table 2: $IC_{50}$ Values of Inhibition of Complement Lysis of EA Cells by Full-Length, N-, P-, or C-Domains, and Synthetic Peptides of CRT

| Protein (with or w/o fusion tag) or N-, P-, or C-domain | hemolysis $IC_{50}$ (nM) | CRT peptides | hemolysis $IC_{50}$ ($\mu$M) |
|---|---|---|---|
| native CRT | 50 | IESKHKSDF | 280 |
| MBP-FL-CRT[b] | 90 | DEEKDFG | 200 |
| free N-domain CRT | 300 | FNYKGKN | 310 |
| MBP-N-domain CRT | 400 | KDIRCKDD | 150 |
| MBP-P-domain CRT | 714 | WDERAKID | 200 |
| MBP-C-domain CRT | no inhibition | GEWKPRQ | no inhibition |
| MBP alone | no inhibition | | |
| Positive Control $C_H2$-Domain-like Peptide | | | |
| AEAKAKA | 750 000 | | |
| Negative Control | | | |
| NNYEPRS | no inhibition | | |

[a] $IC_{50}$ values represent the mean results of three experiments. [b] MBP = maltose fusion protein; FL = full length.

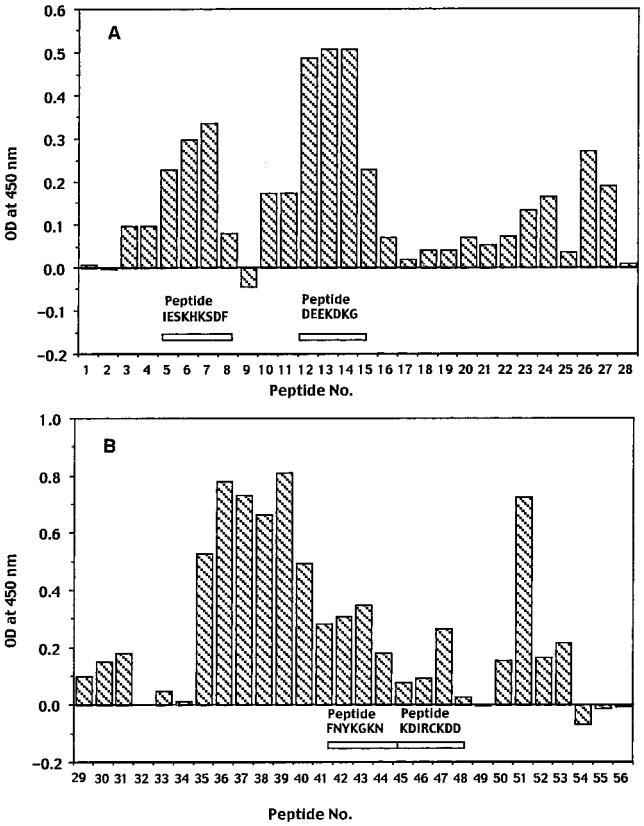


FIGURE 1: Binding of C1q to N-domain CRT peptides. Fifty-six synthetic peptides of 15 amino acids in length, overlapping adjacent peptides by 12 residues and representing the whole 181 amino acid sequence of the N-domain, were synthesized and absorbed onto ELISA plates. C1q (100 ng/well) was added to each peptide-containing well and detected with a rabbit anti-human C1q secondary antibody conjugated to peroxidase. Control background binding was subtracted and the results represent the mean of three separate experiments. Panel A presents the results of C1q binding to the N-terminal half of the N-domain, and panel B, the C-terminal half of the N-domain. The location of the bound peptides containing the IgG $C_H2$-domain-like amino acid sequences are shown below the appropriate OD readings.

Figure 2, a non-$C_H2$-like region peptide present in the P-domain (peptide 79−82), WEPPVIQNPEYKGEWKPR (amino acids 244−261), bound to C1q in addition to the $C_H2$-like domain present in the P-domain, WDERAKID.

