Localisation of the C1q binding site within C1q receptor/calreticulin

G.R. Stuart[a,*], N.J. Lynch[b], J. Lu[c], A. Geick[b], B.E. Moffatt[a], R.B. Sim[a], W.J. Schwaeble[b]

[a]*MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*
[b]*Department of Microbiology and Immunology, School of Medicine, University of Leicester, University Road, Leicester LE1 9HN, UK*
[c]*Department of Biochemistry, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*

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Abstract C1q receptor (C1qR/collectin receptor) is located on many cell types. Binding of C1q to these cells elicits numerous responses. Protein sequencing has shown that C1qR is almost identical to calreticulin (CaR), an abundant multifunctional protein. Radioiodinated C1qR and CaR bind to C1q with identical characteristics. Three recombinant C1qR/CaR domains (N-, C-terminal domains and central P-domain) were expressed using the Thiofusion system, and used to study the interaction with C1q. Both the N- and P-domains were implicated in C1q binding. A region, termed the S-domain, spanning the N and P intersection was expressed, and showed concentration-dependent binding to C1q, demonstrating that the C1q binding site lies within this region.

Key words: C1q receptor; Calreticulin; C1q binding site; Collagen; Complement

1. Introduction

A cell surface receptor which binds to the collagenous region of C1q, the recognition subunit of the first component of complement, has previously been isolated [1]. Protein sequencing studies of this molecule, the C1q receptor (C1qR or collectin receptor), have shown [1] that it is almost identical to calreticulin (CaR), a highly conserved abundant calcium-binding protein that was initially identified in skeletal muscle sarcoplasmic reticulum [2]. C1qR and CaR are both 47-kDa proteins (from mass spectroscopy [3]) with an apparent molecular weight of 56 kDa on SDS-PAGE. Immunohistochemical studies have shown that C1qR is located on the surface of leukocytes, endothelial cells, fibroblasts, platelets and specialised epithelia [4]. Binding of C1q to C1qR on these cell types elicits a range of immunological responses, such as phagocytosis, enhanced cytokine and antibody production and antibody-dependent cell cytotoxicity [5,6]. C1qR also binds to the collectin proteins SP-A, MBL, CL43 and conglutinin [7,8]. The mode of attachment of C1qR/cell surface CaR to the plasma membrane remains uncertain.

A number of proteins with identical or highly homologous sequence to CaR have recently been identified in different cellular compartments, indicating that CaR has a highly diverse functional repertoire [9]. At present, over 40 functions have been described for CaR; however, the number of significant roles in vivo is likely to be much lower. CaR or CaR-like molecules have been suggested to act as: a heat shock/stress protein [10], chaperone [11,12], steroid hormone receptor [13,14], Mg^{2+} or Zn^{2+} binding protein [15,16] and PDI binding protein [17]. CaR also affects replication of rubella virus RNA [18] and intracellular Ca^{2+} homeostasis [19–21]. CaR may also participate in the normal immune response and in autoimmunity [22]. Cell surface CaR may trigger cell spreading [23]. CaR, like C1qR, binds to C1q and the collectins [24].

The interaction between C1qR and C1q is ionic strength-dependent [25], and involves a region of charged residues on the collagen stalks of the C1q ligand [26]. However the region within C1qR that binds to the ligand has yet to be elucidated. Despite the minor reported sequence differences between C1qR and CaR (Fig. 1) we show that the two proteins interact in an identical fashion with C1q and collagens, indicating that there is a common C1q binding region within C1qR and CaR. Due to the high degree of similarity between CaR and C1qR, the protein will be referred to as C1qR/CaR.

