Functional characterization of a recombinant form of the C-terminal, globular head region of the B-chain of human serum complement protein, C1q

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The first step in the activation of the classical pathway of the complement system by immune complexes involves the binding of the six globular heads of C1q to the Fc regions of IgG or IgM. The globular heads of C1q are located C-terminal to the six triple-helical stalks present in the molecule; each head is considered to be composed of the C-terminal halves (3×136 residues) of one A-, one B- and one C-chain. It is not known if the C-terminal globular regions, present in each of the three types of chain, are independently folded modules (with each chain having distinct binding properties towards immunoglobulins) or whether the different binding functions of C1q are dependent upon a globular structure which relies on contributions from all three chains. As a first step towards addressing this question, we have expressed the globular head region (residues 87–226) of the C1q B-chain (ghB) as a soluble fusion protein with maltose-binding

protein (MBP) in *Escherichia coli*. The affinity purified fusion protein, designated MBP–ghB, behaved as a dimer on gel filtration and bound preferentially to aggregated IgG rather than to IgM. It could also inhibit C1q-dependent haemolysis of both IgG- and IgM-sensitized erythrocytes. After its release from MBP, by use of Factor Xa, the free ghB exhibited a tendency to aggregate and come out of solution. Since MBP is known to be a monomeric molecule, the dimerization of the MBP–ghB fusion polypeptide is probably brought about by the ghB region, perhaps through hydrophobic interactions within the ghB region. The functional behaviour of MBP–ghB indicates that the globular regions of C1q may adopt a modular organization, i.e. each globular head of C1q may be composed of three structurally and functionally independent domains, thus retaining multivalency in the form of a heterotrimer.

INTRODUCTION

The classical pathway of the complement system is a major defence and clearance system in the blood circulation which is activated by immune complexes. The first component of complement C1 is a complex of three glycoproteins, C1q, C1r and C1s. The efficient activation of C1 takes place on interaction of C1q with the Fc regions of IgG or IgM antibodies present in the immune complexes. The human C1q molecule (460 kDa) is composed of 18 polypeptide chains (6 A-, 6 B- and 6 C-chains). The A-chain (223 residues), B-chain (226 residues) and C-chain (217 residues) each have a short (3–9 residue) N-terminal region (containing a half-cystine residue involved in inter-chain disulphide-bond formation), followed by a collagen-like sequence of approx. 81 residues and a C-terminal globular region of approx. 135 residues [1,2]. The only inter-chain disulphide bridges are located between the half-cystine residues at positions A-4 and B-4, in the A- and B-chains, and between half-cystine residues at position C-4 in pairs of C-chains. The inter-chain disulphide bonding yields 6 A–B dimer subunits and 3 C–C dimer subunits. The collagenous sequences in the A- and B-chains of an A–B subunit form a triple-helical collagen-like structure with the equivalent sequence in one of the C-chains present in a C–C subunit, which is held together by both covalent and non-covalent bonds. Three of these structural units are then con-

sidered to associate, via strong non-covalent bonds in the fibril-like central portion, to yield the hexameric C1q molecule [3]. Comparison of the mature chains shows that there are four conserved cysteines in each chain (at positions 4, 135, 154 and 171, as per the B-chain numbering). The cysteines at positions 4 are involved in the inter-chain disulphide bridges, yielding the A–B and C–C subunits, while the other three cysteines in each chain are considered to yield one intra-chain disulphide bond and one free thiol group within the C-terminal globular region. Whereas the C1r and C1s binding sites are located in the collagen region of C1q, the binding sites for the Fc regions of IgG and IgM reside in the globular heads [4–6]. The evidence for the interaction of globular head regions of C1q with IgG comes from experiments in which the globular heads, from which the collagenous stalks were removed by collagenase digestion, were shown to inhibit the reaction between immune complexes and C1q [7]. In their monomeric forms, IgG and IgM have only a weak affinity for C1q in the circulation, allowing for co-existence of IgG/IgM and C1q, without activating the complement cascade. Binding of the C1q globular head regions requires the aggregation of IgG or its assembly as an array on a multivalent antigen. At least two IgG molecules must be bound to an antigen and spaced sufficiently close to each other to be able to bind more than one globular head of the same C1q molecule [8,9]. The binding affinity of monomeric IgG for C1q has been estimated as