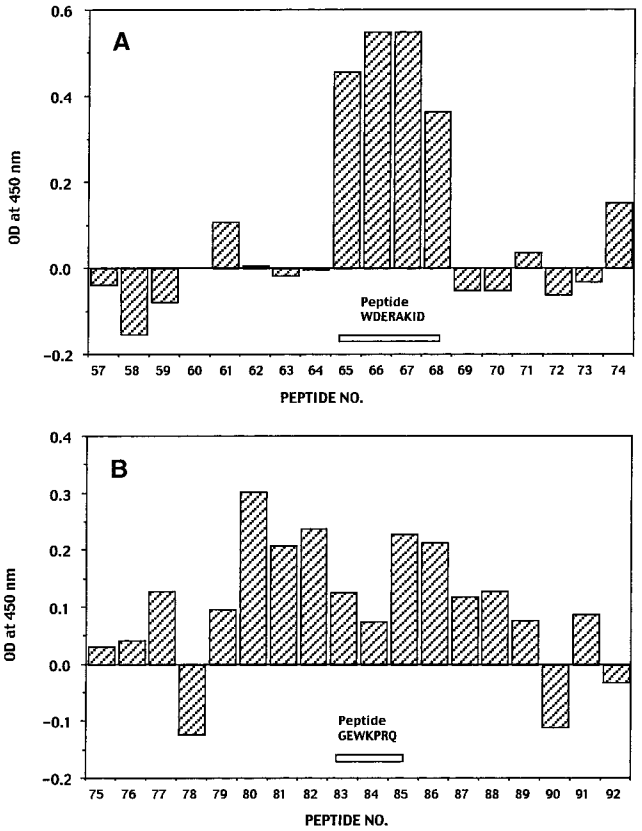


FIGURE 2: Binding of C1q to P-domain CRT peptides. Thirty-five synthetic peptides of 15 amino acids in length, overlapping adjacent peptides by 12 residues and representing the whole 110 amino acid sequence of the P-domain, were synthesized and absorbed onto ELISA plates. Details of the assay conditions were then the same as in Figure 1.

*Binding of ghC1q to Selected Peptides of Calreticulin and to the IgG $C_H2$-Domain-Like Control Peptide of IgG.* We have previously shown that the N- and P-domain regions of CRT bind to the globular head of C1q under half salt and physiological salt conditions (*10*). From comparisons with the C1q binding region within the $C_H2$ moiety of IgG, the N- and P-domains of CRT contained six potential $C_H2$-like regions. The ability of ghC1q to bind to these peptides under physiological salt conditions was examined. As shown in Figure 3, the binding of ghC1q to peptide-coated microtiter wells, in the presence of PBS at 37 °C, differed for individual peptides. In relative terms, peptides 5 (KDIRCKDD), and 3 (DEEKDKG) and the positive control $C_H2$ peptide (AKEKAKA) displayed the best binding. Peptides 4 (FNYKGKN) and 6 (WDERAKID) demonstrated partial binding. In contrast, peptide 2 (IESKHKSF) bound only weakly, and peptide 7 (GEWKPRQ) demonstrated only background binding.

*Saturation Binding and Competitive Inhibition of Biotin-Labeled C1q Binding to IgG by Unlabeled C1q.* The direct binding of biotin-labeled C1q to IgG-coated microtiter wells took place in the presence of PBS/Tween 20. C1q was biotinylated and added to IgG coated wells and reached saturation with 8.0 nM C1q (375 ng of biotin−C1q/200 ng of IgG; Figure 4A). The plating of 10-fold more IgG (2000 ng/0.1 mL) to the wells did not result in an increase in C1q binding, suggesting 200 ng/well was enough to lead to saturation binding of IgG to each well. Biotin-labeled C1q

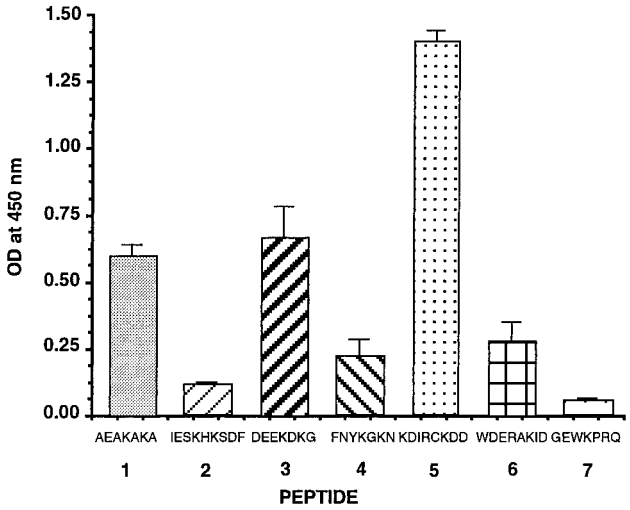


FIGURE 3: Binding of the globular head of C1q to $C_H2$-domain-like control peptide (AEAKAKA) and selected CRT peptides under physiological salt conditions. A single concentration of peptide was coated to wells (250 $\mu$M) and incubated with ghC1q (70 nM; 1 $\mu$g in 100 $\mu$L/well) in the presence of physiological strength PBS. Bound ghC1q was determined in each case by incubation with rabbit anti-human C1q $(Fab)'_2$ antiserum and peroxidase-conjugated anti-rabbit $(Fab)'_2$ antiserum, as described under Experimental Procedures. Results represent the mean of three experiments ± SD. Binding of ghC1q to wells that were not coated with peptides accounted for an $A_{450nm}$ of ≤0.05 and was subtracted from the individual values presented.

was competed with increasing concentrations of unlabeled C1q for binding to IgG at 50% saturation conditions. Figure 4B illustrates that the binding of biotin−C1q was competed out with increasing concentrations of unlabeled C1q and not by a negative control protein, egg lysozyme. These data substantiate the specificity interaction of C1q with IgG. Half-maximal inhibition ($IC_{50}$) was achieved with 7.5 nM unlabeled C1q. This represented only a 1.5-fold molar excess of unlabeled vs labeled C1q. A maximum inhibition of 90% of binding was achieved by approximately a 20-fold molar excess (109 nM) of unlabeled C1q.