A cDNA clone (phCaR-1) was isolated from a human umbilical vein endothelial cell cDNA library cloned in the expression vector CDM8. The coding sequence of phCaR-1 was identical to the previously published CaR sequence [27]. Clone phCaR-1 was used to produce recombinant protein segments to be tested for C1q binding function. These N-, P- and C-domains, as described below, have previously been used to localise CaR function within the molecule [28]. The amino-terminal N-domain contains the binding regions for PDI [17], Zn^{2+} [29] and α-integrins [23]. The proline-rich central P-domain contains the high affinity Ca^{2+} binding site [30]. The acidic C-domain contains the ER-retention terminal KDEL signal [27]. This domain also contains a putative glycosylation site and the low affinity Ca^{2+} binding site [30]. Our studies have localised the C1q binding site across the intersection of the N- and P-domains. Within this region we identified and expressed a subfragment (the S-domain) and showed that it represents the C1q binding site of C1qR/CaR.

2. Materials and methods

2.1. Purification and radioiodination of native C1qR and native calreticulin

Native C1qR was purified from human U937 cells as previously described [1]. Briefly, U937 cells were lysed in 10 mM sodium phosphate, 2 mM EDTA (pH 7.4) containing non-ionic detergent, followed by chromatography on DEAE-Sephacel, FPLC mono-Q ion exchange and FPLC Superose-6 gel filtration (Pharmacia, St. Albans, Herts., UK). C1qR fractions were further purified on a TSK-gel DEAE-NPR high performance liquid chromatography column. Pure C1qR fractions were stored in 10 mM sodium phosphate, 2 mM EDTA (pH 7.4) at −20°C. Native C1qR was also purified from human tonsil lymphocytes (obtained from the E.N.T. Department, Radcliffe Infirmary, Oxford) by the same method.

Native CaR was purified from human tonsils as previously described [31] with modifications. Briefly, 100 g human tonsils were homogenised and CaR precipitated with 85% saturation of ammonium sulphate (pH 4.0). Redissolved protein was fractionated on a high-load Q-Sepharose column (Pharmacia) and then, as for C1qR,

*Corresponding author. Fax: (44) (1865) 275729.

Abbreviations: C1qR, C1q receptor; CaR, calreticulin; SP-A, lung surfactant protein A; MBL, mannose binding lectin

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further purified by FPLC Superose-6 gel filtration and TSK gel DEAE-NPR HPLC. Pure CaR fractions were stored in 10 mM sodium phosphate, 2 mM EDTA (pH 7.4) at −20°C.

Native C1qR and CaR samples (25 μg in 10 mM sodium phosphate buffer, 2 mM EDTA (pH 7.4)) were iodinated with ^{125}I by the Iodogen method [32]. Radiolabelled fractions were stored at 4°C.

2.2. Purification and radioiodination of C1q

C1q was purified as previously described [33] and radioiodinated (40 μg, in 100 mM sodium borate (pH 8.5)) by the method of Bolton and Hunter [34]. This method of iodination causes less damage to large, oxidation-sensitive molecules such as C1q than the Iodogen method [35,36]. Radiolabelled fractions were pooled and stored in 10 mM potassium phosphate, 0.25% (w/v) gelatin at 4°C.

Soluble collagens (in 0.1 M acetic acid) were a generous gift from Prof. R. Timpl, Max-Planck-Institut für Biochemie, Martinsried, Germany. Acid-soluble type I collagen was purified from calf skin; pepsin-solubilized collagens type III, V and VI were purified from human placenta; bacterial collagenase-solubilized type IV collagen was prepared from EHS tumour cells.

2.3. Binding of native C1qR and native CaR to collagens and C1q

The solubilized collagens were neutralized to pH 7.0 with NaOH. Microtitre plates were coated with the collagens, C1q or BSA (100 μl: 10 μg/ml in 35 mM $NaHCO_3$, 15 mM Na_2CO_3 pH 9.6) for 2 h at 37°C. After washing with 10 mM potassium phosphate, 0.5 mM EDTA pH 7.4, non-specific interactions were blocked by incubation with the same buffer containing BSA (10 mg/ml). Radiolabelled native C1qR or native CaR samples (2×10^5 cpm per well), in 10 mM potassium phosphate (pH 7.4) containing different concentrations of NaCl, were added to the wells and incubated overnight at 4°C. Wells were washed three times with the phosphate buffer and bound radioactivity eluted with 200 ml 4 M NaOH per well.

