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Modular Organization of the Carboxyl-Terminal, Globular Head Region of Human C1q A, B, and C Chains

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The first step in the activation of the classical complement pathway, by immune complexes, involves the binding of the globular heads of C1q to the Fc regions of aggregated IgG or IgM. Located C-terminal to the collagen region, each globular head is composed of the C-terminal halves of one A (ghA), one B (ghB), and one C chain (ghC). To dissect their structural and functional autonomy, we have expressed ghA, ghB, and ghC in Escherichia coli as soluble proteins linked to maltose-binding protein (MBP).

The affinity-purified fusion proteins (MBP-ghA, -ghB, and -ghC) bound differentially to heat-aggregated IgG and IgM, and also to three known C1q-binding peptides, derived from HIV-1, HTLV-I, and β-amyloid. In the ELISAs, the MBP-ghA bound to heat-aggregated IgG and IgM as well as to the HIV-1 gp120 peptide; the MBP-ghB bound preferentially to IgG rather than IgM, in addition to binding β-amyloid peptide, whereas the MBP-ghC showed a preference for IgM and the HTLV-I gp21 peptide. Both MBP-ghA and MBP-ghB also inhibited C1q-dependent hemolysis of IgG- and IgM-sensitized sheep erythrocytes. However, for IgM-coated erythrocytes, MBP-ghC was a better inhibitor of C1q than MBP-ghB. The recombinant forms of ghA, ghB, and ghC also bound specifically to apoptotic PBMCs. We conclude that the C1q globular head region is likely to have a modular organization, being composed of three structurally and functionally independent modules, which retains multivalency in the form of a heterotrimer. The heterotrimeric organization thus offers functional flexibility and versatility to the whole C1q molecule. The Journal of Immunology, 2003, 171: 812–820.

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Approximately 27% of the residues, which are conserved between the A, B, and C chains of human gC1q, including three cysteine and several hydrophobic and neutral residues, form the gC1q scaffold and impart upon it a largely β-sheet structure (18).

Several CCP-activating ligands, including immune complexes (IC), bind via the gC1q domain. Binding of C1q to IC (containing IgG or IgM), is considered to induce a conformational change in the CLR, which leads to the autoactivation of C1r that, in turn, activates C1s. Human C1q shows only weak binding to the Fc regions of nonaggregated IgG (functional affinity constant, $K = 4 \times 10^4$ to $5 \times 10^5$ M$^{-1}$) (19–21). Upon presentation of multiple, closely spaced Fc regions (as found in IC), the strength of binding of the hexameric C1q to IgG increases dramatically ($K = 10^7$ to $10^9$ M$^{-1}$) (22). The gC1q recognizes the C2y2 domain of IgG or the Cα3 domain of IgM through ionic interactions. Three charged residues—Glul, Lys$^230$, and Lys$^322$—in the Cγ2 domain (which are highly conserved in different IgG isotypes) and Asp$^{356}$, Asp$^{417}$, Ghu$^{415}$, and His$^{420}$ in the Cα3—are critical to gC1q-Ig interaction (23–24). However, the role and contributions of the C-terminal globular regions of C1q A (gA), B (gB), and C (gC) chains in binding to IgG, IgM, and other non-Ig activators of the CCP have not been precisely defined.

We have expressed the gA, gB, and gC as soluble fusion proteins and examined their interactions with heat-aggregated IgG and IgM, three known C1q-binding peptides derived from HIV-1 gp41, human T lymphotropic virus type 1 (HTLV-I) gp21, and β-amylloid (Aβ42), and apoptotic cells. Our results appear to suggest a modular organization of the gC1q, consistent with the idea that the globular region of each chain may fold and function with a certain degree of autonomy. It is likely that gA, gB, and gC have evolved as functionally specialized domains or modules, which together as a heterotrimer, confer versatility and flexibility to an intact C1q molecule.

Materials and Methods

Human C1q and synthetic peptides

Human C1q was purified from human serum as described previously (25). The purity of C1q was assessed by SDS-PAGE (15% w/v) under reducing conditions, which it appeared as three bands, corresponding to the A, B, and C chains of 34, 32, and 27 kDa, respectively. The HIV-1 gp41 peptide 601–613 (GIWGCSGKLICTT) was kindly provided by Dr. G. J. Arlaud (Institute de Biologie Structurale Jean-Pierre Ebel, Grenoble, France). The HTLV-I gp21 peptide 400–429 (CRPFPNTNSHYPILQERPPLENYR LTGWGL) was purchased from Mimotopes (Clayton, Victoria, Australia). The β-amylloid peptide, Aβ42–42, and apoptotic cells. Our results appear to suggest a modular organization of the gC1q, consistent with the idea that the globular region of each chain may fold and function with a certain degree of autonomy. It is likely that gA, gB, and gC have evolved as functionally specialized domains or modules, which together as a heterotrimer, confer versatility and flexibility to an intact C1q molecule.

