Existence of Different but Overlapping IgG- and IgM-Binding Sites on the Globular Domain of Human C1q[†]

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ABSTRACT: C1q is the first subcomponent of the classical complement pathway that binds antigen-bound IgG or IgM and initiates complement activation via association of serine proteases C1r and C1s. The globular domain of C1q (gC1q), which is the ligand-recognition domain, is a heterotrimeric structure composed of the C-terminal regions of A (ghA), B (ghB), and C (ghC) chains. The expression and functional characterization of ghA, ghB, and ghC modules have revealed that each chain has some structural and functional autonomy. Although a number of studies have tried to identify IgG-binding sites on the gC1q domain, no such attempt has been made to localize IgM-binding site. On the basis of the information available via the gC1q crystal structure, molecular modeling, mutational studies, and bioinformatics, we have generated a series of substitution mutants of ghA, ghB, and ghC and examined their interactions with IgM. The comparative analysis of IgM- and IgG-binding abilities of the mutants suggests that the IgG- and IgM-binding sites within the gC1q domain are different but may overlap. Whereas Arg^{B108}, Arg^{B109}, and Tyr^{B175} mainly constitute the IgM-binding site, the residues Arg^{B114}, Arg^{B129}, Arg^{B163}, and His^{B117} that have been shown to be central to IgG binding are not important for the C1q−IgM interaction. Given the location of Arg^{B108}, Arg^{B109}, and Tyr^{B175} in the gC1q crystal structure, it is likely that C1q interacts with IgM via the top of the gC1q domain.

C1q is the first subcomponent of the classical complement pathway (CCP) and a charge pattern recognition molecule of innate immunity. C1q binding to the IgG- or IgM-containing immune complex leads to the autoactivation of C1r, which, in turn, activates C1s. C1r and C1s, the two serine protease proenzymes, together with C1q constitute C1, the first component of the classical pathway. The activation of the C1 complex ($C1q + C1r_2 + C1s_2$) subsequently leads to the activation of the C2−C9 components of the classical pathway and formation of the terminal, membrane attack complex (MAC) (*1*, *2*). In addition to being the target recognition protein of the classical pathway, C1q is also involved in a number of other immunological processes, including maintenance of immune tolerance via clearance of apoptotic cells, phagocytosis of bacteria, neutralization of viruses, cell adhesion, and modulation of immune cells, thus playing a crucial role in linking innate and adaptive immunity (*3*, *4*).

The primary subunit structure of the human C1q molecule includes an N-terminal triple-helical collagen region leading into the ligand-binding globular domain (gC1q[1] domain), which is a heterotrimeric structure composed of C-terminal regions of A, B, and C chains (*5*, *6*). Six of such structural subunits give rise to the native hexameric C1q that appears like a bunch of tulips (*7*, *8*). C1q can recognize, via its gC1q domain, a broad range of target self- and non-self-molecules, including IgG or IgM, acute phase proteins such as C-reactive

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[1] Abbreviations: gC1q, globular head region of C1q; MBP, maltose-binding protein; ghA, ghB, and ghC, recombinant form of the carboxy-terminal gC1q region of the A, B, and C chains, respectively; CRP, C-reactive protein; PTX3, long prototypic pentraxin 3.

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protein (CRP) and long prototypic pentraxin 3 (PTX3), bacterial porins, viral envelope proteins, lipids, and apoptotic cells. These interactions result in a number of physiologically crucial events such as inflammation and apoptosis (*3*). The versatile ability of the gC1q domain to engage such a wide array of ligands is helped by the modular organization of individual globular head modules of A (ghA), B (ghB), and C (ghC) chains. Individually expressed recombinant modules of the gC1q domain have been shown to have differential binding properties, although they exist as a heterotrimer (*9*, *10*). This notion of the gC1q domain being modular in nature has been reaffirmed by the crystal structure solved at 1.9 Å resolution (*11*) that has revealed a compact, spherical heterotrimer with a pseudo-3-fold symmetry. The three modules within the gC1q domain (ghA, ghB, and ghC) show clear differences in their electrostatic surface potentials, in addition to an equatorial position of ghB. Thus, the modular organization of the heterotrimeric assembly together with different surface charge patterns and the spatial orientation of individual modules enable gC1q to interact with a diverse range of ligands (*3*, *4*, *8*).

The best-examined C1q ligand, IgG, binds the gC1q domain via Glu^{318}, Lys^{320}, and Lys^{322} residues from the murine IgG2b (*12*) in addition to another motif consisting of Asp^{270}, Lys^{322}, Pro^{329}, and Pro^{331} (*13*). Site-directed mutagenesis studies involving substitution mutation within recombinant forms of ghA, ghB, and ghC modules have revealed that Arg^{B114}, His^{B117}, Arg^{B129}, and Arg^{B163} are important for IgG binding (*14*), consistent with chemical modification and molecular-modeling studies (*11*, *15*−*17*). In comparison to the C1q−IgG interaction, little is known about the complementary binding site on the gC1q domain for IgM. This interaction is predominantly ionic in nature, reversible, and sensitive to salt concentration (*18*). The gC1q-binding site within the IgM molecule is considered to reside within Cμ3 and probably Cμ4 domains that consist of a number of charged motifs (*19*−*23*).

The corresponding IgM-binding site on the gC1q domain is not known. No molecular-modeling study concerning the interaction between C1q and IgM is available at present. Individual globular head modules have been shown to bind IgM differentially (*9*, *24*) in a Ca^{2+}-dependent manner (*9*, *24*, *25*). In the present study, we have used a series of substitution mutants of recombinant ghA, ghB, and ghC modules and examined their ability to bind IgM directly and compete with the corresponding wild-type module and C1q. Our results suggest that the IgG- and IgM-binding sites within the gC1q domain of human C1q are overlapping but distinct.

MATERIALS AND METHODS

Buffers. Sodium carbonate (SC) buffer (0.035 M $NaHCO_3$ and 0.015 M Na_2CO_3 at pH 9.6), citric-phosphate buffer (0.025 M citric acid and 0.05 M Na_2HPO_4), phosphate-buffered saline (PBS) (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, and 0.14 M NaCl at pH 7.4), citric-phosphate buffer for the pH range of 3.0−8.5 (0.05 M sodium citrate, 0.05 M Na_2HPO_4, 0.14 M NaCl, and 0.05% Tween 20), and SC buffer for the pH range of 8.5−12.0 (0.05 M $NaHCO_3$, 0.05 M Na_2CO_3, 0.14 M NaCl, and 0.05% Tween 20) were used.

Purified Proteins and Antibody Conjugates. C1q was purified from human plasma using affinity chromatography by IgG-Sepharose as described (*14*). The purity of C1q was assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (15%, w/v) (*26*) under reducing conditions, where C1q appeared as three bands (corresponding to the A, B, and C chains of 34, 32, and 27 kDa, respectively). Goat anti-rabbit IgG−horseradish peroxidase (HRP) conjugate, rabbit anti-mouse IgG−HRP conjugate, goat anti-rabbit IgG−AP conjugate, mouse anti-MBP antibodies, *o*-phenylenediamine dihydrochloride (OPD), and pNPP were purchased from Sigma-Aldrich. Rabbit anti-human C1q polyclonal antibodies were purchased from DAKO.