2.4. Prokaryotic expression of recombinant C1q receptor/calreticulin domains

A 1.9-kb cDNA clone for CaR (phCaR-1) was isolated from a human umbilical vein endothelial cell library (kindly donated by Dr B. Seed, M.I.T., Boston, MA, USA) in the eukaryotic expression vector CDM8 [37]. Sequence analysis revealed that phCaR-1 comprised the complete coding sequence for CaR with absolute identity to the previously published CaR sequence [27]. The Thiobond expression system was used to produce N-, P-, C- and S-domains of C1qR/CaR (representing the N-terminal region, the proline-rich central region, the C-terminal region, and a region spanning the intersection of the N- and P-domains (Table 1)). The individual recombinant domains of human C1qR/CaR were expressed as thioredoxin fusion proteins in *E. coli* using the plasmid pTrxfus (Invitrogen BV, Leek, The Netherlands).

Oligonucleotide primer pairs were generated for each recombinant C1qR/CaR domain. Sense and antisense primers were modified to include sites for *Xba*I and *Sal*I digests, respectively. Clone phCaR-1 was used as a template for PCR. PCR amplification products were ligated into pCRII (Invitrogen). Plasmid DNA was prepared, double digested with *Xba*I/*Sal*I and separated on an agarose gel. C1qR/CaR cDNA fragments were cut from the gel, extracted using the Sephaglas Bandprep kit (Pharmacia), and ligated into pTrxfus that had been linearised with *Sal*I and *Xba*I. The constructs were transformed into *E. coli* strain GI724 and plasmid DNA isolated and sequenced.

Single colonies carrying each construct were inoculated into RMG-Amp medium (1×M9 salts, 2% casamino acids, 0.5% glucose, 1 mM $MgCl_2$ and 100 μl/ml ampicillin) and grown overnight at 30°C. Induction medium (RMG-Amp) was inoculated with the cultures and incubated at 30°C until OD_{550} reached ~0.5. L-Tryptophan (100 μg/ml) was added to induce protein expression and the cells grown for 4 h at 37°C before harvesting. Cells expressing the N-domain were resuspended in TSB (200 mM NaCl, 100 mM Tris-HCl, 1 mM EDTA pH 7.0), 0.1 mM PMSF, and lysed by three cycles of sonication, rapid freezing and thawing. Following centrifugation, the clear supernatant (8 mg/ml of protein) was bound to Thiobond resin (Invitrogen) and purified protein eluted with TSB, 2-mercaptoethanol (5–500 mM). The recombinant P-, C- and S-domains were purified after osmotic shock as the thioredoxin fusion partner localises protein in an osmotically sensitive compartment between the cell wall and plasma membrane in *E. coli*. [38]. Samples were assayed for recombinant C1qR/CaR by SDS-PAGE [39] and by Western blotting with rabbit antisera to whole native C1qR (raised against human C1qR purified from U937 cells [1]), whole native CaR (raised against human CaR purified from native tonsils (Section 2.1)), with rabbit antisera with specificity for CaR C-terminal region (raised against a GST fusion protein containing the final 18 residues of human CaR), and for recombinant CaR N-terminal region (raised against a GST fusion protein containing residues 7–18 of recombinant human CaR: kindly donated by Dr P. Eggleton, MRC Immunochemistry Unit). The amino acid sequence of each expressed recombinant C1qR/CaR domain is shown in Table 1.

C1qR peptide	CaR sequence similarity
YKGRQT	Two KG-QT sequences
KPADMS-S	KPEDWDKP (residues 232-239)

Fig. 1. Reported sequence differences between C1q receptor and calreticulin. C1qR proteolysis fragment peptides sequences [1], not found in the cDNA-derived amino acid sequence of CaR [27]. A third reported sequence difference [1] (DNQSENMS) is now believed to be a purification artefact.