Construction of vectors for the intracellular expression of the globular head region of C1q A, B, and C chains as fusion proteins

The plasmid pMal-c, which codes for maltose-binding protein (MBP) under the Pm promoter, was used for protein expression (New England Bios- labs, Beverly, MA). The following primers were used to PCR-amplify the DNA sequences corresponding to gA, gB, and gC: gA forward primer (FP) 5'-GCCGAGGAGCCTAAGGGCAGCAGGCCG-3' and gA reverse primer (RP) 5'-GCCCCGCTGCACGTTCCGAGCGGGAAGAATG3'; gB FP 5'-GCCCCGAGGCTCTAGGAGGCCATGCTGATGGAGAATG3'; gB RP 5'-GCCGAGGAGCCTAAGGGCAGCAGGCCG-3' and gB reverse primer (RP) 5'-GCCCCGCTGCACGTTCCGAGCGGGAAGAATG3'; gC FP 5'-GCCGAGGAGCCTAAGGGCAGCAGGCCG-3' and gC RP 5'-GCCGAGGAGCCTAAGGGCAGCAGGCCG-3'. Plasmid pMal-c was double-digested with SalI and PstI and each PCR product was cloned as a SalI-PstI fragment. The expressed polypeptide sequence corresponded to residues 88-223 of gA, 90-226 of gB, and 87-217 of gC. The recombinant vectors, containing the gA, gB, and gC sequences linked to MBP, were designated pKBM-A, pKBM-B, and pKBM-C, respectively.

Expression and purification of gA, gB, and gC

The recombinant fusion proteins, MBP-gA, -gB, and -gC, were expressed using E. coli BL21 transformed with pKBM-A, pKBM-B, and pKBM-C, respectively. The bacterial cells were grown in 1 L Luria-Bertani medium containing ampicillin (100 μg/ml) to anOD$_562$ = 0.5 at 37°C, induced with 0.4 mM isopropyl β-D-thiogalactoside for 3 h, and centrifuged (3,000 x g, 4°C, 15 min). The cell pellet (2–5 g) was suspended in 50 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.2% v/v Tween 20, 1 mM EDTA, and 5% v/v glycerol) containing lysozyme (100 μg/ml) and PMSF (0.1 mM), and incubated over ice for 30 min. The cell lysate was then sonicated at 60 Hz for 30 s with an interval of 1 min (15 cycles) to disrupt the cells and shear the bacterial chromosomal DNA. After centrifugation (16,000 x g for 30 min), the supernatant was collected and diluted 5-fold using buffer I (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.2% v/v Tween 20, 1 mM EDTA, and 5% v/v glycerol) and passed through an amylose resin column (50 ml). The column was washed first with buffer I (150 ml), followed by buffer II (250 ml of buffer II without Tween 20). Each fusion protein was then eluted with 100 ml of buffer II containing 10 mM maltose.

To further remove minor contaminating proteins and DNA, the fusion protein was applied to a Q-Sepharose ion exchange column. The bound fraction was extensively washed with column buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl to remove MBP-bound maltose. The fusion protein was subsequently eluted using a 0.1–1 M NaCl gradient. To examine their oligomeric forms, the fusion proteins were individually loaded over a Superose 12 gel filtration column equilibrated with buffer containing 20 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.4. When required, fusion proteins were dialyzed against factor Xa buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM CaCl$_2$, 5% v/v glycerol), and cleaved with factor Xa (New England Bioslabs; 1 U per 50 μg of the fusion protein) at 4°C.

Biotinylation of proteins

Proteins were biotinylated using an EZ-Link NHS-LC biotinylation kit (Pierce, Rockford, IL). The concentration of N-hydroxysuccinimidobiotin used was 25-fold molar excess to the protein solution in PBS, pH 7.2 (protein concentration: 0.4 mg/ml for C1q, 0.22 mg/ml gC1q, 0.25 mg/ml for CLR, 0.9 mg/ml for gA, 1.2 mg/ml for gB, 1 mg/ml for gC, and 1 mg/ml for MBP). The reaction was conducted at room temperature for 3 h followed by extensive dialysis against appropriate buffers to remove free biotin. The biotinylation efficiency was determined using an HABA assay (Pierce) and found to be 72% for C1q, 65% for gC1q, 68% for CLR, and between 75 and 81% for MBP and MBP-containing globular head fragments of each chain as fusion proteins. The hemolytic activity of human C1q was not altered significantly following biotinylation (~8% compared with the unlabeled C1q at the same concentration). The biotinylation of recombinant proteins did not alter their ability to compete with unlabeled native human C1q in the hemolytic assays.

