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Human C1qRp Is Identical with CD93 and the mNI-11 Antigen But Does Not Bind C1q

Eamon P. McGreal,* Nobunao Ikewaki, † Hiroyasu Akatsu, ‡ B. Paul Morgan,* and Philippe Gasque‡*

It has been suggested that the human C1qRp is a receptor for the complement component C1q; however, there is no direct evidence for an interaction between C1q and C1qRp. In this study, we demonstrate that C1q does not show enhanced binding to C1qRp-transfected cells compared with control cells. Furthermore, a soluble recombinant C1qRp-Fc chimera failed to interact with immobilized C1q. The proposed role of C1qRp in the phagocytic response in vivo is also unsupported in that we demonstrate that this molecule is not expressed by macrophages in a variety of human tissues and the predominant site of expression is on endothelial cells. Studies on the rodent homolog of C1qRp, known as AA4, have suggested that this molecule may function as an intercellular adhesion molecule. Here we show that C1qRp is the Ag recognized by several previously described mAbs, mNI-11 and two anti-CD93 Abs (clones X2 and VIMD2b). Interestingly, mNI-11 (Fab′) has been shown to promote monocyte-monoocyte and monocyte-endothelial cell adhesion interactions. We produced a recombinant C1qRp-Fc chimera containing the C-type lectin-like domain of C1qRp and found specific binding to vascular endothelial cells in sections of inflamed human tonsil, indicating the presence of a C1qRp ligand at this site. This interaction was Ca²⁺ independent and was not blocked by our anti-C1qRp mAb BIIG-4, but was blocked by the proadhesive mAb mNI-11. Collectively, these data indicate that C1qRp is not a receptor for C1q, and they support the emerging role of C1qRp (here renamed CD93) in functions relevant to intercellular adhesion. The Journal of Immunology, 2002, 168: 5222–5232.

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C1qRp is a 126-kDa transmembrane glycoprotein reported to be expressed by monocytes, neutrophils, platelets, microglia, and endothelial cells (1, 2). C1qRp was originally described as a putative receptor for complement component C1q, a large serum protein that is responsible for activation of the classical pathway of complement after interaction with IgG or IgM immune complexes (3–5). As well as a role in activating the classical pathway, C1q has also been shown to stimulate multiple responses in a variety of cells in vitro, which is suggestive of the presence of specific cell surface receptors (reviewed in Ref. 6).

In humans, a small number of C1q-deficient patients have been identified, 90% of whom have developed the autoimmune disease systemic lupus erythematous (SLE)† (7). C1q-deficient mice also develop an SLE-like disease that is similar in many respects to that observed in humans and that is characterized by a deficiency in apoptotic cell clearance and pronounced glomerulonephritis (8). It has been demonstrated that autoantigens targeted in SLE are clustered in two populations of surface blebs present on apoptotic keratinocytes (9). Because C1q has been shown to bind directly to such surface blebs (10) and C1q can also stimulate enhanced phagocytosis by monocytes (11–13), C1q-deficient individuals might lack an essential clearance mechanism, which leads to this autoimmune disorder.

It has been reported that C1qRp is the C1q receptor mediating enhanced phagocytosis by monocytes and that this activity was neutralized by specific anti-C1qRp mAbs (4). To date, however, there is no direct evidence of an interaction between C1q and C1qRp.

Previously, we have demonstrated that the rat and mouse homologs of C1qRp, each known as AA4, are not expressed by macrophages in a variety of tissues, precluding a role for this molecule in modulating the function of these professional phagocytes (14, 15). Moreover, the predominant site of AA4 expression in rat tissues was on vascular endothelium.

Studies characterizing the mouse homolog of C1qRp (AA4) have likewise suggested a function for this molecule beyond the phagocytic response (16, 17). Mouse AA4 was found to be expressed by primitive hematopoietic stem cells in the developing embryo (16). A distinct and tightly regulated AA4 expression pattern was also observed on vascular endothelial cells during embryonic development and angiogenesis, leading to the suggestion that AA4 might play a role as a homing receptor involved in intercellular adhesion.

The aim of our study was to clarify the cellular and molecular properties of C1qRp.

Materials and Methods

General reagents and primary Abs

Reagents were obtained from Sigma-Aldrich (Poole, U.K.) or Fisher Scientific (Loughborough, U.K.) unless otherwise stated. All cell culture and molecular biology reagents were obtained from Life Technologies (Paisley,
U.K.) unless otherwise stated. Purified human C1q was obtained from Dr. G. Arlaud (Institut Biologie Structurale, Grenoble, France) or was purchased from Sigma-Aldrich.

Mouse mAb mNI-11 was generated as previously described (18). Monoclonal Ab anti-human C3aR (clone BI1G1, mouse IgG2a) was generated in house (by P.G.). Other Abs used were as follows: rabbit polyclonal anti-human C1q (DAKO, Cambridge, U.K.); rabbit polyclonal anti-factor VIII-related protein (Neomarkers, Fremont, CA); rat IgG2a anti-mouse CD23, clone 2G8 (Serotec, Oxford, U.K.); mouse IgG2a anti-CD11b (CR3), clone M175 (Dr. Y. Horesji, Academy of Sciences of the Czech Republic, Prague); mouse IgG1 anti-CD11c (CR4), clone 3.9 (Dr. N. Hogg, Imperial Cancer Research Fund, London, U.K.); mouse IgG1 anti-CD21 (CR2), clone OKB7 (Ortho Diagnostic Systems, Bucks, U.K.); and mouse IgG1 anti-CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1)), clone JCT/0A (DAKO). Mouse IgG1 mAbs against human CD93, clones X2 and VIMD2b, were purchased from Serotec and BD Pharmingen (Cowley, Oxford, U.K.), respectively.

Cells and cell culture

All cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.) or from in-house stocks. The Chinese hamster ovary (CHO) cell line expressing rat or mouse AA4 was generated as previously described (14, 15). The CHO cell line expressing rat decay accelerating factor (DAF) (CD55)-rat IgG2a Fc was kindly provided by Dr. C. L. Harris (Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff, U.K.).

