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Isolation, cDNA Cloning, and Overexpression of a 33-kD Cell Surface Glycoprotein that Binds to the Globular "Heads" of C1q

By Berhane Ghebrehiwet, Boon-Leong Lim, Ellinor I. B. Peerschke, Anthony C. Willis, and K. B. M. Reid

From the Departments of Medicine and Pathology, State University of New York, Stony Brook, New York 11794-8161; and the Medical Research Council Immunochemistry Unit, Department of Biochemistry, Oxford University, Oxford OX1 3QZ, UK

Summary

This work describes the functional characterization, cDNA cloning, and expression of a novel cell surface protein. This protein designated gClq-R, was first isolated from Raji cells and was found to bind to the globular "heads" of C1q molecules, at physiological ionic strength, and also to inhibit complement-mediated lysis of sheep erythrocytes by human serum. The NH$_2$-terminal amino acid sequence of the first 24 residues of the C1q-binding protein was determined and this information allowed the synthesis of two degenerate polymerase chain reaction primers for use in the preparation of a probe in the screening of a B cell cDNA library. The cDNA isolated, using this probe, was found to encode a pre-pro protein of 282 residues. The NH$_2$-terminus of the protein isolated from Raji cells started at residue 74 of the predicted pre-pro sequence. The cDNA sequence shows that the purified protein has three potential N-glycosylation residues and is a highly charged, acidic molecule. Hence, its binding to C1q may be primarily but not exclusively due to ionic interactions. The "mature" protein, corresponding to amino acid residues 74–282 of the predicted pre-pro sequence, was overexpressed in Escherichia coli and was purified to homogeneity. This recombinant protein was also able to inhibit the complement-mediated lysis of sheep erythrocytes by human serum and was shown to be a tetramer by gel filtration in nondissociating conditions. Northern blot and RT-PCR studies showed that the C1q-binding protein is expressed at high levels in Raji and Daudi cell lines, at moderate levels in U937, Molt-4, and HepG2 cell lines, and at a very low level in the HL60 cell line. However, it is not expressed in the K562 cell line. Comparison of gClq-R NH$_2$-terminal sequence with that of the receptor for the collagen-like domain of C1q (cClq-R) showed no similarity. Furthermore, antibodies to gClq-R or an 18-amino acid residue-long NH$_2$-terminal synthetic gClq-R peptide did not cross-react with antibodies to cClq-R. Anti-gClq-R immunoblotted a 33-kD Raji cell membrane protein, whereas anti cClq-R recognized a molecule of $\sim$60 kD. The NH$_2$-terminal sequence of gClq-R appears to be displayed extracellularly since anti-gClq-R peptide reacted with surface molecules on lymphocytes, polymorphonuclear leukocytes, and platelets, as assessed by flow cytometric and confocal laser scanning microscopic analyses. In addition, all or part of the gClq binding domain may reside within the 24 amino acid stretch of the NH$_2$-terminal sequence of gClq-R since the 18 amino acid residue long-synthetic peptide corresponding to this region inhibited serum C1q hemolytic activity. The data presented in this report suggest that there are at least two types of C1q-R which appear to be expressed on the same type of cells and these receptors individually or in concert may contribute to the diversity of C1q-mediated responses.

C$_1$q binds to a variety of cells such as B cells, monocytes, macrophages, neutrophils, eosinophils, fibroblasts, platelets, endothelial and smooth muscle cells (1–9). The biological responses elicited by C$_1$q are also as diverse as the cell types that express the receptor (1). It enhances FcR- and CR1-mediated phagocytosis in monocytes and macrophages (10, 11), stimulates Ig production by B cells (12, 13), activates platelets to express $\alpha$IIb/$\beta$_3 integrins, P-selectin, and procoagulant activity (14), and plays a role in the activation of tumor cytotoxicity of macrophages (15, 16). Most of the above actions of C$_1$q are considered to be mediated by a C$_1$q receptor that binds to the collagen-like regions...
of C1q (1). This C1q receptor is a single chain, acidic glycoprotein with a molecular mass of \( \sim 60 \text{kD} \) (1). However, this receptor has a relatively low affinity to the collagen-like regions of C1q and binds at a very low ionic strength (10 mM potassium phosphate buffer) (17). In 1989, a high affinity receptor, which binds to the globular heads of C1q, was reported to be present, along with the low affinity receptor, on human diploid fibroblasts (18), thereby indicating that at least two types of C1q receptors might be involved to impart the vast array of C1q-mediated biologic functions.

In this paper and in a previous report (19), a cell surface protein, which has a high affinity for the C1q globular heads, has been characterized. By the use of NH2-terminal protein sequence information and PCR techniques, a full-length cDNA clone was isolated and the primary sequence of this protein has been predicted. Northern blot and RT-PCR studies were carried out to demonstrate the different levels of expression of this protein in different cell lines. Moreover, a recombinant form of this protein has been produced in Escherichia coli and shown to be able to inhibit complement-mediated lysis of sheep erythrocytes by human serum.

Materials and Methods

Chemicals and Reagents

The following chemicals and reagents were purchased from the sources indicated: FCS (Hyclone Laboratories, Logan, UT); RPMI 1640, 10× antibiotic-antimycotic mixture ( Gibco BRL, Gaithersburg, MD); Emulphogene BC 720 (polyoxyethylene-10 tridecyl ether), FITC-conjugated goat anti-rabbit IgG, KLH, DMSO (Sigma Chemical Co., St. Louis, MO); NHS-LC-biotin (Sulfo-NHS-LC-biotin) (Pierce, Rockford, IL); Con A-Sepharose PD-10 columns (Pharmacia, Biotech, Inc., Uppsala, Sweden); Mono-Q, TSKgel DEAE-NPR columns (TosoHaas, Montgomeryville, PA); [125I]-Na (Amersham, Aylesbury, UK); and polyvinylidene difluoride (PVDF) membranes, (Immobilon-P; Millipore Corp., Bedford, MA).