2.5. Interaction of recombinant C1qR/CaR domains with C1q

Binding experiments with the recombinant C1qR/CaR domains were performed throughout in low salt (10 mM potassium phosphate, 0.5 mM EDTA (pH 7.4)) in order to maximise the interaction of the protein domains with C1q. Fig. 2 demonstrates that the highest levels of binding are achieved at low ionic strength.

2.5.1. Solid phase binding assays. Microtitre plates were coated with the recombinant C1qR/CaR domains and with three controls, native C1qR (purified as described in Section 2.1), BSA and Thioredoxin, (8 μg/ml in 35 mM $NaHCO_3$, 15 mM Na_2CO_3 pH 9.6) for 2 h at 37°C. Non-specific interactions were blocked as described in Section 2.3. Any free –SH groups in the samples, due to the presence of the thioredoxin fusion protein, were blocked by a brief washing step using phosphate buffer containing 2 mM iodoacetamide. After further washing, serial dilutions of radioiodinated C1q (in 10 mM potassium phosphate, 0.5 mM EDTA, pH 7.4) were added to the wells and incubated overnight at 4°C. Wells were washed three times with phosphate buffer and bound radioactivity eluted as described in Section 2.3.

2.5.2. Competitive inhibition of native C1qR-C1q interaction by recombinant C1qR/CaR domains. A microtitre plate was coated with native C1qR (0.9 μg per well in 35 mM $NaHCO_3$, 15 mM Na_2CO_3 pH 9.6). Non-specific binding was blocked by incubation with 10 mM potassium phosphate, 0.5 mM EDTA (pH 7.4) containing BSA (10 mg/ml). Serial dilutions of the recombinant domains, thioredoxin, native C1qR and BSA (maximum quantity = 9 μg/well) were prepared in 10 mM potassium phosphate, 0.5 mM EDTA, 2 mM iodoacetamide (pH 7.4). Each dilution (100 μl) was then incubated for 1 h at 37°C with a constant level of radiolabelled C1q and loaded onto the plate. Following overnight incubation at 4°C, the wells were washed, bound radioactivity eluted using 4 M NaOH and then measured.

2.5.3. Haemolytic assays. The ability of native C1qR and the recombinant C1qR/CaR domains to bind to C1q and interfere with binding of $C1r_2C1s_2$ was also determined by haemolysis assays. Sheep erythrocytes (E) and rabbit anti-sheep erythrocytes (A) were provided by TCS, Botolph Claydon, Oxon, UK. EAC1q cells were made as previously described [40,41]. C1q deficient serum was obtained from a patient with homozygous C1q deficiency. EA and EAC1q cells were incubated, at 37°C for 1 h, with serial dilutions of C1q-deficient serum, in order to establish the minimum serum concentration required to cause complete lysis of EAC1q. EAC1q cells (100 μl) were then preincubated, at 37°C for 1 h, with native C1qR, or recombinant C1qR/CaR constructs (100 μl: 100 μg/ml). The cells were then incubated with the appropriate dilution of C1q-deficient serum at 37°C for 1 h. The extent of lysis was established by the addition of cold $DGVB^{++}$ (1 ml), centrifugation, and measurement of the OD_{412} of the supernatant. Controls for 100% lysis comprised 100 μl cells and 1.1 ml water. Initially, fixed concentrations of native C1qR and recombinant C1qR/CaR domains were tested. Based upon this evidence, the concentration dependence of inhibitory effects was then tested by

