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Localization of ligand-binding sites on human C1q globular head region using recombinant globular head fragments and single-chain antibodies

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Abstract

As a charge pattern recognition molecule, human C1q can bind a range of immunoglobulin and non-immunoglobulin ligands via its carboxy-terminal globular domain and activate the classical complement pathway. Each globular domain has a heterotrimeric organization, composed of the carboxy-terminal halves of one A (ghA), one B (ghB), and one C (ghC) chain. Recently, we have found that the recombinant forms of individual ghA, ghB and ghC bind differentially to IgG, IgM, gp41 peptide 601–613 of human immunodeficiency virus-1 (HIV-1), gp21 peptide 400–429 of human T cell lymphotrophic virus-I (HTLV-I), β-amyloid peptide, and apoptotic cells, suggesting a modular organization of the globular domain. This paper examines the interaction of ghA, ghB and ghC with two known C1q ligands: *Klebsiella pneumoniae* porin OmpK36 and salivary agglutinin. In addition, we have used a panel of recombinant single-chain antibodies (scFv) specific for ghA, ghB and ghC in order to map sites on the heterotrimeric globular domain which are likely to interact with IgG1, IgG3, IgM, OmpK36, salivary agglutinin and gp41 loop peptide. The combined use of recombinant ghA, ghB, ghC and single-chain antibodies has revealed at least three ligand-binding sites on the globular domain of C1q: one is IgG- and OmpK36-specific, the second (IgM-binding site) is most likely overlapping with IgG/OmpK36 binding site, and the third (the gp41-binding site) seems to be located at the junction between the collagen and globular domains.

Keywords: Complement; C1q; Recombinant module; Single chain antibody; Therapeutic

1. Introduction

C1q plays a key role in the recognition of a broad range of immunoglobulin and non-immunoglobulin ligands and subsequent activation of the classical complement pathway. The human C1q molecule (460 kDa) is composed of 18 polypeptide chains (6A, 6B, and 6C). The A chain (223 residues), B chain (226 residues), and C chain (217 residues) each have a short (3–9 residues) N-terminal region, followed by a collagen domain (~ 81 residues) and a carboxy-terminal globular domain (~ 135 residues) [1]. In the model of Reid and Porter [2], the inter-chain disulfide bonding yields 6A–B and 3C–C dimer subunits. The collagen domain in the A and B chains of an A–B subunit forms a triple-helical structure with the equivalent sequence in one of the C chains present in a C–C subunit. They form a structural unit of the composition ABC–CBA, three of such units then associate non-covalently to yield the hexameric C1q molecule [2,3].

C1, the first component of the classical complement pathway, is a complex of three glycoproteins: C1q, C1r and C1s. C1r and C1s interact to form a tetrameric proen-

Abbreviations: ghA, ghB, ghC, the recombinant globular head region of human C1q A, B and C chains, respectively; MBP, maltose-binding protein; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; HTLV, human T cell lymphotrophic virus; OmpK36, outer membrane protein K36 of *Klebsiella pneumoniae*; gp41, glycoprotein 41 of HIV-1; gp21, glycoprotein 21 of HTLV-I; scFv, recombinant single-chain antibodies; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TNF, tumor necrosis factor; SP-A, surfactant protein A; SP-D, surfactant protein D; gp-340, glycoprotein 340; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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zyme complex, $C1r_2-C1s_2$, which makes contacts with the collagen domain of C1q [4,5]. Binding of C1q to its ligands via the globular domain is considered to induce a conformational change in the collagen domain, which leads to the auto-activation of C1r which, in turn, activates C1s. The activated C1 complex then cleaves components C2 and C4 in the classical complement pathway. After C1 activation and removal of activated $C1r_2-C1s_2$ by C1 inhibitor, the collagen domain is considered to interact with putative cell surface receptors [4].

The globular domain of each stalk of C1q is composed of the carboxy-terminal halves of one A (ghA), one B (ghB), and one C chain (ghC), which is organized as a heterotrimer. In order to understand the functional contributions of each chain within the heterotrimeric globular domain, Kishore et al. [6] recently expressed ghA, ghB and ghC modules in *Escherichia coli* as soluble fusion proteins linked to maltose-binding protein (MBP) and examined their interaction with a few known ligands of C1q. In the enzyme-linked immunosorbent assay (ELISA), the ghA bound heat-aggregated IgG and IgM, in addition to binding specifically to glycoprotein 41 (gp41)-derived peptide 601–613 of human immunodeficiency virus-1 (HIV-1); the ghB bound preferentially to IgG rather than IgM, in addition to binding β-amyloid peptide, whereas the ghC showed preference for IgM as well as glycoprotein 21 (gp21)-derived peptide of human T cell lymphotrophic virus I (HTLV-I). Both ghA and ghB also inhibited C1q-dependent hemolysis of IgG- and IgM-sensitized sheep erythrocytes. However, for IgM-coated erythrocytes, the ghC was a better inhibitor of native human C1q than the ghB. The recombinant ghA, ghB and ghC also bound specifically to apoptotic cells. These results suggested that each chain of the C1q globular domain might have a certain degree of functional autonomy [5,6].

In the present study, we have examined the interaction of recombinant ghA, ghB and ghC with two non-immunoglobulin activators of the classical complement pathway: porin outer membrane protein K36 (OmpK36) of *Klebsiella pneumoniae* and human salivary agglutinin. In addition, we have used a panel of recombinant single-chain antibodies (scFv), generated via phage display technology, specific for ghA, ghB or ghC in order to identify conformational sites within the heterotrimeric globular domain which are likely to be involved in the interaction of C1q with IgG1, IgG3, IgM, OmpK36, salivary agglutinin and HIV-1 gp41 peptide 601–613.

2. Materials and methods

2.1. Purified proteins and peptides

C1q was isolated from human plasma using IgG-Sepharose and Mono-S columns [7]. The purity of C1q was assessed by 15% weight/volume (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions where it appeared as three bands, corresponding to the A, B, and C chains of 34, 32, and 27 kDa, respectively. The porin OmpK36 from *K. pneumoniae* [8] and salivary agglutinin [9,10] were purified as previously described. The gp41 peptide 601–613 of HIV-1 (sequence: GIWGCSGKLICTT) was kindly provided by Dr. G.J. Arlaud, Grenoble, France.

2.2. Expression and purification of the globular head region of human C1q A, B and C chains

The globular head regions of the A chain (ghA, residues 88–223), the B chain (ghB, 90–226) and the C chain (ghC, 87–217) were expressed in *E. coli* as fusion proteins linked to MBP [6,11,12].

