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Deciphering Complement Receptor Type 1 Interactions with Recognition Proteins of the Lectin Complement Pathway

Mickaël Jacquet, Monique Lacroix, Sarah Ancelet, Evelyne Gout, Christine Gaboriaud, Nicole M. Thielens, and Véronique Rossi

Complement receptor type 1 (CR1) is a membrane receptor expressed on a wide range of cells. It is involved in immune complex clearance, phagocytosis, and complement regulation. Its ectodomain is composed of 30 complement control protein (CCP) modules, organized into four long homologous repeats (A–D). In addition to its main ligands C3b and C4b, CR1 was reported to interact with C1q and mannan-binding lectin (MBL) likely through its C-terminal region (CCP22–30). To decipher the interaction of human CR1 with the recognition proteins of the lectin complement pathway, a recombinant fragment encompassing CCP22–30 was expressed in eukaryotic cells, and its interaction with human MBL and ficolins was investigated using surface plasmon resonance spectroscopy. MBL and L-ficolin were shown to interact with immobilized soluble CR1 and CR1 CCP22–30 with apparent dissociation constants in the nanomolar range, indicative of high affinity. The binding site for CR1 was located at or near the MBL-associated serine protease (MASP) binding site in the collagen stalks of MBL and L-ficolin, as shown by competition experiments with MASP-3. Accordingly, the mutation of an MBL conserved lysine residue essential for MASP binding (K55) abolished binding to soluble CR1 and CCP22–30. The CR1 binding site for MBL/ficolins was mapped to CCP24–25 of long homologous repeat D using deletion mutants. In conclusion, we show that ficolins are new CR1 ligands and propose that MBL/L-ficolin binding involves major ionic interactions between conserved lysine residues of their collagen stalks and surface exposed acidic residues located in CR1 CCP24 and/or CCP25. The Journal of Immunology, 2013, 190: 3721–3731.

The online version of this article contains supplemental material.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CCP, complement control protein; CD, circular dichroism; CR1, complement receptor type 1; LHR, long homologous repeat; MASP, mannan-binding lectin–associated serine protease; MBL, mannan-binding lectin; PIEMP1, Plasmodium falciparum erythrocyte membrane protein 1; PiRH4, Plasmodium falciparum reticulocyte binding protein homolog 4; RU, resonance unit; sCR1, soluble CR1; SPR, surface plasmon resonance.

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CR1 is a multimodular membrane glycoprotein composed of an extracellular stretch of 30 complement control protein (CCP) modules, with the 28 N-terminal CCPs organized into four long homologous repeats (LHR-A, -B, -C, and -D) of 7 CCPs each, a transmembrane domain and a short cytoplasmic tail (16–18). LHR-A contains the major C4b binding site located in CCPs 1–3, also involved in PRR binding, whereas LHR-B and LHR-C contain homologous C4b/C3b binding sites in CCPs 8–10 and 15–17, which are also candidates for interaction with PTEMPI (19–21). C1q and MBL have been proposed to bind LHR-D, but the localization of their interaction sites to particular CCP modules has not been described so far (12, 13). Interestingly, LHR-D carries all known Knops blood groups Ags (resulting from single nucleotide polymorphisms), the most common Knops (Kna/b), McCoy (McCa/b), and Swain–Langley (SI1/2) being located in CCPs 24–25 (22).

The fact that MBL was characterized previously as a ligand of CR1 prompted us to investigate the possibility that ficolins, which are structurally and functionally related to MBL and share the capacity to activate the lectin complement pathway and to interact with cell surface proteins such as calreticulin (23) and CD91 (24), could also interact with CR1. For this purpose, a recombinant fragment encompassing CCP modules 22–30 of CR1 was produced in eukaryotic cells, and its interaction with MBL and ficolins was investigated using surface plasmon resonance (SPR) spectroscopy. The CR1 binding site for MBL/ficolins was mapped using deletion mutants and shown to involve the CCPs 24 and 25 of LHR-D.

Moreover, we show that MBL and L-ficolin share a common binding site for CR1, located in the collagen stalks, most probably at or in close proximity to the MASP interaction site.

Materials and Methods

Proteins and reagents

Oligonucleotides were purchased from Eurogentec (Liège, Belgium). Restriction enzymes were from New England Biolabs (Beverly, MA). Recombinant soluble human CR1 was purchased from R&D Systems Europe (Lille, France). Acetylated BSA and diisopropylphosphorofluoridate were from Sigma-Aldrich (St. Quentin Fallavier, France).

Recombinant human L- and H-ficolins were produced in Chinese hamster ovary cells (25) and purified using a one-step affinity chromatography on N-acetylcysteine–Sepharose for L-ficolin (25) and on acetylated BSA–Sepharose for H-ficolin. Elution from the acetylated BSA–Sepharose column (prepared by coupling 125 mg acetylated BSA to 15 ml CNBr-activated Sepharose 4B [GE Healthcare, Vélizy, France]) was carried out using 1 M sodium acetate and 5 mM EDTA (pH 7.4). Recombinant MBL and its K55A and K55E variants, produced in Freestyle 293-F cells (In Vitrogen Life Technologies, St. Aubin, France) and purified as previously described (26) were provided by NatImmune (Copenhagen, Denmark). The molar concentrations of dimeric MASP-3, tetrameric MBL, tetrameric L-ficolin, and tetrameric H-ficolin were estimated using M₀ values of 175,200, 305,400, 406,300, and 396,000 and absorbance coefficients at 280 nm (A₁% 1 cm) of 13.5, 7.8, 17.6, and 19.4, respectively (25, 27).