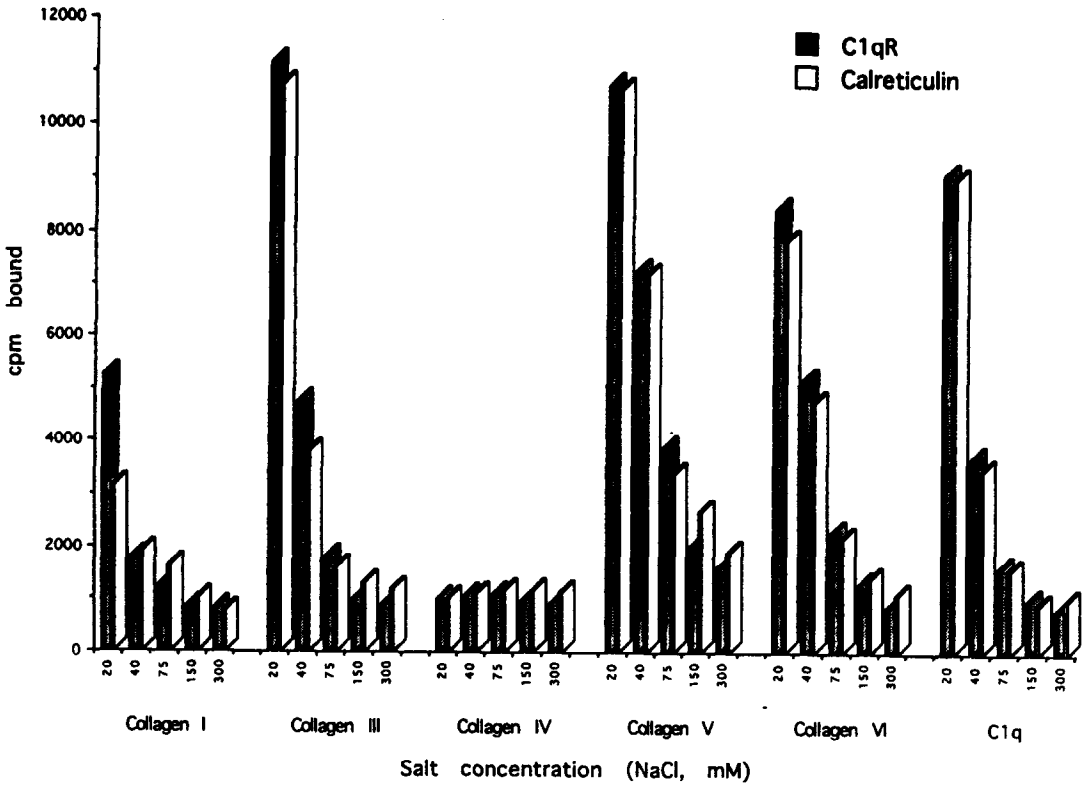

Fig. 2. Binding of native C1q receptor and native calreticulin to C1q and collagens. Radioiodinated C1qR or CaR samples were incubated with solid phase bound C1q or collagens at varying ionic strength conditions, as described in Section 2.3.

the use of varying concentrations of native C1qR and recombinant C1qR/CaR domains.

3. Results

3.1. Interaction of native C1qR and CaR with C1q and collagens

Constant concentrations of radioiodinated native C1qR and CaR were incubated with C1q or Collagens on a microtitre plate in increasing salt concentrations, as described in Section 2.3. Both proteins interact identically, in a salt-dependent fashion, with C1q, and with collagens III, V, VI and, to a lesser extent, collagen I (Fig. 2). Collagen IV showed no binding at pH 7.4, but there was low level salt-independent binding at pH 5.5 (not shown). Excess soluble collagens caused near-complete inhibition of the interaction of C1q with radiolabelled C1qR or CaR, indicating that the collagens bind via an identical, or an overlapping site, to C1q. (J. Lu, unpublished).

3.2. Interaction of recombinant C1qR/CaR domains with C1q

N-, P-, C- and S-domains of recombinant C1qR/CaR were expressed as thioredoxin fusion proteins. Correct expression was verified by SDS-PAGE and Western blotting. The domains were subjected to a number of functional assays, using the 12-kDa thioredoxin fusion partner as a negative control.

3.2.1. Solid phase binding assays. Fig. 3 summarises the results of three separate direct binding experiments. In each case, concentration-dependent, saturable binding (not shown) of radioiodinated C1q was observed for native C1qR, the P-domain and, to a lesser extent, the N-domain. The C-domain showed no binding above the BSA background.