Abbreviations used: ghB, C-terminal globular head region of the B-chain of C1q; MBP, maltose-binding protein; MBP–ghB, fusion protein containing maltose-binding protein and the C-terminal, globular head region of C1q B-chain; EA_{IgG}, sheep erythrocytes coated with IgG; EA_{IgM}, sheep erythrocytes coated with IgM; TMB, 3,3',5,5'-tetramethylbenzidine; IPTG, isopropyl β -D-thiogalactoside.

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between 4×10^3 and $5 \times 10^4 \text{ M}^{-1}$ [10–12], but this increases up to 1000-fold, once the IgG is presented in an aggregated form, to 10^7 – 10^8 M^{-1} [13]. IgM has a low affinity for C1q in free solution (between $2.5 \times 10^4 \text{ M}^{-1}$ [14] and $5 \times 10^5 \text{ M}^{-1}$ [15]), similar to that between an isolated C γ 2 domain and one site on C1q [16]. Upon activation, IgG and IgM are believed to form three and two salt bridges respectively with each C1q globular domain [14]. The precise binding region of the IgG molecule for C1q has been proposed as being located in the C-terminal half of the C γ 2 domain of IgG, and specifically to the three residues, Glu-318, Lys-320 and Lys-322 [17], which are highly conserved in the different IgG isotypes. The C γ 3 region of the IgM molecule has been identified as a binding site for the C1q globular head region [18]. The actual number of binding sites on the intact C1q molecule for IgG has been estimated to be 12–18 [11].

However, the complementary binding sites on the C1q globular head region for IgG and IgM have not been identified. Whether a specific class of immunoglobulin is bound selectively by either of the A- or B- or C-chains of C1q, or by a combination of all three, is unknown. All three individual chains have been proposed as being important for IgG binding, as judged by studies involving carbodi-imide cross-linking of complexes of rabbit IgG and human C1q [19]. In the present study, we have expressed the C-terminal, globular head region of the C1q B-chain (ghB) fused to maltose-binding protein (MBP), and investigated whether a single type of module from the heterotrimeric C1q globular head region retains some biological functions. Solid-phase binding studies indicated that MBP–ghB had a greater ability to bind to IgG than IgM. In addition, MBP–ghB, in its dimeric form, was found to inhibit C1q-dependent haemolysis of IgG-sensitised sheep erythrocytes (EA_{IgG}) with a greater efficiency than IgM-sensitised erythrocytes (EA_{IgM}), indicating that the MBP–ghB competed with whole C1q for binding IgG and IgM on the cell surface, and thereby preventing complement activation. The functional behaviour of MBP–ghB suggests that the C1q globular head region may have modular organization, that is, the C-terminal regions of the A-, B- and C-chains could be structurally and functionally independent modules. The finding that the recombinant form of the C1q globular head (MBP–ghB) is an inhibitor of C1q-mediated complement activation opens up the possibility of blocking the classical pathway of complement activation at a very early stage in the activation cascade.

MATERIALS AND METHODS

Preparation of native C1q

Haemolytically active C1q was isolated from human serum using a modification of the the method described previously by Reid [20] involving the three chromatographic steps, including DNA–cellulose, Mono-Q and gel filtration. Its purity was assessed by SDS/PAGE on a 15% (w/v) polyacrylamide gel under reducing conditions. As a precaution, C1q was centrifuged at 4 °C at 14000 *g* for 15 min immediately before use, to eliminate any aggregates which may have formed during storage.