Construction of the CR1 CCP22–30 and CR1 CCP22–30 size variants expression plasmids

The expression plasmids coding for CR1 CCP22–30 (amino acid residues 1395–1969 of CR1 F allotype) and its size variants were generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Massy, France), according to the manufacturer’s protocol for simple mutations or a modified protocol for the large insertions/deletions (28).

The template containing the DNA coding sequence for the soluble part of CR1 was a generous gift from Prof. J. Cohen (Université de Reims Champagne-Ardenne, Reims, France) (29). First, BglIII and KpnI restriction sites were introduced, respectively, at the 5’ and 3’ ends of the CCP 22–30 coding sequence. The BglII/KpnI restriction fragment was purified and cloned into the BamHI–KpnI sites of the pNT-Bac vector (30), in-frame with the melittin signal peptide. Finally, a fusion 6× His-tag was introduced at the C-terminal end of CR1 CCP22–30 by site-directed mutagenesis. The resulting vector pNT-Bac_C1R1 CCP22–30_His was used as a template for the deletion of CCP modules to obtain the CR1 CCP22–30_His size variants. A DNA fragment corresponding to CR1 CCP22–30 was also cloned into the pCDNA3.1 mammalian expression vector, in-frame with the native signal peptide of CR1 (that was inserted by site-directed mutagenesis). The sequences of all constructs were verified by dsDNA sequencing (GATC Biotech, Mulhouse, France).

Production and purification of CR1 CCP22–30_His and its deletion variants in insect cells

The insect cells Spodoptera frugiperda (Sf21) and Trichoplusia ni (High Five) were routinely grown and maintained as described previously (31), and recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen). The bacmid DNA was purified using the Qiagen midprep purification system (Qiagen, Courtabeuf, France) and used to transfect Sf21 insect cells with cellfection in S900 II serum-free medium, as recommended by the manufacturer (Invitrogen). Recombinant virus particles were collected 4–6 days later and amplified as described previously (31). High Five cells (1.75 × 10⁶ cells/175-cm² tissue culture flask) were infected with the recombinant viruses at a multiplicity of infection of 2 in S900 II serum-free medium at 27°C for 72 h. The culture supernatants containing the CR1 CCP22–30 fragment or its size variants were collected by centrifugation, supplemented with 1 mM diisopropylphosphorofluoridate, and purified immediately or stored at −20°C until use.

The same procedure was used for the purification of CR1 CCP22–30_His and its size variants. Insect cell culture supernatants (250 or 500 ml) were dialyzed against 50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole buffer (pH 7.4), centrifuged, and loaded at a flow rate of 2.5 ml/min onto a 20-ml HIS-Select Nickel Affinity (Sigma-Aldrich)–packed column (2.5 × 10 cm) equilibrated in the same buffer. After washing, proteins were eluted with a 150 mM imidazole buffer. Fractions containing the recombinant protein were identified by SDS–PAGE analysis, pooled, and dialyzed against PBS (pH 7.2). The purified proteins were concentrated to 0.7–2.5 mg/ml by ultrafiltration (Vivaspin Ultra4, Millipore, Molsheim, France) and stored at −20°C. The concentrations of the purified CR1 CCP22–30_His variants were estimated using the following absorption coefficients A₁% 1 cm at 280 nm calculated in silico using the PROTPARAM program on Exasy Server (http://web.expasy.org/protparam), and experimental molecular weights were determined by mass spectrometry: CR1 CCP22–30, 12.0 and 11.156; Δ22–23, 12.4 and 56132; Δ22–25, 13.9 and 38894; Δ27–30, 10.1 and 40107; Δ25–26, 12.5 and 57083; Δ26–30, 9.8 and 32708; Δ29–30, 9.7 and 56567; and CCP25–26, 9.4 and 15434.

Production and purification of untagged CR1 CCP22–30

FreeStyle 293-F cells grown in Freestyle 293 Expression Medium (Invitrogen) were transiently transfected using 293fectin (Invitrogen) as recommended by the manufacturer. The medium was harvested 72 h post-transfection. The following procedure was used to purify untagged CR1 CCP22–30 from the 293-F or High Five cells supernatants. The supernatant was dialyzed against 40 mM NaCl and 25 mM sodium phosphate (pH 7.5), and loaded at 1.5 ml/min onto a Q-Sepharose Fast Flow column (GE Healthcare) (2.8 × 10 cm) equilibrated in the same buffer. Elution was carried out by applying a 1-l linear gradient to 500 mM NaCl in the same buffer. The recombinant protein, which was identified in the flow through fractions by SDS–PAGE analysis, was concentrated and applied onto a Superose 12 10/30GL column (GE Healthcare) at a flow rate of 0.5 ml/min in 150 mM NaCl and 50 mM sodium phosphate (pH 7.5). The purified protein was concentrated by ultrafiltration to 0.5–1.5 mg/ml.