2.3. Single chain antibodies

Clones secreting C1q-specific Fv fragments were generated from large human Vaughan (V) [13] and Griffin (G) [14] libraries. Clones scFv2 (V), scFv3 (V), scFv4 (V), scFv8 (V) and scFv10 (V), generated from the Vaughan library, were provided by Dr. G. Winter (M.R.C. Center for Protein Engineering, Cambridge, UK). Clones scFvM (G) and scFv7 (G) were obtained from the Griffin library and their antigenic specificity was determined [15]. All scFv antibodies were purified by metal affinity chromatography [16]. Polyclonal antibodies against recombinant ghA, ghB and ghC were raised in rabbits [6,11].

2.4. Biotinylation of proteins

Proteins were biotinylated using EZ-link biotinylation kit (Pierce Biotechnology). The concentration of *N*-hydroxy-succinimido-biotin used was 25-fold molar excess to the protein solution in PBS, pH 7.2. The reaction was carried out at room temperature for 3 h followed by extensive dialysis against appropriate buffers to remove free biotin.

2.5. Interaction of C1q, ghA, ghB and ghC with porin OmpK36

OmpK36 in 0.1 M sodium carbonate buffer, pH 9.6 (0–10 μg/well) was coated on microtitre wells overnight at 4 °C, followed by blocking with phosphate-buffered saline (PBS) containing 2% w/v bovine serum albumin (BSA). C1q (2 μg/well) in PBS was added and incubated for 2 h at 37 °C. Following extensive washing, rabbit anti-human C1q polyclonal antibody (1 μg/well) in PBS containing 0.05% volume/volume (v/v) Tween 20 was added and incubated for 2 h at 37 °C. The color was developed using goat anti-rabbit IgG-alkaline phosphatase conjugate and *p*-nitrophenyl phosphate as substrate. Insulin was used as a negative control. The interaction between OmpK36 and recombinant C1q globular head fragments was examined by incubating biotinylated ghA, ghB, ghC or MBP (0–3 μg/well) with

OmpK36 (2 μg/well)-coated wells for 2 h at 37 °C. Following washing, the amount of recombinant proteins bound were determined using extravidin conjugated to alkaline phosphatase. In order to determine the ability of ghA, ghB or ghC to inhibit binding of OmpK36 to C1q, biotinylated OmpK36 (2 μg/well) was pre-incubated with various concentrations of recombinant proteins (0–10 μg/well) and then applied to C1q (2 μg/well)-coated wells. After washing, bound OmpK36 was detected as described above.

2.6. Interaction of C1q, ghA, ghB or ghC with salivary agglutinin

Salivary agglutinin (5 μg/well) in carbonate buffer was coated on microtitre wells overnight at 4 °C and then blocked with PBS containing 2% w/v BSA. After washing, C1q (0–5 μg/well) in PBS containing 0.05% v/v Tween 20 was added to the wells and incubated for 2 h at 37 °C. Bound C1q was probed using goat anti-rabbit IgG-alkaline phosphatase conjugate, as described above. The recombinant ghA, ghB or ghC were coated overnight (0–10 μg/well), washed and blocked. Biotinylated salivary agglutinin (3 μg/well) was added to the wells (2 h at 37 °C) and the color was developed using extravidin-alkaline phosphatase conjugate. In order to assess the ability of ghA, ghB or ghC to inhibit C1q binding to salivary agglutinin, biotinylated salivary agglutinin (3 μg/well) was pre-incubated with various concentrations of ghA, ghB or ghC (1–10 μg/well) and then applied to C1q (1.5 μg/well)-coated wells. Bound salivary agglutinin was detected using extravidin-alkaline phosphatase conjugate.

2.7. Binding of scFv to ghA, ghB and ghC

C1q, ghA, ghB or ghC (5 μg/well) were added to scFv (5 μg/well)-coated wells. After blocking and washing the wells as described above, rabbit anti-human C1q polyclonal antibodies or rabbit anti-ghA, ghB or ghC polyclonal antibodies (1 μg/well) were added to the appropriate wells and incubated at 37 °C for 2 h. The binding was subsequently detected using alkaline phosphatase-conjugated goat anti-rabbit IgG polyclonal antibodies. MBP was used as a negative control protein. In another ELISA, different amounts of solid-phase C1q, ghA, ghB or ghC (0–10 μg/well) were allowed to interact with biotinylated scFv (2 μg/well) for 2 h at 37 °C and the bound scFv was detected with extravidin conjugated to alkaline phosphatase.

2.8. Detection of inhibitory activity of scFv on the interaction between C1q and heat-aggregated IgG or IgM

Microtitre wells were coated overnight at 4 °C with heat-aggregated IgG1 (3 μg/well), IgG3 (3 μg/well) or IgM (5 μg/well), blocked and washed. C1q (1.5 μg/well) was co-incubated with scFv at different concentrations (0–12 μg/well) before being added to the immunoglobulin-coated wells. After incubation at 37 °C for 2 h, the amount of bound C1q was measured using rabbit anti human C1q polyclonal antibodies, followed by goat anti-rabbit polyclonal antibodies conjugated to alkaline phosphatase.

2.9. Detection of inhibitory activity of scFv on the interaction between C1q and OmpK36

Microtitre wells, coated with C1q (1.5 μg/well), were blocked and washed. ScFv (0–10 μg/well) was applied in the presence of biotinylated OmpK36 (3 μg/well) and the color was developed using extravidin-alkaline phosphatase conjugate.

2.10. Detection of the inhibitory activities of scFv on the interaction between C1q and salivary agglutinin

Biotinylated salivary agglutinin (1.5 μg/well), together with various scFv (0–10 μg/well), was applied to the C1q-coated wells (1.5 μg/well) and incubated for 2 h at 37 °C. The amount of salivary agglutinin bound to C1q was estimated as described above.