Construction of plasmid encoding the C-terminal, globular head region of C1q B-chain

The plasmid pMAL-c2 (6148 bp) (New England Biolabs, Hitchin, MA, U.S.A.), which contains the *E. coli* *malE* gene under the inducible P_{ac} promoter, which codes for MBP fused in-frame to the *lacZ* gene, was used as expression vector. Sites between *malE* and *lacZ* are available for cloning, which interrupts

the *malE*–*lacZ* fusion and allows blue/white selection on 5-bromo-4-chloroindol-3-yl galactopyranoside (X-gal) plates. This expression vector encodes *E. coli* MBP followed by a Factor Xa cleavage site and a polylinker region for subcloning. The forward primer, 5'-GAACCCGGGGACTACAAGGCCACC-3', and the reverse primer corresponding to Bluescript plasmid were used to PCR-amplify a DNA fragment corresponding to amino acids 87–226 of the B-chain of C1q (this includes the last Gly-Xaa-Yaa triplet of the C1q collagenous region). The full-length cDNA of the B-chain subcloned in the Bluescript plasmid was used as a template. The PCR product (~ 450 bp in size) was cleaved with *Sma*I and *Eco*RI and subcloned into pBC/SK cloning vector restriction digested with *Sma*I and *Eco*RI. The ghB insert was then cleaved with *Xba*I and *Hind*III and subcloned into pMal-c2, restriction digested with *Xba*I and *Hind*III. The recombinant plasmid containing the ghB region fused with MBP was designated pKBM-b.

Expression and purification of the C-terminal region of the globular head of C1q B-chain

E. coli BL21 containing the recombinant plasmid pKBM-b was grown in Luria–Bertini broth (1 litre) containing ampicillin (100 µg/ml) at 37 °C, with vigorous agitation on a shaker to an *A*₆₀₀ of 0.8. Isopropyl β-D-thiogalactoside (IPTG; 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 an additional 3 h and harvested by centrifugation. Cells (~ 3 g of cell pellet) were suspended in 50 ml of lysis buffer [20 mM Tris/HCl (pH 8.0)/0.5 M NaCl/0.25% (v/v) Tween 20/1 mM EGTA/1 mM EDTA/5% (v/v) glycerol]. Lysozyme and PMSF were added to final concentrations of 100 mg/ml and 0.1 mM respectively. All subsequent steps of purification were carried out at 4 °C. The cell suspension was incubated over ice for 30 min and the lysate was then sonicated at 60 Hz for 30 s with an interval of 1 min (10 cycles) to shear the bacterial chromosomal DNA. After centrifugation at 16000 *g* for 30 min, the supernatant was collected and diluted 5-fold using column buffer [20 mM Tris/HCl (pH 8.0)/0.1 M NaCl/0.25% (v/v) Tween 20/1 mM EDTA/5% (v/v) glycerol] and loaded onto an amylose resin column (50 ml bed volume; New England Biolabs, MA, U.S.A.) equilibrated with the column buffer. The column was washed successively with 3 bed vol. of column buffer containing Tween 20, 5 bed vol. of column buffer without Tween 20, and 5 bed vol. of Factor Xa buffer [20 mM Tris/HCl (pH 8.0)/0.1 M NaCl/2 mM CaCl₂/5% (v/v) glycerol]. The fusion protein was eluted with 100 ml of Factor Xa buffer containing 10 mM maltose (~ 30 mg of fusion protein per litre of bacterial culture). In order to remove maltose bound to MBP, the fusion protein was applied to a Mono-Q ion-exchange column. The bound fraction was extensively washed with column buffer (20 mM Tris/HCl (pH 8.0)/100 mM NaCl) in order to wash off the maltose bound to the MBP before elution. The fusion protein was subsequently eluted using a 0.1–1 M NaCl gradient, with the peak fractions eluting at 0.6 M NaCl. The fusion protein was subjected to gel-filtration chromatography using a Superose 12 HR 10/30 column (Pharmacia Biotech Inc.) equilibrated with 20 mM Tris/HCl (pH 8.0)/0.1 M NaCl. After dialysis against Factor Xa buffer, the concentration of the fusion protein was adjusted to 1 mg/ml. Factor Xa (1 unit/ml; New England Biolabs) was added (1 unit of Factor Xa per 200 µg of MBP–ghB) to the fusion protein and incubated overnight at 4 °C to release the ghB module from the fusion partner. After its release from MBP, by use of Factor Xa, the ghB exhibited a