Expression and purification of recombinant MASP-3

A DNA segment encoding the human MASP-3 signal peptide plus the 709-aa residues of the mature protein was amplified by PCR using the pFastBac-MASP-3 plasmid (32) as a template, according to established procedures. The amplified MASP-3 DNA was cloned into the NheI and EcoRI restriction sites of the pCDNA3.1 plasmid. Transient transfection of 293-F cells with the pCDNA3.1 vector encoding MASP-3 was performed as described above for untagged CR1 CCP22–30. The median was harvested 72 h post-transfection, and diisopropylphosphorofluoridate was added to a final concentration of 1 mM. The recombinant protein was purified using a one-step affinity chromatography on a Clq–Sepharose column (prepared by coupling 30 mg purified serum-derived Clq (33) to 15 ml CNBr-activated Sepharose 4B according to the manufacturer’s protocol). Briefly, the supernatant was loaded onto the Clq–Sepharose column equilibrated in 50 mM triethanolamine-HCl, 145 mM NaCl, and 2 mM CaCl₂ (pH 7.4), and MASP-3 was eluted using the same buffer containing 5 mM EDTA instead of CaCl₂. The purified protein was dialyzed against...
50 mM triethanolamine-HCl, 145 mM NaCl, and 2 mM CaCl₂ (pH 7.4) and concentrated to ~1 mg/ml by ultrafiltration. SPR analyses and data evaluation

All experiments were performed on a BIACore 3000 instrument (GE Healthcare). Recombinant soluble CR1 (sCR1), CR1 CCP22–30, and its size variants were covalently immobilized on CMS sensor chips in 10 mM HEPES, 145 mM NaCl, and 0.005% surfactant P20 (pH 7.4) (HBS-P) using the amine coupling chemistry according to the manufacturer’s instructions (GE Healthcare). The protein ligands were diluted in 10 mM sodium acetate at the following concentration and pH values prior to immobilization: sCR1, CR1 CCP22–30 and its deletion variants: 25, 20, and 5 µg/ml (pH 4.2); MBL and BSA: 25 µg/ml (pH 4); L-ficolin: 21 µg/ml (pH 5). Binding was measured at a flow rate of 20 µl/min in 150 mM NaCl, 10 mM HEPES (pH 7.4), and 0.005% surfactant P20 (HBS-P), containing 2 mM EDTA (HBS-EP) or 2 mM CaCl₂ and 10 mM mannose, to prevent unwanted interaction between the high-mannose–type N-linked carbohydrates of the recombinant fragment produced in insect cells and the C-type lectin domains of MBL. Sixty microliters of each analyte at desired concentrations were injected over 13,700–14,900 resonance units (RU) of immobilized sCR1 and 2,500–6,000 RU of CR1 CCP22–30. A flow cell submitted to the coupling steps without immobilized protein was used as blank, and the specific binding signal was obtained by subtracting the background signal over the blank surface. The immobilization levels for the CR1 CCP22–30 size variants varied between 900 RU (CR1 CCP25–26) and 3700 RU (CR1 CCP22–30ΔΔ25–26), according to the relative m.w. of each size variant. For the determination of the binding capacity of the size variants, several sensorchips were prepared, each with immobilized CR1 CCP22–30 (as a reference) and one or two more variants, given that a sensorchip has only four flow cells, with one reserved for the blank. For competition assays, MBL or L-ficolin was incubated for 20 min at room temperature with recombinant MASP-3 in HBS-P containing 2 mM CaCl₂ and 10 mM mannose before injection. Binding of sCR1 and CR1 CCP22–30 to immobilized MBL (13,000 RU) and L-ficolin (17,000 RU) was measured in HBS-EP at a flow rate of 20 µl/min, using immobilized BSA (10,200 RU) as reference. Regeneration of the surfaces was achieved by 10-µl injections of 1 M NaCl, 10 mM EDTA.

Data were analyzed by global fitting to a 1:1 Langmuir binding model of both the association and dissociation phases for at least five analyte concentrations simultaneously, using the BIAevaluation 3.2 software (GE Healthcare). Buffer blanks were subtracted from the data sets used for kinetic analysis (double referencing). The apparent equilibrium dissociation constants (Kᵩ) were calculated from the ratio of the dissociation and association rate constants (kᵩ/kᵠ). χ² values were below 7 in all cases.

SDS-PAGE, N-terminal sequence, and MALDI-TOF mass spectrometry analyses

Recombinant CR1 CCP22–30 and its size variants were analyzed by SDS-PAGE under nonreducing or reducing conditions using Tris-HCl gels containing 10% polyacrylamide. N-terminal sequence determination was performed using an Applied Biosystems gas-phase sequencer model 492 coupled online with an Applied Biosystems Model 140C HPLC system. MALDI-TOF mass spectrometry analyses were performed using a Bruker Daltonics Autoflex mass spectrometer.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a thermostated Jasco J-810 spectropolarimeter using a 1-mm pathlength cuvette (Hellma France, Paris, France) at a temperature of 20°C. The CR1 fragments were dialyzed against 20 mM sodium phosphate and 130 mM NaF (pH 7.3) and used at concentrations of 10–20 µM. Five spectra were acquired in the far-UV region (200–250 nm) at a scan rate of 50 nm/min.

Electron microscopy analyses

CR1 CCP22–30 samples were diluted to 20–140 nM in 50 mM Tris-HCl and 150 mM NaCl (pH 7.4), applied to carbon-coated mica, and negatively stained with 2% sodium silicotungstate (pH 7). A grid was placed on top of the carbon film, which was subsequently dried in air prior to transmission electron microscopy observation. Images were taken under low-dose conditions with a CM12 FEI electron microscope operating at 120 kV.