Cultured Cells

Raji cells were used to prepare membrane proteins, and were grown in RPMI 1640 containing 10% (vol/vol) heat-inactivated (3 h, 56°C) FCS and 1% (vol/vol) of a 100× antibiotic-antimycotic mixture that contained 10,000 U/ml penicillin G, 25 μg/ml amphotericin B, and 100,000 μg/ml streptomycin sulfate and maintained in an atmosphere of 95% air and 5% CO2. When large quantities of Raji cells were required, the cells were grown in 2-liter roller bottles as described earlier (20). Other cell lines such as Daudi, U937, Molt-4, and HL60 were grown under similar conditions.

Purified Proteins

Human C1q used in these studies was purified by the method of Reid (21). For comparison, C1q was also purified from a pool of freshly obtained sera according to the procedures of Yonemasu and Stroud (22) followed by purification on Con A-Sepharose 4B as described (23).

The collagen “stalks” of C1q (cC1q) were prepared by pepsin digestion of the purified C1q molecule following the procedure of Reid (24) and the globular “heads” of C1q were prepared by collagenase digestion as described (25).

Radioiodination

Except for C1q, which was labeled by the method of Bolton and Hunter (26), all other proteins were radioiodinated by the iodogen method (27) with 1 mgI of NaI125I as described elsewhere (3, 28). After labeling, the free 125I was removed by gel filtration using PD-10 (Sephadex G-25) columns equilibrated with the appropriate buffer system in which the labeled protein was to be kept.

Purification of C1q-R

The purification of C1q-R in these studies used a combination of two published methods (2, 3) with some minor modifications. These methods were: (a) affinity purification on C1q-Sepharose CL-4B, (b) ion-exchange separation on FPLC using a Mono-Q column, and (c) HPLC purification using a TSKgel DEAE-NPR column as described (3, 28).

C1q-Sepharose Affinity Chromatography. Raji cell membranes were prepared from 20-liter cultures (4 × 1010 cells) as described in detail earlier (2, 20). Half of the solubilized membrane solution was then applied to a column packed with 3 ml of C1q-Sepharose CL-4B (3 mg C1q/ml Sepharose) equilibrated with 10 mM sodium phosphate, pH 7.4, containing 20 mM NaCl, 0.1% Emulphogene BC720, and a cocktail of enzyme inhibitors (2, 20). After washing (5× column volume), the bound proteins were eluted with the same buffer containing 1 M NaCl. The eluted material was dia lyzed against equilibrating buffer, and a 0.5-ml aliquot was radio labeled and then tested for C1q-binding activity by a solid phase radioligand binding assay described below. Preliminary studies showed that the eluted material contained most of the C1q-binding activity.

FPLC on Mono-Q Column. The C1q-binding material eluted from the C1q-Sepharose CL4B column was mixed with 100 μl (~1010 cpm) of the radiolabeled sample and applied to a 1-ml Mono-Q column (9, 17). The column was washed until cpm/ml <100, and then the bound proteins were eluted with a linear NaCl concentration gradient (0–600 mM NaCl). C1q-containing fractions were identified by solid phase binding assay (see below), pooled, and concentrated.

HPLC on DEAE Column. The concentrated, C1q-binding fraction from the Mono-Q column was dialyzed against 50 mM Tris, pH 8.0, and then subjected to HPLC using a 1-ml TSK gel DEAE-NPR column as described (9, 17). The bound proteins were eluted using first a 0–500-MM NaCl gradient in equilibrating buffer (10 min) and then a 500 mM–1 M gradient (3 min).

Solid Phase Binding Assay

Solid phase radioligand assays were performed using 125I-labeled C1q-R, or C1q-R-enriched material by a previously described method (29). Briefly, 50 μl, 5 μg/ml each (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6) of C1g, cC1q, or gC1q were applied to duplicate wells of flat-bottomed strip plate-8 (Costar Corp., Cambridge, MA) and incubated for 2 h at 37°C. Wells coated with either 1%, BSA, 100 μg/ml IgG, or 100 μg/ml properdin, served as irrelevant or positively charged protein controls. After incubation, the wells were washed (3 × 300 μl) with TBST (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20). Then 50 μl of 125I-labeled C1q-R in TBB (20 mM Tris-HCl, pH 7.5, containing 100 μg/ml BSA) containing a fixed or increasing amounts of counts per minute were added and further incubated.
Aldarion was given as above 2 wk later and, thereafter, injections were made every 2 wk in IFA until a total of four such injections were made. The rabbits were then rested for 4-6 wk before a final injection was given and the rabbits bled and the serum collected. Hemolytic Assays

Hemolytic assays were performed on 1.5-mm-thick slabs according to the method of Laemmli (30). Samples were either run unreduced or reduced and alkylated by boiling for 5 min in the presence of 0.1 M dithiothreitol and 0.2 M iodoacetamide. After running, the gels were stained with Coomassie Brilliant Blue, de-stained and dried. Gels containing radio labeled samples were exposed to Kodak X-OMAT AR film at -80°C and then analyzed by autoradiography. Samples for Western blot analysis (31) were first run on SDS-PAGE, then electrotransferred to polyvinyl difluoride (PVDF) nitrocellulose membranes, blocked with 5% nonfat milk containing TBST, and the bound proteins probed with predetermined dilutions of rabbit antibodies in the above buffer. The bound antibodies were visualized by horseradish peroxidase-conjugated goat anti-rabbit IgG followed by reaction with 4-chloro-1-naphthol substrate.