tendency to aggregate and come out of solution. Attempts to denature ghB with 6 M urea and slowly renature it using decreasing concentrations of urea, down to zero molar urea solution, did not help recover the protein in the soluble fraction.

Western blot analysis of the recombinant ghB of human C1q

The recombinant protein, ghB (2 µg), free from MBP, was suspended in sample buffer containing 0.01 % (w/v) Bromophenol Blue, 10 % (v/v) glycerol and 20 mM dithiothreitol reducing agent and incubated for 5 min at 100 °C. The sample was electrophoresed on a 15 % (w/v) polyacrylamide gel and subsequently electrotransferred on to a PVDF transfer membrane (Immobilon-P; Millipore Corp, Bedford, MA, U.S.A.) using a Semiphor transfer unit. After 2 h blocking with 2 % (w/v) BSA, the blot was probed with diluted (1:5000) rabbit anti-human C1q, or rabbit preimmune serum (1:2000), for 45 min at 37 °C. The blot was then washed in PBS containing 0.04 % (v/v) Tween 20 and incubated with 1:10000 diluted goat anti-rabbit IgG-alkaline phosphatase conjugate for 45 min at 37 °C. After washing for 1 h in PBS containing 0.04 % (v/v) Tween 20, the blot was developed using the substrates *p*-Nitro Blue Tetrazolium chloride (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) dissolved in the alkaline phosphatase buffer (100 mM Tris/100 mM NaCl/5 mM MgCl₂). The reaction was stopped by PBS buffer containing 20 mM EDTA. The control protein, MBP, was not recognized by the antibodies used (blot not shown).

Protein sequencing

To confirm that the protein expressed was the C-terminal globular head region of C1q B-chain, an Edman degradation was carried out using an Applied Biosystems 470A protein sequencer and on-line Applied Biosystems 120A analysis of the amino acid derivatives.

Binding specificities of MBP-ghB for IgG and IgM

The relative abilities of heat-aggregated IgG, or IgM, to be bound by solid-phase MBP-ghB were assessed. The immunoglobulins were heat-aggregated by incubating a 20 mg/ml solution at 63 °C for 30 min, and the soluble fractions were used. Recombinant MBP-ghB (0–3 µg/ml), MBP alone, and C1q (0–1 µg/ml) in sodium carbonate buffer, pH 9.6, were coated on to wells of polysorb ELISA plates and left overnight at 4 °C. After blocking with 2 % (w/v) BSA for 2 h and washing, the plates were incubated with either 10 µg/ml of IgG or 20 µg/ml of IgM in TBS-NTC [50 mM Tris/HCl/150 mM NaCl/0.05 % (w/v) NaN₃/0.05 % (v/v) Tween 20/5 mM CaCl₂] at 21 °C for 2 h. The wells were washed and goat anti-human IgG and IgM conjugated to peroxidase (Sigma, Poole, Dorset, U.K.) (1:1000 dilution) were added to the appropriate wells. After incubation for 1 h at 37 °C, the microtitre wells were washed three times. A volume of 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) in aqueous dimethylformamide mixed with one tenth volume of hydrogen peroxide was added to each well. After incubation at room temperature for 20 min, the colour reaction was terminated by adding 150 µl of 1 M H₂SO₄ to each well and the absorbance was read at 450 nm.