Results

Production and biochemical characterization of the recombinant CR1 CCP22–30 fragment

A fragment of CR1, encompassing the nine C-terminal CCP22–30 modules of the ectodomain (LHRD + CCP29–30) was produced in a baculovirus/insect cells system and purified from the cell culture supernatant by ion-exchange and size-exclusion chromatography, as described in Materials and Methods. The same fragment with a C-terminal 6× His-tag was also produced and purified using a one-step nickel-affinity chromatography. The mean yields of purified proteins from 1-l insect cells culture supernatant were 0.76 and 3.3 mg for the untagged and His-tagged proteins, respectively. SDS-PAGE analysis of the purified fragments yielded in both cases a single band migrating with an apparent mass of 80 kDa under reducing conditions, as illustrated for the His-tagged protein (Fig. 1A). The protein migrated much faster under nonreducing conditions, which accounts for the presence of 18 disulfide bridges (two in each CCP module). N-terminal sequence analysis of the fragments yielded the single sequence Asp-Leu-His-(Cys)-Lys-Thr-Pro-Glu.., corresponding to the segment His¹³⁹⁵–Glu¹⁴⁰⁰ of human CR1 preceded by two Asp-Leu residues expected to be added at the N-terminus because of in-frame cloning with the melittin signal peptide (30). Mass spectrometry analysis yielded heterogeneous peaks centered on mass values of 70,498 ± 70 Da and 71,156 ± 70 Da for the untagged and tagged fragments, respectively, accounting for the polypeptide chains (predicted masses, 63,679 and 64,502 Da) plus extra masses (6,819 ± 70 and 6,654 ± 70 Da) likely corresponding to N-linked carbohydrates. High-mannose N-linked saccharide chains found on recombinant proteins produced by High Five cells usually comprise 2 N-acetylgalactosamine and four to eight mannose residues (calculated masses, 1055–1704 Da). The predicted values could thus account for the presence of four to six N-linked glycans. The CCP22–30 fragment contains eight predicted N-glycosylation sites (34), but it is likely that all positions are not occupied by carbohydrates, in accordance with previous carbohydrate analyses on full-length CR1 suggesting N-glycosylation of 14 among 25 predicted sites (34).

FIGURE 1. Characterization of the recombinant CR1 CCP 22–30 His fragment. (A) SDS-PAGE analysis of 4 µg CR1 CCP22–30 under reducing (R) and nonreducing (NR) conditions. The positions of the m.w. markers are indicated. (B) CD spectroscopy of CR1 CCP22–30. A spectrum was recorded in the far-UV (200–260 nm) and collected six times. The mean values for each wavelength were calculated. The maximal ellipticity is indicated by an arrowhead. (C) Electron microscopy analysis of CR1 CCP22–30 (∼75000) after negative staining with 2% sodium silicotungstate.
CD spectroscopy was used to assess the correct folding of the fragments. As illustrated in Fig. 1B for the His-tagged protein, the spectrum was characterized by a maximal molar ellipticity value at ∼230 nm, in accordance with CD spectra described for other complement proteins containing CCP modules, such as C4BP α-chain, complement factor H, CR2, and a CR1 fragment (CCP15–17) (35–37). The presence of the peak at 230 nm provides evidence for the correct folding of the CR1 CCP22–30 fragment. Indeed, this peak is generated by an invariant tryptophan residue located between the third and fourth Cys residues of the CCP modules (35) and is lost upon disruption of disulfide bonds (38).

Size-exclusion chromatography showed that the CCP22–30 fragments eluted much earlier than expected from their mass (at a position corresponding to a globular protein of 189 kDa; data not shown), a behavior previously observed with proteins assembled from tandem CCP modules such as factor H (39) and CR2 (37). The elongated shape of the fragment was confirmed by electron microscopy analysis of negatively stained samples showing flexible strings of beads with a length estimated to 25–30 nm, as shown in Fig. 1C for the His-tagged fragment. Given that the mean size of a single CCP module is ∼3.6 nm (34), our electron microscopy data are compatible with the expected size of a molecule containing nine CCP modules.

The chemical and biophysical data presented above show that both untagged and 6× His-tagged recombinant CCP22–30 fragments secreted by insect cells are correctly folded glycoproteins with an elongated shape. Comparable results were obtained for the recombinant untagged fragment produced in 293-F mammalian cells (data not shown).

**FIGURE 2.** SPR analysis of the interaction of MBL, L-ficolin, and H-ficolin with immobilized sCR1 and CR1 CCP22–30. Sixty microliters of 20 nM MBL, 10 nM L-ficolin, and 30 nM H-ficolin were injected over 13,700 RU immobilized sCR1 (A) and 5,700 RU immobilized CR1 CCP22–30 (B) in 150 mM NaCl, 2 mM EDTA, 20 mM HEPES (pH 7.4), and 0.005% surfactant P20 (HBS-EP) at a flow rate of 20 µl/min. MBL (10 nM) (C) and L-ficolin (10 nM) (D) were injected over immobilized His-tagged CR1 CCP22–30 produced in High Five (HF) insect cells (3353 RU) and CR1 CCP22–30 produced in 293-F mammalian cells (4040 RU) in the above conditions. The specific binding signals shown were obtained by subtracting the background signal over a reference surface with no protein immobilized.
It has been shown previously that the MBL–sCR1 interaction was sensitive to ionic strength (13). In accordance with these observations, only 13% of MBL binding to CR1 CCP22–30 was maintained when the experiment was performed in a running buffer containing 250 mM NaCl, and the interaction was abolished in the presence of 400 mM NaCl. The interaction with L-ficolin was also salt sensitive, although to a lesser extent, because 73 and 23% of the binding were observed in the presence of 250 and 400 mM NaCl, respectively. Complete elution of bound L-ficolin could be achieved by a pulse injection of 1 M NaCl.