Site-Directed Mutagenesis and Cloning of Single-Residue Mutants of ghB. In this study, we used a number of single-residue mutants described previously as follows: Arg^{A162}-Ala/Glu, Arg^{B114}Ala/Gln/Glu, His^{B117}Ala/Asp, Arg^{B129}Ala/Glu, Arg^{B163}Ala/Glu, and Arg^{C156}Ala/Glu (*14*) and Lys^{B136}Glu and Tyr^{B175}Leu (*15*). Additionally, two mutants Arg^{B108}Glu and Arg^{B109}Glu were generated by site-directed mutagenesis using an overlapping polymerase chain reaction (PCR) approach (*27*). The PCR products incorporating the mutations were digested with *Xba*I and *Hin*dIII and subcloned into the pMal-c2 expression vector (New England Biolabs, Beverly, MA). The following custom-made primers (Invitrogen, Paisley, U.K.) were used to generate mutations. Primers for Arg^{B108}Glu were FP, AACGTCCCCCTGGAGCGGGACCAGACC; RP, GGTGTGGTGCCGGTCCAGGGGGACCTT. Primers for Arg^{B109}Glu were FP, AACGTCCCCCTGCGCGAGGACCAGACCATC; RP, GATGGTCTGGTCCTCGCGCAGGGGGACGTT. The incorporation of point mutations were confirmed by automated DNA sequencing (ABI Prism 3100 analyzer; Applied Biosystems) using bacteriophage M13 and maltose-binding protein (MBP)-specific malE primers.

Intracellular Expression and Purification of the Wild Type and Point Mutants of rghA/B/C as Fusion Proteins Linked to MBP. In this study, we used globular head regions of the A, B, and C chains (ghA, 88−223; ghB, 90−226; ghC, 87−217) and a number of single-residue mutants. The recombinant wild type and mutant versions were expressed as MBP fusion proteins in *Escherichia coli* and purified using amylose-affinity chromatography. The expressed proteins were additionally purified by ion-exchange chromatography on Q-Sepharose as in ref *9*. The mutant variants were tested using enzyme-linked immunosorbent assay (ELISA) for their ability to bind anti-human C1q, chain-specific antibodies, and anti-MBP monoclonal antibodies.

ELISA To Assess pH Dependence of the Interaction of IgM with C1q, ghA, ghB, and ghC. IgM (1 μg/well) was coated on the microtiter wells and incubated with C1q, ghA, ghB, and ghC (2 μg/well) overnight at 4 °C in citric-phosphate buffer for the pH range of 3.0−8.5 or SC buffer for the pH range of 8.5−12.0. After washing, the amount of bound protein was detected by incubation with anti-MBP antibodies (1:1000) and rabbit anti-mouse IgG−HRP conjugate. The OPD substrate system was used, and A_{490} was measured. The results were presented in arbitrary units, where the minimal observed binding was accepted as 0 and the maximal was accepted as 1. The analysis of the pH dependence was made by fitting the data plots with the closest sigmoid curve, using the data analysis software Microcal Origin 6.0. The inflection points of the curves represent the effective pK_a.

To compare the pH dependence of the interaction of ghB with IgM and IgG, the plates were coated with 1 μg/well IgG or IgM and incubated overnight with 2 μg/well ghB. The amount of bound recombinant protein was detected by incubation with anti-MBP antibodies (1:1000) and rabbit anti-mouse IgG−HRP conjugate. The OPD substrate system was used, and A_{490} was measured. The results are presented in optical density units.

Interaction of Recombinant gC1q Modules and Their Substitution Mutants with Heat-Aggregated IgG. Microtiter wells coated with different concentrations of C1q globular head wild type or corresponding mutant protein in carbonate buffer (pH 9.6), washed, blocked with PBS containing 2% (w/v) bovine serum albumin (BSA) for 2 h, and incubated with heat aggregated human IgG (HAIgG) (5 μg/well) in PBS containing 0.05% Tween 20 for 2 h at 37 °C. After washing, the color was developed using goat anti-human IgG−HRP conjugate and OPD was used as a substrate (data not included).

In the second detection system, the microtiter wells were coated with HAIgG (1 μg/well) in 0.2 carbonate buffer at pH 9.6 overnight at 4 °C. After the wells were blocked with PBS containing 2% (w/v) BSA and subsequent washing, the wells were incubated for 2 h at 37 °C with different concentrations of recombinant proteins (0.0313, 0.0616, 0.125, 0.25, 0.5, and 1 μg/well) in PBS at pH 7.6 containing 0.05% Tween 20 and the amount of bound recombinant protein was detected by incubation with mouse anti-MBP antibodies (1:1000) and rabbit anti-mouse HRP conjugate. Color was developed using the OPD substrate system. MBP was used as a negative control.

Interaction of Recombinant C1q Modules or Their Mutants with IgM. Two types of ELISA were used. The first involved coating of microtiter wells with IgM (1 μg/well) in SC buffer at pH 9.6 for 2 h at 37 °C, with any residual binding sites blocked with 1% (w/v) BSA in PBS for 1 h at 37 °C and washed with PBS at pH 7.4 containing 0.05% Tween 20 (PBST). The recombinant modules (wild type or mutants) at different concentrations (4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 μg/well) were added and incubated overnight in PBST at 4 °C. After the wells were washed with PBST, they were incubated with mouse anti-MBP antibodies. The amount of bound recombinant protein was detected by incubation with rabbit anti-mouse IgG−HRP conjugate or goat anti-rabbit IgG−AP conjugates, respectively. Color was developed using appropriate substrate (OPD/pNPP), and A_{490}/A_{405} was measured. MBP was used as a negative control. Alternatively, ELISA plates coated with different concentrations of recombinant proteins were probed with IgM (2 μg/well). The amount of bound IgM was detected by goat anti-human anti-IgM IgG−HRP conjugate (data not included).

Competitive ELISA. The inhibitory effects of mutant proteins ($Arg^{B108}Glu$, $Arg^{B109}Glu$, and $Lys^{B136}Glu$) on the interaction between ghB and HAIgG or IgM were examined as follows: the microtiter wells were coated with ghB (2 μg/well), blocked, and washed; HAIgG (or IgM) (at 1 μg/well) and different amounts of mutant proteins (1.25, 2.5, 5, and 10 μg/well) were mixed and added to the wells; and the amount of bound HAIgG or IgM was detected by incubation with anti-human IgG−HRP conjugate or goat anti-human IgM−HRP conjugate. OPD was used as a substrate.

Alternatively, a competitive ELISA was set up to analyze the inhibitory effect of the recombinant gC1q mutants on the C1q−IgM (IgG) binding. IgM (2 μg/well) or HAIgG (1 μg/well) was coated on the microtiter wells in SC buffer and then blocked with 2% BSA in PBS for 1 h at 37 °C. C1q (1.5 μg/well) and globular heads (the wild types or the corresponding mutants) (10 μg/well) were mixed and added to the wells. The amount of bound recombinant protein was detected with mouse anti-MBP antibodies and rabbit anti-mouse IgG, conjugated with HRP. Color was developed using the OPD substrate system, and A_{490} was measured. In control experiments, the decrease of C1q binding in the presence of different amounts of recombinant globular C1q heads (1.25, 2.5, 5, and 10 μg/well) was detected with anti-C1q antibodies specific to the collagen region. The inhibitory effect for the wild-type recombinant proteins was considered as 100% and compared to the values for the corresponding mutants. The percentage reduction of IgM (or HAIgG) binding capacity of the mutants was calculated by subtracting the inhibitory capacity of the corresponding mutant from that of the wild type. The obtained data are presented as the mean ± standard deviation (SD) of triplicate measurements.