The ability of C1q to inhibit binding of IgG to MBP-ghB was also tested. Aliquots of MBP-ghB (10 µg/ml) were coated onto ELISA plates. C1q at various concentrations (0, 2, 5, 15 and 50 µg/ml) was incubated with 10 and 20 µg/ml heat aggregated

IgG for 30 min at 4 °C. Then the C1q-treated IgG preparations were added to the wells as described above.

Inhibition of C1q-dependent haemolysis by MBP-ghB

C1q-deficient human serum (Sigma) was diluted 1:40 in DGVB⁺⁺ [isotonic Veronal-buffered saline containing 0.1 mM CaCl₂, 0.5 mM MgCl₂, 0.1 % (w/v) gelatin and 1 % (w/v) glucose] and various concentrations of purified C1q were added back in a final volume of 100 µl before incubation at 37 °C for 1 h. C1q haemolytic assay was performed by adding 100 µl of EA_{IgG} or EA_{IgM} (10⁷ cells) in DGVB⁺⁺ and incubating for another hour. 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 A₄₁₂ values of 100 µl aliquots of the supernatant were measured to monitor haemoglobin release.

The assay for the inhibition of C1q-dependent haemolysis by MBP-ghB was performed as follows. Aliquots of EA cells (10⁷/100 ml) sensitized with IgG or IgM were preincubated for 1 h at 37 °C with 0.75, 1.25, 2.5, 5 and 10 µg of the MBP-ghB fusion protein or MBP. The pretreated cells were then gently pelleted by centrifugation at 3000 *g* for 2 min, washed and resuspended in 100 µl of DGVB⁺⁺. Each aliquot of EA was added to a mixture, composed of 1 µg of C1q in 10 µl, 2.5 µl of C1q-deficient serum and 87.5 µl of DGVB⁺⁺. After 1 h incubation at 37 °C, the unlysed cells were pelleted and the amount of haemoglobin released was determined spectrophotometrically from the A₄₁₂. Total haemolysis was assessed as the amount of haemoglobin released upon cell lysis with water. The C1q-dependent haemolytic activity was expressed as a percentage of total haemolysis.

RESULTS

Expression and purification of ghB

The globular head of C1q B-chain was expressed as a fusion to *E. coli* MBP. The expression and purification of the fusion protein containing MBP were performed as described previously [21,22]. As shown in Figure 1 (a), upon induction with 0.4 mM IPTG, the MBP-ghB accumulated as an over-expressed protein of ~ 60 kDa (lane 3). After one-step affinity purification on an amylose resin column, followed by ion-exchange chromatography using Mono-Q (lane 4), the ghB module was cleaved from its fusion partner MBP using Factor Xa protease (lane 5). After cleavage and storage overnight, the free ghB showed a tendency to aggregate. The precipitate could be harvested by high-speed centrifugation at 16000 *g* for 15 min and run on SDS/PAGE (lane 6). It migrated as a ~ 16 kDa band under reducing conditions. The recombinant ghB module (free from MBP) was recognized by rabbit anti-human C1q antisera, as judged by the Western blot (Figure 1c). MBP alone did not react with anti-C1q antiserum. N-terminal amino acid sequencing of the ghB confirmed that the right C-terminal globular head region of the C1q B-chain was expressed. The amino acid sequence of the recombinant protein read as T S G F P GDYKATQKIAFSAT. The first five residues are vector-derived, the sixth residue is B87 of the B-chain sequence, the sequence of the following thirteen residues is consistent with the known B-chain sequence [1].