3.2.2. Competitive inhibition of native C1qR-C1q interaction. The results of competitive inhibition studies are shown (Fig. 4). The N-domain and the P-domain show concentration-dependent inhibition of binding of radiolabelled C1q to immobilised native C1qR. As expected, soluble native C1qR also demonstrated inhibition (not shown). Thioredoxin and the C-domain had no inhibitory effect, demonstrating that they do not bind to C1q.

3.2.3. Haemolytic assays – inhibition of C1 assembly and activation. Haemolytic assays were also used to investigate whether the recombinant domains would disrupt C1q function. EAC1q cells are mixed with C1q-deficient serum.

Table 1
Sequence and position of C1q receptor/calreticulin domain constructs

Domain	Construct sequence		Residues expressed
N	Thioredoxin	PLEPAVYF.....ESGSLVDL Q	18–196
S	Thioredoxin	PLDIRCKD.....QIDNPVDLQ	160–283
P	Thioredoxin	PLEDDWDF.....DPSIYVDLQ	197–306
C	Thioredoxin	PLDNFGVL.....QAKDEL	309–417

Constructs were expressed with Thioredoxin fusion products as described in Section 2. The amino acid sequences were deduced from the nucleotide sequence of human cDNA [30]. Residues underlined are human C1qR/CaR; others are vector or primer derived.

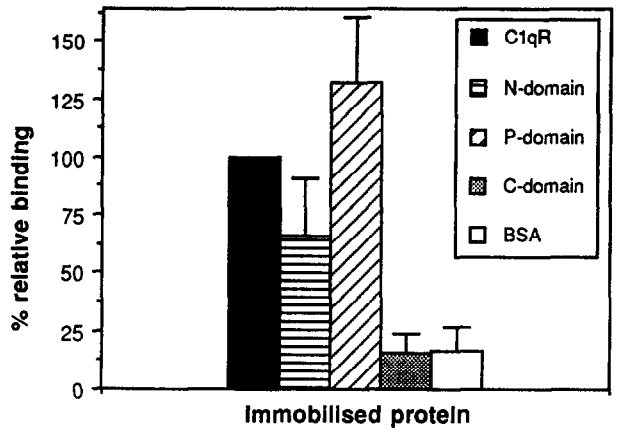

Fig. 3. Binding of C1q to recombinant C1q receptor/calreticulin domains. Recombinant C1qR/CaR domains were expressed in *E. coli* and purified as described in Section 2.4. Solid-phase bound domains, with appropriate controls, were incubated with radioiodinated C1q. Binding levels obtained in three separate experiments, at saturation, were measured and calculated as % (bound/loaded). These levels were then standardised against the results for native C1qR, taken as 100%.

$C1r_2C1s_2$ in the serum binds to C1q, becomes activated, and subsequent activation of the complement system causes cell lysis. Species that interfere with the C1q-$C1r_2C1s_2$ interaction will inhibit lysis. Preliminary results (not shown) using a constant concentration of recombinant C1qR/CaR domains demonstrated that the P-domain has a stronger inhibitory activity than the N-domain. The C-domain did not show inhibition. On the basis of these results, a concentration dependence assay was performed. As shown in Fig. 5, concentration-dependent inhibition of cell lysis was observed for the N- and P-domains. The S-domain, which overlaps parts of the N- and P-domain, also clearly shows significant concentration-dependent inhibition, demonstrating that it binds to C1q. The thioredoxin and BSA controls did not show significant inhibition of C1 formation and activation.

4. Discussion

Sequence analysis of the 56-kDa surface receptor for C1q, MBL, CL43 and SP-A at the protein level revealed a primary structure almost identical to calreticulin [1]. Based upon these results, we determined and compared the binding characteristics of native C1qR and native CaR for aggregated C1q. Both C1qR and CaR preparations exhibit identical binding profiles, attaining strongest binding activity at low salt strength, pointing to the predominantly ionic nature of the interaction. Four recombinant subfragments of C1qR/CaR were expressed as thioredoxin fusion proteins, three of which are subfragments representing previously described CaR domains. Each single subfragment was recognised by polyclonal antisera raised against purified native C1qR, as well as by antisera raised against native and recombinant CaR. Several recent reports [23,42] describe the presence of CaR on the outer surface of cell membranes. These and the results presented here provide strong evidence to indicate that C1qR is a membrane-associated form of calreticulin.