Inhibition of C1q-Dependent Hemolysis by the Wild-Type ghB and Its Mutants. SRBC (E), sensitized with either IgG (EA_{IgG}) or IgM (EA_{IgM}), was prepared in $DGVB^{2+}$ [dextrose gelatin veronal buffer containing 2.5 mM sodium barbital, 71 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% (w/v) glucose, and 0.1% (w/v) gelatin at pH 7.4]. The addition of human C1q (1 μg) to C1q-deficient serum (1/40 dilution in $DGVB^{2+}$) was sufficient to lyze >90% EA cells (EA_{IgG} or EA_{IgM}) in the assay described below. This concentration of human C1q was subsequently used to determine if the pretreatment of EA_{IgG} or EA_{IgM} with ghB or mutant proteins protected EA_{IgG}/EA_{IgM} from C1q-mediated hemolysis. The EA_{IgG} or EA_{IgM} (10^7 cells per 100 μL) was pretreated with a range of concentrations of ghB or mutant proteins (1.25, 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 $DGVB^{2+}$. Each aliquot of pretreated EA_{IgG} cells was added to a mixture composed of 1 μg of C1q in 10 μL of $DGVB^{2+}$ buffer, 2.5 μL of C1q-deficient serum, and 87.5 μL of $DGVB^{2+}$. After 1 h of incubation at 37 °C, the reaction was stopped by transferring the tubes to ice and adding 600 μL of ice-cold $DGVB^{2+}$. The unlysed cells were pelleted by centrifugation, and the A_{412} of the supernatants was read. Total hemolysis (100%) was taken as the amount of hemoglobin released upon cell lysis with water. The C1q-dependent hemolytic activity was expressed as a percentage of the total hemolysis. MBP was used as a negative control protein.

RESULTS

pH Dependence of the Interaction between Recombinant gC1q Modules and IgM. We have analyzed the pH dependence of the interaction between C1q (and its recombinant derivatives) with IgM in a wide pH range from 3.0 to 12.0 (Figure 1a). The curve trends for IgM binding of the native C1q, ghA, and ghC are comparable (with maximum binding at pH about 5.0), while the curve for ghB is quite different. The inflection points of the curves for C1q, ghA, and ghC are at around pH 4.0 and 6.0. Only the ghB results could be fitted to three sigmoid curves (with inflection points at pH

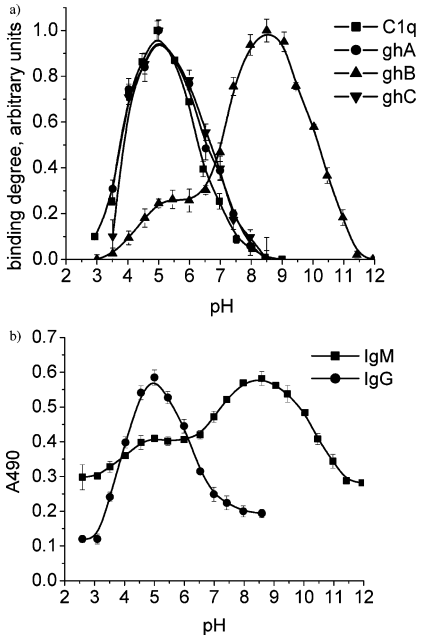

FIGURE 1: Comparative analysis of the pH dependence of the interaction of C1q and its recombinant modules with IgG and IgM. (a) pH dependence of the interaction of C1q, ghA, ghB, and ghC with IgM. C1q, ghA, ghB, and ghC were incubated with IgM-coated wells using buffer with a pH range between 3 and 12. The results are presented as arbitrary units. The data shown are means ± SD of triplicate measurements. (b) pH dependence of the interaction of ghB with IgM and IgG. The ghB module was incubated with IgM- or HAIgG-coated wells using buffer with a pH range between 2.5 and 12. The results are presented in optical density units. The data shown are means ± SD of triplicate measurements.

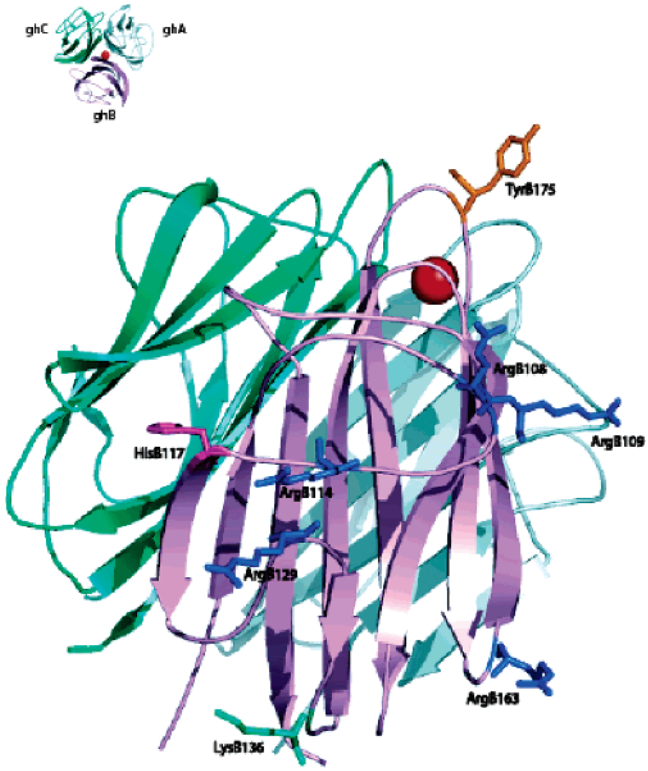

FIGURE 2: Positions of the amino acid residues substituted using site-directed mutagenesis. Ribbon representation of gC1q with mutated residues Arg^{B108}, Arg^{B109}, Arg^{B114}, His^{B117}, Arg^{B129}, Lys^{B136}, Arg^{B163}, and Tyr^{B175} represented in different colors. Arg residues are colored in dark blue; Lys residues are colored in light blue; His residues are colored in violet; and the Tyr residue is orange. The calcium ion is represented as a red ball. (Inset) Top view of gC1q domain, with ghA, ghB, and ghC modules depicted. The figure is generated using PyMol.

4.0, 7.0, and 10.0), corresponding to three independent ionization processes, and its maximum binding to IgM was attained in the alkaline pH range (at pH 9.0). Because we have already analyzed the pH dependence of the interaction of the recombinant globular head fragments of C1q with heat-aggregated IgG (*28*), we wished to know if the data, obtained for IgM (Figure 1a), were similar to that for the ghB−IgG over the same pH range. It was found that the curve for ghB−IgG binding (Figure 1b) was similar to ghA and ghC for their binding to both, IgM and IgG (Figure 1a). In contrast to ghA and ghC, the ghB exhibited differences in IgM and IgG binding that were dependent upon pH. Thus, we focused our attention mainly on the amino acid residues within ghB that were responsible for IgM binding, assuming that this C1q chain might be differentially involved in IgG and IgM binding.