Binding specificities of MBP-gA, -gB, or -gC for heat-aggregated IgG and IgM

C1q, MBP-gA, -gB, -gC, and MBP (0.125, 0.25, 0.5, 0.75, and 1 μg in 100 μl per well for IgG binding; 0.25, 0.5, and 1 μg in 100 μl per well for IgM binding) in carbonate buffer, pH 9.6, were coated to the microtiter wells overnight at 4°C. After blocking with PBS containing 5% w/v BSA and 0.5%Tween 20 and 5 mM CaCl$_2$ at 37°C. Following a 2-h incubation, the plates were washed and either goat anti-human IgG or IgM conjugated to alkaline phosphatase (AP) (1/10,000 dilution) was added to the respective wells. After 2 h, the color was developed using p-nitrophenyl phosphate and A$_405$ was measured.

Inhibition of C1q-dependent hemolysis by MBP-gA, -gB, and -gC

The hemolytic assays were conducted as previously described (26, 27). SRBC (EA), sensitized with either IgG (EA$_{IgG}$) or IgM (EA$_{IgM}$), were pretreated with buffer or heat-aggregated IgG (DGVB containing 2.5 mM sodium barbital, 71 mM NaCl, 0.15 M NaCl, 0.5 mM MgCl$_2$, 2.5% v/v glycerol, 0.1% v/v gelatin, pH 7.4). The addition of human C1q (1 μg) to C1q-deficient serum (1/40 dilution in DGVB) containing 0.5% EA cells (EA$_{IgG}$ or EA$_{IgM}$). This concentration of human C1q was subsequently used to examine whether the pretreatment of EA$_{IgG}$ or EA$_{IgM}$ with MBP-gA, -gB, or -gC protected SRBC from C1q-mediated hemolysis.
The EAγδ + EAγδm (10^7 cells per 100 μl) were pretreated with a range of concentrations of MBP-ghA, -ghB, or -ghC (0.75, 2.5, 5, and 10 μg each) for 1 h at 37°C. Cells were centrifuged and the pellet was washed and resuspended in 100 μl of DGBV-B. Each aliquot of pretreated EA cells was added to a mixture, composed of 1 μg of C1q in 10 μl of buffer, 2.5 μl of C1q-deficient serum, and 87.5 μl of DGBV-B. Following a 1-h incubation at 37°C, the reaction was stopped by transferring the tubes to ice and adding 600 μl of ice-cold DGBV-B. The unlysed cells were pelleted by centrifugation and the A412 of the supernatant was read. Total hemolysis (100%) was assessed as the amount of hemoglobin released upon cell lysis with water. The C1q-dependent hemolytic activity was expressed as percentage of total hemolysis. MBP was used as a negative control protein.

**Interaction between MBP-ghA, -ghB, or -ghC with HIV-1 gp41 peptide 601-613**

A synthetic peptide, corresponding to residues 601-613 of the HIV-1 gp41 (1 μg in 100 μl per well) was coated to microtiter wells in the carbonate buffer overnight at 4°C and subsequently blocked with Veronal-buffered saline (VBS) (5 mM sodium barbital, pH 7.4, 150 mM NaCl, 0.15 mM CaCl₂, 1 mM MgCl₂ and 1% w/v BSA) for 1 h. After washing with VBS containing 0.1% v/v Tween 20, various amounts of C1q, MBP-ghA, -ghB, -ghC, or MBP were added in 100 μl of PBS/NTC buffer and incubated for 4 h. After washing with VBS containing 0.1% v/v Tween 20, rabbit anti-human C1q polyclonal IgG (1/2500 dilution in VBS) was added to the wells and the amount of bound proteins were probed using goat anti-rabbit IgG-HRP conjugate, followed by addition of o-phenylenediamine (OPD) (ODP) as substrate. In an alternative ELISA, immobilized peptides were probed with biotinylated C1q, MBP-ghA, -ghB, or -ghC using streptavidin-HRP conjugate.

In the competitive ELISA, biotinylated C1q (1 μg in 100 μl per well) was coincubated with different concentrations of C1q, MBP-ghA, -ghB, -ghC, or MBP (1, 2, 3, 4, and 5 μg in 100 μl per well) before being added to the wells previously coated with the peptide. Following a 3-h incubation at room temperature, the amount of bound C1q was detected using streptavidin-HRP conjugate.