**NH2-terminal Amino Acid Sequencing and Peptide Synthesis**

The NH2-terminal sequence information of gClq-R was generated using a protein sequencer and analyzer (models 470A and 120A; Applied Biosystems, Inc., Foster City, CA) as described elsewhere (28, 32). An 18-mer synthetic peptide (gClq-Ras) derived from the NH2-terminal sequence of gClq-R was synthesized on a peptide synthesizer (model 430A; Applied Biosystems, Inc.) at the Center for Analysis and Synthesis of Macromolecules (Department of Medicine, State University of New York, Stony Brook, NY). The peptide was then purified by gel filtration on HPLC.

**Antibody Production**

Antibody to gClq-R was prepared in rabbits according to the following schedule. Purified gClq-R (10-20 µg) in 200 µl PBS was mixed with 200 µl each of complete and IFA and injected at three sites: one subcutaneous and two intramuscular. Booster injection was given as above 2 wk later and, thereafter, injections were made every 2 wk in IFA until a total of four such injections were made. The rabbits were then rested for 4-6 wk before a final injection was given and the rabbits bled and the serum collected and tested by ELISA against purified gClq-R.

Antibody to gClq-R peptide was generated by injection of 500 µg of KKL-conjugated peptide following the immunization protocol described above. Conjugation of gClq-R peptide to KKL was accomplished by the glutaraldehyde method as described (33). The IgG fraction from each antiserum was prepared using the Immunopure A/G IgG purification kit following the manufacturer's recommendations (Pierce). Antipeptide antibodies were further purified by affinity purification on KKL-Sepharose CL4B.

**Hemolytic Assays**

The effect of gClq-R18, KKL-gClq-R18, or the soluble, recombinant form of gClq-R (r-gClq-R) was assessed by incubating various amounts of these molecules with 10 µl normal human serum (NHS) for 60 min at room temperature in a total volume of 100 µl GVB (Veronal-buffered saline, pH 7.4, containing 0.15 mM CaCl2, 1 mM MgCl2, and 0.1% gelatin). At the end of the incubation, the volume of the reaction mixtures was brought to 950 µl GVB, and 50 µl of EA (10%/ml, sheep erythrocytes [E] sensitized with IgM anti-sheep E [A]) was added and further incubated for 60 min at 37°C. The reaction was then stopped, immediately centrifuged, and free hemoglobin in the supernatant assessed spectrophotometrically at 412 nm.

**Confocal Laser Scanning Microscopy**

PBL, monocytes, and PMN were purified by a combination of Ficoll-Hypaque sedimentation (to separate PMNs from mononuclear cells) and plastic adherence in autologous serum (to separate monocytes from lymphocytes), as described (34). For confocal laser scanning microscopic (CLSM) analysis, the cells were suspended on a polystyrene dish, then incubated (1 h, 37°C) with 1 ml of either IgG anti-gClq-R or IgG from preimmune rabbit serum. After incubation, the cells were washed (2 x 2 ml) with cold GVB by centrifugation (800 g, 4°C), the pellet resuspended in 0.3 ml GVB and further incubated (1 h, 37°C or 4°C) with 50 µl of a 1 mg/ml each of either IgG anti-gClq-R or IgG from preimmune rabbit serum. The antibody to gClq-R was prepared in rabbits according to the method of Laemmli (30). The antibody sequence was determined by automated protein sequencing (Table I). Based on this analysis, two degenerate PCR primers (BH-S and BH-3) (Table 2) were designed and synthesized on a DNA synthesizer (model 38LA; Applied Biosystems, Cheshire, UK). The antisense primer (BH-3) was used as the primer for the first strand cDNA synthesis from total RNA. All the components in the reverse transcription reaction were from a kit (cDNA Synthesis System Plus; Amersham International, Amersham, Bucks, UK), except for total RNA and primer. In a 10-µl reaction: 3.0 µl H2O; 2.0 µl 5x first strand reaction buffer; 0.5 µl sodium pyrophosphate solution; 0.5 µl RNase inhibitor; 1.0 µl deoxynucleoside triphosphate mix; 1.0 µl BH-3 primer (1 µg/ml); 2.0 µl Raji cell total RNA (10 µg/ml); and 10 U AMV reverse transcriptase were mixed together. After incubation at 42°C for 40 min, the tube was stored at -20°C for subsequent PCR amplification.

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<th>Receptor type</th>
<th>NH2-terminal sequence</th>
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<tr>
<td>cClq-R</td>
<td>EPAVYFKEQFLDGDDG</td>
</tr>
<tr>
<td>gClq-R</td>
<td>LHTDGDKAFVDFLSDEIKEE RKIQ</td>
</tr>
</tbody>
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The underline sequence in gClq-R peptide was used for the construction of two degenerate oligonucleotide probes as well as the generation of a synthetic peptide (gClq-Ras).

1. Abbreviations used in this paper: CLSM, confocal laser scanning microscopy; IPTG, isopropyl β-D-thiogalactopyranoside; NHS, normal human serum.
Table 2. Sequences of PCR/Library Screening Oligonucleotides