Bacterial Expression of Substitution Mutants of ghA, ghB, and ghC Modules. Using PCR-based site-directed mutagenesis, a number of mutant forms of gC1q modules were generated, including Arg^{A162}Ala/Glu, ArgB^{114}Ala/Gln/Glu, His^{B117}Ala/Asp, Arg^{B129}Ala/Glu, Arg^{B163}Ala/Glu, and Arg^{C156}-Ala/Glu (*14*) and Tyr^{B175}Leu and Lys^{B136}Glu (*15*). Additional mutants, Arg^{B108}Glu and Arg^{B109}Glu, were generated to examine the IgG- and IgM-binding properties of ghB. The relative positions of the residues that were subjected to substitution mutagenesis within the three-dimensional structure of the gC1q domain are in Figure 2. All of the mutants were expressed to a level comparable to the corresponding wild-type modules following induction with 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h and accumulated intracellularly as overexpressed soluble proteins of ~60 kDa, judged by SDS−PAGE under reducing conditions (data not shown). The expressed proteins were purified using affinity chromatography (amylose column) and a Q-Sepharose column as ~95% pure soluble fraction, as described previously (*9*, *14*). All expressed and purified mutants reacted immunologically to human C1q and ghB chain-specific antisera (data not shown).

Contribution of Arg^{B108}, Arg^{B109}, and Tyr^{B175}, Which Are Located at the Apex of the gC1q Domain, to C1q Binding to IgG or IgM. We examined the interaction of ghB or single-residue mutants (Arg^{B108}Glu, Arg^{B109}Glu, and Tyr^{B136}Leu) with IgG or IgM by directly coating either immunoglobulins (probed with ghB) or the recombinant proteins (probed with IgG/IgM). In either case, the results were comparable, consistent, and dose-dependent. The substitution of Arg^{B108} and Arg^{B109} with Glu resulted in ~30 and ~22% reduction in IgG binding, respectively, compared to ghB (Figure 3a). The IgM-binding ability of the two mutants was also reduced down to ~26% for Arg^{B108}Glu and ~34% for Arg^{B109}Glu, respectively (Figure 3b). In the competitive ELISA, the mutant proteins were allowed to compete with the wild-type ghB for IgG or IgM binding (parts c and d of Figure 3). The mutant Arg^{B108}Glu showed ~30 and 26% reduction in binding IgG and IgM, respectively, when compared to the inhibitory activity of ghB. Arg^{B109}Glu reduced the binding of ghB to IgG and IgM by ~20 and ~36%, respectively. Comparable results were obtained when the Arg^{B108} and Arg^{B109} mutants were allowed to compete with C1q for IgM

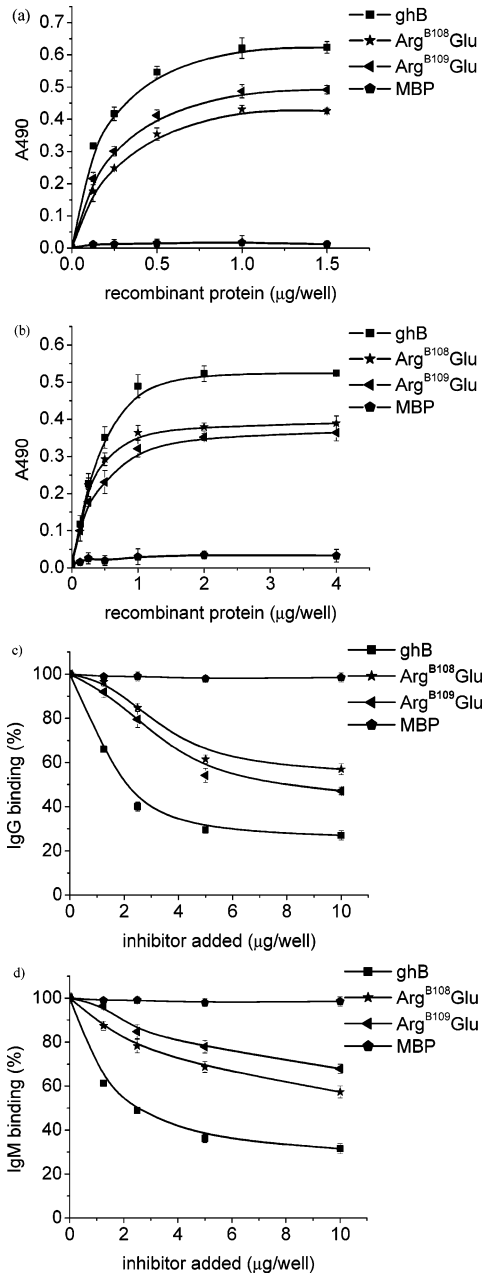

FIGURE 3: Interaction of the wild type and mutant forms of Arg^{B108}Glu and Arg^{B109}Glu with heat-aggregated IgG and IgM (a) Direct ELISA for IgG binding. HAIgG was coated on the microtiter wells and incubated with ghB or mutant forms (Arg^{B108}Glu and Arg^{B109}Glu) at different concentrations. The amount of bound recombinant protein was detected by incubation with mouse anti-MBP Ab and rabbit anti-mouse HRP conjugate. The data shown are means ± SD of triplicate measurements. (b) Direct ELISA for IgM binding of the wild type and mutant forms of Arg^{B108}Glu and Arg^{B109}Glu. The recombinant globular head proteins (wild type or mutants) at different concentrations were incubated with IgM-coated microtiter wells. The amount of bound recombinant protein was detected by incubation with goat anti-mouse HRP conjugate. The data shown are means ± SD of triplicate measurements. (c) Inhibitory activity of the point mutants Arg^{B108}Glu and Arg^{B109}Glu on the interaction between ghB and heat-aggregated IgG. HAIgG and different amount of ghB mutants were preincubated and added to the ghB (wild type) coated wells. The amount of bound HAIgG was detected with anti-human IgG−HRP conjugate using the OPD substrate system. (d) Inhibitory activity of the point mutants Arg^{B108}Glu and Arg^{B109}Glu on the interaction between ghB and IgM. IgM and different amount of ghB mutants were preincubated and added to the ghB-coated wells. The amount of bound IgM was detected as in c with the exception that the goat anti-human IgM−HRP conjugate was used.

Table 1: Inhibitory Effect of Recombinant gC1q (Wild Types and Mutants) on the C1q and IgM (and IgG) Interaction

inhibitor (10 μg/well)	decrease in IgM binding (%)	SD ±%	decrease in IgG binding (%)	SD ±%
ghA	0	1.2	0	1.7
Arg^{A162}Ala	0.5	2.0	16	2.2
Arg^{A162}Glu	0	1.4	25	2.1
ghB	0	2.7	0	2.1
Arg^{B108}Glu	24.3	2.1	31	2.5
Arg^{B109}Glu	35.5	1.9	20.1	1.7
Arg^{B114}Ala	2	2.3	41	1.8
Arg^{B114}Glu	1.5	1.9	49.5	2.5
Arg^{B114}Gln	0	1.3	43.5	1.9
His^{B117}Ala	2.1	0.3	19.5	1.8
His^{B117}Asp	7	3.8	24.3	2.1
Arg^{B129}Ala	4	0.9	25.2	2.2
Arg^{B129}Glu	9.5	1.5	34.5	2.9
Lys^{B136}Glu	36.8	2.6	20	2.2
Arg^{B163}Ala	2.3	1.8	26.5	1.9
Arg^{B163}Glu	1.9	2.1	31.1	2.2
Tyr^{B175}Leu	43.2	2.3	18.7	1.9
ghC	0	1.8	0	1.9
Arg^{C156}Ala	0	1.9	18.7	2.1
Arg^{C156}Glu	0.6	1.7	22.5	1.8