**Detection of interaction between MBP-ghA, -ghB, or -ghC with HTLV-I gp21 peptide 400-429**

The HTLV-I gp21 peptide 400-429 (1 μg in 100 μl per well) was coated in carbonate buffer at 4°C overnight and probed with different concentrations of biotinylated C1q (1–5 μg in 100 μl per well), MBP-ghA, -ghB, or -ghC (2, 4, 6, and 8 μg each in 100 μl per well). The microtiter wells were blocked with 1% w/v BSA (37°C for 1 h) and washed with PBST (PBS + 0.1% v/v Tween 20) three times. Following incubation with streptavidin-HRP conjugate (1/1000 dilution, 37°C for 2 h), the color was developed using OPD as substrate and read at A405.

For competitive inhibition studies, the microtiter wells were coated with the peptide 400-429 (1 μg in 100 μl) and blocked. Different concentrations of biotinylated C1q, MBP-ghA, -ghB, and -ghC (2, 4, 6, and 8 μg each in 100 μl per well) were then allowed to compete with biotinylated C1q (2.5 μg in 100 μl per well) before being added to each well (37°C for 2 h). The color was developed using the same substrate, and the absorbance was read at A405.

**ELISA for the detection of interaction between MBP-ghA, -ghB, or -ghC with AβI-42**

Direct binding ELISA experiments were performed by coating 1 μg of AβI-42 peptide in 100 μl per well in carbonate buffer overnight and probed with different concentrations of biotinylated C1q (1–5 μg/well), MBP-ghA, -ghB, or -ghC (0.5, 1, 1.5, 2.0, and 2.5 μg/well). The microtiter wells were blocked with 1% w/v BSA (37°C for 1 h) and washed with PBST three times. Following incubation with streptavidin-HRP conjugate (1/1000 dilution, 37°C for 2 h), the color was developed using OPD as substrate and read at A405.

For competitive inhibition studies, different concentrations of nonbiotinylated C1q, MBP-ghA, -ghB, and -ghC (0.5–2.5 μg in 100 μl per well) were added to the peptide-coated (1 μg) wells and incubated (37°C for 2 h) following coincubation with biotinylated C1q (1 μg in 100 μl). After incubation at 37°C for 2 h and washing with PBST, the streptavidin-HRP conjugate (1/2000) and the OPD system were used to develop color.

**Labeling of apoptotic cells with MBP-ghA, -ghB, and -ghC**

Apoptosis was induced by irradiation of PBMCs with a calibrated UV lamp at a dose of 2500 mJ/cm² at a density of 1 × 10⁶/ml in 6-well plates in PBS. Irradiated cells were cultured for a further 6 h in PBS and stained with either FITC-annexin V and propidium iodide (PI) to monitor cell death, or with C1q. Different amounts of biotinylated C1q, MBP-ghA, -ghB, -ghC were incubated with 1 × 10⁶ irradiated PBMC for 30 min at 4°C. Cells were washed twice in PBS containing 1% w/v BSA (PBS + BSA) and bound proteins detected with FITC-conjugated anti-biotin mAb (Sigma-Aldrich, St. Louis, MO). Cells were analyzed by flow cytometry (FACS: BD Biosciences, Mountain View, CA) in the presence of PI. Apoptotic cells were detected on the basis of scatter profile and PI staining. For competition assays, irradiated PBMCs were incubated with either buffer alone, or 5 μg of MBP, MBP-ghA, -ghB, or -ghC in PBS + BSA for 30 min at 4°C. After washing twice in PBS/BSA, cells were incubated with biotinylated C1q (1 μg) and analyzed by FACS.

**Results**

The ghA, ghB, and ghC modules of human C1q can be expressed as soluble fusion proteins.

The globular head regions of C1q A, B, and C chains were expressed as MBP fusion proteins in E. coli BL21 cells containing pKBM-A, pKBM-B, and pKBM-C, respectively. Following induction with 0.4 mM isopropyl β-D-thiogalactoside for 3 h, each fusion protein appeared as overexpressed protein band (~60 kDa, as judged by SDS-PAGE under reducing conditions. The majority of the MBP fusion proteins bound to the affinity column and eluted as ~95% pure soluble fractions (~18 mg of MBP-ghA, ~22 mg of MBP-ghB, and ~30 mg of MBP-ghC per liter of bacterial culture) (Fig. 1, lanes 2, 3, and 4). Further purification was achieved using a Q-Sepharose anion-exchange column (fusion proteins bound at 0.1 M NaCl and eluted as a sharp peak at ~0.6 M NaCl.)
before factor Xa protease digestion to release the globular head fragments free from MBP (Fig. 1). The ghA (lane 5) and ghB (lane 6) migrated as bands of ~18 kDa whereas the ghC band migrated as ~16 kDa (lane 7). However, the free globular regions (ghA, ghB, and ghC) showed tendencies to aggregate and, therefore, the MBP fusions of each globular region were used for functional assays.