Molecular cloning of C1qRp and recombinant C1qRp-Fc fusion proteins

The full-length (FL) C1qRp cDNA was cloned by RT-PCR on mRNA extracted from the THP-1 monocyte cell line using protocols previously described (14).

Two fragments corresponding to bp 1–807 (primers C1qRp-up 1/C1qRp-do 1) and bp 675–1959 (primers C1qRp-up 2/C1qRp-do 2) were amplified using the following primers: C1qRp-up 1, 5'-GCT CTG GAA GAC CGG GAT GCC CAC CTC CAT (C1qRp FL-IgG1); C1qRp-up 2, 5'-TGG GGA AGG TGA CAT GGA AGG TAA GGA CG (675); C1qRp-do 1, 5'-GCC CCC ATT GTT GAA GTC GG (807); and C1qRp-do 2, 5'-GCC GGA TCC TGA GCA GTC TGT CCC ACC AGG TGT (1595).

C1qRp-up 1 and C1qRp-do 1 were designed with XbaI and BamHI restriction sites (in bold italics), respectively. Both of these fragments were used as the template for an overlapping PCR protocol to obtain the C1qRp-cDNA. Overlapping PCR was performed as follows: 100 ng of each of the C1qRp fragments obtained was added to a PCR (50-μl final volume) containing 10× buffer (NEB, Hitchin, Hertfordshire, U.K.), 200 μM dNTP, 2.5 mM MgCl2, and 0.75 U of Pwo DNA polymerase (Hybaid, Ashford, Middlesex, U.K.). One cycle of denaturation at 94°C for 30 s and annealing/extension at 72°C for 3 min was conducted before the addition of C1qRp primers (C1qRp-up 1 and C1qRp-do 1). This was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 3 min with a final step at 72°C for 15 min. The PCR product was purified and digested with restriction enzymes before ligation into the high-expression vector pDR_EF1a (a gift from Dr. I. Anegon, Institut National de la Santé et de la Recherche Médicale Unité 437, Nantes, France) (19).

To generate a soluble recombinant form of human C1qRp for Ab production, DNA encoding the first 235 (amino terminus) amino acids of the mature protein, including all the β-type lectin-like domain (CTLD), was amplified by PCR, using Pwo DNA polymerase, from the plasmid containing the FL human C1qRp cDNA. Primers used for amplification incorporated restriction sites enabling ligation into the human IgG1 (Fc tail) expression vector Signal pIg (R&D Systems, Abingdon, U.K.) after digestion of the PCR product with XbaI and BamHI (Amersham Pharmacia, Little Chalfont, U.K.) and of the vector with Nhel and BamHI. Primers were as follows (5'-3') with restriction sites in bold italics: plg.C1qRpHupU 5'-TCT AGA AGC GGA GTC GAC AGG GAC GGC-3' and plg.C1qRpHdoU 5'-TGG CAT GGC CTC TTC GAC AGG GAC GGA GGC AGC AGG-3'.

Ligation into the multiple cloning site resulted in addition of five amino acids to the amino terminus of the mature protein (Asp-Lys-Leu-Ala-Arg). To obtain high levels of secretion driven by the strong elongation factor 1α (EF1-α) promoter, DNA encoding the C1qRp_FL-C1qRp fusion protein was subcloned into the expression vector pDR_EF1α.

To generate a soluble recombinant chimeric containing the Fc domain of rat IgG2a (C1qRpFL-IgG2a), PCR was performed on plasmid pDR_EF1α containing the FL human C1qRp cDNA using primer pairs C1qRp FL-Fc 5'-GCC GGA TCC CTT TGG CCC GTC AGT GCT CAC ATC (XbaI) and C1qRp FL-Fc 3'-GCT CTA GAA GAC CGG GAT GCC CAC CTC CAT (BamHI) to amplify the entire extracellular domain of C1qRp from bp 1 to bp 1740. PCR was similarly performed using C1qRp FL-Fl 5'-C1qRp FL-Fl 5'-CGC GGA TCC GCT GAC ACA GAG GGG GCC GCG (BamHI) to generate a C1qRp_C1qRp_FL-IgG2a chimera containing the N-terminal 258 amino acids. Restriction enzyme sites are noted as bold italicized bases in the primer sequence. PCR was performed as previously described using Pwo polymerase. Purified PCR products were digested with the appropriate restriction enzymes before ligation into a high-efficiency expression vector (pDR_EF1α) containing the CH2, CH3, and hinge domains of rat IgG2a Fc (kindly provided by Dr. C. L. Harris). Automated sequencing of all plasmid constructs was conducted in house using an ABI model 377 DNA sequencer (Applied Biosystems, Warrington, U.K.).

Transfection of cells

CHO and mouse myeloblast C2C12 cells were transfected by incubation at 40% confluency in a 25-cm² flask with 4 μg of pDR_EF1α plasmid containing the insert of interest and 20 μg/ml of Lipofectamine Plus (Life Technologies) in OptiMEM serum-free medium. CHO and C2C12 cells were cultured in HAM F-12 and DMEM, respectively, and all culture media were supplemented with 10% FCS. Transfected cells were selected on the basis of resistance to hygromycin B and, once selected, all transfected cell lines were maintained in the appropriate medium containing 100 μg/ml hygromycin B and 5% ultra low Ig FCS (Invitrogen, San Diego, CA). A total of 5 × 10⁴ mouse T lymphocyte cell line (EL4) were electro- porated with 10 μg of pDR_EF1α-C1qRp or pDR_EF1α-C3aR (P.G.) using a Bio-Rad gene pulser (Hemel, Hemstead, U.K.). Electroporation was performed at 276 V and 975 μF. Cells were gradually brought to room temperature and finally resuspended in 15 ml of RPMI 1640/10% FCS. Cells were selected for several days using hygromycin B (750 μg/ml), until a homogenous population of C1qRp- or C3aR-positive cells was established.