2.11. Detection of inhibitory activity of anti-C1q scFv antibodies on the interaction between C1q and gp41 peptide 601–613 of HIV-1

Various amounts of biotinylated scFv (0–5 μg/well), together with 0.5 μg of peptide 601–613, were applied to

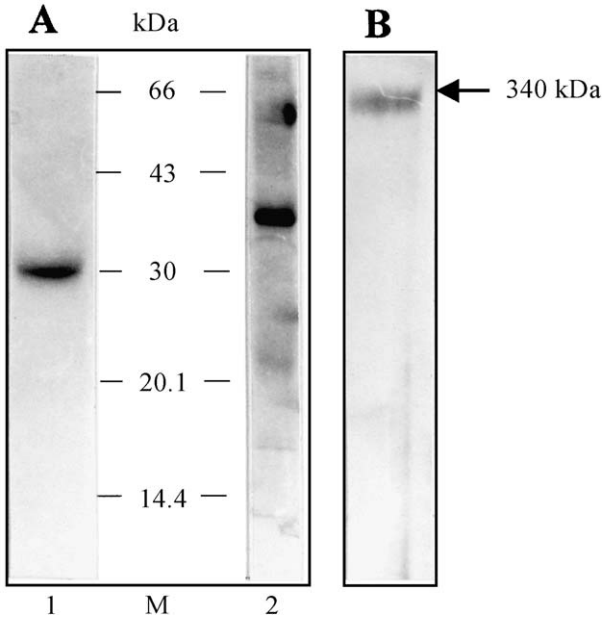

Fig. 1. Purified preparations of *K. pneumoniae* OmpK36, salivary agglutinin and scFv10 (V). (A) 11% w/v SDS-PAGE (under reducing conditions) of scFv10 (V) following affinity chromatography on His-Trap column (lane 1, purified scFv migrated at ~ 30 kDa) and OmpK36 porin after gel filtration on Sephacryl S 200 (lane 2, ~ 36 kDa); M, molecular weight markers. (B) 7.5% w/v SDS-PAGE of purified salivary agglutinin under reducing conditions. (lane 1, ~ 340 kDa).

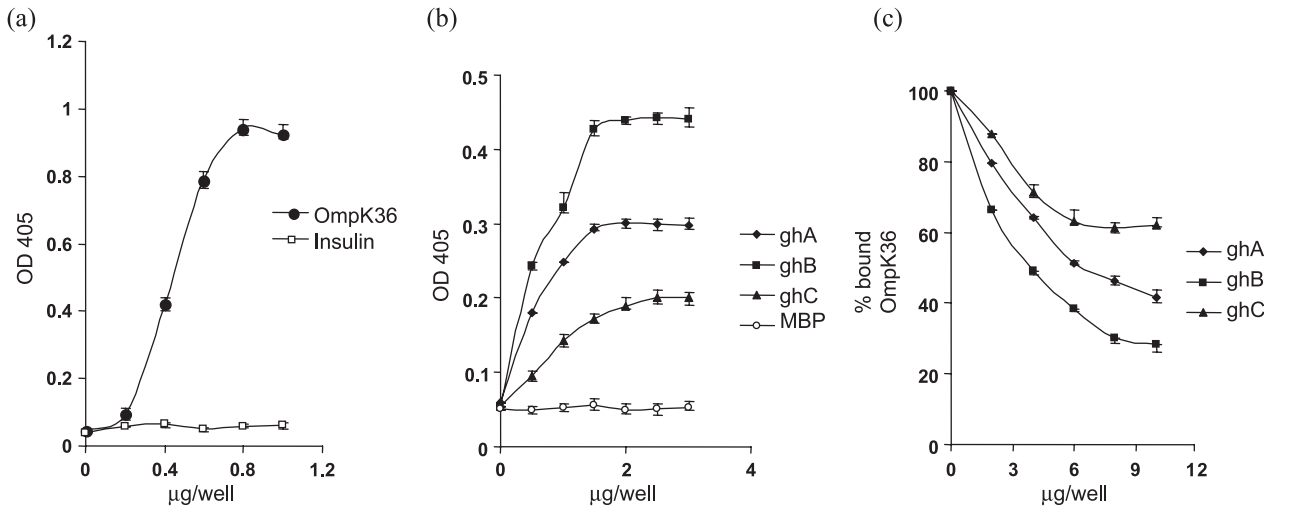

Fig. 2. Interaction between OmpK36 and human C1q, ghA, ghB or ghC. (a) Human C1q was added to OmpK36-coated wells (0–10 µg/well) and detected using rabbit anti-human C1q polyclonal antibodies followed by goat anti-rabbit polyclonal antibodies conjugated to alkaline phosphatase. (b) Biotinylated ghA, ghB or ghC were added to OmpK36-coated wells and probed using extravidin-alkaline phosphatase conjugate. (c) To assess inhibition of C1q–OmpK36 interaction, biotinylated OmpK36 was co-incubated with ghA, ghB or ghC (0–10 µg/well) before being added to C1q-coated wells. MBP and insulin were used as negative controls. The data shown are means ± standard deviations of triplicate measurements.

C1q (2 µg/well)-coated wells and incubated for 3 h at room temperature. After washing, the amount of bound scFv was detected using extravidin-alkaline phosphatase conjugate. In another ELISA, the microtitre wells were coated overnight at room temperature with peptide 601–613 (1 µg/well) [17]. C1q (1.5 µg/well) was pre-incubated with different amounts of scFv (0–5 µg/well) before being added to the peptide-coated wells. Following incubation at 37 °C for 3 h, the