The recombinant globular head modules ghA, ghB, and ghC (free from MBP) were recognized by rabbit anti-human C1q antisera, as judged by Western blot. Polyclonal Abs, raised in rabbit against each module, also recognized the whole C1q molecule in the Western blot and ELISA. The N-terminal amino acid analysis revealed the sequences: GSIEGRPKDQ for ghA, TSGFPGDYKATQKIAFSAT for ghB, and GSIEGRPKQK for ghC (with the sequences of each globular sequence given in italics) that confirmed the identity of each recombinant protein. The fusion proteins, when loaded onto a Superose 12 gel filtration column (Pharmacia, Piscataway, NJ) in the buffer containing 20 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.4, eluted between 120 and ~68 kDa molecular size markers, suggesting that the fusion proteins were either dimers or monomers which may be retarded during gel permeation. MBP alone, as expected, eluted as a monomer of ~42 kDa. A small proportion of MBP-ghC also eluted on a gel filtration column as a large aggregate that was separated from the more homogeneously eluting fraction for use in various assays. When run on a 12% SDS-PAGE under nonreducing conditions in the presence of iodoacetamide, the majority of the MBP fusion proteins migrated at ~60 kDa, suggesting that the MBP-containing C1q globular head fragments were not cross-linked via aberrant disulfide bonding.

**MBP-ghA, -ghB, or -ghC bind heat-aggregated IgG and IgM differentially**

Different concentrations of C1q, MBP-ghA, -ghB, or -ghC, coated to the microtiter wells, were allowed to bind heat-aggregated IgG or IgM, and then probed with goat anti-human IgG or IgM conjugated to AP. C1q, MBP-ghA, and MBP-ghB bound heat-aggregated IgG in a dose-dependent manner, whereas MBP-ghC showed comparatively weaker binding ($A_{405} < 0.3$) that did not change with an increase in its concentration (Fig. 2A). C1q, MBP-ghA, and MBP-ghB bound IgM well, compared with MBP-ghB and MBP alone (Fig. 2B). The binding of the MBP-ghA and MBP-ghC to heat-aggregated IgM was comparable, although 4-fold lower than C1q. The MBP-ghB showed relatively weak background binding, which was not dose-dependent (26). It is worthwhile to note that the amount of the recombinant MBP fusion proteins in molar terms is higher than C1q for the same concentration of proteins by weight. For example as in Fig. 2A, a comparison between C1q (~21 nM) and MBP-gh (~167 nM) binding to aggregated IgG at 1 $\mu$g/100 $\mu$L reveals nearly an 8-fold excess of recombinant proteins. Because the native C1q has 18 globular heads (which are clustered) and the recombinant ghA, ghB, and ghC are single polypeptide chains, it is difficult to compare such interactions with respect to multiple heads of C1q vs single heads of recombinant modules.

**MBP-ghA, -ghB, and -ghC differentially inhibit C1q-dependent hemolysis**

To examine the inhibitory effects of MBP-ghA, -ghB, and -ghC on C1q-dependent hemolysis, SRBC (E) were sensitized with hemolysin (A; anti-sheep erythrocyte IgG or IgM fraction) to yield $EA_{120}$ or $EA_{120M}$ Cells. Reconstitution of C1q-deficient serum with 1 $\mu$g of C1q was found to completely lyse (>95%) the sensitized EA cells. $EA_{120G}$ and $EA_{120M}$ cells were pretreated with various concentrations of MBP-ghA, MBP-ghB, MBP-ghC, or MBP alone (0.625–10 $\mu$g) before reconstituting C1q-deficient serum with exogenous human C1q. As shown in Fig. 3A, ~4 $\mu$g of MBP-ghA or MBP-ghB were required to reduce C1q-mediated hemolysis to 50%, whereas 10 $\mu$g of MBP-ghC failed to achieve 50% inhibition for $EA_{120G}$. To achieve >50% inhibition of $EA_{120G}$, ~4 $\mu$g of MBP-ghA and ~10 $\mu$g of MBP-ghC were required, whereas 10 $\mu$g of MBP-ghB failed to achieve 50% inhibition (Fig. 3B). Therefore, the potencies of the three recombinant proteins as inhibitors of C1q, based on C1q-dependent hemolysis of sensitized sheep erythrocytes, are in the order ghA > ghB > ghC for $EA_{120G}$, and ghA > ghC > ghB for $EA_{120M}$. Normal serum (1/20 dilution) and C1q-deficient serum (1/40 dilution) were used as control for complete and background lysis, respectively. MBP did not interfere with C1q-dependent hemolysis.