Gel-filtration analysis of recombinant protein

The MBP-ghB and MBP alone were applied to a Superose 12 column (Figure 2). A series of proteins of known molecular mass

Figure 1 SDS/PAGE (15%, w/v) analysis of (a) the various steps involved in the purification of the recombinant C-terminal region of the C1q B-chain, and (b) purified fusion protein MBP-ghB, examined under reduced and non-reduced conditions, and (c) Western blot of ghB

(a) Lane 1, molecular-mass markers (97.4, 66, 45, 31, 21.5 and 14.5 kDa); lane 2, *E. coli* total extract of uninduced cells; lane 3, total extract 3 h after induction with 0.4 mM IPTG; lane 4, fusion protein affinity purified on an amylose resin column and eluted with 10 mM maltose; lane 5, cleavage of the fusion protein using Factor Xa protease; lane 6, free ghB tended to aggregate and could be separated as a pellet/precipitate from MBP by high-speed centrifugation (16 000 *g*) for 15 min. (b) Lane 1, molecular-mass markers (97.4, 66, 45, 31, 21.5 and 14.5 kDa); lane 2, reduced fusion protein; lane 3, non-reduced fusion protein. (c) The ghB module, free from MBP, was electrophoresed on a 15% (w/v) SDS/PAGE under reducing conditions, transferred to PVDF membranes and probed with a rabbit anti-human C1q polyclonal antibody.

were run through the column. The MBP-ghB eluted as a dimer of apparent molecular mass ~ 120 kDa, whereas MBP eluted as a monomer (~ 42 kDa).

Figure 2 Elution profiles of gel-filtration chromatography of MBP-ghB

The MBP-ghB and MBP alone were adjusted to a concentration of 1 mg/ml in the column buffer containing 20 mM Tris (pH 8.0) and 100 mM NaCl. Bovine thyroglobulin (BTG) 669 kDa, alcohol dehydrogenase (ADH) 150 kDa, BSA 67 kDa and soya bean trypsin inhibitor (SBTI) 20.3 kDa were used as molecular size standards. Their approximate elution positions are shown. The proteins were fractionated at a flow rate of 0.5 ml/min using Superose 12 column (HR 10/30) attached to an FPLC (Pharmacia) system.

Interaction of IgG and IgM with MBP-ghB

Different concentrations of C1q and MBP-ghB were coated on to microtitre wells, while control wells were coated with BSA or MBP. Heat-aggregated IgG or IgM were then added to the wells. After incubation at 21 °C for 2 h, the amount of IgG and IgM bound to immobilized C1q and MBP-ghB was quantified by ELISA. The results shown in Figure 3 indicate that both aggregated IgG and IgM bound in a saturable manner to C1q-coated plates, whereas only background levels of IgG and IgM bound to MBP- and BSA-coated wells ($A_{450} < 0.1$). Analysis of IgG and IgM binding by MBP-ghB revealed that IgG was bound preferentially, and in a dose-dependent manner, compared with IgM. In some experiments, heat-aggregated IgG and IgM (1 and 4 μ g/ml) were bound to the plates, then C1q or MBP-ghB were allowed to bind. Under low ionic conditions, MBP-ghB bound to IgG, but not significantly to IgM (results not shown). To explore further the interaction between IgG and MBP-ghB, heat-aggregated IgG was incubated with increasing concentrations of C1q for 30 min at 4 °C, then applied to wells coated with MBP-ghB. IgG was competitively inhibited (by 15–30%), on prior incubation with C1q, from binding to MBP-ghB in a dose-dependent manner (results not included).

Inhibition of C1q-dependent haemolytic activity by MBP-ghB

In view of the MBP-ghB binding to immunoglobulins in the solid-phase ELISA, a C1q-dependent haemolytic assay was performed to study a possible inhibitory effect of MBP-ghB on C1q-mediated complement activation. Sheep red blood cells (E) were sensitized with haemolysin (A), comprising purified anti-sheep blood cell immunoglobulin (IgG or IgM), to yield EA_{IgG} or EA_{IgM} sensitized cells. The C1q-dependent haemolytic assay 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 (1 μ g/ml) back to C1q-deficient serum was sufficient to completely lyse the EA cells (coated with IgG or IgM). This concentration was then chosen as the standard in a series of

Figure 3 Binding of heat-aggregated IgG and IgM to solid-phase-bound C1q and MBP-ghB