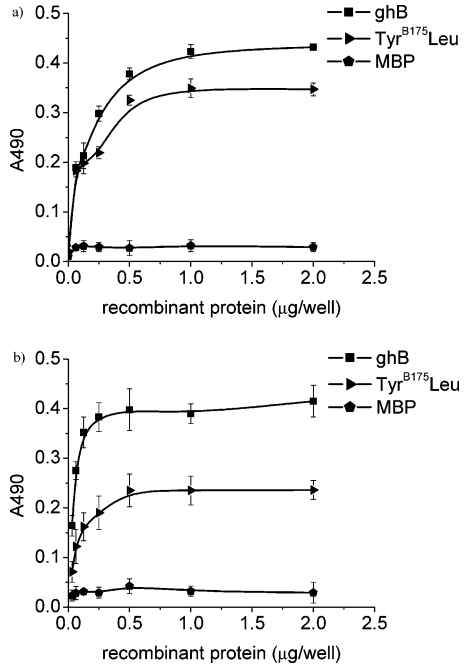

FIGURE 4: Binding of wild-type ghB module and mutant Tyr^{B175}Leu to HAIgG (a) and IgM (b). The wild-type ghB or mutant Tyr^{B175}Leu (at different concentrations) was incubated with HAIgG (a) or IgM (b) coated wells. The immunoglobulin binding was detected by mouse anti-MBP antibodies and rabbit anti-mouse IgG−AP conjugate. The data shown are means ± SD of triplicate measurements.

(IgG) binding (Table 1). The Arg^{B108}Glu and Arg^{B109}Glu were less potent inhibitors of the C1q−IgM interaction (∼24 and 33% reduction, respectively). Substitution of Tyr^{B175} to Leu resulted in ∼40% reduction of IgM binding (Figure 4a), compared to a mere 15−17% decrease in IgG binding (Figure 4b). The inhibitory assay demonstrated that the Tyr^{B175} mutant gives inhibition up to ∼43% for IgM and ∼19% for aggregated IgG.

In comparison to ∼70% inhibition of C1q-dependent hemolysis of EA$_{IgG}$ by ghB at the maximum concentration tested (10 μg), the mutants Arg^{B108}Glu (∼40%) and Arg^{B109}Glu (∼49%) were weaker inhibitors (Figure 5a). For EA$_{IgM}$

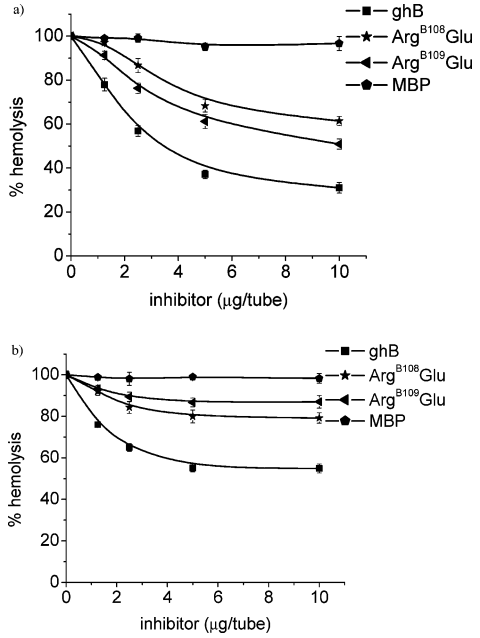

FIGURE 5: Inhibition of C1q-dependent hemolysis of SRBC sensitized with IgG (a) or IgM (b) by Arg^{B108}Glu and Arg^{B109}Glu. SRBCs, sensitized with either IgG (EA_{IgG}) (a) or IgM (EA_{IgM}) (b), were pretreated with recombinant proteins to assess if EA_{IgG}/EA_{IgM} cells were protected from C1q-mediated hemolysis. After reconstitution of C1q-deficient serum, the reaction was stopped, the incubation mixture was centrifuged, and the A_{412} of the supernatants were read. Total hemolysis (100%) was taken as the amount of hemoglobin released upon cell lysis with water. The C1q-dependent hemolytic activity was expressed as a percentage of the total hemolysis. MBP was used as a negative control protein.

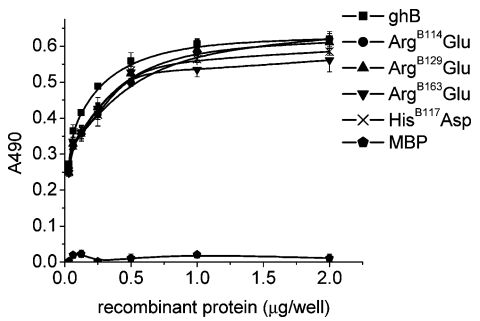

FIGURE 6: Binding of recombinant ghB and mutants Arg^{B114}Glu, Arg^{B129}Glu, Arg^{B163}Glu, and His^{B117}Asp with IgM. The recombinant globular head proteins (wild type or mutant ones) at different concentrations were incubated with IgM-coated microtiter wells, and the binding was detected using anti-MBP monoclonal antibodies. The data shown are means ± SD of triplicate measurements.

cells, the inhibitory activity of ghB was found to be ~45%, as compared to ~20% by Arg^{B108}Glu and ~13% by Arg^{B109}-Glu (Figure 5b). MBP did not interfere with C1q-dependent hemolysis.

Contribution of Arg^{A162}, Arg^{B114}, His^{B117}, Arg^{B129}, Arg^{B163}, and Arg^{C156} Located at the Side Surface of the ghB Module in the C1q−IgM Interaction. To examine the inteaction of Arg^{A162}Ala/Glu, Arg^{B114}Ala/Gln/Glu, His^{B117}Ala/Asp, Arg^{B129}Ala/Glu, Arg^{B163}Ala/Glu, and Arg^{C156}Ala/Glu with IgM, two ELISA assays were set up by coating either IgM or recombinant proteins. In either case, all tested mutants bound IgM in a dose-dependent manner (Figure 6). When the Arg^{A162}, Arg^{B114}, Arg^{B129}, Arg^{B163}, and Arg^{C156} residues were substituted with either Ala or Glu, no significant change in the IgM-binding activity was observed. Similarly, the

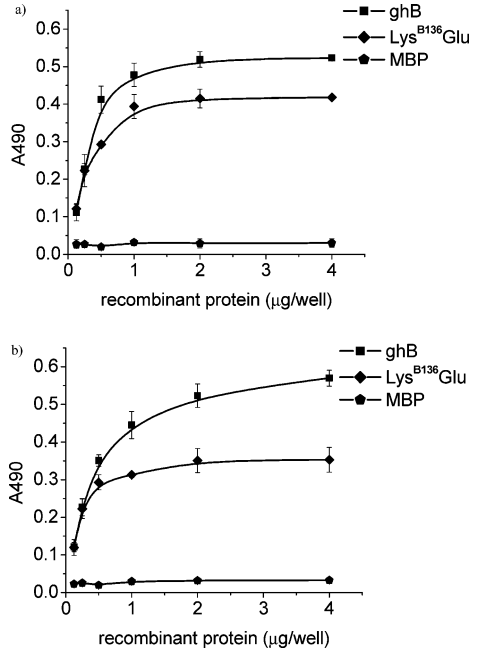

FIGURE 7: Binding of ghB and mutant Lys^{B136}Glu with HAIgG (a) and IgM (b). HAIgG (a) or IgM (b) coated wells were incubated with various amounts of ghB or mutant Lys^{B136}Glu. The binding was probed using anti-MBP monoclonal antibodies. The data shown are means ± SD of triplicate measurements.