*Competitive Inhibition of Biotin-Labeled C1q to IgG by Unlabeled Calreticulin Peptides and Control Peptide.* A positive control peptide based on the C1q binding motif, AEAKAKA, together with CRT-derived peptides, were used to competitively inhibit C1q binding to IgG-coated wells. Since polyclonal and monoclonal anti-C1q antibodies used in ELISA often lead to unacceptable background levels, biotin-labeled C1q was used to monitor directly C1q binding to IgG. As shown in Figure 5A, the C1q binding motif as well as the CRT peptides competitively inhibited C1q binding to the IgG-coated wells in a dose-dependent manner. The degree of inhibition differed for individual peptides, with maximum inhibition being achieved with peptides 6 (WDERAKID), 3 (DEEKDKG), 2 (IESKHKSDF), and 5 (KDIRCKDD), but a 45 000-fold molar excess was needed of these peptide inhibitors to maximally inhibit C1q from binding to IgG by up to 70%. A similar molar excess of the positive control peptide (AEAKAKA) resulted in a 50% reduction in C1q binding to IgG. In contrast, peptide 4 (FNYKGKN) showed only a limited (10%) inhibition of binding, and peptide 7 (GEWKPRQ) did not inhibit C1q binding to IgG at any of the concentrations used.

*Competitive Inhibition of Biotin-Labeled Calreticulin Binding to C1q by Unlabeled Calreticulin Peptides and*

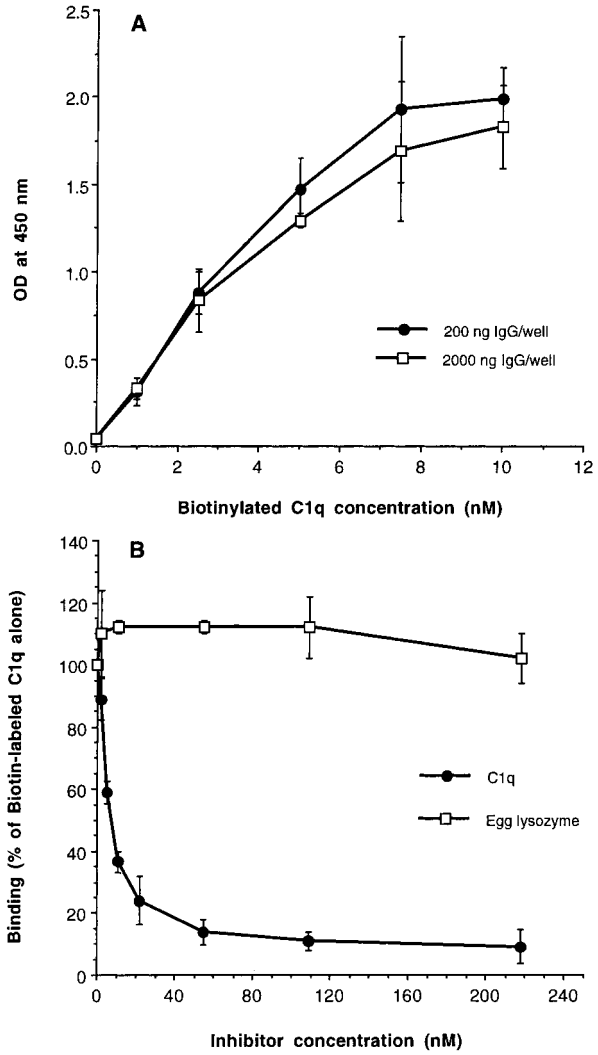


FIGURE 4: Binding of biotinylated C1q to IgG. (A) Saturation binding of increasing concentrations of biotin-labeled C1q (50−500 ng/0.1 mL; 1.09−10.1 $\mu$M) to IgG-coated wells [200 or 2000 ng $(0.1\ mL)^{-1}\ well^{-1}$]. Bound biotin−C1q was determined as described in Figure 3. Results represent the means of triplicate experiments. The vertical bars indicate ± 2 SD. (B) Competitive inhibition of biotin-labeled C1q binding to IgG. ELISA microtiter wells coated with IgG (200 ng) were incubated with 5.45 nM biotin-C1q (250 ng/0.1 mL of PBS-Tween 20) combined with increasing concentrations (0−218 nM) of unlabeled C1q as described under Experimental Procedures. Egg lysozyme was used as a negative control. Bound biotin−C1q was detected by HRP conjugated strepavidin. Results are expressed as percentage of the specific binding determined in the absence of inhibitor. Data represent the means of quadruplicate experiments and error bars indicate ± 2 SD.