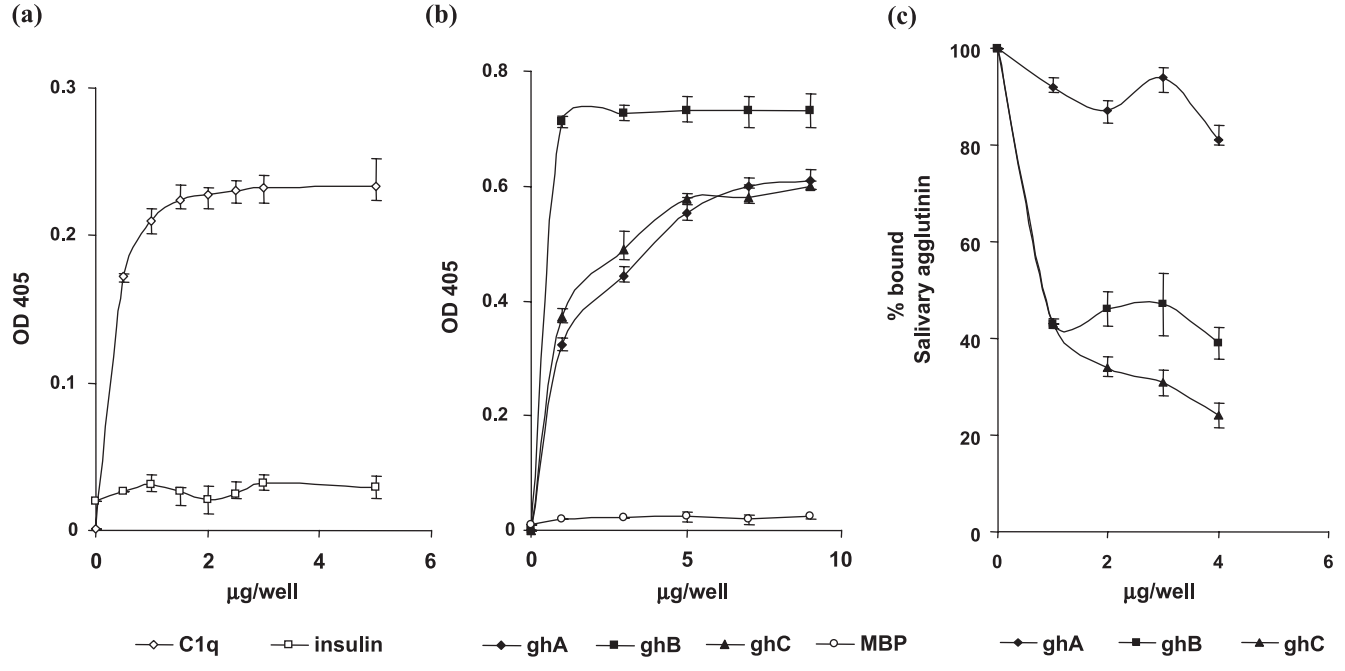

Fig. 3. Binding of human C1q, ghA, ghB or ghC to salivary agglutinin. (a) C1q was incubated with salivary agglutinin-coated wells and probed first with rabbit anti-human C1q polyclonal antibodies (1 µg/well) and then with goat anti-rabbit polyclonal antibodies conjugated to alkaline phosphatase. (b) Biotinylated salivary agglutinin (3 µg/well) was incubated with ghA, ghB or ghC-coated wells. The amount of salivary agglutinin bound was determined using extravidin-alkaline phosphatase conjugate. (c) Inhibition of C1q-binding to salivary agglutinin by ghA, ghB or ghC. Biotinylated salivary agglutinin (3 µg/well) was pre-incubated with recombinant ghA, ghB or ghC (0–10 µg/well) before being added to C1q-coated wells. C1q bound salivary agglutinin was detected using extravidin-alkaline phosphatase conjugate. MBP and insulin were used as negative control proteins. The data shown are means ± standard deviations of triplicate measurements.

amount of bound C1q was detected using rabbit anti-human C1q and alkaline phosphatase-conjugated goat anti-rabbit IgG polyclonal antibodies.

3. Results

3.1. The recombinant ghB is the most efficient module in binding K. pneumoniae porin OmpK36

K. pneumoniae OmpK36, purified via membrane extraction and gel filtration, migrated at ~36 kDa on SDS-PAGE under reducing conditions (Fig. 1). Human C1q, ghA, ghB and ghC bound solid-phase OmpK36 in a specific and saturable manner (Fig. 2a,b). The ghB bound best compared to ghA and ghC. In order to examine the abilities of ghA, ghB and ghC to inhibit binding of OmpK36 to C1q, biotinylated OmpK36 was co-incubated with various concentrations of ghA, ghB or ghC and then applied to C1q-coated microtitre wells. As shown in Fig. 2c, the ghB was found to be the best inhibitor, demonstrating ~50% inhibition at 4.5 μg compared to 7 μg of ghA. The maximal inhibition by ghC was ~40% at the highest concentration tested (10 μg).

3.2. The ghB and ghC modules are the best inhibitors of C1q–salivary agglutinin interaction

Salivary agglutinin, fractionated from human saliva, migrated at ~340 kDa on SDS-PAGE (Fig. 1) similar to previously reported glycoprotein-340 (gp-340) purified from lung washings using a maltose-agarose column (Fig. 1). C1q (Fig. 3a) as well as ghA, ghB and ghC (Fig. 3b) bound salivary agglutinin in a dose-dependent manner (C1q>ghB>ghC>ghA), the ghB being the best binder. However, in the competitive ELISA where biotinylated salivary agglutinin was pre-incubated with various concentrations of ghA, ghB and ghC before being transferred to C1q-coated wells, both ghB and ghC modules showed comparable inhibitory activity. The ghA weakly inhibited the C1q–salivary agglutinin interaction (Fig. 3c).

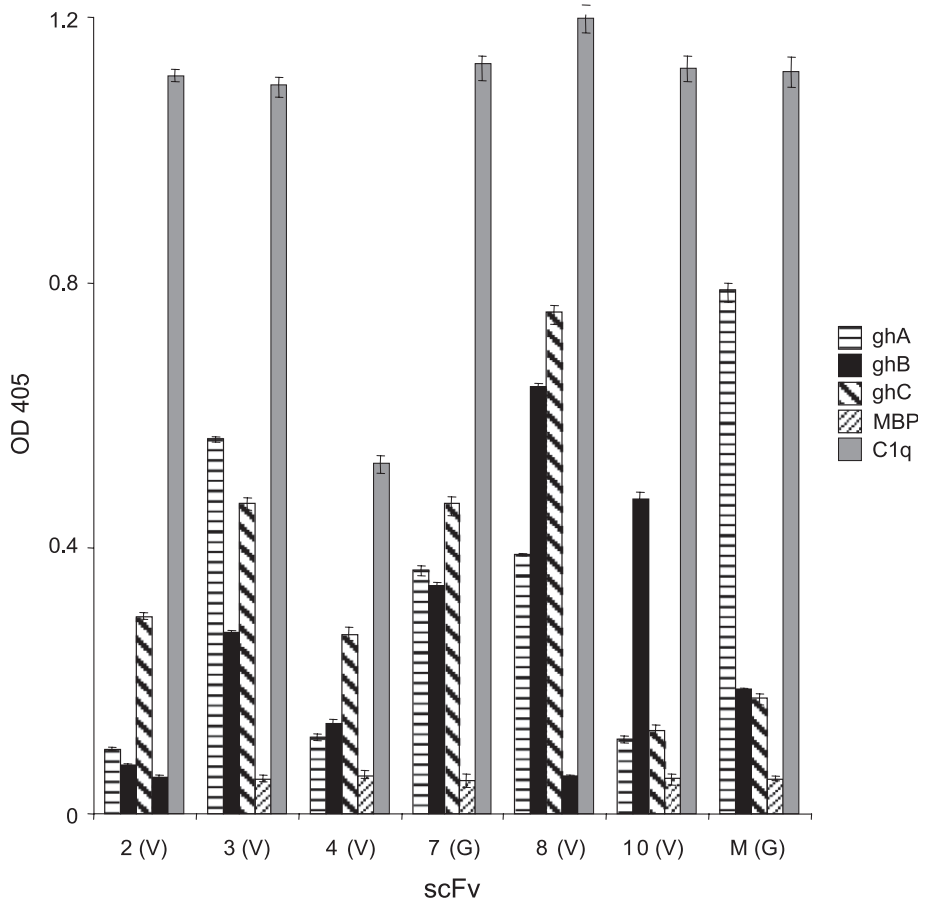

Fig. 4. Binding specificity of scFv to C1q, ghA, ghB and ghC. C1q, ghA, ghB or ghC were incubated in scFv-coated wells and probed with rabbit anti-human C1q, anti-ghA, anti-ghB or anti-ghC polyclonal antibodies (1 μg/well), followed by goat anti-rabbit polyclonal antibodies conjugated to alkaline phosphatase. MBP was used as a negative control protein. The data shown are means ± standard deviations of triplicate measurements.