**The ghA module preferentially binds HIV-1 gp41 peptide 601-613**

C1q (Fig. 4A) and MBP-ghA (Fig. 4B) bound HIV-1 gp41 peptide 601-613 specifically, whereas background level binding was...
shown by MBP-ghB and MBP-ghC (Fig. 4B). When recombinant globular head fragments were used to compete with C1q for binding to peptide 601-613, the MBP-ghA appeared to compete with C1q preferentially and dose-dependently (Fig. 4C, B).

The ghC module shows a preference for HTLV-I gp21 peptide 400-429

When the HTLV-I gp21 peptide 400-429 (1 μg/well) was coated on microtiter wells and probed with biotinylated C1q, MBP-ghA, -ghB, or -ghC, the C1q and MBP-ghC bound specifically with background binding of MBP-ghA and MBP-ghB similar to MBP alone (Fig. 5, A and B). In a competitive ELISA, where nonbiotinylated C1q, MBP-ghA, -ghB, or -ghC were allowed to inhibit binding of biotinylated C1q to the peptide, MBP-ghC inhibited ~40% binding at a concentration of 50 μg/ml (5 μg/well; Fig. 5C).

The β-amyloid peptide specifically interacts with the ghB module

The Aβ1-42 bound native C1q, gC1q, and CLR in a dose-dependent manner in the order C1q > gC1q > CLR (Fig. 6A). The MBP-ghB showed highly specific, preferential and dose-dependent binding to solid-phase Aβ1-42 (Fig. 6B). In a competitive ELISA, where varying concentrations of nonbiotinylated C1q, MBP-ghA, -ghB, and -ghC were allowed to inhibit binding of biotinylated C1q (1 μg) to Aβ1-42 (1 μg), the MBP-ghB inhibited ~50% binding at 20 μg/ml (2 μg/well; Fig. 6C). These results
suggest that like the CLR domain, the gC1q might have additional binding sites for Aβ/H1006-1-42, which appears to be located within the ghB module.

**FIGURE 5.** Binding of HTLV-I gp21 peptide 400-429 to human C1q and MBP-ghA, -ghB, or -ghC. The microtiter wells were coated with HTLV-I gp21 peptide 400-429 in carbonate buffer and probed with biotinylated C1q (A) or MBP-ghA, -ghB, and -ghC (B). After blocking and washing the wells, the binding was detected using streptavidin-HRP conjugate and OPD as substrate. C, Inhibition of C1q binding to HTLV-I gp21 peptide 400-429 by MBP-ghA, -ghB, or -ghC. Microtiter wells were coated with HTLV-I gp21 peptide 400-429 and blocked. Different concentrations of nonbiotinylated C1q, MBP-ghA, -ghB, and -ghC were used as inhibitors of biotinylated C1q before being added to the wells and incubated at 37°C for 2 h. Bound C1q was subsequently probed with streptavidin-HRP conjugate.

**FIGURE 6.** A, Binding of β-amyloid peptide (Aβ/H1006-1-42) to human C1q, gC1q, and CLR. The peptide (1 μg/well) was coated onto the microtiter wells, which were then probed with biotinylated human C1q, gC1q, or CLR. The color was developed using streptavidin-HRP conjugate and OPD as substrate. B, Binding of Aβ/H1006-1-42 to MBP-ghA, -ghB, or -ghC. The solid phase Aβ/H1006-1-42 (1 μg) was probed with a range of concentrations of biotinylated MBP-ghA, -ghB, and -ghC (37°C, 2 h). The color was developed using streptavidin-HRP conjugate. C, Inhibition of C1q binding to Aβ/H1006-1-42 by MBP-ghA, -ghB, or -ghC. The recombinant fusion proteins, C1q, MBP-ghA, -ghB, and -ghC were allowed to inhibit binding of biotinylated human C1q (1 μg/well) to Aβ/H1006-1-42 (1 μg/well). The ghB module inhibited >50% C1q binding to Aβ/H1006-1-42 at a concentration of 20 μg/ml (2 μg/well).

**MBP-ghA, -ghB, or -ghC bind apoptotic cells specifically**

C1q bound apoptotic PBMCs in a dose-dependent manner, consistent with previous reports (28, 29). Biotinylated C1q bound to 62% (±0.12) of the apoptotic cells at a 5 μg/ml concentration and
to 28\% (±0.071) of the apoptotic cells at 0.2 µg/ml (Fig. 7A). When apoptotic cells were labeled with various concentrations of biotinylated MBP-ghA, -ghB, or -ghC, all three recombinant globular head fragments bound to apoptotic cells, in a similar manner to that of C1q (Fig. 7A). Binding of recombinant proteins to apoptotic cells was specific, because nonbiotinylated MBP-ghA, -ghB, or -ghC (5 µg each) inhibited binding of biotinylated C1q (1 µg) by 73, 70, and 79\%, respectively (Fig. 7B). MBP alone had no effect on binding of C1q to apoptotic cells.