<table>
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<th>Name</th>
<th>Sequence (5' → 3')</th>
<th>Direction</th>
<th>Position</th>
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<tr>
<td>BH-S</td>
<td>5'-ACIGYGGIGAYAARGCITTT</td>
<td>Sense</td>
<td>a.a. 76–82</td>
</tr>
<tr>
<td>BH-3</td>
<td>5'-YTGIAVTYTICTYTCYTC</td>
<td>Antisense</td>
<td>a.a. 92–97</td>
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<tr>
<td>KS-1</td>
<td>5'-CAGGAATTCCTGTAGTAGGAATTAGGG</td>
<td>Sense</td>
<td>ntd. 331–352</td>
</tr>
<tr>
<td>KA-1</td>
<td>5'-TGGAATTCACTACACTGGAATCAACA</td>
<td>Antisense</td>
<td>ntd. 324–343</td>
</tr>
<tr>
<td>KS-2</td>
<td>5'-AACCTCGCAAGGCGAGAA</td>
<td>Sense</td>
<td>ntd. 521–539</td>
</tr>
<tr>
<td>BM-1</td>
<td>5'-GTAGGATTTGTTCATCTGGCCA</td>
<td>Antisense</td>
<td>ntd. 967–988</td>
</tr>
<tr>
<td>BN-1</td>
<td>5'-GCCATGGCTCTGCACACCGACGGAGAC</td>
<td>Sense</td>
<td>ntd. 297–315</td>
</tr>
<tr>
<td>Fl1</td>
<td>5'-ACTCCTGGAGCCCGTCAGTAT</td>
<td>λgt11 primer</td>
<td></td>
</tr>
<tr>
<td>R11</td>
<td>5'-GACCAAATCGGTAATGGTAGGCGAC</td>
<td>λgt11 primer</td>
<td></td>
</tr>
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Added restriction sites are underlined: EcoRI(KS-1 and KA-1); Ncol(BN-1).
a.a., amino acid; I, Inosine; ntd., nucleotide; R, A/G; Y, C/T.

Generation of the First Exact Match Oligonucleotide Probe by PCR Purification. The first strand cDNA generated as described above was used as template and the two degenerate, inosine-containing oligonucleotides (BH-S and BH-3) were used as primers in the PCR reaction. A PCR reaction was performed with 30 cycles in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT) using a cycle of denaturation for 0.5 min at 94°C, annealing for 0.5 min at 48°C, and an extension at 72°C for 0.5 min. The reaction mixture (50 μl) contained 25 pmol of each primer, 0.25 mM dNTP, 1.25 U Taq polymerase (Promega, Southampton, UK) with the manufacturer's buffer system, together with 1.0 μl first strand cDNA. The PCR product (5 μl) was electrophoresed on a 4% (wt/vol) agarose gel (Nusieve; FMC Bioproducts Europe, Vallensbaek, Denmark). The expected 66-bp product was obtained.

Subcloning and DNA Sequencing. After electrophoresis, the 66-bp fragment was cut out from the gel and extracted from the gel slice by centrifugation through glass wool (35). The DNA fragment (6 μl of eluant) was blunted-ligated to the HindIII site of plBluescript SK (0.1 μg) using T4 DNA ligase (1 unit) in a 10-μl reaction volume overnight at room temperature. The E. coli strain XL-Blue 1 was used as the host in the transformation of cells with the ligation mixture. After transformation, the cells were plated on LAT plates (LB plates with 100 μg/ml ampicillin and 10 μg/ml T7 polymerase (Promega), Southampton, UK) with the manufacturer's buffer system, together with 1.0 μl first strand cDNA. The PCR product (5 μl) was electrophoresed on a 4% (wt/vol) agarose gel (Nusieve; FMC Bioproducts Europe, Vallensbaek, Denmark). The expected 66-bp product was obtained.

Subcloning the cDNA Insert Encoding the gClq-R into Plasmid pGex-2T. First, a Ncol site was created in the pGex-2T plasmid (36). pGex-2T plasmid was digested by SmaI, desphosphorylated by alkaline phosphatase, and ligated to a Ncol linker (10 mer) by T4 DNA ligase. Plasmid (pGex-2TN) with the Ncol site inserted was selected, cultured, and purified by alkaline lysis method. Second, in order to create a Ncol site on the gClq-R protein insert for subcloning, a PCR primer (BN-1) was synthesized. A 50–μl PCR reaction was carried out using BN-1 and T7P (a plBluescript primer) as primers and plBluescript containing the gClq-R cDNA insert as a template. PFU DNA polymerase (Promega) was used to replace Taq polymerase so as to secure a high fidelity. The amplified PCR product was then precipitated and digested by Ncol and BamHI. The digested insert was purified by gel and subcloned into the Ncol site and BamHI site of pGex-2TN. The plasmid that contained the right insert was isolated (pGex-2TNH) and transformed into E. coli strain NM554.

Overexpression of Clq-binding Protein (Residues 74-282 of the Amino Acid Sequence Predicted from the cDNA/Clone) in E. coli Employing pGex-2T Vector. A colony carrying pGex-2TNH was inoculated into a 100-ml LA (LB plus 50 μg/liter Ampicillin) and grown overnight at 37°C. The next morning, the 100-ml culture was inoculated into 900 ml LA. After 1 h at 37°C, protein expression was induced by 0.1 mM IPTG, the culture allowed to grow for 7 h at 37°C, and the cell harvested by centrifugation at 5,000 g for 15 min. For every liter of culture, 15 ml MTPBSN (150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, 0.05% NaN3, 0.1% Triton X-100, pH 7.3) was used to resuspend the cell pellet. The cells were lysed by sonication (3 x 1 min) and the supernatant generated by centrifugation at 12,000 g for 15 min contained soluble proteins, including GST-gClq-R fusion product. Glutathione agarose beads (5 ml, sulphur linkage; Sigma Chemical Co.) were pre-
pared by preswelling in MTPBSN, then washing twice in the same buffer. The supernatant was allowed to pass through a column (10 mm × 150 mm) of the beads and the eluant was discarded. The column was washed by 50 ml MTPBSN to elute nonbinding proteins. The fusion protein was eluted by competition with free glutathione using 20 ml 50 mM Tris-HCl, pH 8.0, containing 5 mM reduced glutathione (Sigma Chemical Co.) (final pH 7.5, freshly prepared). The eluent (20 ml) was made 150 mM with respect to NaCl and 2.5 mM with CaCl₂ before thrombin digestion. The concentration of the fusion protein at this stage was estimated to be 1.6 mg/ml. Thrombin was added to a concentration of 3.2 μg/ml and the digestion was allowed to proceed at room temperature for 2 h on a rotating platform. Complete digestion was achieved, as judged by SDS-PAGE carried out after reduction of disulfide bonds.