Purification of recombinant C1qRp-Fc fusion proteins

The C1qRp chimera containing the Fc portion of human IgG1 was purified from transfected CHO tissue culture supernatants (TCS) using protein A Sepharose chromatography as previously described (14). Despite the high affinity of protein G for the Fc domain of rat IgG2a, we were unable to obtain highly purified rat IgG2a chimeras. We found that the preparation was contaminated with bovine IgG present in the FCS. Hence, the recombinant proteins were purified by affinity chromatography using 10 mg of anti-C1qRp mAb BiG-4 and were coupled to 2.5 g of cyanogen bromide (CNBr)-activated Sepharose (Amersham Pharmacia) according to the manufacturer's instructions. Briefly, immunoreactive choristins were passed over a CNBr-Sepharose column, to which 10 mg each of human and bovine Igs were passed and were slowly washed over the BiG-4 column at 4°C. The column was washed with 10 volumes of PBS before elution using 50 mM diethylamine in PBS. Fractions of interest were dialyzed overnight against PBS. Average protein yield was 1–2 mg of recombinant protein per liter of TCS as measured by spectrophotometry at 280 nm.

Generation and affinity purification of a polyclonal anti-C1qRp Ab

A New Zealand White rabbit was immunized s.c. six times with C1qRp-IgG1 chimera (100 μg purified protein/injection). The first immunization was prepared as a 1:1 emulsion in CFA, and all subsequent immunizations were prepared as emulsions in IFA (1:1 ratio of C1qRp-IgG1 chimeric:adjuvant). Test bleeds were taken at several intervals and tested for immuneactivity against CHO-C1qRp by flow cytometry. When the titer of the anti-C1qRp was optimal, the animal was exsanguinated and serum was purified from whole blood. Contaminating rabbit anti-human Ig and CD3-Fc were both removed by passage of the antiserum over CNBr-Sepharose column, to which 10 mg each of human and bovine Igs had been coupled. The breakthrough from this column was subsequently passed over an affinity column generated by coupling 10 mg of C1qRp-IgG1 chimera to 2 g of CNBr-Sepharose. Rabbit anti-human C1qRp was eluted with 50 mM diethylamine (pH 7). Ab concentration was determined by spectrophotometry at 280 nm, and appropriate fractions were pooled and dialyzed against PBS.

Generation of monoclonal anti-C1qRp Abs

Adult BALB/c mice were immunized five times s.c. with 0.4 ml of C2C12-C1qRp (10⁷ cells/ml) in PBS with 10% pertussis vaccine (Wyeth Vaccines, Maidenhead, U.K.) or with C1qRp_C1qRp_FL-IgG1 fusion protein (100 μg/mouse). The immunization protocol for the C1qRp-human Fc chimera was identical with that for the generation of the polyclonal Ab.

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Two days after the final immunization, fusion of splenocytes with the immortalized SP2/0 myeloma cell line was performed by standard methods (20). Immediately after fusion, hybridomas were maintained in RPMI 1640 supplemented with 15% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, sodium pyruvate, glutamate, and hypoxanthine/aminopterin/thymidine. Supernatants from hybridoma exhibiting cell growth were screened by flow cytometry for immunoreactivity against CHO-C1qRp and a CHO cell line transfected with an empty vector. Hybridomas displaying specific immunoreactivity were cloned by the limiting dilution technique, and screening and cloning was continued until the mAbs BIIG-3 and BIIG-4 were established. BIIG-3 and BIIG-4 were established from mice immunized with C2C12-C1qRp and C1qRpCTLD-IgG1, respectively. The mAb isotype was determined using the “IsoStrip” isotyping kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions.

Protein analysis

Western blotting under nonreducing conditions on 7.5% polyacrylamide gels was performed essentially as described previously (14) on conditioned TCS from CHO cells expressing C1qRpFL-IgG2a and C1qRpCTLD-IgG2a. Blots were probed with HRP-conjugated rabbit anti-rat IgG Fc Abs (Sigma-Aldrich) at 1:1000 in PBS/milk overnight at 4°C. Prestained molecular mass markers (NEB) were used as molecular mass standards. The purity and integrity of the affinity-purified C1qRp-rat Fc chimera was also assayed by silver stain after purification of purified protein on a 7.5% SDS-PAGE gel under nonreducing conditions or after reduction by heating to 95°C for 10 min in the presence of 2-ME.

Tissue processing and immunohistochemistry

Human tissues were obtained postoperatively from the Department of Surgery (University of Wales College of Medicine) and the Fukushimura Hospital (Dr. H. Akatsu, Aichi, Japan) in accordance with local ethical approvals. Tissues were snap frozen in isopentane precooled on dry ice before sectioning at 10 μm on a cryostat (Shandon, U.K.). Sections were captured on poly-L-lysine-coated glass slides (Surgipath, Peterborough, U.K.) and allowed to air dry for at least 3 hours before use. Frozen sections were fixed in acetone for 5 min and briefly allowed to dry air before two washes in PBS. Sections were blocked PBS/1% BSA for 10 min before incubating with the appropriate Fc-fusion protein for 1 h at room temperature. In blocking experiments, Fc-fusion proteins were presaturated with 40 μg/ml mAbs (mNI-11 or BIIG-4) for 30 min at room temperature, or they were spiked with a final concentration of 10 mM EDTA before incubation with the tissue section.

For double immunofluorescence staining, rabbit anti-factor VIII-related protein (VIII-related protein (FLI)) was coincubated with the C1qRp-Fc. In other experiments, sections were incubated with a combination of anti-C1qRp mAb BIIG-4 (4 μg/ml) and rabbit anti-CD35 (1:300) or rabbit anti-α-1-anti-chymotrypsin (1:300) pAb in PBS/1% BSA. Sections were also incubated with a combination of rabbit anti-C1qRp (1:300) and mouse mAb anti-CD11b, CD11c, CD21 or CD31 (all at 4 μg/ml) in PBS/1% BSA. Sections were washed three times in PBS and saturated once more in PBS/1% BSA before the addition of the appropriate secondary Ab. According to the primary Ab combination used, sections were incubated for 1 h at room temperature with TRITC-conjugated donkey anti-rabbit Ig or FITC-conjugated donkey anti-mouse or donkey anti-rat Ig secondary Abs diluted to 1.5 μg/ml in PBS/BSA (all secondary Abs had minimal cross-reactivity to Ig species other than the target and were obtained from Jackson Immunoresearch Laboratories, West Grove, PA). Sections were washed three times in PBS, and slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) before viewing under UV fluorescent microscopy (DMLB; Leica, Heidelberg, Germany).