Discussion

A distinctive, heterotrimeric gC1q domain, situated at the C-terminal end of the triple-helical CLR, characterizes the structural organization of C1q. As a charge pattern recognition protein, C1q is known to interact with a range of ligands via gC1q. However, the role and contributions of ghA, ghB, and ghC in binding to IgG and IgM have not been precisely defined. We generated the recombinant forms of ghA, ghB, and ghC to understand whether the C-terminal globular regions of human C1q A, B, and C chains are functionally autonomous modules (with ghA, ghB, and ghC having distinct binding properties), or the ability of C1q to bind its ligands is dependent upon a combined, globular structure. In addition to aggregated IgG and IgM, we also included in our study some of the known non-Ig targets of C1q, such as peptide 601-613 derived from the transmembrane envelope glycoprotein gp41 of HIV-1, the gp21 peptide 400-429 from HTLV-I, β-amyloid peptide, and apoptotic PBMC.

Based on the Acrp-30 numbering, four residues are conserved throughout the members of C1q and TNF families: Tyr163, Gly159, Phe237, and Leu242 (14). Each residue seems important for the effect on binding of C1q to apoptotic cells. Perturbation of all three chains. Alternatively, it may also reflect on the requirement of multiple contact sites between gC1q and IgM. These observations are consistent with the results of C1q-dependent hemolytic assays where both ghA and ghB could also inhibit C1q-dependent hemolysis of IgG- and IgM-sensitized sheep erythrocytes. However, for IgM-coated erythrocytes, the ghC was a better inhibitor of C1q than the ghB.

C1q has been shown to interact with residues 601-613 (loop region) of the envelope protein gp41 of HIV-1, which facilitates infection of complement-receptor bearing cells, instead of viral lysis (32–34). HIV-1-infected B cells, which may serve as an alternative reservoir for HIV-1, also activate CCP in a gp41-dependent manner (35). Structurally, the gp41 loop region exhibits an absence of charged residues and an abundance of exposed hydrophobic side chains (characteristics of protein-protein interaction sites). When modeled on the gp41 structure of simian immunodeficiency virus, the HIV-1 gp41 loop region forms a cleft, which is likely to be complementary to the binding of gC1q (36). We found a specific interaction between ghA and peptide 601-613 compared with ghB and ghC (37). In the competitive ELISA, there was a dose-dependent decrease in binding of C1q to peptide 601-613 when MBP-ghA was used as an inhibitor of this interaction. The inhibitory effect of MBP-ghA was maximally 50\% implicating a prominent role of the gC1q domain, especially ghA, in the interaction with gp41. Because HIV-1-induced CCP activation is stronger upon shedding of gp120, the gp120 and gC1q are likely to have overlapping binding sites within the gp41 loop region. Previously, Lys608, Leu609, and Ile610 within peptide 601-613 have been identified as central to interaction between gp41 and C1q (38). The Ala substitution of all hydrophobic residues (including the 4-carbon aliphatic moiety of the Lys608 side chain) within the loop abolishes C1q-gp41 interaction. Because this interaction involves hydrophobic contacts, it is curious that only ghA binds to peptide 601-613 preferentially. It will be interesting to examine whether ghA can interfere with the gp41-C1q interaction and thus inhibit complement-dependent enhancement of viral infection.

C1q has been shown to bind and inhibit the infectivity of the cell-free HTLV-I virion via its interaction with the extramembrane region of gp21, a transmembrane protein considered responsible for fusion between the virus and target cell membrane (39, 40). Although the C1q binding site has been mapped to residues 400-429 and 426-460 of gp21, only peptide 400-429 has been shown to inhibit the syncytium formation induced by cocultivation of HTLV-I-bearing C91/PL cells with indicator MOLT-4 cells (41). The N-terminal region of gp21 bears a 29-residue long hydrophobic stretch (a feature of a fusion peptide) and an anchorage domain,
corresponding to a hydrophobic sequence (residues 446-465) located near the C terminus of the gp21. Thus, gp21 peptide 400-429 corresponds to the site between the anchorage domain and the fusion domain of gp21. Because syncytium formation induced by virus-bearing cells involves binding to the virus receptor and folding of the envelope leading to the exposure of the fusion domain and membrane fusions, the interaction between peptide 400-429 and C1q may directly affect the fusion process required for syncytium formation. Our result suggests that the C1q-HTLV-I gp21 interaction is likely to take place via the gC1q domain and the ghC module is likely to have a central role.