The kinetic parameters of the interactions were determined by recording binding of varying concentrations of MBL and L-ficolin to immobilized sCR1 and its CCP22–30 fragment (Fig. 3). Data were evaluated by global fitting using a 1:1 Langmuir interaction model as described under Materials and Methods and are reported in Table I. MBL and L-ficolin bound to sCR1 with comparable association and dissociation rate constants, yielding resulting apparent \( K_D \) values in the same range (0.76–0.92 nM). L-ficolin bound to sCR1 and CR1 CCP22–30 with comparable kinetic and dissociation constants, whereas MBL interacted with the fragment slightly faster than with full-length CR1. In all cases, the apparent equilibrium dissociation constants were in the nanomolar range, indicative of tight binding. It has been reported previously that MBL interacted with sCR1 with a \( K_D \) of 3.0–5.2 nM (13). Although this value is slightly higher than that determined in this study, it is compatible with our SPR results, given that the experimental settings (radioiodinated MBL and sCR1-coated plastic wells) and the MBL origin (plasma-derived) were different.

**CR1 interacts with MBL and L-ficolin at or close to the MASP binding site**

Previous studies have suggested that the CR1 binding site of C1q is located in its collagen-like stalks and that the C1s-C1r-C1r-C1s complex interferes with C1q–CR1 interaction (13, 40). To investigate whether CR1 binds to the homologous region within MBL and L-ficolin, we tested the ability of MASP-3 to compete with MBL and L-ficolin for binding to sCR1 and its CCP22–30 fragment. MBL and L-ficolin were preincubated in the presence of varying amounts of recombinant MASP-3, and the mixture was injected over immobilized sCR1 and CR1 CCP22–30. Because the interaction of the MASP-3 dimer with MBL and ficolins is Ca\(^{2+}\) dependent (32), the experiments were performed in the presence of 2 mM CaCl\(_2\) and 10 mM mannose to prevent interaction of CR1 CCP22–30 with the lectin domain of MBL. Recombinant MASP-3 was produced in a mammalian expression system (see Materials and Methods) for the same reason. As shown in Fig. 4A, MASP-3 clearly inhibited MBL binding to sCR1 and its CCP22–30 fragment in a dose-dependent manner, reaching almost maximal inhibition on both surfaces at a MBL:MASP-3 dimer molar ratio of 1. Comparable results were obtained when performing the competition experiments using sCR1 and the recombinant CCP22–30 fragment produced in mammalian cells in the presence of Ca\(^{2+}\) and in the absence of mannose (data not shown), thus confirming that the MBL lectin domain was not involved in the interaction. Similar results were obtained with L-ficolin (Fig. 4B), except that the maximal inhibition was ~70 and 80% for sCR1 and its fragment, respectively. MASP-3 only partially interfered with H-ficolin binding to immobilized sCR1 and CCP22–30, with maximal inhibition of 44% (sCR1) and 38% (CCP22–30) at a H-ficolin:MASP-3 dimer molar ratio of 2 (data not shown).

We have previously generated MBL mutants devoid of MASP-binding capacity after mutation of the Lys55 residue of the collagenous region (25). The ability of the MBL K55A and K55E mutants to bind immobilized sCR1 and CR1 CCP22–30 was next compared with that of wild-type MBL. As shown in Fig. 5, no detectable interaction was obtained with these mutants, providing further support to the hypothesis that CR1 binds at or close to the MASP binding site of MBL. Similar results were obtained when

**FIGURE 3.** Kinetic analysis of the interaction between MBL or L-ficolin and immobilized sCR1 or CR1 CCP22-30. (A) MBL was injected at the indicated concentrations over immobilized sCR1 (14,700 RU) or CR1 CCP22–30 (5,200 RU) in HBS-EP. (B) L-ficolin was injected at the indicated concentrations over immobilized sCR1 (14,700 RU) and CR1 CCP22–30 (2,500 RU) in HBS-EP and HBS-P, respectively. Fits are shown as dotted lines and were obtained by global fitting of the data using a 1:1 Langmuir binding model. The results shown are representative of three to eight experiments (see number of experiments in Table I).
the MBL K55E mutant was injected over sCR1 in the presence of Ca2+, thus providing further evidence that the carbohydrate recognition domains of MBL do not contribute to the interaction.

**Location of the binding site in CR1 CCP22–30**

With a view to determine which CCP modules interact with MBL and ficolins, we generated a set of seven deletion mutants by removing CCP modules at both extremities and in the central part of the CR1 CCP22–30 segment (Fig. 6A). The mutated proteins were produced using a baculovirus–insect cells system and purified in the same way as CR1 CCP22–30, using Ni2+-affinity chromatography. SDS-PAGE and mass spectrometry analyses indicated that all fragments were glycosylated, given the diffuse character of the bands (Fig. 6B) and their measured molecular masses (Table II). Although the accuracy of the mass measurements and the glycosylation heterogeneity preclude the exact determination of the number of occupied N-linked glycosylation sites and their location within CCP modules, some information can be inferred from the deduced masses of the glycosylated moieties of the fragments (Table II). For example, the CCP22–23 and 25–26 module pairs seem to contain only one N-linked glycan, which means that only one of the two potential sites (Fig. 6A) is occupied. Comparison of the various fragments also shows unambiguously that the CCP23, CCP24, and CCP27 modules are glycosylated, but it is difficult to draw conclusions about the presence of one or two N-linked glycans in CCP module 24. CD analysis of the fragments yielded in all cases spectra with ellipticity maxima centered on 223–230 nm, typical of CCP modules (41), thus indicating that the deletions did not induce significant changes in the secondary structure of the fragments (data not shown).