Flow cytometry

Adherent cells were harvested by culture in incubation in FACS buffer (PBS containing 1% BSA and 0.01% NaN₃ supplemented with 10 nM EDTA). Cells were washed and resuspended at 10⁶ cells/ml in FACS buffer without EDTA. Cells were incubated with primary Ab alone (0.5 μg/ml in FACS buffer unless otherwise stated) or where appropriate with primary Ab plus 30 μg/ml of either human C1qRpCTLD-IgG1 or control rat AA4CTLD-IgG1 fusion protein for 1 h at 4°C. Cells were then washed twice in cold FACS buffer, incubated with the appropriate FITC- or rPE-conjugated secondary Ab (1/100 in FACS buffer) for 1 h at 4°C, and washed an additional three times before resuspension in FACS buffer and analysis on a FACSCaliber (BD Biosciences, San Jose, CA) flow cytometer.

C1q binding assays

Two assays were adapted from the protocol described by Klickstein et al. (21) to test for C1q binding to C1qRp. First, mouse EL4 cells expressing recombinant human C1qRp or human C3aR (as a control) were harvested from culture, washed thoroughly, and resuspended in PBS/BSA (1% BSA) at a cell density of 2 x 10⁶ cells/ml. Aliquots (100 μl) were dispensed into a round-bottom 96-well plate (Nunc, Roskilde, Denmark). Prior titration revealed that 50 μg/ml C1q gave optimum binding to cells (data not shown). Cells were incubated with or without 50 μg/ml purified C1q for 45 min at 4°C. Cells were washed, resuspended in 100 μl of PBS/BSA with rabbit anti-human C1q (1/1000 dilution), and incubated for 30 min at 4°C. Cells were washed and resuspended in 100 μl of PBS/BSA with rPE-conjugated goat anti-rabbit IgG and were incubated for 30 min at 4°C. Cells were washed once more and resuspended in 200 μl of PBS/BSA before analysis by flow cytometry as described above.

Second, for microtiter plate assay, 50 μl of C1q or mAb mNI-11 diluted to 4 μg/ml in coupling buffer (100 mM NaHCO₃, 100 mM Na₂CO₃ in dH₂O, pH 9) was incubated in 96-well microtiter plates at 4°C overnight. Wells were washed thoroughly and incubated with 50 μl of heat-aggregated Ig (HAI) (prepared by heating a 2 mg/ml solution of human Ig in PBS to 65°C for 30 min), monomeric Ig, C1qRp-FL-IgG2a, or rat IgG2a control Ab (rat anti-mouse CD23 clone 2G8) diluted to 0.05, 0.5, or 5 μg/ml in PBS/1% BSA/0.01% Tween for 45 min at 37°C. Wells were again washed thoroughly and incubated with 50 μl of HRP-conjugated goat anti-human IgG (Sigma-Aldrich) or goat anti-rat IgG (Jackson Immunoresearch Laboratories) diluted 1:1000 in PBS/BSA for 45 min at 37°C. Wells were washed thoroughly and developed using 50 μl of o-phenylenediamine (Dako)/0.01% H₂O₂ prepared per manufacturer’s instructions. Development was arrested with 50 μl of 10% H₂SO₄. Plates were analyzed on a microtiter plate reader (Bio-Rad model 3550-UV) at 490 nm.

Results

Anti-C1qRp monoclonal and polyclonal Abs specifically detect C1qRp

The mAbs BIIG-3 and BIIG-4 were tested by flow cytometry for immunoreactivity against CHO cells expressing C1qRp or CHO cells expressing mouse and rat AA4. Both mAbs specifically stained C1qRp-expressing cells, with no immunoreactivity observed toward cells expressing mouse and rat homologs (Table I). The specificity of the rabbit anti-C1qRp pAb was confirmed in the same manner. The pAb strongly stained CHO cells expressing human C1qRp, with some immunoreactivity observed on CHO cells expressing mouse and rat AA4 (Table I). The specificity of all three Abs was also tested by Western blot. BIIG-4 and polyclonal rabbit anti-C1qRp detected a band of Mr = 100 kDa, corresponding to C1qRp in CHO-C1qRp cell lysates but not in control CHO cell lysates under nonreducing conditions (Fig. 1). No immunoreactivity was obtained with mAb BIIG-3 by Western blot (data not shown). After reduction of proteins within lysates using 2-ME, BIIG-4 no longer detected bands in C1qRp⁸ lysates (data not shown). However, rabbit anti-C1qRp detected a band of Mr = 130 kDa under these conditions (Fig. 1).

Table I. Immunoreactivity of monoclonal and polyclonal Abs against human C1qRp and rodent C1qRp (AA4 Ag)⁸

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>BIIG-3 (FL1)</th>
<th>BIIG-4 (FL1)</th>
<th>Rabbit Anti-C1qRp (FL2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-C1qRp (human)</td>
<td>175.4</td>
<td>775.9</td>
<td>2993</td>
</tr>
<tr>
<td>CHO-AA4 (mouse)</td>
<td>6.8</td>
<td>8.1</td>
<td>312</td>
</tr>
<tr>
<td>CHO-AA4 (rat)</td>
<td>0</td>
<td>0</td>
<td>580</td>
</tr>
</tbody>
</table>

⁸ CHO cell lines expressing human C1qRp (CHO-C1qRp) and the mouse and rat homologs of C1qRp (CHO-AA4 (mouse) and CHO-AA4 (rat)) were incubated with the polyclonal Abs and mAbs (BIIG3, BIIG4) against human C1qRp. Flow cytometry was performed following the addition of FITC-conjugated anti-mouse or rPE-conjugated anti-rabbit Abs. Numbers are the mean of fluorescence intensity.
ELISA using immobilized C1qRP\textsubscript{CTLĐ-IgG1} indicated that BIIG-4 but not BIIG-3 recognized the CTLD of C1qRP (data not shown).