A Mono-Q column (Pharmacia) was employed to purify the recombinant protein. The pH of the sample was adjusted by adding one-twentieth the volume of 1 M Tris, pH 8.6. The sample was applied to a Mono-Q column (1 ml) preequilibrated in buffer A (20 mM Tris, pH 8.5, 0.05% sodium azide) at a flow rate of 0.5 ml/min. A large peak which was not retained on the column, was collected and then the column was washed with buffer A containing 0.4 M NaCl until the base line returned to zero. Another peak was eluted by buffer A containing 0.6 M NaCl. The two peaks were run on a 15% (wt/vol) SDS-PAGE under reducing conditions and the band giving the expected sequence of the gClq-R protein was determined, by NH₂-terminal peptide sequencing to be in the peak eluted with buffer A containing 0.6 M NaCl.

**Gel Filtration Chromatography.** The recombinant protein and the markers were dialyzed into the gel filtration running buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% (wt/vol) NaN₃, pH 7.5) and then loaded onto a Superose 12 column, which was connected to a FPLC system (Pharmacia). The molecular size markers employed were bovine IgG (160 kD), OVA (43 kD), soybean trypsin inhibitor (20.3 kD), and cytochrome C (12.4 kD).

**Purification of Total RNA and Northern Blot Analysis.** Total RNA (10 μg) from each sample was electrophoresed on a formaldehyde-containing 1% (wt/vol) agarose gel. After electrophoresis, the RNA was transferred to a Hybond-N membrane (Amersham) by capillary blotting and fixed onto the membrane by UV cross-linking (XL-1500 UV crosslinker; Spectronics Corporation, AMS, Burford, UK). A high specific activity, single strand DNA probe was generated by using the PCR method, with a 1.9-kb cDNA clone as a template and the antisense primer, BM-1, using 30 cycles of denaturation for 0.5 min at 94°C, annealing for 0.5 min at 53°C, and an extension at 72°C for 45 s. The reaction mixture (50 μl) contained 25 pmol of each primer, 0.25 mM dNTP, 1.25 U Taq polymerase with the manufacturer's buffer system, together with 1.0 μl first strand cDNA. The PCR product (5 μl) was electrophoresed on a 2% (wt/vol) NuSieve agarose gel.

**Results**

**Purification and Characterization of gClq-R from Raji Cells.** The purification of gClq-R, used sequentially, C1q-Sepharose CL-4B affinity chromatography (2), followed by FPLC on Mono-Q and then HPLC using a column of TSK gel DEAE-NPR (28, 32). When the C1q-binding fraction from the FPLC Mono-Q column was subjected to purification on HPLC, the profile depicted in Fig. 1 was obtained. Fractions eluting between 9 and 18 min were coated onto microtiter plates and tested for their ability to bind 125I-C1q by the solid phase radioligand binding assay. Three major regions of activity were observed with the strongest C1q binding noted with fractions corresponding to peak 4. The position where the RO-SS/A-associated calreticulin is expected to elute is marked in Fig. 1 and is close to the position of C1q-R which eluted at ~450 mM NaCl (peak 1).

Of these molecules possess C1q-binding activity. To assess the structure and composition of the molecules contained within this region of C1q-binding activity, 250-μl samples were taken from each region and analyzed by SDS-PAGE and autoradiography. As shown in the composite autoradiogram (inset, Fig. 1), peak 1 (inset A) contained a band of ~60 kD (non-reduced), which corresponds to the molecular weight of C1q-R; peak 2 (inset B) contained a 60-kD band and an additional 80-kD band; peak 3 (inset C) contained predominantly the 80-kD band (non-reduced) whereas peak 4 (inset D) consisted of a single band that migrated with an apparent molecular mass of 33 kD. Solid phase binding studies performed on the 33-kD molecule showed that it binds to C1q very strongly in a dose-dependent and saturable manner (Fig. 2).

Although the binding of this molecule to C1q is enhanced by low ionic strength conditions, significant binding (70%, n = 3) does occur even at physiologic ionic strength. Furthermore, whereas the binding of cC1q-R and calreticulin to whole C1q was indistinguishable from each other, the binding of the 33-kD molecule was in general, two to three times higher than either the cC1q-R or calreticulin (Fig. 2). In addition, both the 60- and 80-kD species in peak 2 (Fig. 1) appeared to bind to C1q as assessed by autoradiographic analysis of these proteins after elution from C1q-coated microtiter plates (data not shown).

**The 33-kD Molecule Binds to Globular Heads of C1q.** To identify the domain of C1q to which the 33-kD molecule binds, solid phase binding studies were performed by binding 125I-33 kD to microtiter plate wells coated with C1q, cC1q, gC1q, or BSA as described in Materials and Methods. As shown in Fig. 3, the 33-kD molecule binds with high affinity to C1q and gC1q but not to cC1q, suggesting that it is a unique molecule with preferential binding to the globular heads of C1q. Because its elution position on the HPLC
Figure 1. HPLC profile of Clq-R purification. Solubilized Raji cell membranes were sequentially subjected to purification on Clq-Sepharose CL-4B and FPLC Mono-Q. Fractions containing Clq-binding activity were pooled and purified on HPLC using TSK gel DEAE-NPR column and proteins were eluted using concentration gradients of 0-500 mM NaCl (10 min) and 500 mM-1 M (3 min). The positions of cClq-R which eluted at 0.45 M NaCl and gClq-R which eluted at about 700 mM NaCl are indicated. (Inset) SDS-PAGE analysis of HPLC peaks. Samples from major peaks (1-4) of HPLC were radiolabeled and analyzed by a 10% SDS-PAGE (with [+] or without [-] reduction) and autoradiography. Lanes: (A) HPLC peak 1, contains cClq-R; (B) HPLC peak 2; (C) peak 3; and (D) HPLC peak 4, shows a single chain 33-kD gClq-R.