*Control Peptide.* To investigate further the importance of the $C_H2$-like peptides of CRT interfering with the whole CRT−C1q interaction directly, the peptides were used to competitively inhibit CRT binding to C1q. As shown in Figure 5B, maximum inhibition was achieved by peptide 5 (KDIRCKDD) at the concentration of $4 \times 10^5$ nM, which represented a 500 000-fold molar excess, Half-maximal inhibition ($IC_{50}$) values were achieved with CRT peptides 2, 3, 4, and 6 when present in 500 000-fold molar excess competition with CRT. Peptide 7 (GEWKPRQ) only marginally inhibited CRT binding to C1q (10−20%) when used at the highest concentration tested. Since a 500 000-fold molar excess of peptides 2, 3, 4, and 6 could not inhibit

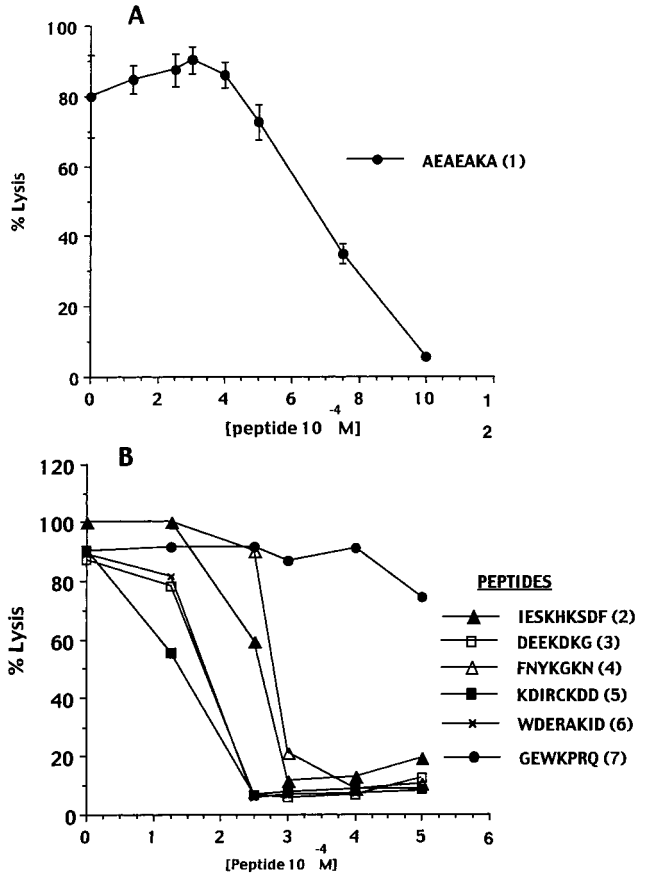


FIGURE 5: Competitive inhibition studies. (A) Inhibition of biotin-labeled C1q binding to IgG. ELISA microtiter wells coated with IgG (200 ng) were incubated with 5.45 nM biotin−C1q combined with increasing concentrations (100−250 000 nM) of unlabeled $C_H2$-domain-like control (AEAKAKA) and CRT peptides as described under Experimental Procedures. Data represent the mean of triplicate experiments and are expressed as mean percentage binding compared with control wells incubated with biotin-labeled C1q alone. (B) Inhibition of biotin-labeled CRT binding to C1q by CRT peptides. Wells were coated with 2 $\mu$g of C1q were incubated with 0.5 nM CRT−biotin with increasing concentrations (16.6−255 $\mu$M) unlabeled $C_H2$-domain-like control (AEAKAKA) and CRT peptides as described under Experimental Procedures. Data represent the mean of duplicate experiments and are expressed as mean percentage binding compared with control wells incubated with biotin-labeled CRT alone.

complete binding of CRT to solid-phase C1q, they probably possess different binding affinities for C1q.