Identification of C1qRP as the Ag recognized by mAb mNI-11

Monoclonal Ab mNI-11 has been reported to induce intercellular adhesion of LPS-stimulated monocyte (U937) cells as well as inducing the adhesion of these cells to an endothelial (HUVEC) cell layer and rapid spread formation in HUVECs (18, 22). The identity of the mNI-11 Ag was unknown. However, based on 1) the molecular mass of the Ag in the presence and absence of reducing agents (∼117 kDa and ∼97 kDa, respectively), 2) its expression pattern on monocyte and endothelial cells, and 3) our hypothesis that C1qRP may play a role in adhesion, we decided to test whether C1qRP was the Ag recognized by mNI-11. FACS analyses (Fig. 2a) revealed that mNI-11 reacted strongly with CHO-C1qRP. The double population of cells (C1qRP\textsuperscript{high} and C1qRP\textsuperscript{low}) observed after mNI-11 staining was identical with that observed with anti-C1qRP mAbs BIIG-4 and BIIG-3. None of the mAbs reacted with CHO-mouse AA4 when compared with control staining.

Previous studies have demonstrated that monomeric F(ab')\textsubscript{2} of mNI-11 induce adhesive events in monocytes and endothelial cells (18, 22). This observation precludes receptor cross-linking as the mechanism underlying the observed adhesive events and suggests that mNI-11 may mimic a naturally occurring ligand for C1qRP. To localize the mNI-11 binding domain within C1qRP, we stained THP-1 with mNI-11 in the absence or presence of the soluble C1qRP\textsubscript{CTLD}-IgG1 protein or a similar protein containing the N-terminal CTLD from the rat homolog of C1qRP (14). Cells were subsequently stained with rPE-conjugated goat anti-mouse IgG before analysis by flow cytometry. The mNI-11 stained THP-1 strongly, and the staining was inhibited by the C1qRP\textsubscript{CTLD}-IgG1, but not by the control AA4 Fc-chimera (Fig. 2b), demonstrating that mNI-11 interacts with a site within the N-terminal CTLD of C1qRP. This finding was confirmed by ELISA using immobilized C1qRP\textsubscript{CTLD-IgG1} (data not shown).
Identification of C1qRp as the Ag recognized by anti-CD93 mAbs

CD93 has been defined by a panel of mAbs that recognizes a 100- to 117-kDa protein expressed by blood cells of the myeloid lineage. The nature and function of the CD93 Ag is unknown; however, similarities of molecular mass and cellular distribution with CD11c with C1qRp was observed and may represent dendritic phagocytic cells in tissues. Moreover, the principal site of C1qRp expression was mainly restricted to endothelial cells on both large and small blood vessels (Fig. 5a). Further immunohistological staining revealed that C1qRp was not expressed by CD35 (CR1) (Fig. 5c) or CD21 (CR2) (Fig. 5d) positive B cells. Similarly, CD8-positive T cells were not stained for C1qRp (data not shown). Background staining was tested in sections stained using an irrelevant mouse mAb (512 IgG1 mouse anti-rat Ctry) or irrelevant rabbit anti-rat Ctry (Fig. 5a, inset).

Expression of recombinant C1qRpFL-Fc and C1qRpCTLD Fc in CHO cells

To further analyze C1qRp/ligand interactions, recombinant proteins consisting of the full extracellular domain of C1qRp or an N-terminal fragment of C1qRp containing the CTLD fused to the CH2, CH3, and hinge regions of rat IgG2a were generated. The predicted molecular masses for C1qRpFL-Fc and C1qRpCTLD-Fc under nonreducing conditions are 240 kDa and 130 kDa, respectively, taking into account primary amino acid sequence and known and predicted glycosylation sites (28, 29) and assuming correct folding of the proteins.

After transfection of CHO cells with pDR_EF1α expression vectors containing C1qRp-rat IgG2a Fc or C1qRpCTLD-rat IgG2a Fc (Fig. 6a), cells were selected using hygromycin B. TCS was collected at regular intervals and analyzed for expression of C1qRp-rat IgG2a Fc fusion proteins by Western blot. Conditioned TCS was separated on a 7.5% gel by SDS-PAGE and blotted to a nitrocellulose membrane before incubation with HRP-conjugated anti-rat Ctry (Fig. 5a) positive B cells. Similarly, CD8-positive T cells were not stained for C1qRp (data not shown). Background staining was tested in sections stained using an irrelevant mouse mAb (512 IgG1 mouse anti-rat Ctry) or irrelevant rabbit anti-rat Ctry (Fig. 5a, inset).

FIGURE 3. C1qRp is recognized by anti-CD93 mAbs. EL4 cells expressing C1qRp or control cells expressing C3aR were prepared for flow cytometry by incubation with anti-C1qRp mAb BIIG-4 or anti-CD93 mAbs X2 and VIMD2b, as well as isotype control Ab. Cells were subsequently stained with an rPE-conjugated anti-mouse Ig Ab and analyzed by flow cytometry.

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Bound proteins were eluted with 50 mM diethylamine and dialyzed overnight against PBS. Silver staining of SDS-PAGE separated chimeric protein under reducing and nonreducing conditions indicated that the protein was intact and migrated at the predicted molecular masses of 240 kDa (nonreduced) and 130 kDa (reduced) (Fig. 6c). No contaminating proteins were evident in the reduced

**FIGURE 4.** C1qRp is predominantly expressed by endothelial cells in a range of normal human tissues. Snap frozen human tissues were sectioned (10 μm) and immobilized on glass slides before staining for C1qRp using BIIG-4 followed by HRP-conjugated anti-mouse secondary Ab. Sections were subsequently counterstained with hematoxylin before dehydration and mounting. Slides were viewed by light microscopy. Background staining was assessed using an irrelevant isotype control Ab (5I2 anti-rat Crry) (a–j, insets). Original magnification for a–e, g, i, and j, ×200; f and h, ×400.