suggests this molecule to be highly acidic, an experiment was performed to demonstrate that the binding of this molecule to Clq, a basic protein, was not charge dependent. To this end, two very basic proteins, human IgG and properdin, were included in the binding assay and the binding of $^{125}$I-gClq-R to these molecules compared with that of Clq and gClq. The result of a representative experiment (n = 3) is depicted in Fig. 4 and shows that $^{125}$I-gClq-R binds to Clq and gClq but not to BSA, IgG, or properdin. Furthermore, gClq-R binds to Clq whose collagen stalks have been occupied by specific anti-stalks IgG (anti-cClq), whereas no binding was observed to IgG anti-cClq alone (Fig. 4).

The NH$_2$-terminal Sequence of gClq-R Contains gClq-Binding Activity. In an effort to raise antipeptide antibody for affinity purification and immunochemical studies, an 18 amino acid residue-long synthetic peptide (gClq-R$_{18}$) of gClq-R in Table 1 (underlined), was synthesized and purified on HPLC. Since a preliminary study had indicated that the peptide binds to Clq (data not shown) it prompted the question: Does this peptide inhibit the hemolytic activity of Clq in serum? To address this question, various concentrations of either gClq-R$_{18}$ or KLH-conjugated peptide were incubated

The NH$_2$-terminal Sequence of gClq-R Is Not Similar to that of cClq-R. To determine if the NH$_2$-terminal amino acid sequence of gClq-R shows any relationship to that of cClq-R, both molecules (i.e., peaks 1 and 4, Fig. 1) were subjected to NH$_2$-terminal amino acid sequence analyses. The results presented in Table 1 show that the two molecules do not appear to be related. Furthermore, within the 24 amino acid residues of the 33-kD gClq-R, there are four aspartic acid and three glutamine acid residues suggestive of its acidic nature.

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Figure 2. Binding of \( ^{125}\text{I} \alpha \text{Clq-R} \) to Clq, and influence of ionic strength or metal ions on binding. Binding of radiolabeled \( \alpha \text{Clq-R} \) to increasing concentrations of Clq was done in PB or PBS (150 mM NaCl) with or without 0.15 mM CaCl\(_2\) and 1 mM MgCl\(_2\). For comparison, the binding of radiolabeled cClq-R and the RO-SS/A-associated calreticulin are included.

Figure 3. Solid phase binding assay. Radiolabeled gClq-R was incubated (2 h, 37°C) with increasing concentrations of immobilized Clq, cClq, gClq, or BSA in PBS, pH 7.5. After incubation, individual wells were washed five times and counted. Each data point represents a mean of three separate experiments run in quadruplicate.

with 10 μl of NHS or GVB for 1 h at 37°C in a total volume of 100 μg GVB. After incubation, the residual hemolytic activity was determined as described in Materials and Methods. Both the KLH-peptide and the gClq-R\(_{18}\) (data not shown) inhibited the hemolytic activity of NHS. Neither KLH alone nor irrelevant peptides of similar length and charge as the gClq-R\(_{18}\) had such inhibitory activity.

Anti-gClq-R and Antipeptide Antibodies Immunoblot a 33-kD Membrane Molecule. Polyclonal antibodies raised against the 33-kD molecule and its 18 amino acid-NH\(_2\)-terminal peptide were used in immunoblotting studies. These results shown in Fig. 5 demonstrate that both antibodies recognize the same membrane molecule which migrates with an approximate molecular mass of 33 kD.

CLSM Analysis Shows the 33-kD to Be Expressed on the Surface of Many Peripheral Blood Cells. To ascertain that gClq-R was a surface molecule, peripheral blood mononuclear leukocytes were purified by Ficoll-Hypaque centrifugation as described
in Materials and Methods and then analyzed by incubation with either IgG of preimmune rabbit, anti-33-kD IgG or antipeptide IgG. The bound antibodies were detected by incubation with FITC-conjugated F(ab')2 goat anti-rabbit antibody. Approximately 90-95% of the mononuclear cell population stained with various intensities with anti gClq-R antibodies. A representative experiment (n = 4) is shown in Fig. 6 and is presented as 0.5 μm optical sections of a representative stained cell.

Isolation of a cDNA Clone Coding for the Clq-binding Protein. Since the Clq-binding protein was isolated from the Raji cell line, total RNA from Raji cell was used as a template for first strand cDNA synthesis and subsequent PCR amplification, which employed primers BH-S and BH-3 (Table 2). A DNA fragment of the expected size (66 bp) was generated by PCR. This fragment was subcloned and three clones were sequenced. Based on the sequences, two exact match primers (KS-1 and KA-1) were synthesized for use in the screening of a λ gt 11 library. Three positive clones were isolated from the human B cell library and all were found to contain the same cDNA sequence (Fig. 7). The longest clone was 1,139 bp long whereas the other two clones encompassed nucleotides 106-1113, found in the longest clone. The derived amino acid sequence (Fig. 7) confirms the protein sequencing data (amino acid sequence 74-97) and indicates that the Clq-binding protein is synthesized as a pre-pro protein of 282 residues. The mature protein which starts at residue 74 is predicted from cDNA studies to be preceded by a 60 residue-long hydrophobic stretch containing five cysteines. This in turn is predicted to be preceded by a 13 residue-long leader peptide (37). The predicted molecular weight of the 209 residue-long protein is 24.3 kD and contains three potential N-glycosylation sites at residues 114 (Asn-Gly-Thr), 136 (Asn-Asn-Ser), and 223 (Asn-Tyr-Thr).