*Effect of Calreticulin ghC1q Binding Peptides and the Control Peptide on Complement Activation in Whole Serum.* Activation of the classical pathway of complement is brought about by the binding of the globular head region of C1q to the Fc portion of IgG or IgM in the immune complex, which leads to activation of proenzymes C1r and C1s, which are removed by C1 inhibitor. In view of the evidence that regions within the N- and P-domains of CRT bind to C1q, a complement hemolytic assay was used to study the effect of the ghC1q binding peptides identified in this study on complement activation. As the majority of C1q is associated with high affinity to C1r and C1s, which together constitutes the C1 complex, a whole serum assay complement activation assay was chosen initially. It was considered important to determine whether the CRT peptides could inhibit C1q activation before, as well as after, the activation of the components of the C1 complex. In the present study,

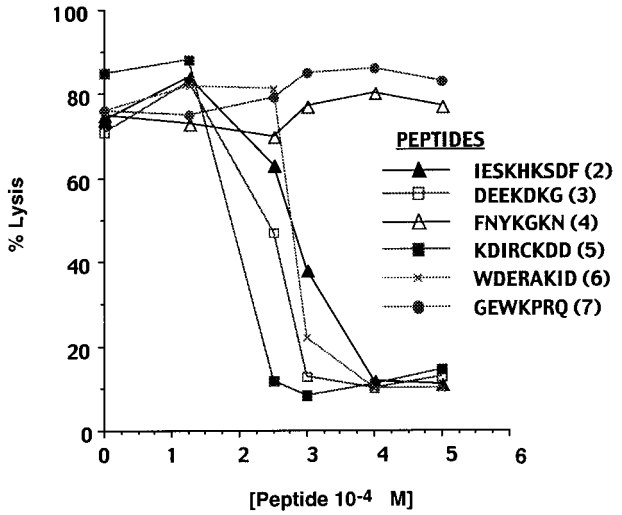


FIGURE 6: Inhibition by CRT peptides of complement activation in whole serum as detected by hemolysis of IgG-sensitized erythrocytes. Different concentrations of CRT peptides [(0−5) × $10^{-4}$ M] were added to 1:50 dilution of whole human serum together and incubated for 1 h at 37 °C. Then $1 \times 10^7$ EA in 100 $\mu$L was added for an additional 1 h at 37 °C. The nonlysed 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. The results are expressed as mean triplicate experiments.

complement activity in whole serum was titrated. The complement activity of a 1:50 dilution of normal serum without the addition of any peptides produced between 75% and 85% hemolysis. This dilution was chosen for all further studies in this series of experiments. Various concentrations of CRT peptides ranging from $1 \times 10^{-5}$ to $5 \times 10^{-4}$ M were incubated with a 1:50 dilution of human serum at 37 °C before addition of the mixtures to EA. The addition of between 1 and $4 \times 10^{-4}$ M concentrations of peptide 2 (IESKHKSDF), 3 (DEEKDKG), 5 (KDIRCKDD), or 6 (WDERAKID) lowered the hemolytic activity of complement from approximately 80% to below 20% (Figure 6). The peptides 7 (GEWKPRQ) and 4 (FNYKGKN) had no effect on hemolytic activity. The IgG $C_H2$ peptide (AEAKAKA) was used as a positive control and inhibited 90% hemolysis of EA when it was used as a competitor at a higher concentration of $7.5 \times 10^{-4}$ M.

*Inhibition of C1q-Dependent Hemolysis by Calreticulin Peptides and the Control Peptide.* To determine if C1q is specifically involved in the inhibition of complement-dependent hemolysis by the CRT C1q binding peptides, a C1q-dependent hemolytic assay was used. The assay requires purified C1q to be added back to C1q-deficient serum in order to reconstitute the C1 complex. In this present study, the addition of 2.5 $\mu$g/mL C1q back to C1q-deficient serum was sufficient to lyse 70−80% of the EA. This concentration was then chosen as the standard in a series of studies to determine whether the ghC1q-binding peptides of CRT could specifically inhibit C1q-dependent hemolysis. Various concentrations of peptides ranging from $1 \times 10^{-5}$ to $5 \times 10^{-4}$ M were incubated with 500 ng of C1q in 1:40 dilution of C1q-deficient serum reconstituted in 100 $\mu$L of $DGVB^{2+}$ for 60 min at 37 °C, before addition of the mixtures to EA. The IgG $C_H2$ peptide (AEAKAKA), which is known to inhibit C1q-dependent hemolysis (*11*), did inhibit hemolysis of EA by up to 95% when it was used as a competitor at a