**FIGURE 5.** C1qRp is abundantly expressed by endothelial cells but not by macrophages in human tonsil tissue. Frozen human tonsil tissue was sectioned and immobilized on glass slides before double immunofluorescence staining for a variety of cell-specific markers and C1qRp. a, Sections were stained with BIIG-4 (mouse anti-C1qRp) and rabbit anti-α 1 anti-chymotrypsin, a macrophage-specific marker. b, Confirmation of the endothelial specific localization of C1qRp was provided by staining of sections with rabbit anti-C1qRp and mouse anti-CD31 (PECAM-1). The specificity of the staining was further confirmed by double staining of sections with BIIG-4 and rabbit anti-CR1 (B-cells/subset of macrophages) (c), rabbit anti-C1qRp and mouse anti-CR2 (B cells and sparse follicular dendritic cells) (d), rabbit anti-C1qRp and mouse anti-CR3 (macrophages/dendritic cells) (e), and rabbit anti-C1qRp and mouse anti-CR4 (macrophages/subset of dendritic cells) (f). No staining was obtained with irrelevant Abs (rabbit anti-rat Crry and mouse anti-rat Crry (clone 5I2); a, inset). Original magnification for a–c, e, and f, ×200; d, ×100.
C1qRp alone does not support the binding of C1q to cells

C1qRp was originally proposed as either a receptor for C1q or part of a coreceptor/signaling complex for C1q. However, to date C1q binding to C1qRp has not been demonstrated.

We tested the ability of a purified C1q preparation to bind to EL4 cells expressing high levels of C1qRp (EL4-C1qRp) or to control EL4 cells expressing rC3aR (EL4-C3aR) (Fig. 7a), which is a receptor for complement anaphylatoxin C3a but not C1q. C1q (50 μg/ml) in PBS/BSA was incubated with either EL4-C3aR or EL4-C1qRp. Cells were subsequently stained for C1q by indirect immunofluorescence using a rabbit polyclonal anti-C1q followed by an rPE-conjugated goat anti-rabbit Ig (Sigma-Aldrich). C1q bound to similar degrees to both EL4-C1qRp and EL4-C3aR compared with cells to which C1q had not been added (Fig. 7a). No enhanced binding was observed on cells expressing C1qRp compared with control C3aR-expressing cells. C1q binding on untransfected EL4 cells was similar to the binding on transfected cells (data not shown).

C1qRpl-lgG2a does not bind to C1q

Additional experiments were performed that analyzed the interaction of purified recombinant C1qRpl-lgG2a with purified C1q coated on a microtiter plate (Fig. 7c). Because there are no well-characterized ligands for C1qRp, protein integrity was confirmed by its ability to interact with mAb mNI-11 coated on the plate surface. The mNI-11 only recognizes native C1qRp, and data presented in this report suggest that this mAb may interact with a ligand binding site within C1qRp. C1qRpl-lgG2a, presented at concentrations from 0.05 to 5 μg/ml, interacted strongly with mNI-11 coated on the plate surface (Fig. 7c). A control rat IgG2a Ab presented at the same concentrations did not interact with mNI-11. C1q integrity and functionality was also assessed by its ability to bind HAI. HAI, prepared by heating to 63°C and presented at concentrations ranging from 0.05 to 5 μg/ml, interacted with C1q coated on a plate surface at a concentration of 4 μg/ml (Fig. 7c). Monomeric human IgG, which does not have a natural affinity for C1q, did not interact under the same conditions. Having confirmed the integrity of the reagents and the validity of the method for analysis of C1q/protein interactions, we performed assays to assess C1qRp/C1q interactions.

Purified C1qRpl-lgG2a or control rat IgG2a was added to C1q-coated plates at concentrations ranging from 0.05 to 5 μg/ml in PBS/1% BSA/0.01% Tween. Plates were incubated for 45 min at 37°C and protein interactions were subsequently detected using an HRP-conjugated anti-rat IgG Ab. No interaction was observed between C1qRpl-lgG2a and C1q compared with experiments using control rat IgG2a (Fig. 7c).

Localization of ligands for C1qRp in inflamed human tonsil by affinity cytochemistry

Data presented here have demonstrated that C1qRp is not expressed by human tissue macrophages and does not bind C1q. Consequently, the function of this novel protein remains undefined. Studies characterizing the mouse homolog of C1qRp have suggested that it may be involved in cell-cell interactions and cellular homing (16).

The exclusive expression of C1qRp by circulating leukocytes and vascular endothelial cells is similar to that of other adhesion molecules such as CD31 (PECAM-1) (23).

To function as an intercellular adhesion molecule, C1qRp would be expected to interact with a ligand expressed by other cell types. The existence of endogenous ligands for C1qRp was investigated by staining human tonsil tissue sections with C1qRp/Fc chimeras.

Frozen sections of human tonsil tissue were fixed and incubated with C1qRpl-lgG2a or control rat DAF (CD55)-lgG2a. Sections were subsequently incubated with fluorescently conjugated donkey anti-rat IgG, which had been preabsorbed with Ig from a variety of closely related species (Jackson ImmunoResearch Laboratories). UV light microscopic evaluation of the sections revealed intense and specific C1qRp-lgG2a binding to vascular endothelial cells (Fig. 8a). Control rat DAF-lgG2a protein showed no significant staining in semiserial sections (Fig. 8b). To confirm that C1qRp was binding to endothelial cells, double immunofluorescence staining was performed with C1qRpl-lgG2a and polyclonal rabbit anti-FVIII related protein, an endothelial specific marker. Sections were subsequently stained with minimal cross-reactive Abs to rat IgG (FITC) and rabbit IgG (TRITC). UV-light microscopic
analysis of the sections demonstrated colocalization of C1qRp and FVIII related protein staining (Fig. 8, c and d; arrowheads orientate the figures with respect to colocalization of staining), confirming that C1qRp bound specifically to endothelial cells. Interestingly, not all FVIII related protein + endothelial cells were stained using C1qRpFL-IgG2a protein.