Northern Blot Analysis. Total RNA from various cell lines (38) was analyzed and is shown in Fig. 8. After stringent washing (0.1 x SSC, 0.1% SDS, 65°C, 15 min) and exposure for 7 d, strong signals were seen in both B cell lines (Daudi and Raji, Fig. 8), whereas weaker signals could be seen in HepG2 and Molt-4 cell lines. No signal was seen in K562 and only a smear could be seen in HL60. In another Northern blot carried out using the same conditions (data not shown), the U937 cell line (monocyte cell line) yielded a weaker signal than the Raji cell line but a comparable signal to that obtained with HepG2 and Molt-4 cell lines. The size of the mRNA for the Clq-binding protein is ~1.5-1.6 kb.

RT-PCR. Using primers BM-1 and KS-2, a PCR fragment of 468 bp should be generated from first strand cDNAs of positive cell lines. The PCR products were run into a 2% (wt/vol) NuSieve gel and the results are shown in Fig. 9. In the positive control, plasmid containing the Clq-binding protein cDNA was used as template in the PCR reaction, whereas there is no first strand cDNA template in the negative control. There are strong signals in HepG2, Molt-4, Daudi, Raji, and U937 cells, but none in K562 cells. There is a weak signal from HL60, showing that it has a low level of the Clq-binding protein mRNA which is not detectable by Northern blotting.

Production of Recombinant Protein and its Properties. The
Leukocyte labeled with Anti-gClq-R peptide

Transmitted light Anti-gClq-Rp (FITC) Composite A & B

Figure 6. Confocal laser scanning microscopic imaging (CLSM). Mononuclear leukocytes were incubated (30 min, 37°C) in GVB containing 0.02% NaN₃ with IgG preimmune rabbit serum, IgG anti-gClq-R, or anti-gClq-R peptide. The bound antibodies were detected by goat FITC-IgG anti-rabbit and analyzed by CLSM. Approximately 90-95% of the cells stained with both antibodies. The image in the top panel (B) is a 0.5 μm section of a representative cell stained with anti-gClq-R and (A) is the transmitted light image of the section in (B), whereas (C) represents a composite image of (A) and (B). The bottom panel is a composite of a series of 0.5-μm sections taken from the center of the cell ("equator") upwards to the "pole" (top left to right and bottom left to right).

Figure 7. cDNA and derived amino acid sequences of the Clq-binding protein. Nucleotide sequences are numbered (right) and amino acid sequences are numbered (left). Amino acid residue number one is the first methionine of the putative translated sequence. The derived amino acid sequence confirms the NH₂-terminal protein sequencing data obtained from the mature protein isolated from Raji cells (amino acid sequence residues 75-97, underlined). (*) The stop codon tag at nucleotides 924-926; (double underline) the three potential N-glycosylation sites. These sequence data are available from EMBL/GenBank/DDBJ under accession number X75913.
Fig. 8. Northern blot analysis. Total RNA (10 μg) from various cell lines were analyzed. The samples were HepG2 cells (lane 1); Molt-4 cells (lane 2); K562 cells (lane 3); Daudi cells (lane 4); Raji cells (lane 5); blank (lane 6); and Kb60 cells (lane 7). The 18S and 28S rRNA were taken as size markers. The size of the signal is ~1.55 b.

mass of 27-28 kD on SDS-PAGE (even though the molecular weight calculated from the amino acid sequence is only 24.3 kD). In gel filtration studies using a Superose 12 column, the recombinant Clq-binding protein was eluted at a position corresponding to 97.2 kD which is consistent with it being a tetramer of a 24.3-kD polypeptide chain.

Recombinant gClq-R (rgClq-R) Inhibits Clq Hemolytic Activity. The recombinant, soluble form of gClq-R generated by expression in E. coli was used to see its effect on Clq hemolytic activity in serum by incubating various concentrations of the recombinant molecule with NHS as described above.

As shown in Fig. 10, the rgClq-R was capable of inhibiting the hemolytic activity of Clq in serum in a dose-dependent manner. To verify the specificity of this inhibition, rgClq-R was first preincubated with Clq before addition to Clq-depleted serum. Whereas, addition of Clq preincubated with rgClq-R did not reconstitute the hemolytic activity of Clq-depleted serum, the addition of Clq alone did, suggesting that addition of rgClq-R to serum indeed prevents the binding of Clq to immune complexes (data not shown). The gClq-R molecule does not, however, activate complement as assessed by C4 titration (data not shown).

Discussion

The existence of two distinct types of Clq receptors had been suggested earlier by Bordin et al. (6, 18) who showed that human diploid fibroblasts express a low affinity Clq-R for the collagen-like domain and a small subpopulation expressing high affinity Clq-R which binds to the globular region of Clq. The present report describes the purification, functional characterization, cDNA cloning, and overexpression of this molecule henceforth referred to as the gClq-R.

The gClq-R molecule was isolated to homogeneity using the same purification procedures for cClq-R and employed a combination of two previously published methods (2, 28, 32). Functional and immunochromatographic characterization of the gClq-R molecule revealed that: (a) it is a unique protein that does not share cross-reactive epitopes with cClq-R; (b) it binds with high affinity to the globular heads of Clq even when the collagen tail of Clq is saturated by anti-cClq IgG; (c) both the purified and recombinant forms of the molecule in-
hibit the hemolytic activity of C1q in serum and this inhibitory activity may reside within the 24 amino acid residues of the NH2 terminus; and (d) anti-gC1q-R antibodies blot a 33-kD molecule from Raji cells as well as other types of cells. Furthermore, CLSM analyses of peripheral blood monocytes and lymphocytes (Fig. 6) and other blood cells (8) with anti gC1q-R antibodies show that gC1q-R is expressed on the surface of these cells, although the degree of expression was found to differ from cell type to cell type. These results indicate that gC1q-R is coexpressed with cC1q-R and is likely to be ubiquitously distributed (1).