His^{B117}Ala mutant did not show an altered binding to IgM, compared to the wild-type ghB module. Only a slight decrease in the IgM binding was observed for His^{B117}Asp (~10%) and Arg^{B129}Glu (~13%). Furthermore, mutant forms of ghA and ghC (Arg^{A162}Ala/Glu and Arg^{C156}Ala/Glu) did not show any significant reduction in IgM binding (data not included). The inhibitory activity of the mutants on the C1q−IgM interaction was compared with the inhibitory activity of the wild-type recombinant globular head modules (Table 1). The obtained results correlate well with the data obtained from the direct binding assays. These data are very interesting because the above-mentioned residues have been shown to be involved in IgG binding (*14*).

Contribution of Lys^{B136} in the C1q−IgM Interaction. The contribution of Lys^{B136} in IgG1, CRP, and PTX3 binding was recently reported (*16*). The mutant was analyzed for IgM-binding ability. Here, we compared IgM- and HAIgG-binding properties of the Lys^{B136}Glu mutant. The obtained results indicated that the substitution Lys^{B136}Glu resulted in up to ~35% decrease in the IgM binding (Figure 7a). The influence of Lys^{B136}Glu on HAIgG binding was less effective; the decrease of HAIgG-binding reaches up to ~20% (Figure 7b). In the competitive ELISA, the mutant proteins Lys^{B136}Glu and the wild-type ghB were allowed to compete with C1q for IgM binding (Table 1). The inhibitory capacity of the mutant was found to be consistent with that observed in the direct binding assay. In the C1q-dependent hemolytic assay, Lys^{B136}Glu (10 μg) was a weaker inhibitor (~50%) than ghB (70%) (Figure 8a). For EA_{IgM} cells, this mutant inhibited ~13%, compared to ~46% by ghB and Lys^{B136}Glu (Figure 8b).

DISCUSSION

In the present study, we have attempted for the first time to identify residues present on the gC1q domain, which may

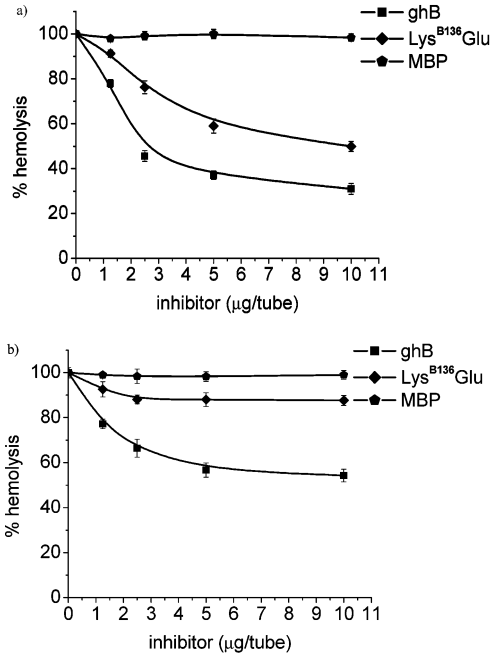

FIGURE 8: Inhibition of C1q-dependent hemolysis of SRBC sensitized with IgG (a) or IgM (b) by Lys^{B136}Glu. The inhibition of hemolysis was performed as it was described in Figure 5. The ghB and mutant Lys^{B136}Glu were used in this assay.

be crucial for the C1q−IgM interaction. Although the native gC1q structure exists as a heterotrimer, it has been difficult to generate the recombinant form of ghA, ghB, and ghC holding together as one structure. However, when the recombinant modules are expressed individually, they have been shown to be functionally active (*9*, *10*). Thus, the availability of these modules has opened up possibilities to engineer mutations, characterize them, and then extrapolate the data in the heterotrimeric context (*14*, *15*). We generated several substitution mutants that have been highlighted to be important for C1q−ligand interactions via chemical modification and mutational studies (*14*), crystal structure and molecular modeling (*11*), bioinformatics (*3*), and in silico theoretical calculations of electric moment vectors (*25*).

The availability of the X-ray crystal structure of the gC1q domain of human C1q has been of immense value in the design of mutational studies. The structure shows that the gC1q domain has a compact, spherical heterotrimeric organization with a noncrystallographic pseudo-3-fold symmetry. Each of the individual globular head modules, with their N and C termini emerging at the base of the trimer, has a jellyroll topology consisting of a 10-stranded β sandwich made up of two five-stranded antiparallel β sheets (*11*). The gC1q is held together predominantly by nonpolar interactions, with contributions from a series of interactions along its 3-fold axis that include hydrogen bonds, a well-exposed Ca^{2+} ion located near the apex, and main-chain polar interactions. Additional lateral interactions, which are hydrophobic at the base and polar and hydrophilic toward the apex, further stabilize the heterotrimeric assembly. The three modules within gC1q, ghA, ghB, and ghC, show clear differences in their electrostatic surface potentials. The modules ghA and ghC both show a combination of basic and acidic residues scattered on their external face, whereas module ghB shows a predominance of positive charges, especially a continuous patch of arginine residues (Arg^{B101}, Arg^{B114}, and Arg^{B129}), that have been implicated in the C1q−IgG interaction (*14*, *16*). Molecular modeling based on the crystal structure of the gC1q of human C1q has also revealed that, within the ghB module, which is the most accessible of the three modules because of its equatorial position, Arg^{B129} and Glu^{B162} seem central to the C1q−IgG interaction and additional ionic interactions are provided by Arg^{B114} and Arg^{B161}. The modeling has also proposed that Tyr^{B175} (ghB) and Lys^{A200} (ghA) can potentially form complementary CRP-binding sites. In addition, it has recently been reported that the residues Tyr^{B175} and Arg^{B109} from the apex of the gC1q heterotrimer are important in C1q binding to CRP, IgG1, and fucoidan, respectively (*15*, *17*). Furthermore, an analysis of ghA, ghB, and ghC sequences individually, using a computer program called ConSurf, has identified a number of functionally critical residues that are highly variable within the gC1q-containing C1q family members and map within the potential binding area on the gC1q crystal structure (*3*).

Recently, we have found that the exposed Ca^{2+} within the gC1q heterotrimer primarily influences the target recognition properties of C1q toward IgG, IgM, CRP, and PTX3 (*25*). At pH 7.4, the loss of Ca^{2+} leads to changes in the direction of the electric moment from a coaxial position in the calcium-saturated holo form (toward the gC1q apex) to one perpendicular to the molecular axis in the calcium-depleted apo form (toward the equatorial side of the B chain). Thus, two planes, normal to the electric moment vectors in the holo form (the holo plane) and in the apo form (the apo plane), can be defined with potential importance for target recognition. Some of the mutated residues as well as residues that have been demonstrated to be important for the ligand-binding belong to these planes *(3*, *11*, *17*).