FIGURE 8. Localization of ligands for C1qRp in human tonsillar tissue. Frozen human tonsillar tissue was sectioned (10 μm), dried, and fixed before affinity cytochemistry. Sections were incubated at room temperature for 1 h with C1qRpFL-IgG2a (a), control rat DAF-IgG2a (b), or C1qRpFL-IgG2a Fc and rabbit anti-FVIII related protein simultaneously (c and d). Sections were washed and incubated with FITC-conjugated donkey anti-rat IgG or TRITC-conjugated donkey anti-rabbit IgG Abs that had been treated to remove any cross-reactivity with IgG from closely related species. Original magnification for a, ×400; b–d, ×200.
The N-terminal domain of C1qRp, containing the CTLD, is both necessary and sufficient for binding to endothelial cells

After the observation that C1qRp binds to endothelial cells, we proceeded to determine which domain within C1qRp was responsible for ligand binding. It has already been demonstrated that the mAb mNI-11 binds to a site within the CTLD of C1qRp and it has previously been reported that the interaction of mNI-11 (Fab') with monocytes and endothelial cells induces adhesive events, suggesting that this mAb interacts with a specific ligand binding domain within C1qRp. To test whether mNI-11 interacts with the ligand binding domain responsible for C1qRp binding to endothelial cells, affinity cytochemistry was performed as described. Before incubating tissue sections with C1qRp FL-IgG2a, the recombinant protein was presaturated with either mNI-11, BIIG-4, or an isotype control Ab for 30 min at room temperature. Sections were subsequently stained in the same manner as above.

Evaluation of the stained sections revealed that mAb mNI-11 completely inhibited C1qRp binding to endothelial cells, whereas BIIG-4 (Fig. 9), which also binds to a region within the CTLD of C1qRp, had no effect on binding (Fig. 9). Isotype control Ab did not affect the staining pattern (data not shown).

With the knowledge that the N-terminal portion of C1qRp, including the CTLD but not containing the EGF-like domains, was responsible for C1qRp/ligand interactions on endothelium, it was also important to test whether this region of C1qRp alone was sufficient for this interaction. A rat IgG2a Fc fusion protein, consisting of only the first 258 aa of C1qRp containing the CTLD(C1qRpCTLD-IgG2a) was used to stain tonsil sections as described above. The Fc-C1qRpCTLD displayed an identical pattern of staining on endothelial cells to the FL construct and, predictably, mNI-11 but not BIIG-4 was able to inhibit the staining, confirming that this region is both necessary and sufficient for C1qRp binding to endothelium. Inclusion of 10 mM EGTA, a calcium chelator, had no effect on C1qRp/endothelium interactions (Fig. 9).

Discussion

Data presented in this report demonstrate that C1qRp, previously characterized as a putative receptor for complement component C1q, does not interact with C1q under physiological conditions.

The least well characterized of the complement receptors are those proposed for C1q. The original studies demonstrating an interaction between C1q and various cell types (30, 31) have since been complemented by many in vitro assays indicating that C1q can modulate the phenotype of these cells. C1q has variously been shown to enhance Ig secretion by B cells (32), to induce adhesion molecule expression by platelets and endothelial cells (33, 34), and to enhance phagocytosis in monocytes (12, 13), which is suggestive of the presence of specific cell surface receptors. However, the identification of these receptors has proven difficult.

The ability of C1q to enhance monocyte phagocytosis has been investigated in detail by Tenner and coworkers (3, 4). In an effort to identify the cell surface receptor mediating this response, mAbs which could inhibit the response were generated after immunization of a mouse with C1q binding fractions from a monocyte cell line lysate (4). Several Abs capable of inhibiting the enhanced phagocytosis recognized a cell surface protein of $M_r = 126$ kDa (reducing conditions) expressed by monocytes, neutrophils, platelets, and endothelial cells (1, 4). Molecular characterization of C1qRp has shown it to be a heavily $O$-glycosylated, type I transmembrane protein consisting of an N-terminal domain with homology to C-type lectin domains, a tandem array of EGF-like domains, a single transmembrane domain, and a short cytoplasmic tail (1). Despite the ability of anti-C1qRp mAbs to inhibit the phagocytic enhancement, there was no clear evidence that C1q interacts directly with C1qRp, and the authors of these original studies suggested that C1qRp might be a part of a signaling complex involved in C1q-mediated events rather than a true receptor for C1q (4).

In this study, we present several lines of evidence that indicate that C1qRp is not a true C1q receptor. We have studied the interaction of C1q with C1qRp using two different strategies. First, we added C1q to cells expressing recombinant C1qRp. When compared by flow cytometry to cells expressing a control protein (C3aR), we did not observe any enhanced binding, indicating that specific hyperexpression of C1qRp at the cell surface did not confer an increased C1q binding capacity. Because it was possible that any enhanced C1q binding to these cells might have been masked by the presence of an endogenous C1q binding molecule on the cell surface, we also analyzed the ability of a soluble recombinant C1qRp-Fc chimera to bind to C1q presented in an aggregated form on microtitre well surfaces. This system was considered ideal for these studies because the original report characterizing the ability of C1q to enhance monocyte phagocytosis was performed by adding monocytes to microtiter wells onto which C1q had been coated, indicating a requirement for aggregated C1q (12). Furthermore, the C1qRp constructs used here consisted of two C1qRp molecules linked to an Fc tail; thus, the receptor was presented in a bivalent manner, which may be preferable for ligand interactions.
to a receptor presented in monomeric form. No interaction between C1qRp presented at concentrations from 0.05 to 5 μg/ml was observed when added to C1q coated at a concentration of 4 μg/ml. In contrast, HAI, which is well known to interact with C1q, did show considerable binding at 0.5 up to 5 μg/ml. A recent report published while this paper was in revision (35) has demonstrated that C1qRp is the Ag recognized by a panel of CD93 mAbs, which confirmed our own data. However, the report presented data that indicated an interaction between biotinylated C1q (C1qbio) and C1qRp/CD93 transfected cells when C1q was presented at a concentration similar to that tested in our study. This interaction was only observed under conditions of low ionic strength and not under physiological conditions. Furthermore, the authors were unable to reduce C1qbio binding by prior saturation of the cells with unlabeled C1q, indicating that the interaction was probably nonspecific. The authors suggested that binding under physiological conditions might only be possible by interaction of CD93 with aggregated C1q; however, the data presented here have demonstrated that this is not the case. The ability of C1q to interact with many proteins under conditions of low ionic strength is perhaps not surprising given the positively charged nature of this molecule (isoelectric point ~ 10.3) and its ability to interact electrostatically with proteins such as Ig. Because C1qRp is negatively charged (isoelectric point ~ 4) (4), presumably due to the presence of sialic acid, an interaction with C1q would be expected at low ionic strength.