The cDNA for the C1q-binding protein encodes a pre-pro protein of 282 residues, as determined from the first methionine, whereas NH2-terminal sequence of the mature protein isolated from Raji cell begins with residue 74 (Leu) (Fig. 7). It is uncertain if this is due to proteolytic cleavage during protein purification or if this protein has an unusually long signal peptide of 73 residues. It seems probable that residues 1-13, or 1-7, form the signal peptide of the C1q-binding protein since they give scores of 5.5 and 4.5, respectively, in the SIGCLEASE command of the GCG program (39). The mature protein (residues 74-282) is highly charged and is very acidic, with a calculated pl of 4.15. Out of 209 residues in the mature protein, there are 28 glutamic acid, 20 aspartic acid, 16 lysine, 5 histidine, and 4 arginine residues. In contrast, the first 73 residue-long stretch of the pre-pro protein does not have any glutamic acid, aspartic acid, lysine, or histidine residues, but does have 11 arginine residues. The mature protein has only one cystine and thus should not have any intra-chain disulfide bonding.

Since the mature protein contains many charged residues distributed all through the sequence, it is unlikely that it contains a transmembrane domain. Rather, the NH2-terminal 73 residue-long stretch, which is too long for a signal peptide, is more likely to contain a transmembrane and a cytoplasmic domain, in addition to a signal peptide. The mature protein purified from the Raji cell line has an apparent molecular mass of 33 kD in SDS-PAGE, much bigger than the calculated molecular weight from the primary sequence (24 kD). This could be explained partly by three potential N-glycosylation sites and partly by its highly acidic nature. For instance, although the recombinant protein has a calculated molecular mass of 24.3 kD and should be smaller than its fusion partner, glutathione-S-transferase (26 kD), it had a larger apparent molecular weight (~27.5 kD) than GST. On the other hand, the recombinant protein behaved as a tetramer of the 24.3-kD polypeptide chain on gel filtration in nondissociating conditions, i.e., as a protein of 97.2 kD.

Northern blot studies and RT-PCR showed that this protein was expressed in a variety of different types of cell lines. Daudi and Raji cells, which are both B cell lines, express a higher amount of mRNA than the other cell lines. The protein is also expressed in the U937 (a monocyte cell line), Molt-4 (a T cell line) and HepG2 cell lines (a hepatocyte cell line), but at a lower level than in the B cell lines. It was shown by RT-PCR that HL-60 (a cell line derived from human promyelocytic leukemia, which is able to differentiate into neutrophil) expresses a low level of C1q-binding protein mRNA which was not detected by the Northern blotting study. However, both Northern blotting and RT-PCR confirmed that there is no expression in K562 cell line (a cell line derived from human myelogenous leukemia, which is highly undifferentiated and of the granulocytic series). Furthermore, in previous studies (8, 19), Western blot analyses showed that this protein is also expressed on eosinophils and neutrophils. On the other hand, a protein, apparently identical to the C1q-binding protein described in this paper, has been purified from HeLa cell line (a fibroblast cell line), and was initially identified as being a pre-mRNA splicing factor, designated as p32 or SF2 (40, 41). However, the claims that it was a pre-mRNA splicing factor, were withdrawn by the same group in their subsequent paper (42). The cDNA clone encoding p32 (41) lacked the first 90 nucleotides seen at the 5' end of the cDNA clone encoding the C1q-binding protein (Fig. 7) and, therefore, it was concluded that p32 lacked an initiating methionine and a signal peptide. However, both these features are seen in the amino acid sequence predicted for the C1q-binding protein (Fig. 7) and are consistent with this protein being secreted from the cell and found on the cell membrane.

Whether this novel molecule plays a primary or auxiliary role within the broad scheme of C1q-C1q-R interaction cannot be ascertained from the present studies. However, the existence of a multi-receptor system may explain in part the participation of C1q-R in such a vast array of cellular responses. It is shown that gC1q-R is able to bind to the globular heads of C1q, even when it is associated with the C1r2-C1s2 complex. The binding of gC1q-R to C1 complexes can inhibit the binding of C1 to IgM sensitized erythrocyte and thus block the lysis of the erythrocyte by complement. Of course, under in vivo conditions, the relative ratio of gC1q-R and C1 is not the same as in in vitro experiments and, it is possible that C1 is able to bind to immune complexes, activate complement, and cross-link the immune complexes to the C1q-binding protein on effector cell surfaces, like B cell, T cell, monocytes, etc. The binding may result in various biological responses. That gC1q-R participates in a variety of biological responses is evident from a number of experiments. Aggregated C1q-mediated platelet activation which leads to the induction of α1-integrins and expression of procoagulant activity (14) can be inhibited by either preincubation of the platelets with anti gC1q-R antibody or, preexposure of the aggregated C1q with gC1q-R (43). In addition, the gC1q-R and cC1q-R, both of which are expressed on eosinophils, have been shown recently to mediate chemotaxis and chemokinesis (8). These results clearly demonstrate that both receptors alone or in conjunction with other cell surface molecules are involved in various C1q-mediated cellular responses. Since the C1q molecule is postulated to circulate in plasma with its globular heads potentially available for binding, it is not clear how a gC1q-R-bearing cell avoids binding to C1q. One possible hypothesis is that the conformational change of C1q followed by binding to cC1q-R must take place before the gC1q-R is engaged to induce secondary messages.
References