3.3. Specificity of recombinant scFv towards ghA, ghB and ghC

We affinity-purified seven scFv antibodies using Nickel column [15], verified purity and size via SDS-PAGE (Fig. 1) and examined their specificities towards recombinant ghA, ghB and ghC. C1q, ghA, ghB and ghC were added to scFv-coated wells and then probed with respective polyclonal antibodies (Fig. 4). The scFv8 (V) bound ghB and ghC, whereas scFv7 (G) bound more significantly to ghA and almost equally to ghC and ghB. The scFv3 (V) recognized ghA and ghC. The scFv10 (V) and scFvM (G) were found to be specific to ghB and ghA, respectively (Fig. 4). Two other antibodies, scFv2 (V) and scFv4 (V), did not bind ghA, ghB or ghC above background levels.

The abilities of scFv3 (V), scFv8 (V), scFv10 (V) and scFvM (G) to bind recombinant C1q globular head modules were confirmed by another experiment where different amounts of solid-phase C1q, ghA, ghB or ghC were probed with biotinylated scFv (Fig. 5). These scFv antibodies were used as inhibitors of the interactions between C1q and IgG1, IgG3, IgM, OmpK36, salivary agglutinin and gp41 peptide 601–613.

3.4. Inhibition of C1q–IgG and C1q–IgM interactions by scFv antibodies

The abilities of scFv3 (V), scFv8 (V), scFv10 (V) and scFv M (G) to inhibit C1q–immunoglobulin interaction were examined by adding different amounts of scFv, together with C1q, to the microtitre wells previously coated with IgG1, IgG3 or IgM. Based on the detection of C1q bound to immunoglobulin, the scFv10 (V) was found to be the best inhibitor of C1q–IgG1 interaction, followed by scFv3 (V) and scFv8 (V) (Fig. 6a). All three scFv were inefficient inhibitors of C1q–IgG3 interaction since only scFv10 (V) showed ~ 50% inhibitory activity (Fig. 6b). The ghA- and ghC-specific scFv3 (V) appeared to be the best inhibitor of

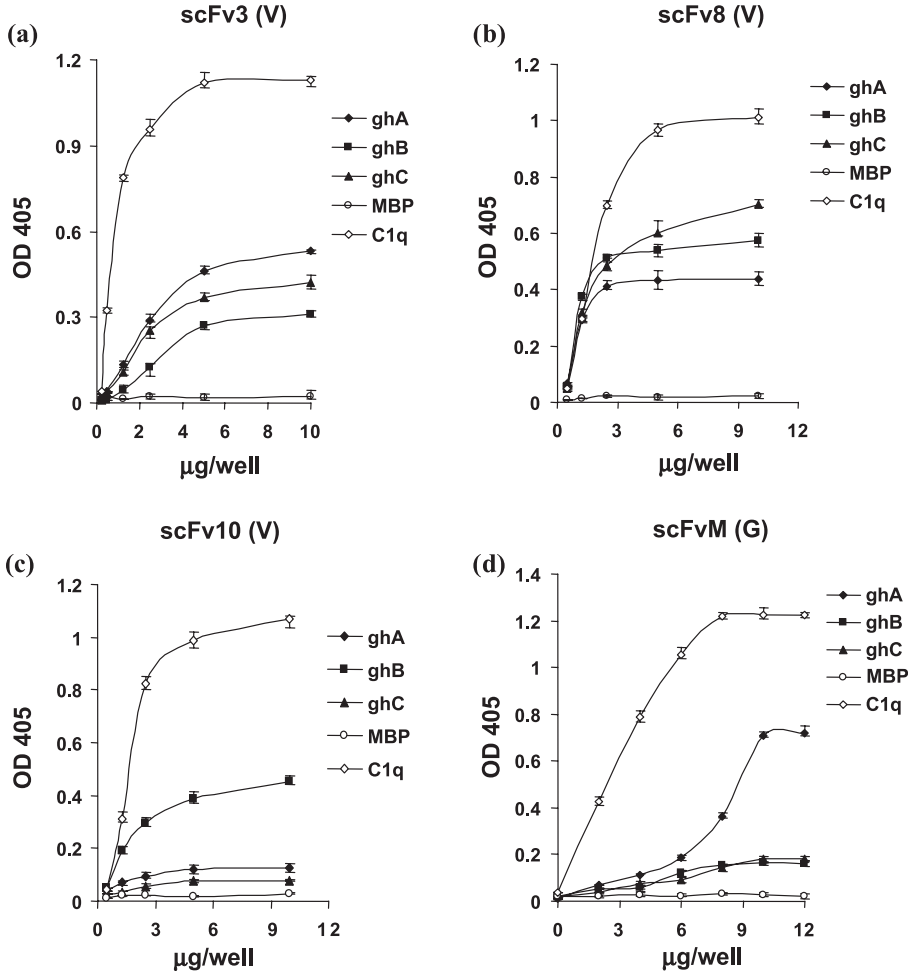

Fig. 5. Interactions of scFv3 (V), scFv8 (V), scFv10 (V) and scFvM (G) with ghA, ghB and ghC. Solid-phase C1q, ghA, ghB or ghC (0–10 µg/well) were incubated with biotinylated scFv3 (V) (a), scFv8 (V) (b), scFv10 (V) (c) and scFvM (G) (d), and then detected with extravidin-alkaline phosphatase conjugate. MBP was used as a negative control protein. The data shown are means ± standard deviations of triplicate measurements.