C1q has the ability to bind the fibrils of β-amyloid (Aβ1-42), which accumulate around cerebral blood vessels. It is the major protein component of the senile or neuritic plaques in the brains of patients with Alzheimer’s disease and is known to activate the CCP (42, 43). Recently, it has been shown that C1q interaction with fibrils of the Aβ1-42 is likely to take place through the gC1q region (44), contrary to an earlier report suggesting an involvement of residues 14-26 within the C1q A chain CLR in C1q-Aβ1-42 interaction (45). Our results provide evidence for a highly specific and preferential interaction of the ghB module with β-amyloid fibrils. However, we also noticed a dose-dependent binding of CLR to Aβ1-42 (Fig. 6A), compared to the other two peptides examined, i.e., HIV-1 gp41 peptide 601-613 and HTLV-I gp21 peptide 400-429 (data not included). We think that both gC1q and CLR are capable of engaging β-amyloid fibrils and such interactions should be viewed in the context of fibril aggregation and the activation of the CCP.

C1q is considered to protect against autoimmunity by serving as “opsonin” in the efficient recognition and physiological clearance of cells undergoing programmed death by apoptosis. The apoptotic cells generate discrete subcellular structures, called “apoptotic blebs”, that contain either nuclear or cytoplasmic constituents, many of which are targeted by autoantibodies in systemic lupus erythematosus (SLE) patients. The finding that C1q can bind specifically to the surface blebs of apoptotic cells (28), and that the common autoantigens targeted in SLE can be found in high concentrations on the surface of the apoptotic cells (46), have led to the view that C1q deficiency may cause SLE as a result of an impaired clearance of apoptotic cells (47–49). C1q has been shown to bind directly to apoptotic blebs of vascular endothelial cells and PBMCs via the ghC1q domain (29), which causes CCP activation and deposition of C4 and C3 on the surface of these cells and on cell-derived blebs (50). Thus, appropriate recognition of apoptotic cells by gC1q and targeted clearance of the molecular contents of surface blebs to complement receptors via exposed CLR (for example, CR1 on the surface of erythrocytes) may be critical for the maintenance of immune tolerance. The recombinant ghA, ghB, and ghC showed specific, dose-dependent binding to the apoptotic PBMC and competed with C1q in binding to the apoptotic cells. Our results are consistent with a recent study where globular head-specific mAbs as well as native gC1q (prepared following collagenase digestion of native human C1q) were able to inhibit binding of C1q to apoptotic cells (29). The recombinant globular head modules may prove to be useful reagents in identifying potential autoantigens, which are likely to be clustered within apoptotic blebs.

In summary, the expression and functional characterization of the individual globular head regions has allowed examination of the modular organization of the C-terminal regions of human C1q. The specificity as well as selectivity of individual globular heads potentially offers enormous flexibility and versatility to the intact C1q molecule, which is considered to perform broad spectrum immune functions by engaging a range of ligands, cell types, and putative cell surface receptor molecules. The recognition of a C1q/TNF superfamily, whose members have a wide range of functions in host defense, inflammation, apoptosis, autoimmunity, cell differentiation, and homeostasis, emphasizes the possibility that the globular regions of C1q may fulfill more binding functions than previously envisaged. The recombinant ghA, ghB, and ghC therefore could be useful reagents in identifying TNF-related functions (15, 16). It should also be possible to re-examine the interaction of gC1q with a range of ligands, either via direct binding studies, or as inhibitors of whole C1q molecule. Such studies have consistently been influenced by difficulties of generating reasonable amounts of relatively soluble gC1q domains, prepared by collagenase digestion of native C1q.

However, there are significant quantitative differences in molar terms between C1q and recombinant globular heads wherever interaction of native and recombinant proteins with ligands have been examined. A direct comparison between C1q and recombinant fragments of globular heads is further compounded by the fact that intact C1q is likely to have up to 18 binding sites (or at least 6 interacting sites: 6 × ghA, 6 × ghB, and 6 × ghC) compared with a single site within the recombinant ghA, ghB, and ghC. It is also likely that avidity and kinetics of C1q binding to its ligands will be different because of multivalency of globular regions in the intact molecule. Therefore, it is important to consider these issues while interpreting the results of direct binding and competitive inhibition described in the present study.

Future work would involve reconstitution of ghA, ghB, and ghC, following denaturation and renaturation procedure, to generate a soluble and stable heterotrimer, as is considered to be present in the native molecule. If successful, such a “recombinant” heterotrimeric gC1q molecule should provide opportunities to engineer point mutations within ghA, ghB, or ghC modules and study the effects of single residue mutations within a heterotrimeric organization. At a functional level, these recombinant gC1q fragments could also be used as “C1q inhibitors” to block the CCP at the first step of its activation (15, 16, 27, 51, 52).

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