The ability of the deletion mutants to interact with MBL and L-ficolin was investigated using SPR spectroscopy, as described for the parent CR1 CCP22–30 molecule. Equimolar amounts of the different constructs were immobilized and the binding signals of MBL and L-ficolin, recorded as described under Materials and Methods, are presented in Supplemental Fig. 1. A summary of the data, expressed as percentage of the reference signal obtained with immobilized CCP22–30, is presented in Fig. 6C. Deletion of the CCP modules located at the N- or C-terminal ends (DCCP22–23 and DCCP27–30) had no or little impact on the MBL and L-ficolin binding capacities. In contrast, removal of the central CCP module pairs 24–25 (DCCP22–25) and 25–26 (DCCP25–26) reduced the interaction in a significant manner. Interestingly, the CCP22–25 fragment (DCCP26–30) had a higher binding capacity than the CCP22–26 (DCCP27–30) fragment, suggesting that deletion of

### Table I. Kinetic and dissociation constants for binding of MBL and L-ficolin to immobilized sCR1 and CR1 CCP22–30

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<th>Immobilized sCR1</th>
<th>Immobilized CR1 CCP22–30</th>
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<tr>
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<td>$k_a$ (M$^{-1}$ s$^{-1}$)</td>
<td>$k_d$ (s$^{-1}$)</td>
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<tr>
<td>MBL</td>
<td>7.15 ± 0.91 × 10$^3$</td>
<td>5.19 ± 0.54 × 10$^{-4}$</td>
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<tr>
<td>L-ficolin</td>
<td>7.72 ± 0.60 × 10$^3$</td>
<td>6.65 ± 0.99 × 10$^{-4}$</td>
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Values are expressed as mean ± SE.

*Number of separate experiments on different sensorchips.

**FIGURE 4.** MASP-3 prevents the interaction between MBL or L-ficolin and immobilized sCR1 and CR1 CCP22–30. MBL (A) or L-ficolin (B) was incubated 20 min at room temperature in the absence or presence of recombinant MASP-3 at the indicated molar ratios. The mixture was then injected in HBS-P containing 2 mM CaCl$_2$ (HBS-CaP) over immobilized sCR1 (14,900 RU) and in HBS-CaP containing 10 mM mannose over immobilized CR1 CCP22–30 (3,400 RU for MBL and 4,300 RU for L-ficolin). The concentrations of soluble analytes were as follows: MBL, 20 nM (sCR1) and 10 nM (CCP22–30); L-ficolin, 5 nM (sCR1) and 10 nM (CCP22–30). MBL and L-ficolin are considered as tetramers and MASP-3 as a dimer.
Results

The CCP26 module enhanced the interaction with both MBL and L-ficolin. These results suggest that the binding site for MBL and L-ficolin involves both CCP 24 and 25 of CR1. The fact that the CCP25–26 fragment has a low binding capacity suggests that the CCP24 module plays an essential role in the interaction. However, it should be kept in mind that covalent immobilization of the short CCP25–26 fragment on the sensor chip might restrict access to the binding site for MBL and L-ficolin. Surprisingly, deletion of CCP29–30 resulted in an important decrease in the binding signal. It seems unlikely that these modules are directly involved in the interaction because other fragments devoid of these modules have a binding capacity comparable to or even higher than that of the CCP22–30 fragment. A hypothesis might be that the conformation of the immobilized CCP22–28 fragment is not favorable to the interaction with MBL and L-ficolin. It should also be mentioned that, although MBL and L-ficolin yielded essentially comparable interaction profiles, small differences were observed, the most noteworthy regarding ΔCCP27–30. Indeed, removal of CCP27–30 yielded reduced L-ficolin binding (61% of the reference value) but had no significant effect on the interaction with MBL (Fig. 6C, Supplemental Fig. 1), which suggests slight differences in the binding sites for MBL and L-ficolin.

Discussion

We show in this study for the first time, to our knowledge, that, in addition to MBL, ficolins are ligands of CR1. Both L- and H-ficolins interacted with immobilized sCR1, although the binding level of H-ficolin was much weaker (Fig. 2A, 2B). This observation may be related to the shorter collagen-like segment of H-ficolin (11 GXY triplets compared with 15 for L-ficolin and 19 for MBL). Indeed, longer collagen-like regions are expected to bring more flexibility, which could facilitate interactions involving the collagen stalks. Although no structural data are available regarding soluble ficolins, recent studies using solution X-ray scattering of MBL oligomers revealed highly flexible molecules with near planar fan-like structures in some instances (42, 43). Moreover, MBL bound to surface ligands was shown to adopt a stretched conformation, as shown by atomic force microscopy (44). MBL and H-ficolin bound more to the CR1 CCP22–30 fragment than to full-length CR1, which might result from increased accessibility of their binding site in the absence of the rest of the CR1 molecule. However, the fact that comparable binding levels were obtained in the case of L-ficolin likely reflects slightly different requirements for CR1 binding among the three recognition proteins.