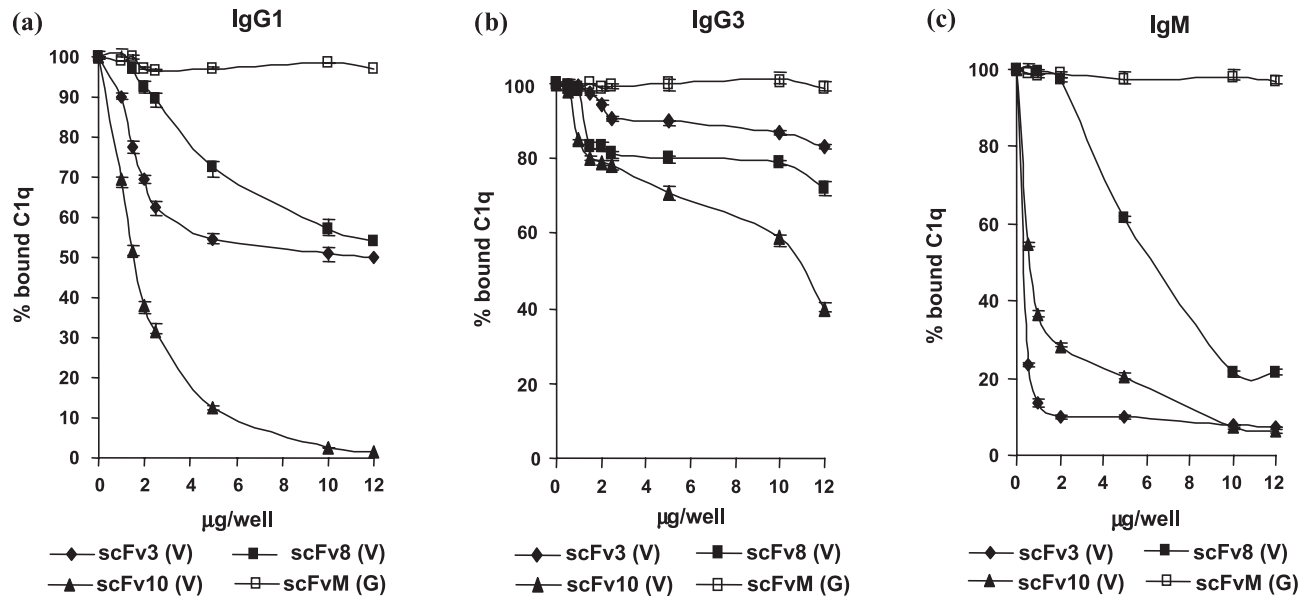

Fig. 6. Inhibition of interactions between C1q and IgG1, IgG3 or IgM by scFv3 (V), scFv8 (V), scFv10 (V) and scFvM (G). C1q (1.5 μg/well) was pre-incubated with each scFv (0–12 μg/well) and added to wells coated with (a) IgG1 (3 μg/well), (b) IgG3 (3 μg/well), or (c) IgM (5 μg/well). The amount of immunoglobulin-bound C1q was detected using rabbit anti-human C1q polyclonal antibodies, followed by goat anti-rabbit polyclonal antibodies conjugated to alkaline phosphatase. The data shown are means ± standard deviations of triplicate measurements.

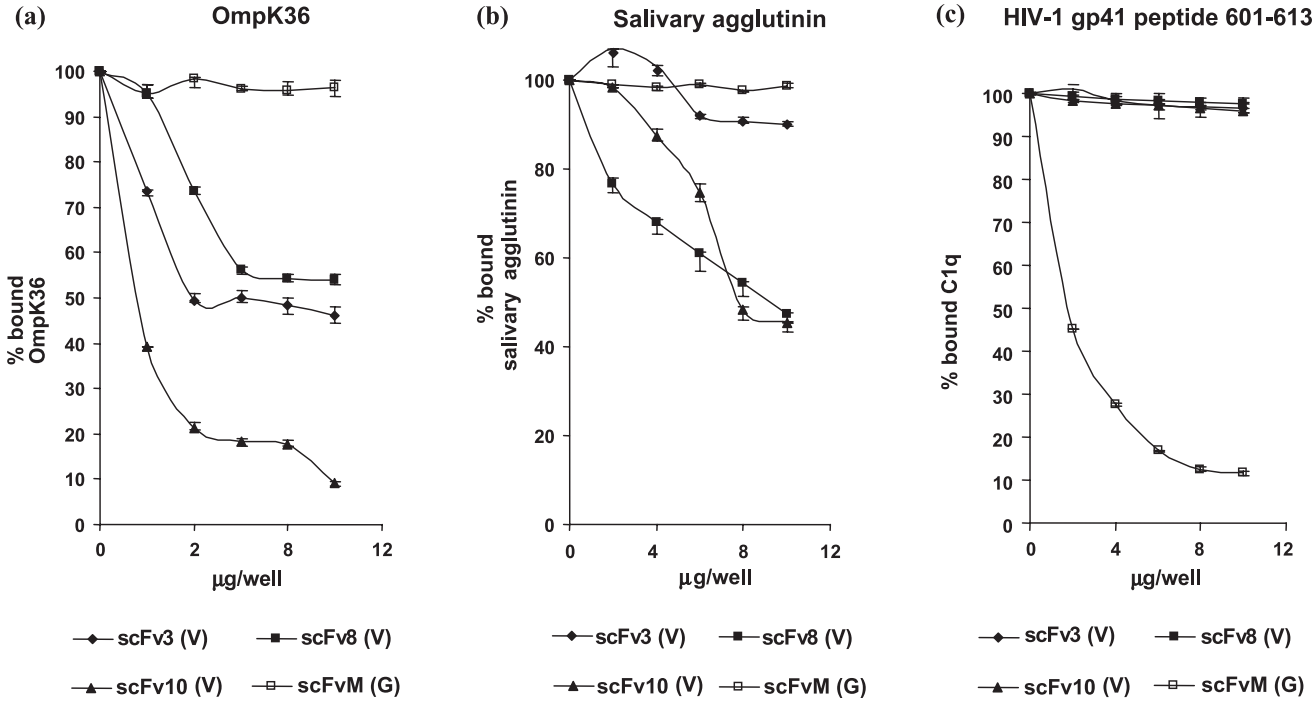

Fig. 7. Inhibition of interactions between C1q and porin OmpK36, salivary agglutinin or gp41 peptide 601–613 of HIV-1 by scFv3 (V), scFv8 (V), scFv10 (V) and scFvM (G). C1q (1.5 μg/well) was coated on microtitre wells and scFv (0–10 μg/well) was added in the presence of (a) biotinylated OmpK36 (3 μg/well), or (b) biotinylated salivary agglutinin (1.5 μg/well). C1q-bound OmpK36 or salivary agglutinin was measured using extravidin-alkaline phosphatase conjugate. (c) Microtitre wells were coated with gp41 peptide 601–613 (1 μg/well). C1q (1.5 μg/well) was pre-incubated with scFv (0–5 μg/well) and then added to the peptide-coated wells. The amount of bound C1q was detected using rabbit anti-human C1q polyclonal antibodies. The data shown are means ± standard deviations of triplicate measurements.

C1q–IgM interaction, followed by scFv10 (V) and scFv8 (V) (Fig. 6c). The scFvM (G) did not inhibit interaction of C1q with either IgG or IgM.