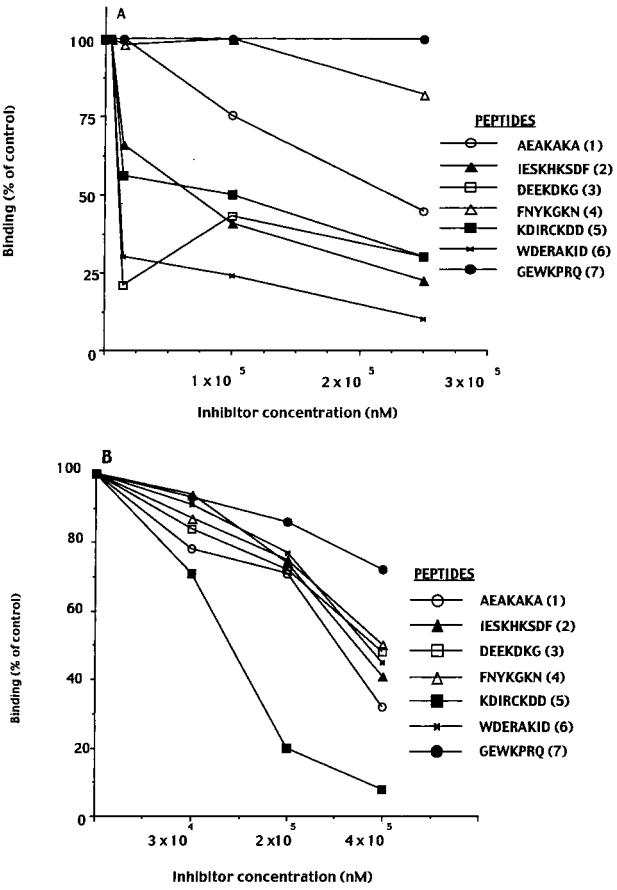


FIGURE 7: Inhibition by $C_H2$-domain-like (AEAKAKA) and CRT peptides of the C1q-dependent hemolysis of IgG-sensitized erythrocytes. (A) Different concentrations of AEAKAKA peptide were added to a 1:40 dilution of C1q-deficient serum together with suboptimal amounts of C1q, and the samples were incubated for 1 h at 37 °C. Then $1 \times 10^7$ EA in 100 $\mu$L was added for an additional 1 h at 37 °C. The nonlysed 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. The results are expressed as mean ± SD. (B) Inhibition of the C1q-dependent lysis of EA by the CRT peptides. Six peptides from the N- and P-domain of CRT that show sequence similarity to putative C1q-binding regions in the $C_H2$ domains of IgG were employed as inhibitors in the assay described above. The results represent the mean of triplicate experiments performed on two separate occasions.

concentration of $1 \times 10^{-3}$ M (Figure 7A). The addition of CRT peptides 2 (IESKHKSDF), 3 (DEEKDKG), 5 (KDIRCKDD), 6 (WDERAKID), and 4 (FNYKGKN) at concentrations of $(2.25-4) \times 10^{-4}$ M lowered the hemolytic activity of complement from approximately 80% to below 20% (Figure 7B). The peptide 7 (GEWKPRQ) had no effect on hemolytic activity.

## DISCUSSION

The release of the abundant intracellular ER protein CRT, from dead and dying cells can result in its presence in the vascular circulation, where it is known to induce an autoantigenic response. A number of independent studies have established that a large number of sera screened from SLE patients contain autoantibodies against CRT (*3, 21, 22*). The production of autoantibodies against CRT may depend on the levels of CRT detected in the sera. Relatively low levels of CRT have been detected in approximately 20% of normal sera (*23*), while much higher levels have been observed in up to 50% of SLE patients (*3*). We have previously shown that, as well as inducing an antigenic response, CRT also has the potential to bind to C1q (*4*) and interfere with C1q-mediated inflammatory processes in SLE (*2, 3, 10*). The interaction of CRT may be consequential in the etiology of immune complex formation and deposition, which are particularly important features in the disease state of SLE patients. In these earlier studies, the ghC1q binding site(s) was established to be located on the N-terminal half of the protein, in the regions of the molecule known as the N-and P-domains. Under physiological salt conditions, the globular head region of C1q binds specifically to both immune complexes and CRT. The ghC1q binding site on mouse IgG2b is known to contain three charged residues, Glu318, Lys320, and Lys322, which orientate in a linear position to one another and which are relatively conserved in other antibody isotypes (*11*). In this study, examination of the CRT protein sequence revealed six short amino acid sequences with similar motifs to the ghC1q binding site on IgG (Table 1). Sequence alignments of a number of forms of CRT from different species revealed that most of these sites were highly conserved (Figure 1). To further characterize whether some of these potential binding sites on the CRT molecule can interact with ghC1q and inhibit C1q−IgG-mediated responses, a series of short peptides of 7−9 amino acids in length were synthesized. These were then examined for their ability to (i) bind to the globular head region of C1q, (ii) inhibit C1q binding to solid-phase human IgG, (iii) inhibit CRT binding to solid-phase C1q, and (iv) inhibit complement-dependent lysis of EA cells. Some of the peptides constructed (Table 2) had more than three charged residues present. This was of interest since the charged nature of the C1q binding motif, with one acidic or polar and two basic residues spaced two amino acids apart, implies an electrostatic interaction between C1q and IgG. One of the potential ghC1q binding peptides comprising amino acids 256−262 (GEWKPRQ) contained a proline residue. Computer modeling of the extended conformation of this peptide with the proline in the cis and trans positions suggests that the peptide could not align the charged side-chains in a linear arrangement in fluid phase (not shown). Other control peptides containing proline residues were also unable to inhibit EA lysis.