The analysis of the pH dependence of the interaction between C1q (and its recombinant derivatives) with IgM in a wide pH range from 3.0 to 12.0 indicated that C1q, ghA, and ghC demonstrated similar behavior in the binding of IgM and IgG, while the curve for ghB was quite different. While the results for ghA and ghC could be fitted into two sigmoids, the results for ghB could be fitted into three sigmoids (with inflection points at about 4.0, 7.0, and 10.0), corresponding to three independent ionization processes with the maximum binding at the alkaline pH range (about 9.0). A potential role for Lys and Tyr residues in the ghB−IgM binding, belonging either to IgM or ghB, could be speculated from the alkaline part of the pH dependence of the ghB−IgM interaction. Because no such residues were reported to be involved in the formation of the C1q-binding site for IgM (*21*), we logically considered that Tyr^{B175}, which has previously been reported as important for IgG1 and pentraxins binding, may contribute to IgM binding as well.

The differences in the pH curves indicated that the maximum ghB−IgG binding is achieved under acidic conditions, while the maximum ghB−IgM binding is reached in the alkaline pH range. C1q as well as ghA and ghC exhibit maximum IgM binding in the acidic pH range. These observations suggest that ghA and ghC are more important for the C1q−IgM interaction at the lower pH (under conditions of inflammation), while ghB has a leading role in the IgM binding at higher pH (during apoptosis). Thus, IgM may have a greater role in complement-mediated clearance of apoptotic cells than IgG (*28*). The different IgG- and IgM-binding behavior of ghB under different pH

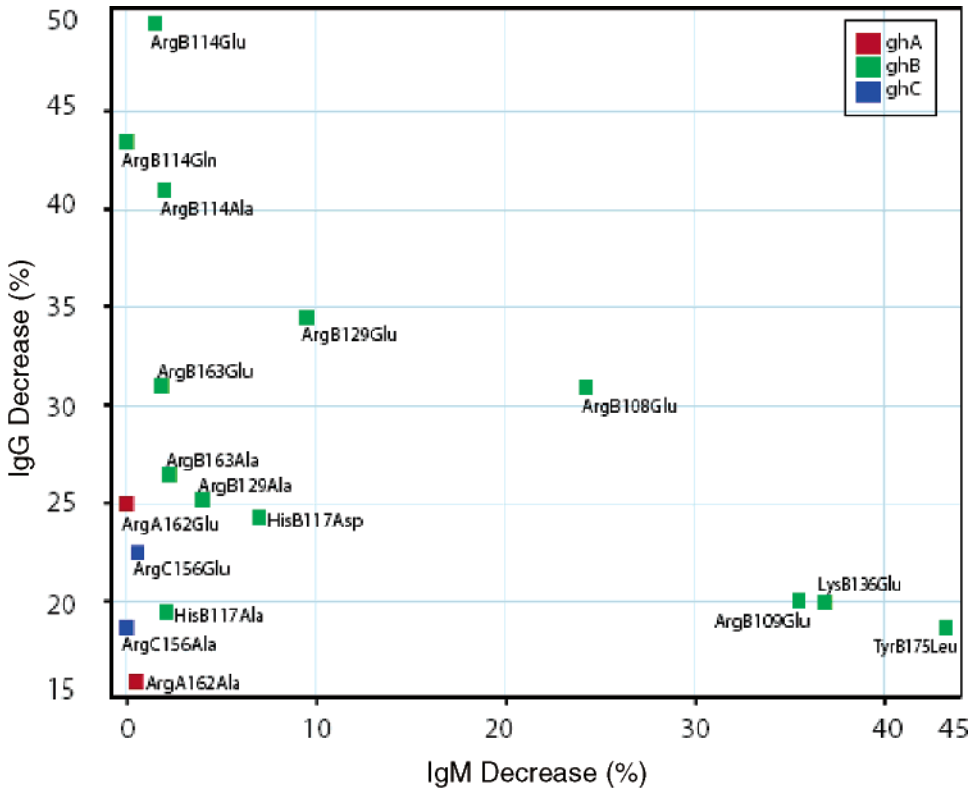

FIGURE 9: Scatter plot showing the obtained percent decreases in IgG and IgM binding. Data for all mutants is shown in the plot. It is clearly seen that mutations in Arg^{B108} and Arg^{B109} have big effects on binding by IgG and IgM, and possibly, both form the overlapping region of overlap between the IgG and the IgM-binding site on C1q. Lys^{B136} is also seen to have a big effect, but it is possible that this is a remote effect because this residue is far removed from the top of C1q. Tyr^{B175}, which is located on the top of the C1q globular head, also shows a tendency to affect binding by both molecules, although the effect seen for IgM is far more than that seen for IgG.

conditions provided an important direction to the mutational analysis using ghB. It this study, we have analyzed the immunoglobulin-binding properties of recombinant globular head modules of C1q and several corresponding mutants under physiological conditions (pH ~ 7.0) to compare the results reported here and those already published (*9*, *10*, *14*, *15*, *25*).

The comparative analysis of IgM- and IgG-binding activity of all of the tested mutants has revealed that residues Arg^{B108} and Arg^{B109}, which belong to both the apo and holo planes of the C1q molecule, are keen candidates for participation in the recognition of IgG, IgM, and other targets. It seems that these residues are involved to a different extent in the interaction with the two tested ligands IgG and IgM. The results show that Arg^{B108} is more important for IgG binding, while the contributions of Arg^{B108} and Arg^{B109} to the ghB−IgM binding were comparable. Most likely these residues are important for the initial stage of IgG and IgM recognition. Tyr^{B175} is most likely involved in the formation of the IgM-binding site of C1q, while its contribution to IgG binding is very little (~15−19% decrease of the Tyr^{B175}Leu−IgG interaction, when compared to that of the wild-type ghB−IgG). The location of Tyr^{B175}, near Arg^{B108} and Arg^{B109} at the top of the gC1q heterotrimer, indicates that the gC1q domain interacts with IgM via the top of the molecule. Tyr^{B175} can participate in both hydrogen bonding with its hydroxyl group and hydrophobic/stacking interactions via its aromatic ring. In the case of the Tyr^{B175}Leu mutant, although the hydrogen-bonding potential of Tyr^{B175} is impaired, the hydrophobic interaction remains intact. Given the significant decrease in its IgM-binding ability, it is likely that Tyr^{B175} forms hydrogen bond with a complementary partner from IgM.

The mutational analysis, presented in the scatter plot, has also suggested that the residues that have previously been implicated in IgG binding (Arg^{B114}, His^{B117}, Arg^{B129}, and Arg^{B163}) (*14*) do not participate in the formation of the IgM-binding site of the gC1q domain (Figure 9). While Arg^{A162} and Arg^{C156} may be involved in IgG binding, these residues do not appear to contribute to the IgM recognition. Lys^{B136} seems to be quite important for C1q binding to IgG as well as IgM (this study), in addition to CRP and PTX3 (*15*). This residue is located very far from the top of the C1q, near the collagen arm.

Considering the overall structure of IgM, it is very unlikely that the top as well as the side surfaces of the gC1q domain are engaged in ligand binding simultaneously. Most likely, the IgM recognition is achieved by the top of the gC1q domain, where Arg^{B108}, Arg^{B109}, and Tyr^{B175} are located. If this is the case, no direct binding of Lys^{B136} with this ligand will occur. The influence of this residue on IgM (and other previously tested ligands) binding could be the exertion of a remote effect.