3.5. Inhibition of C1q–OmpK36 interaction by scFv

Different amounts of scFv were added, together with biotinylated OmpK36, to C1q-coated wells and the amount of bound OmpK36 was estimated using extravidin-alkaline phosphatase conjugate. Of the four scFv antibodies tested, scFv3 (V), scFv8 (V) and scFv10 (V) were able to inhibit C1q–OmpK36 interaction (Fig. 7a). However, the ghB-specific scFv10 (V) was found to be the best inhibitor. The inhibitory activities of all the scFv tested were very similar to those obtained for the C1q–IgG1 interaction.

3.6. Inhibition of interaction between C1q and salivary agglutinin by scFv

The single-chain antibodies scFv3 (V), scFv8 (V), scFv10 (V) and scFvM (G) were also examined for their abilities to inhibit the C1q–salivary agglutinin interaction. A range of concentrations of scFv was applied, in the presence of biotinylated salivary agglutinin to C1q-coated wells and the amount of bound salivary agglutinin, was measured. As shown in Fig. 7b, scFv10 (V) and scFv8 (V) inhibited ~ 46% binding of C1q to salivary agglutinin. These results were consistent with those in Fig. 3c where ghB and ghC inhibited C1q binding to salivary agglutinin most efficiently. The scFv3 (V) and scFvM (G) had no significant inhibitory effects on the C1q–salivary agglutinin interaction.

3.7. Inhibition of the C1q and HIV-1 gp41 peptide 601–613 interaction by scFv

The ability of the selected scFv antibodies to inhibit the interaction between C1q and gp41 peptide 601–613 was examined in two ways. In the first assay, different amounts of biotinylated scFv, together with 0.5 μg peptide, were applied to C1q-coated wells and the bound scFv was detected using extravidin conjugated to alkaline phosphatase. In the second ELISA, different amounts of scFv antibodies were added to the peptide-coated wells in the presence of C1q (Fig. 7c). The scFvM (G) was the only single chain antibody that interfered with the C1q–peptide interaction (~ 90% inhibition at 10 μg).

4. Discussion

C1q has a characteristic heterotrimeric globular domain situated at the carboxy-terminal end of the triple-helical collagen domain. Such globular domains are also found in a variety of non-complement proteins including human type VIII and type X collagen, precerebellin, chipmunk hibernation proteins, multimerin, adiponectin, saccular collagen, and EMILIN [4,5]. The crystal structure of the recombinant globular domain of mouse adiponectin has revealed a tertiary structure and trimeric organization that is closely related to that of tumor necrosis factor (TNF), suggestive of an evolutionary link between the TNF and C1q families (and hence a C1q/TNF superfamily) [18,19]. The chains containing these globular modules appear to form either a homotrimer (as in type X collagen, multimerin, Acrp-30, precerebellin and saccular collagen) or a heterotrimeric structure (as in C1q and the hibernation protein).

The C1q globular domain recognizes the Cγ2 domain of IgG and the Cμ3 domain of IgM primarily through ionic interactions. Three charged residues: Glu^{318}, Lys^{320} and Lys^{322} in the Cγ2 domain and Asp^{356}, Asp^{417}, Glu^{418} and His^{420} in the Cμ3 are considered to participate in the interaction with globular domain [20,21]. Chemical modification studies have implicated arginine residues, especially Arg^{114}, Arg^{129} and Arg^{163} in the B chain, Arg^{162} in the A chain, and Arg^{156} in the C chain, which are quite central to the C1q–IgG interaction [22]. However, the role and contributions of the carboxy-terminal globular regions of A, B and C chains in binding to IgG, IgM and other C1 activators have not been clearly defined.

Kishore et al. [6] recently generated the recombinant forms of ghA, ghB and ghC and addressed the question whether each chain within C1q globular domain existed as a functionally autonomous module (with ghA, ghB and ghC having distinct binding properties), or the ability of C1q to bind its ligands depended upon a combined heterotrimeric structure that relies on the collective contributions from ghA, ghB and ghC. It appears that the carboxy-terminal globular region of each chain has a certain degree of functional independence within the heterotrimer. The ghA was found to be the most efficient module in interacting with IgG and IgM, whereas ghB and ghC showed individual preference for IgG and IgM, respectively. In addition, the ghA, ghB and ghC showed preferential binding towards certain non-immunoglobulin ligands of C1q, such as peptide 601–613 derived from transmembrane protein gp41 of HIV-1, peptide 400–429 derived from envelope protein gp21 of HTLV-I and β-amyloid peptide [6]. The ghA interacted specifically with gp41 peptide 601–613 of HIV-1, consistent with the structural model [23,24]. However, the ghC module appeared to be specific for the gp21 peptide 400–429 of HTLV-I, which corresponds to the site between the anchorage domain and the fusion domain of gp21 [25]. Very interestingly, the ghB module showed specific, preferential and dose-dependent interaction with β-amyloid peptide 1–42, which is the major protein component of the senile or neuritic plaques in the brains of patients with Alzheimer's disease [26–28]. In addition, the ghA, ghB and ghC showed specific, dose-dependent binding to the apoptotic cells and competed with C1q in binding to the apoptotic cells [29]. A recent study, which has examined the inter-

action of ghA, ghB and ghC with long pentraxin 3 [30], also appears to substantiate the view that the C1q globular domain has a modular organization.

In the present study, we have examined the interaction of ghA, ghB and ghC with bacterial porin OmpK36 of *K. pneumoniae* and salivary agglutinin: two non-immunoglobulin ligands of C1q. The direct interaction of OmpK36 with the C1q globular domain and subsequent activation of the classical complement pathway is followed by C3b binding and C5b-9 deposition [31], which constitutes a part of innate immunity against this opportunistic pathogen [8]. Although all three chains of C1q were involved in binding OmpK36, the ghB appeared to have a leading role. We also examined C1q interaction with salivary agglutinin, a high molecular weight component of saliva, which binds *Streptococcus mutans* and *Helicobacter pylori* [9,10]. Recently, salivary agglutinin has been shown to be a glycoform of pulmonary gp-340. The gp-340 is also known to bind lung surfactant protein A (SP-A) and D (SP-D) [32,33]. Salivary agglutinin and gp-340 have identical primary sequences containing 13 scavenger receptor cysteine-rich domains [34]. It is likely that these proteins are scavenging opsonins in and around mucosa and in the presence of SP-A, SP-D and C1q, the clearance of the pathogens is expedited either via phagocytic (SP-A and SP-D) or complement-dependent (C1q) mechanisms [35,36]. All three C1q globular head regions appeared to be involved in salivary agglutinin binding, ghB and ghC being the most important chains. It is intriguing that SP-D has been shown to bind gp-340 via its globular lectin domains in a carbohydrate-independent manner, suggesting protein–protein interaction between SP-D and gp-340 [32]. Whether the SP-D lectin domain and the C1q globular domain compete for the same site on salivary agglutinin/gp-340 remains to be examined.