In the present study we have shown that ghC1q is capable of binding to some, but not all, of the $C_H2$-like CRT peptides. The results indicate that peptides 1, 3, and 5 bind specifically to ghC1q. Peptide 2 does not bind to C1q but it is effective in the competitive hemolytic binding assay. This suggests that it might interact with IgG directly. Interestingly, peptide 7 (GEWKPRQ), which contains the proline residue, showed only background binding, emphasizing the importance of a linear arrangement of the charged residues for binding. Thus the interaction of the CRT linear peptides may interfere with C1q interaction with IgG. Normally, the majority of C1q (approximately 90%) in the circulation is complexed, in a calcium-dependent manner, to C1r and C1s and binding to monomeric IgG is negligible. When immunoglobulins are in a multimeric conformation during infection, inflammation, or autoimmune disease (or coated to erythrocytes), C1 becomes activated and C1q binds with high affinity to immunoglobulins, leading to complement activation. There are six heads on C1q that can potentially bind to the Fc

portion of an antibody, but as individual heads they bind with an affinity of 100 $\mu$M (*24*). However, when several heads of C1q are presented with multivalent Fc sites, for example, when binding to aggregated IgG, the binding affinity is enhanced 10 000 times to 10 nM (*25*). It would therefore be expected that short CRT peptides would have a much lower affinity for C1q than the multivalency of IgG molecules presented on the surface of microtiter wells. Our results are consistent with this hypothesis. By use of purified peptides of CRT in a competitive ELISA, a number of the CRT peptides were able to compete out C1q binding to solid-phase multimeric human IgG (Figure 5A), but only when presented in much higher molar excesses than that required when unlabeled C1q is used to block the binding of biotin-labeled C1q to IgG on microtiter wells (Figure 4B). To further study the ability of CRT peptides to compete with IgG for globular head C1q binding, a series of in vitro EA hemolytic studies were undertaken. That selective CRT peptides were in fact blocking the C1q−IgG interaction was confirmed when several of the peptides were shown to be potent inhibitors of C1q-dependent hemolytic activity, when used at a concentration of $(2.5-5) \times 10^{-4}$ M. Moreover, the same peptides could inhibit complement activation even when whole serum was presented to the IgG-coated erythrocytes. This suggests the CRT peptides can bind to C1q when present as part of the C1 complex, as well as to free C1q. The $IC_{50}$ of CRT peptides required to inhibit C1q-dependent complement lysis of EA cells ranged from 150 to 310 $\mu$M, which is comparable with values reported for the positive control peptide, which mimics the binding site on the $C_H2$ domain of IgG (AEAKAKA $IC_{50} = 150$ $\mu$M) in an earlier study (*11*). In our hands the AEAKAKA peptide was less inhibitory than the CRT peptides, requiring 750 $\mu$M before $IC_{50}$ was achieved. The differences in these results may reflect the different assay systems employed in the earlier investigation. In our previous studies, the N- and P-domain peptides were used in inhibition of complement lysis assays. The N-domain used in these earlier studies contains four of the $C_H2$-like peptides employed in this study, while the other two peptides used here are located in the P-domain. The N-domain of CRT, containing multiple $C_H2$-like binding regions, was able to inhibit complement lysis by 50% (see Table 2) at a much lower molar concentration than the P-domain, which contains two $C_H2$-like binding regions. Furthermore, one of the two proposed interaction sites on the P-domain is the proline-containing peptide region GEWKPRQ, which is unable to inhibit complement lysis or bind to the globular head of C1q. One might expect that full-length CRT would inhibit complement lysis most effectively. As shown in Table 2, both native and recombinant full-length CRT did indeed have the lowest $IC_{50}$ values (50 nM and 90 nM, respectively) in comparison to any of the individual domains or peptides. This implies that the ghC1q binding motifs are accessible on the intact molecule, in either a recombinant or native form.

We propose that any one or several of these fragments contain some of the actual interaction sites for C1q on CRT. Additional evidence in support of this comes from inhibition studies of CRT binding to C1q (Figure 5B), although one cannot precisely determine binding affinity constants because it is difficult to assess the amount of protein coupled to the microtiter plate. To varying degrees the $C_H2$-like CRT peptides, when present in large molar excess, were capable of inhibiting full-length CRT binding to solid-phase C1q. However, 2−5-fold more peptide was required to block native CRT binding to C1q than the concentrations required to block C1q binding to solid-phase IgG. This would imply that native CRT has a higher binding affinity for C1q than IgG. This observation, together with the fact that inhibition of CRT binding to C1q could not be abolished completely, suggests other non-$C_H2$-like binding regions present on the whole molecule may also participate in the strong interaction between C1q and CRT. Until CRT structural data becomes available we have to assume that the identified region assumes a linear conformation on an accessible surfacial location within the context of the complete folded protein architecture. The second basic assumption in the present study is that the protein−protein interaction is primarily of electrostatic character, which renders it reasonably strong but nonspecific.