Molecular modeling based on the crystal structure of the gC1q of human C1q has revealed that the ghB is the most accessible of the three modules because of its equatorial position (*11*). It has a predominantly positively charged outer surface distinguished by the presence of several basic amino acids from ghB (Arg^{B101}, Arg^{B114}, and Arg^{B129}), two of which have already been described as important for the IgG−C1q interaction. We think that, at the very first stage of the recognition of IgG and IgM, C1q uses the apex, where

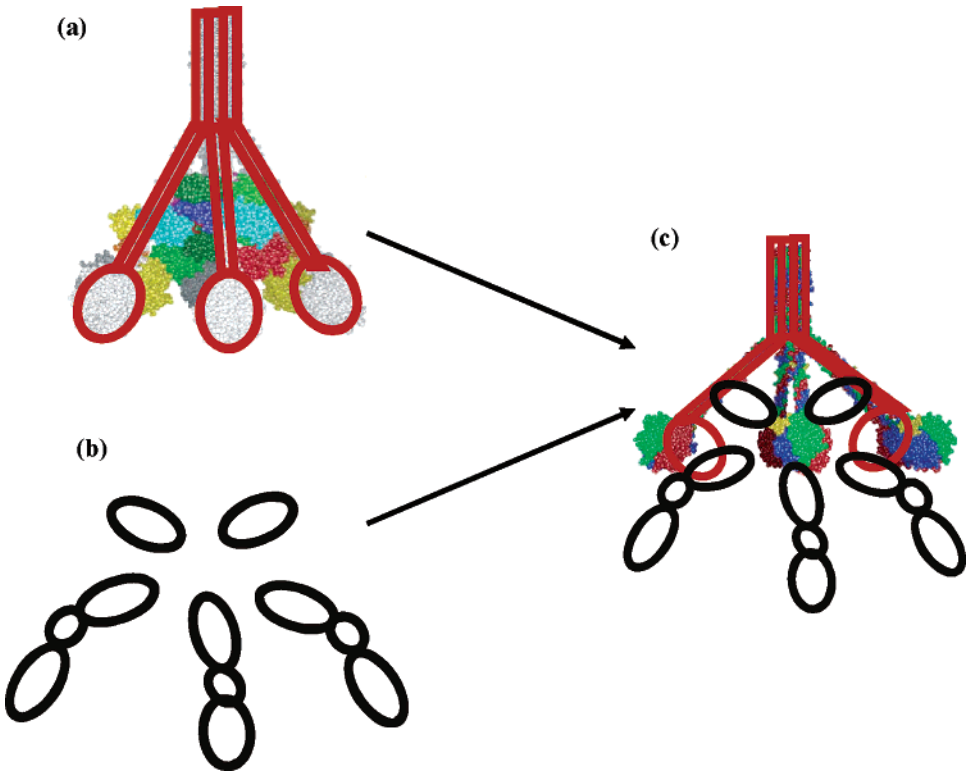

FIGURE 10: Proposed model for the gC1q−IgM interaction. C1q binds two [or three with slight movement of the C1q head, which is between the two presented in the figure (not shown)] IgM Cμ3 domains, being in closed (nonactive) conformation. To shift toward active (open) conformation, without tearing off all bonds to the target, gC1q heads have to rotate. The angle between the stalks increases from 28° to 45°. This mechanical stress activates the enzymes. This model is based on steric assumptions and the fact that amino acid residues from the gC1q apex and not from side surfaces of ghA, ghB, and ghC are important for the interaction. Lys^{B136} probably exerts a remote effect and is not involved in a binding site. The C1q models in open and closed conformation are taken from ref *29* with permission. (a) Nonactive (closed) conformation of C1q. The angle between the C1q stalks is 28°. (b) IgM, drawn in complement-activating staple conformation. The Fab regions are rotated to 60° out of the plane to remove all steric conflicts. (c) Active conformation of C1q. After the rotation of C1q heads, they are bound again to the same two or three IgM Cμ3 domains but with participation of different residues. The angle between the C1q stalks increases up to 45°.

Arg^{B108}, Arg^{B109}, and Tyr^{B175} are located. The differences in binding the two immunoglobulins appear later. It is very likely that, following the rotation of the gC1q because of Ca^{2+} release (*25*), Asp^{270} and Lys^{322} of IgG form salt bridges with Arg^{B129} and Glu^{B162}, respectively, with additional ionic interactions provided by Arg^{B114} and Arg^{B161} (*11*). However, the geometry of IgM, which is very different from that of IgG, does not allow for the involvement of residues from the side surface of the B chain during the C1q−IgM interaction.

According to Perkins' model (*19*), two adjacent heads of C1q are involved in the interaction with two Cμ3 domains on two adjacent subunits of IgM, when C1q is in the active "closed" conformation (angle between the collagenous arms being 28°). The open conformation of C1q, with an angle of 40−45° is nonactive, because the C1q heads are too far apart for two adjacent C1q heads to attach to Cμ3 domains on adjacent subunits in IgM. Thus, the base-arm angle is flexible and can vary from 40−45° to 30° from equilibrium at any moment. Gaboriaud and co-workers (*11*, *29*) suggested that C1 in the closed conformation is nonactive and C1q is complement activating in the open conformation. On the basis of these facts and our own results, we suggest that two or three heads of C1q are involved in the interaction with the pentameric molecule of IgM. We propose that it is most likely that two or three nonadjacent heads have to be involved in binding Cμ3 domains of IgM, which are in the closed (nonactive) conformation. To shift toward the active (open) conformation, without tearing off all bonds to the target, gC1q heads have to rotate without tearing the links to the Arg^{B108}−Arg^{B109} axis (Figure 10). The angle between the stalks increases from 28° to 45°. The mechanical stress because of this rotation is able to activate the complement. This model is based on steric assumptions and the experimental data, reported above, which indicated that the amino acid residues from the gC1q apex but not from the side surfaces of the individual C1q modules are important for IgM binding. It is also evident that the IgM-binding site within the gC1q domain is different from that for the IgG; however, the two sites are overlapping. The charged residues from the apex of the heterotrimeric gC1q (Arg^{B108}, Arg^{B109}, and Tyr^{B175}) participate in the recognition of both IgG and IgM, while Arg^{B114}, Arg^{B129}, Arg^{B163}, and His^{B117} are exclusively involved in IgG binding. We may speculate that, during the interaction with different ligands (e.g., IgG, CRP, and IgM) and Ca^{2+} release, a rotation of the gC1q around an axis, involving Arg^{B108} and Arg^{B109} residues, could occur, resulting in a reorientation of gC1q from one plane to another (*25*). Thus, Arg^{B108} and Arg^{B109} are likely to be central to the initial recognition process involving both IgG and IgM.

C1q is a rigid molecule formed by distinctive independent modules composed mainly of β sheets. Therefore, great mobility of the individual modules within such a structure is not anticipated. Thus, the recognition versatility of C1q

is achieved by charged surfaces of each module with specific spatial orientation and therefore can contribute differentially to different recognition processes. There are sufficient data to suggest that the gC1q ligand-binding sites can be formed in several different ways that correspond to the specific features of the ligand (*9*, *10*, *14*, *15*, *24*, *25*). For instance, C1q binding to IgM, CRP, and PTX3 is realized by the contribution from all three globular modules. In the case of other ligands, the combination of two or binding of even one module independently is sufficient (e.g., gp41 of HIV). The ligand-binding sites can be overlapping (as for IgG and IgM), very similar (as for IgG and OmpK36), or completely different (as for IgG and gp41 of HIV). Thus, a pool of charged amino acid motifs could participate to a different extent in the binding of the different C1q ligands. The present study highlights the issue that C1q can use its exposed surfaces carefully while liaising with various ligands.

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