Having investigated the interaction of individual ghA, ghB and ghC with IgG, IgM, gp41 peptide of HIV-1, gp21 peptide of HTLV-I, β-amyloid peptide, apoptotic cells (previous study; Ref. [6]), and also with OmpK36 of *K. pneumoniae* and salivary agglutinin (this study), we sought to view the interaction of C1q with its ligands in the context of a heterotrimeric organization of the globular domain. Therefore, we screened a panel of C1q-specific scFv antibodies, generated by phage display technology using Griffin's and Vaughan's libraries (Table 1) for their conformation-dependent specificity towards ghA, ghB and ghC (as well as native C1q and globular domain). We further examined their abilities to interfere with the interaction of C1q with IgG1, IgG3, IgM, OmpK36, salivary agglutinin and gp41 peptide 601–613 of HIV-1.

Four scFv were selected for the proposed mapping of binding site(s) within the globular domain. Three of these antibodies, scFv3 (V), scFv8 (V) and scFv10 (V), bound the IgG3-captured C1q globular domain and strongly inhibited C1q-dependent hemolysis of sensitized sheep erythrocytes. They did not recognize the individual C1q polypeptide chains (following separation by SDS-PAGE under reducing conditions and Western blot) suggesting conformational specificity (recognition of conformational epitopes located within the globular domain). The scFvM (G) bound the globular as well as the collagen domains in addition to binding the recombinant ghA, suggesting that it probably recognized sequential antigenic determinant(s) within the C1q "neck" region: the junction between the globular and the collagen domains (residues 81–97). In addition to recognizing native C1q and globular domain, the antibodies scFv3 (V), scFv8 (V) and scFv10 (V) also interacted with recombinant globular head fragments (scFv3 with ghA and ghC, scFv8 with ghB and ghC, and scFv10 exclusively with ghB), suggesting that the ghA, ghB and ghC modules expressed in *E. coli* were probably correctly folded. The ability of scFv to bind more than one recombinant globular head fragment could be due to the recognition of similar or identical epitopes within the C1q globular domain. Alternatively, participation of multiple globular head regions in the formation of one epitope is also a possibility.

The ghB-specific scFv10 (V) at 10 μg concentration inhibited >95% of the C1q–IgG1 interaction, and >50% of the C1q–IgG3 interactions. These results are consistent with previous reports which have described the globular head region of C1q B chain as a key player during C1q–IgG interaction [6,11,12,37]. The inhibition by scFv3 (V) and scFv8 (V) of C1q–IgG/IgM interactions seemed to suggest that their epitopes may be located close to the scFv10 epitope, and most likely, these epitopes are overlapping. Given that (i) recombinant ghA, ghB and ghC differ in their abilities to bind IgM and ghA and ghC bind

Table 1
Summary of the features of human C1q-specific recombinant scFv antibodies (based on Refs. [13,15])

Clone	C1q binding	IgG–C1q complex binding	Globular domain binding	Collagen domain binding	C1q Western blot	Inhibition of hemolysis	ghA	ghB	ghC
scFv2 (V)	++	+	++	–	–	+/–	–	–	+
scFv3 (V)	++	+	++	–	–	+	+++	+/–	+++
scFv4 (V)	+	–	+	–	+	–	–	–	+/–
scFv8 (V)	++	+	++	–	–	+	+	+++	+++
scFv10 (V)	++	–	++	–	–	+	–	++	–
scFv7 (G)	++	+	+	–	+	+/–	+	+	++
scFvM (G)	++	+	++	+	+ (A chain)	–	+++	+/–	+/–

IgM more strongly than ghB [6,11,12], (ii) scFv3 (V) preferentially binds ghA and ghC (ghA>ghC), and (iii) scFv3 (V) inhibits the C1q–IgM interaction more effectively than other scFv antibodies, it appears that IgG and IgM binding sites within the globular domain, though overlapping, are not identical.

The ghB-specific scFv10 (V) was the most potent inhibitor of the C1q–OmpK36 interaction, consistent with the observation that ghB plays a leading role in OmpK36 binding. OmpK36 and enterobacterial porins in general have charged residues in the extracellular domains or loops, which are probably involved in binding to C1q [31]. There are several species-specific C1q-binding motifs known within IgG [20,38]. The motif Glu^{318}, Lys^{320} and Lys^{322} [20] is similar to a conserved motif of one negative and two positive side chains (Lys^{278}, Lys^{280} and Asp^{281}) found in the OmpK36. It appears that the nature of interaction between porins and C1q is similar to that of C1q and IgG. The OmpK36 and IgG binding sites on globular domain are probably identical. However, it is worth noting that biotinylated OmpK36 still binds C1q indicating that residues other than lysine are involved in C1q recognition. The scFv8 (V), which preferentially binds ghB and ghC, was the most potent inhibitor of the C1q–salivary agglutinin interaction. It is likely that the binding site for salivary agglutinin on the globular domain is located close to the immunoglobulin-binding sites.

The scFvM (G), obtained from the Griffin library, has interesting binding properties since its epitope seems to be located within residues 81–97 of ghA. This epitope is probably sequential, as opposed to another scFv with similar specificity reported previously [39], which binds a conformational antigenic determinant. We have also reported that the gp41 peptide 601–613 binding site on C1q does not overlap with the IgG-binding site and the ghA constitutes a significant part of gp41-binding site [37]. Thus, scFvM (G), which binds ghA with strong affinity, was used as an inhibitor of the interaction between C1q and peptide 601–613. It appears that at least one of the gp41-binding sites on C1q is located within the A chain, and this binding site does not overlap with the IgG- or IgM-binding site.

In summary, the combined use of recombinant forms of globular head regions of human C1q and C1q-specific scFv has revealed at least three ligand-binding sites within the globular domain: one is IgG- and OmpK36-specific, the second (IgM-binding site) is most likely overlapping, but not identical with the IgG/OmpK36 binding site, and the third (the gp41-binding site) seems to be located at the junction between collagen and globular domains. The binding properties of scFv towards ghA, ghB and ghC are consistent with their potencies as 'inhibitors' of C1q. The scFv3 (V), scFv8 (V) and scFv10 (V), which strongly inhibited the C1q–immunoglobulin interaction and could potentially block the classical complement pathway at the first step of its activation, appear to be attractive therapeutic candidates as 'C1q inhibitors' [5,12,40,41].

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Note added in proof

A recent paper on the interaction of C1q and factor H with phospholipids has further confirmed the modular nature of the C1q globular domain: L.A. Tan, U. Kishore, B.B. Yu, R.B. Sim, Complement activation by phospholipids and its regulation, Eur. J. Immunol., in press.

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