L-ficolin and MBL bound to sCR1 with comparable nanomolar affinities, in a range similar to that previously reported for MBL (13). Previous studies have provided evidence for a single CR1 binding site for C1q and MBL, likely located in a segment encompassing the nine C-terminal CCP modules of the ectodomain (LHR-D + CCP29–30), thus differing from the binding sites for C3b/C4b located in LHR-A, -B, and -C (12, 13). However, these experiments were performed by capturing the C-terminal recombinant CR1 fragment by lysates of transfected Chinese hamster ovary cells using an appropriate Ab coated on an ELISA plate. To investigate the MBL/L-ficolin–CR1 interaction at the molecular level, the CCP22–30 fragment of CR1 was expressed in a recombinant form in eukaryotic cells, which allowed production of a glycosylated fragment and subsequent use of the purified protein in SPR interaction studies. MBL and L-ficolin were found to bind to immobilized CR1 CCP22–30 with affinities in the same range as those obtained with sCR1. In accordance with previous studies (13), the interaction with MBL was observed in the presence of EDTA and was thus not contingent upon the Ca2+-dependent lectin activity of MBL. It should be mentioned that we were not able to observe the CR1–MBL/L-ficolin interaction in the reverse configuration of the SPR experiments (i.e., using immobilized MBL or L-ficolin and soluble CR1 or its CCP22–30 fragment) up to concentrations of 100 nM and 1 μM, respectively (data not shown) (see also Materials and Methods). This might arise from the process of covalent immobilization of the ligand onto the sensor chip, possibly restricting access to the soluble interactant or putative conformational changes associated with the binding process, as previously described in the case of calreticulin, the receptor for the collagen fragments of collectins (25). Oriented capture of MBL might also favor the interaction, because solid-phase binding of sCR1 to MBL immobilized through its CRD domain was observed previously, although the affinity was ~10 times lower than that obtained in the reverse configuration (13). It should also be mentioned that the SPR configuration used allows multivalent interactions between oligomeric MBL/ficolin molecules and immobilized CR1 or its CCP22–30 fragment, which are likely strengthened through an avidity phenomenon. This might have physiological consequences because avidity is known to play a dominant role in immune recognition and ligand–receptor interactions, as underlined in a recent review (45).

With a view to explore the CR1–MBL interaction in a cellular context, we also transfected 293-F cells with a plasmid containing a CR1 fragment encompassing CCP modules 22–30, the transmembrane segment and the intracytoplasmic tail. The CR1 fragment could be detected at the cell surface by flow cytometry and

FIGURE 5. Lys55 of MBL is essential for the interaction with sCR1 and CR1 CCP22–30. The binding of the K55E or K55A MBL mutants was analyzed by SPR using wild-type MBL as a reference. Each analyte was injected over immobilized sCR1 (14,900 RU) (A) and immobilized CR1 CCP22–30 (6,000 RU) (B) in 150 mM NaCl, 50 mM triethanolamine-HCl (pH 7.4), and 0.005% surfactant P20 at the following concentrations: 20 nM (sCR1) and 10 nM (CCP22–30).
immunostaining, but we obtained no evidence for specific MBL binding to the transfected cells (data not shown). However, ligand binding to cell surface CR1 is known to induce clustering of the receptor (46), which may not happen at the surface of transiently transfected 293-F cells.

It has been shown previously that the C1s-C1r-C1r-C1s tetramer interferes with C1q binding to CR1, suggesting a steric hindrance effect or direct competition with the receptor for a CCP binding site on the collagen stalks of C1q (40). It was therefore of interest to investigate whether the MASP dimers had a similar effect on the binding of MBL/ficolins to CR1 and its CCP22–30 fragment. Preincubation with MASP-3 was shown to inhibit binding in all cases, although to a lesser extent for H-ficolin, with maximal inhibition obtained at an equimolar ratio of MBL or L-ficolin (considered as tetramers) and MASP-3 dimers. These results are in accordance with our previous findings that isolated trimeric and tetrameric forms of serum-derived MBL are able to accommodate only one MASP-3 dimer (47). Moreover, the affinities of recombinant MASP-3 produced in mammalian cells for MBL and L-ficolin are in the nanomolar range (1.0 and 1.1 nM, respectively, for the MASP-3 monomer and thus 0.5–0.55 nM for the dimer) (N.M. Thielens, unpublished observations), which indicates that most available MBL and L-ficolin are complexed with MASP-3 at the concentrations used in our experiments (10–20 nM MBL and 5–10 nM L-ficolin). These data thus strongly suggest that the CR1 binding site is located at or near the sites occupied by the MASPs on the collagen stalks of MBL and L-ficolin. This hypothesis is further supported by the fact that mutations of MBL lysine 55, which impair binding to the MASPs (25), also abolish the interaction of MBL with CR1 and its CCP22–30 fragment.