Various concentrations of C1q and MBP-ghB were coated on ELISA plates. Fixed concentrations of: top, heat-aggregated IgG (10 µg/well), or bottom, IgM (20 µg/well), were added and probed with top, anti-human IgG, or bottom, anti-human IgM, conjugated to peroxidase. The colour was developed using TMB and A_{450} values were measured. All experiments were performed in duplicate and MBP was used as a control. In all control experiments the A_{450} never exceeded 0.1.

studies to determine if pretreatment of EA_{IgG} or EA_{IgM} with MBP-ghB resulted in inhibition of C1q-dependent haemolysis. Various concentrations of MBP-ghB, ranging from 0.75–10 µg/ml, were added to EA_{IgG} and EA_{IgM} cells and incubated to test if pretreatment of EA cells could protect them from C1q-mediated haemolysis. As shown in Figure 4, the addition of 1.8 µg of MBP-ghB brought haemolysis of EA_{IgG} cells down to < 50 %, whereas it required 10 mg of MBP-ghB to inhibit lysis of IgM-coated EA cells to a similar extent. Normal serum (diluted 1:20) and C1q-deficient serum (diluted 1:40), containing no exogenous C1q, were used as controls for complete and background lysis. The MBP on its own did not interfere with C1q-dependent haemolytic activity at a concentration of 10 µg/100 ml.

DISCUSSION

All three chains of C1q have been implicated in the interaction with the heavy chain of IgG [19,23]. On comparison of the C-terminal regions of the A-, B- and C-chains of C1q (immediately after the collagen-like region, from residue 90 onwards, as based

Figure 4 Inhibition by MBP-ghB of the C1q-dependent haemolysis of IgG- and IgM-sensitized erythrocytes

EA cells (1×10^7), sensitized with IgG (EA_{IgG}) or IgM (EA_{IgM}), were pretreated with various concentrations of MBP, or MBP-ghB, for 1 h at 37 °C, and then spun, washed and resuspended in assay buffer. C1q (1 mg) together with a C1q-deficient serum (diluted 1:40) was then added to the pretreated EA cells for an additional 1 h. The non-lysed cells were pelleted and A_{450} values of 100 ml aliquots of the supernatant were measured spectrophotometrically. □, Control MBP added to EA_{IgG} ; ▲, MBP-ghB added to EA_{IgM} ; △, MBP-ghB added to EA_{IgG} . The percentage lysis was determined relative to complete (100 %) haemolysis. The means of three experiments are presented for each set of experiments.

on the B-chain numbering), approx. 27 % of the residues are completely conserved. The conserved residues include three cysteines and a large number of hydrophobic and neutral residues which form the scaffold of the 135-residues-long C1q C-terminal module and are probably responsible for its largely β -sheet structure, as has been predicted from Fourier-transform IR spectroscopy and analysis of the sequence [24]. Modules of the same type as the C1q C-terminal module are also found in a variety of non-complement proteins, including the C-terminal regions of the human type VIII [25] and type X collagens [26], precerebellin [27], the chipmunk hibernation proteins [28], human endothelial cell protein, multimerin [29], a serum protein, Acrp-30, which is secreted from mouse adipocytes [30], and the sunfish inner-ear-specific structural protein [31]. In several of these proteins, the chains containing these C1q-like C-terminal modules appear likely to form a homotrimeric structure (as in type X collagen, multimerin, Acrp-30 and possibly precerebellin and the inner-ear-specific protein). However, in the other proteins, a heterotrimeric structure is probably formed (as in C1q and the hibernation protein, which both have three types of chain, and the type VIII collagen, which has two $\alpha 1$ and one $\alpha 2$ chains). The C1q molecule is the only one of these proteins for which, to date, a function has been ascribed to the C1q C-terminal module, that is, binding to the Fc regions of IgG and IgM on the formation of immune complexes. The above-mentioned examples are consistent with the speculation that each type of globular head found in the A-, B- and C-chains of C1q may have autonomy as a functional unit.