In order to localise the C1q binding site within C1qR/CaR, the different subfragments of recombinant C1qR/CaR were assessed in direct and indirect assays for binding to activated C1q, using purified native C1qR as a control. The indirect binding assay revealed that C1q efficiently binds to the N-domain, and to a lesser extent to the P-domain (Fig. 3). Direct binding to radiolabelled C1q was demonstrated for the N- and P-domains (Fig. 4). No C1q binding activity was observed for the C-domain. These findings are in agreement with the results of a C1-dependent haemolysis assay, in which the fragments were tested for their potential to inhibit complement activation by binding to C1q (Fig. 5). Here, strong inhibitory activity was localised in the S-domain, a subfragment which spans the N- and P-domain intersection. These

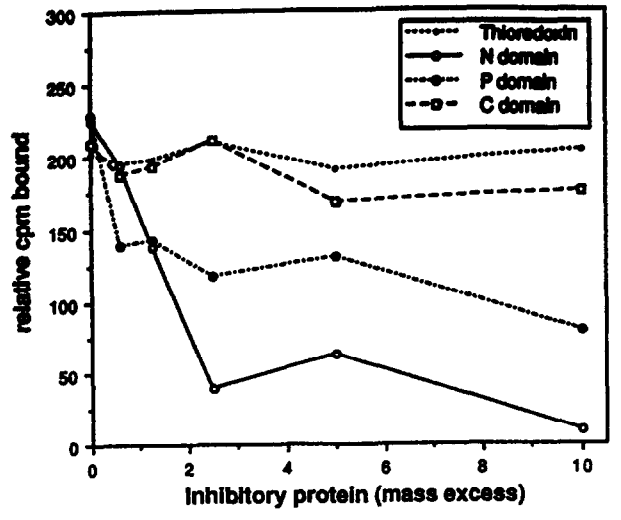

Fig. 4. Inhibition of the native C1qR-C1q interaction by recombinant C1qR/CaR domains. Constant levels of radiolabelled C1q, preincubated with serial dilutions of unlabelled recombinant C1qR/CaR domains or Thioredoxin controls, were bound to, and eluted from, solid-phase bound native C1qR as described in Section 2.5.

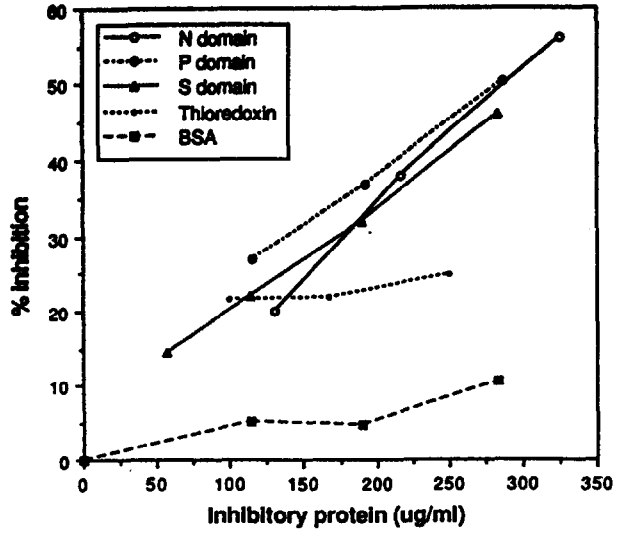

Fig. 5. Inhibition of C1 formation and activation by recombinant C1qR/CaR domains. Haemolytic assays were used to test the concentration-dependence of inhibition of C1 formation using varying concentrations of recombinant C1qR/CaR domains, as described in Section 2.5.

data indicated that the C1q binding site of C1qR/CaR is localised on the S-domain. Site-directed mutagenesis will be employed to further refine this C1q binding site.

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