As for the physiological consequences of the CRT−C1q interaction, because C1q initiates clearance of immune complexes by the classical pathway of complement, the presence of CRT deposits in the vascular circulation or other tissue sites may affect this clearance function of C1q. Recent observations suggest that keratinocytes and other cell types undergoing apoptosis following exposure to ultraviolet light and viral infection (*26*−*28*) generate discrete subcellular structures referred to as surface blebs, which contain high concentrations of CRT (*29*). Moreover it has recently been shown that CRT-containing blebs of apoptotic keratinocytes directly bind to C1q in the absence of antibody (*30*). During active SLE disease, the mechanism of apoptosis is believed to be impaired (*31*) and the surface blebs containing CRT are susceptible to attack by anti-phospholipid antibodies (*32*).

We have observed that recombinant N-domain fragments of CRT are susceptible to forming dimers and other multimeric forms (data not shown), which in turn could increase the potential number of C1q binding sites available and increase the affinity of CRT for C1q. Moreover, the N-domain of CRT is resistant to proteolytic digestion by major serine proteases, elastase and cathepsin G, which are released from leukocytes during inflammation (data not shown), suggesting such fragments if formed in vivo may also be resistant to degradation in an inflammatory environment. Consequently, although CRT is not normally accessible, the binding of C1q to apoptotic surface blebs may arise possibly via CRT after possible release from ER bleb degradation during SLE disease, which in turn may impair immune complex clearance. Alternatively, the binding of C1q on the apoptotic cell surface may enhance removal of the antigen-containing clusters via the phagocytic pathway. These alternative possibilities are undergoing further investigation.

It is, however, intriguing that the C1q binding motif is found so frequently in the CRT protein, and these in turn are highly conserved in versions of the CRT protein found in lower animal species. Several studies have shown that human CRT shares 50−60% amino acid identity with proteins found in the human filarial parasite *Onchocerca volulus* (*33*), the blood fluke *Schistosoma mansoni* (*34*), and malarial protozoa *Plasmodium falciparum* (*35*). The common ghC1q binding motifs present in these ancient forms of CRT derived from parasites may function to prevent complement

activation by the host. Further evidence for this hypothesis comes from studies by Needham and co-workers (*36*), who have observed that the tick species *Amblyomma americanum*, while feeding on its host, secretes CRT together with other anti-complement factors.

Why CRT contains six immunoglobulin $\gamma$ $C_H2$-like C1q binding motifs remains unclear. The likelihood of finding the motif ExKxK with tolerance to Q or T in the first position and K to R substitutions is high, and the motif does appear in a number of other host proteins from both intracellular and extracellular sources.

However, the number of host proteins that bind to C1q are relatively few. They include lactoferrin, moesin, C-reactive protein, histidine-rich glycoprotein, and fibronectin. Of these, lactoferrin, moesin, histidine-rich glycoprotein, and fibronectin possess ExKxK-like motifs. Lactoferrin is believed to have a greater affinity for C1q than IgG (*37*). However, the precise interaction site of C1q with these host proteins is unclear and may involve either the globular head or collagen-like tail of C1q. Also it is not known whether the residues appear in a solvent-exposed and linear conformation. C1q also binds directly to a number of bacterial and viral surface proteins, for instance, streptococcal protein H and certain *Klebsiella pneumoniae* outer surface porins. It is interesting to note that both streptococcal protein H and *Klebsiella* porins also possess the ExKxK motif. The interaction between C1q and proteins that possess linear ExKxK motifs on virus and bacterial surfaces may have important implications in host−parasite relationships, either as a means of targeting of proteins by the innate immune system for clearance or inhibition of activation of the classical complement pathway by the proteins containing the ExKxK-like motifs.

Interestingly, two additional C1q binding sites of a non-$C_H2$-like nature were identified in the N- and P-domains of CRT. The novel C1q binding region identified in the N-domain is also a dominant autoantigenic region of CRT (data not shown). The interaction of C1q with this site may be of importance, as C1q may play a protective role, masking this potential autoantigenic site. Alternatively, C1q association with this antigenic region of CRT may result in epitope spreading, leading to autoantibodies being raised against C1q. These possibilities are currently under investigation.

In conclusion, the globular head binding sites of C1q for CRT have been identified and the biochemical interaction of CRT with C1q in the presence of IgG has been defined. CRT, as well as being a well-defined autoantigen in SLE disease, also appears to play a role in the interference of complement activation by the classical pathway. Further studies should provide insight into the role of CRT in the pathogenesis of immune complex disease and have potential in designing specific molecular structures to regulate complement activation.

## ACKNOWLEDGMENT

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