It is generally accepted that the globular heads of C1q contain the binding sites for the Fc regions of IgG [7,13] as well as IgM. C1q binds to the $C\gamma 2$ domain of IgG and the $C\mu 2$ domain of IgM and the interactions in both cases appear to be primarily ionic. The three charged residues, Glu-318, Lys-320 and Lys-322, in the $C\gamma 2$ domain have been implicated in the binding of C1q to IgG, as judged by a site-directed mutagenesis study of a mouse Ig2b

and antihapten hybridoma antibody [17]. The charged residues Asp-356, Asp-417, Glu-418 and His-420 in the $C\mu 2$ region of IgM are considered to be involved in binding to the C1q heads [18]. Lysine residues thought to be involved in the IgG-binding site within the C1q globular regions have been identified by carbodi-imide cross-linking of complexes of IgG and C1q [19], with all three chains of C1q being implicated in the binding. Chemical modification of C1q using diethyl pyrocarbonate also indicates a role for arginine residues (at A-162, B-114, B-129, C-156) in binding to IgG [32]. It is not known if each of the C-terminal globular regions is an independently folding module (with the A-, B- and C-chain C-terminal globular regions having distinct binding properties) or whether the different binding functions of C1q are dependent upon a combined globular structure that relies on contributions from all three chains. The expression of the C-terminal region of C1q B-chain (residues B-87 to B-226) in *E. coli* has allowed us to address these questions.

C1q bound equally well to both aggregated IgG and IgM, whereas MBP-ghB bound with preferential affinity to aggregated IgG rather than to IgM (Figure 3). In another set of experiments, heat-aggregated IgG and IgM were immobilized to the wells of microtitre plates and binding by C1q and ghB to the immunoglobulins was then assayed under physiological and half-physiological salt conditions. Both IgG and IgM bound C1q at physiological as well as half-physiological ionic strengths. However, the MBP-ghB was only bound by aggregated IgG on the wells under low-ionic-strength conditions and not by IgM under either set of conditions (results not shown). These results indicate that it is possible that the main binding function mediated by the B-chain globular head region, when it is within the intact C1q molecule, is that of binding to IgG aggregates. This experiment, as well as the one described in Figure 4, indicate that ghB may bind preferentially to IgG, rather than IgM, whereas the whole C1q molecule binds equally well to both types of immunoglobulin. It is possible that the globular head regions of the A- or C-chains may be primarily responsible for binding to IgM. In order to study the C1q-IgG or C1q-IgM interactions under more physiological conditions, EA cells were sensitized with either IgG or IgM and the MBP-ghB was tested for its ability to interfere with C1q-dependent haemolysis of the IgG- or IgM-sensitized sheep erythrocytes. The MBP-ghB was able to inhibit lysis of EA cells sensitized with IgG or IgM. However, MBP-ghB was a more potent inhibitor of EA_{IgG} lysis than EA_{IgM}.

The expression and functional characterization of ghB has indicated that each C-terminal region of the three chains may have some independence of structure and function. Protein-protein interactions between the heads of C1q and targets are considered to be mediated by ionic interactions. The dimerization of ghB, as a soluble fusion protein, could be similar to what is seen in the dimerization of the carbohydrate-recognition domain of rat mannose-binding lectin when it is prepared without its α -helical coiled-coil neck region [33]. By analogy, the precipitation of free ghB could be the result of a concentration-dependent hydrophobic interaction between exposed sites on the ghB modules. Attempts to express the globular head regions of A- and C-chains, in soluble forms, are in progress which should possibly allow us to compare each of the recombinant globular heads in terms of binding specificities and affinity towards various ligands. Previously, a synthetic peptide corresponding to residues 189–200 of the B-chain of C1q has been described that binds IgG immune

complexes and inhibits binding of the immune complex to C1q, suggesting that the sequence B-189–B-200 constitutes part of the IgG binding site of human C1q [23]. This observation, together with results presented in this study, suggests a major contribution of ghB in C1q binding to immunoglobulins.

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