This inherent “stickiness” of C1q has proven to be the greatest hindrance to the identification of true C1q receptors as opposed to C1q binding proteins. Why C1qRp was purified by C1q affinity chromatography from monocyte cell lysates in the initial studies (4) is not clear. Because the cell lysates were applied in a lysis buffer containing a mild detergent (permitting the maintenance of macromolecular complexes), it is possible that C1qRp was isolated because it is a component of a larger C1q receptor signaling complex needed for this C1q-mediated event. Blocking C1qRp with a mAb might then attenuate the cellular response. Because C1qRp does not bind C1q, the exact function of this receptor remains undefined. A role for C1qRp in modulating the phagocytic response in vivo, as originally proposed, appears unlikely in that we have demonstrated that this receptor is not expressed by macrophages in a variety of human tissues, including the inflamed tonsil. These data extend our studies on the rodent homolog of C1qRp (14, 15), known as AA4, and complement data reported by Tenner and coworkers while this manuscript was in revision (36). Both of these studies found that C1qRp/AA4 was not expressed by tissue macrophages and, as described here, showed that the major site of C1qRp expression was on vascular endothelial cells. A previous study, describing the molecular characterization of mouse AA4 (16), had also yielded similar results and demonstrated a tightly regulated AA4 expression on hematopoietic stem cells and endothelial cells during embryogenesis. The authors of this study suggested that C1qRp/AA4 might have a role in intercellular adhesion during stem cell homing and angiogenesis.

Although the cellular expression pattern of C1qRp on vascular endothelial cells and leukocytes is consistent with that of other intercellular adhesion molecules such as CD31 (PECAM-1) (37), there is no experimental evidence to support a role for C1qRp as an adhesion molecule.

In this study, we have identified C1qRp as the Ag recognized by mAb mNI-11. The mNI-11 was originally shown to induce the ICAM-1-dependent aggregation of LPS-stimulated monocytes (U937) as well as inducing the adhesion of these cells to an endothelial cell (HUVEC) layer (18). Further studies demonstrated that mNI-11 could also induce rapid spreading of sparsely seeded HUVECs to form tight monolayers (22). Our finding that C1qRp is the mNI-11 Ag is the first demonstration of a role for C1qRp in the adhesive process. We have also shown that the CTLD of C1qRp is the domain specifically recognized by mNI-11, which is of particular importance because F(ab’)- mNI-11 have been shown to induce the same effects as the whole Ab (18), precluding receptor cross-linking as the mechanism for the observed effect and suggesting that mNI-11 may interact with a ligand binding site within C1qRp, delivering a proadhesive signal through the receptor.

We reasoned that if C1qRp truly functions in intercellular adhesion, it must possess a counter receptor/ligand. To facilitate a better understanding of the in vivo function of C1qRp, we used two C1qRp-Fc chimeras to locate endogenous ligands of this receptor. When applied to sections from inflamed human tonsil, C1qRp-Fc bound solely to vascular endothelial cells, which also stained positively for FVIII related protein. Interestingly, C1qRp did not bind to all endothelial cells present within the tissue and preferentially stained vascular structures that were reminiscent of post capillary venules. This staining pattern is consistent with a role for C1qRp as an adhesion molecule involved in leukocyte extravasation in that the majority of leukocyte transendothelial migration is thought to occur at post-capillary venules (38).

To test the nature and specificity of C1qRp/endothelial cell interactions, we also stained tonsil sections with an Fc-chimera that contained only the N-terminal CTLD of C1qRp. An identical pattern of staining to that observed using the FL construct was obtained, demonstrating that the CTLD alone is sufficient to mediate this interaction. Surprisingly, this interaction was not inhibited by inclusion of the Ca2+ chelator EGTA. Although carbohydrate-lectin interactions are classically Ca2+ dependant (39), the CTLD present within C1qRp/AA4 bears only distant homology to proven Ca2+-dependant carbohydrate recognition domains such as those present in mannose binding lectin or macrophage mannose receptor (14).

In view of the suggestion that mNI-11 interacted with the ligand binding site on C1qRp, we further tested binding by presaturating C1qRp-Fc chimeras with mNI-11 or another anti-CTLD mAb (BIIG-4) before staining tonsil tissue sections. The mNI-11 but not BIIG-4 inhibited the interaction, supporting our proposal that this mAb recognizes a specific ligand binding domain within the CTLD of C1qRp. Further characterization of the C1qRp ligands present on vascular endothelial cells and the epitope recognized by mNI-11 will advance our understanding of the functional significance of these interactions.

In light of the findings described in this report we propose that the current nomenclature for C1qRp is inappropriate. C1q does not interact with C1qRp under physiological conditions, and although a wealth of data has demonstrated its involvement in the phagocytic response by monocytes in vitro, its absence on macrophages in human tissue sections indicates that it cannot modulate the function of these professional phagocytes in vivo. In light of data described here and recently by others, we feel that C1qRp should henceforth be referred to as CD93. We have presented data that are suggestive of a role for this protein in mediating or modulating intercellular adhesion. Further studies should confirm whether this is indeed the case.

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References