Interestingly, similar observations have been made previously regarding the interaction of MBL and ficolins with two other cell surface MBL/ficolin-binding proteins, calreticulin (25, 48) and the low-density lipoprotein receptor-related protein CD91 (24). Whereas the MBL/L-ficolin binding sites in CD91 and CRT remain to be identified, site-directed mutagenesis and structural studies demonstrated that the MBL–MASP interface involves major ionic interactions between acidic Ca\(^{2+}\) ligands of the MASP C1r/C1s, sea urchin epidermal growth factor, bone morphogenetic protein domains, and a conserved lysine residue of the collagen-like region of MBL (25–27, 49, 50). Considering also the fact that the interactions are sensitive to high ionic strength, these results

<table>
<thead>
<tr>
<th>CR1 Fragment</th>
<th>Theoretical Mass</th>
<th>Experimental Mass</th>
<th>(\Delta)</th>
<th>No. of Predicted Glycosylation Sites</th>
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<tr>
<td>CCP22–30</td>
<td>64,502</td>
<td>71,156 ± 70</td>
<td>6.654</td>
<td>8</td>
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<tr>
<td>ΔCCP22–23</td>
<td>50,978</td>
<td>56,132 ± 56</td>
<td>5,154</td>
<td>6</td>
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<tr>
<td>ΔCCP22–25</td>
<td>36,493</td>
<td>38,894 ± 39</td>
<td>2,401</td>
<td>3</td>
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<tr>
<td>ΔCCP29–30</td>
<td>50,470</td>
<td>56,867 ± 56</td>
<td>6,097</td>
<td>7</td>
</tr>
<tr>
<td>ΔCCP27–30</td>
<td>35,835</td>
<td>40,107 ± 40</td>
<td>4,272</td>
<td>6</td>
</tr>
<tr>
<td>ΔCCP25–30</td>
<td>29,234</td>
<td>32,708 ± 33</td>
<td>3,474</td>
<td>5</td>
</tr>
<tr>
<td>ΔCCP25–26</td>
<td>51,163</td>
<td>57,083 ± 57</td>
<td>5,920</td>
<td>6</td>
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<tr>
<td>CCP25–26</td>
<td>14,408</td>
<td>15,434 ± 15</td>
<td>1,026</td>
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</tbody>
</table>

\(\Delta\) Difference between the experimental mass determined by mass spectrometry and the calculated mass of the polypeptide chain.
altogether strongly suggest a contribution of ionic bonds in the MBL/ficolin–CR1 interaction, likely involving the conserved lysine residue of the MASp binding site in the collagen stalks and surface-exposed acidic residues of CR1.

With a view to delineate the MBL/L-ficolin binding site within the CR1 CCP22–30 segment, we generated a series of deletion mutants and investigated their MBL/L-ficolin binding properties. The fragments devoid of modules 24 and/or 25 had a reduced binding capacity compared with the full-length segment, whereas the deletion of the other modules had less impact. On the basis of these data, we propose that CCPs 24 and 25 contain the interaction site of CR1 CCP22–30 with the collagen stalks of MBL and L-ficolin. We aligned the sequences of the 30 CCP modules of CR1 (Supplemental Fig. 2) to search for specific acidic residues of CCP24 and 25 potentially involved in MBL/ficolin binding. We have identified Glu1555, a CCP24 residue located in a loop, which is shared by a limited number of CCP modules and contains a glutamic acid in CCP24 exclusively. In addition, this residue may be part of a patch of negative charges contributed by two additional acidic residues located nearby, Asp1553 and Glu1559 (Supplemental Fig. 2). Regarding CCP25, we have identified two close glutamate residues (Glu1595 and Glu1597) (Supplemental Fig. 2). Although the presence of these negatively charged residues, taken individually, is not specific to CCP25, such proximity is unique to CCP25.

The CCP modules of CR1 have been classified into four different types, based on homology criteria (17). Interestingly, the CCPs 22–25 are unique and divergent from the other CCPs of CR1 (17, 51). This divergence suggests a specific role for these modules, which could be in accordance with our experimental hypothesis regarding the involvement of CCPs 24 and 25 in the interaction with MBL and L-ficolin. It is also noteworthy that CCPs 24 and 25 contain all of the known Knops blood group polymorphisms (22). The ethnic-specific distribution of two of these blood group variants, SI1/2 (Lys/Glu1590 at the CCP24–25 junction) and McCα/b (Arg/Gly1601 in CCP25) has been proposed to result from selective pressures in malarial endemic regions of Africa. However, contradictory results have been obtained regarding their association with malarial severity in different African populations (52–55). CR1 interacts with two P. falciparum proteins, the malarial adhesin PfEMP1 (14) and the PfRh4 invasion ligand (15), but the tetramer from the C1 complex, is presently not known. Alternatively, CR1 might interact with locally synthesized MBL and ficolins. MBL/L-ficolin binding likely involves major ionic interactions between conserved lysine residues of their collagen stalks and surface-exposed acidic residues located in CR1 CCP24 and/or CCP25, which remain to be identified. Polymorphisms resulting in substitution of charged residues like some of the Knops blood groups might result in changes in the surface electrostatic properties of CCP modules and impair electrostatic interactions involving these residues or their close vicinity (69).

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**Disclosures**

The authors have no financial conflicts of interest.


SUPPLEMENTAL MATERIAL

**Figure S1. SPR analysis of MBL and L-ficolin binding to the CCP22-30 variants.**
Each panel shows the binding signal obtained by injecting 10 nM MBL or L-ficolin over immobilized CR1 CCP22-30 and one or two more deletion variants on the same sensor chip. Data are expressed as mean ± SE of 3 injections. Comparisons between CCP22-30 and the deletion variants within each chip were made by the paired Student t test. p values < 0.05 (*) are considered significant.

**Figure S2. Sequence alignment of the 30 CCP modules of CR1.**
The sequences of CCP24 and 25 are highlighted in grey. The four conserved Cys residues are in red characters. The negatively charged residues identified in CCP24 and 25 are in blue characters, except for Glu 1555, indicated in white characters with blue highlighting. The beta strands and beta bridges secondary structure elements experimentally observed in CR1 CCP module 16 are indicated as a reference (1).

Fig S1

MBL

L-ficolin