

Mapping of the C3b-binding Site of CR1 and Construction of a (CR1)₂-F(ab')₂ Chimeric Complement Inhibitor

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Summary

CR1/CR2 chimeric receptors in which various short consensus repeats (SCRs) of CR1 were attached to CR2 were transiently expressed on COS cells, and assessed for the binding of polymerized C3b (pC3b) and anti-CR2 by immunofluorescence. Of COS cells expressing chimeras containing SCR 1-4, 1-3, 2-4, 1-2, and 2-3 of the long homologous repeats (LHRs) -B or -C, 96%, 66%, 23%, 0%, and 0%, respectively, bound pC3b. K562 cells were stably transfected with wild-type CR1, deletion mutants of CR1, and the CR1/CR2 chimeras, respectively, and assayed for binding of ¹²⁵I-pC3b. The dissociation constants (K_d) for pC3b of wild-type CR1 and the LHR-BD and -CD constructs were in the range of 1.0–2.7 nM, and of the CR1/CR2 chimeras containing SCRs 1-4, 1-3, and 2-4 of LHR-B or -C were 1.8–2.4, 6–9, and 22–36 nM, respectively. The factor I-cofactor function of the CR1/CR2 chimeras paralleled the C3b-binding function of the constructs. A CR1/immunoglobulin (Ig) chimeric protein was prepared by fusing SCRs 1-4 of LHR-B to the heavy chains of a murine F(ab')₂ anti-nitrophenacetyl (NP) monoclonal antibody. The (CR1)₂-F(ab')₂ chimera, which retained its specificity for NP, was as effective as soluble, full-length CR1 in binding pC3b, serving as a cofactor for factor I-mediated cleavage of C3b, and inhibiting activation of the alternative pathway, indicating that the bivalent expression of these SCRs reconstitutes the alternative pathway inhibitory function of CR1. The feasibility of creating CR1/Ig chimeras makes possible a new strategy of targeting complement inhibition by the use of Ig fusion partners having particular antigenic specificities.

CR1 (CD35) was first purified to homogeneity from detergent-solubilized erythrocyte (E) membranes not as a receptor but as an inhibitor of the alternative complement pathway that resembled factor H in its capacity to decay-dissociate the C3b,Bb convertase and to serve as a cofactor for the cleavage of C3b by factor I (1). This initial study demonstrated that CR1 was even more inhibitory than factor H, especially when C3b was on an alternative pathway-activating surface, a circumstance in which factor H was relatively ineffective (2). Subsequent studies showed that CR1 also promoted the factor I-mediated cleavage of iC3b to C3c and C3dg (3, 4), the dissociation of the classical pathway convertase, C4b, 2a, and the factor I-mediated cleavage of C4b to C4c and C4d (5, 6). Therefore, CR1 is unique in having the combined inhibitory functions of the other members of the regu-

lators of complement activation (RCA)¹ family, the plasma proteins factor H and C4-bp, and the membrane proteins decay-accelerating factor (DAF), and membrane cofactor protein (MCP) (7), and in not being restricted by alternative pathway activators, as are factor H and DAF (8). The remarkable inhibitory capability of CR1 was demonstrated when a recombinant soluble form of the receptor, sCR1, suppressed complement activation in serum by the classical and alternative

¹ Abbreviations used in this paper: BMH, bismaleimidohexane; DAF, decay-accelerating factor; LHR, long homologous repeat; MCP, membrane cofactor protein; NP, 4-hydroxy-3 nitrophenacetyl; pC3b, polymerized C3b; RCA, regulators of complement activation; SCR, short consensus repeat; sCR1, recombinant soluble CR1.

pathways at concentrations that were 100-fold less than the endogenous concentrations of factor H and C4-bp, and *in vivo* in a model of myocardial reperfusion injury (9).

The molecular basis for and biologic reactions subserved by these functions became apparent with the molecular cloning of CR1 (10–12), and the analysis of its function on E and B lymphocytes. CR1 consists of 30 short consensus repeats (SCRs) of 60–70 amino acids that are characteristic of complement proteins that bind C3, C4, and C5. Four groups of seven SCRs are highly homologous, and form long homologous repeats (LHRs). By analysis of recombinant deletion mutants, three of these LHRs were shown to have ligand-binding function: LHR-A for C4b, and LHRs-B and -C for C3b (11). Thus, the basis for CR1 having the functions of factor H and C4-bp is the occurrence of structurally distinct sites for interacting with C3b and C4b. Atkinson and his colleagues (13) have shown recently that substitution of certain residues in SCRs-1 and -2 with residues from corresponding positions in the homologous SCRs-8 and -9 generated C3b-binding function in LHR-A, confirming the observation that the first two SCRs of each LHR determined ligand specificity (11).

The biologic purpose of the inhibitory function of CR1 may be to suppress further complement activation after the amounts of C3b and C4b that have been covalently attached to an activator are sufficient for mediating uptake by cellular receptors. The inhibitory function of CR1 also causes the conversion of C3b to iC3b and C3dg, which are ligands for the signal-transducing receptors CR2 and CR3. This sequence of CR1-mediated uptake of complement-activating complexes, followed by processing and transfer to other receptors, may occur during the clearance of immune complexes by E (14), and may be the functional correlate of the molecular complex between CR1 and CR2 on B lymphocytes (15). Thus, it may be biologically appropriate for CR1 to be the most effective inhibitor among the RCA proteins because the receptor must suppress the complement-activating functions of C3b or C4b whenever these proteins are ligated, whereas factor H and C4-bp, for example, must permit activation by certain stimuli, such as microbial cell walls and immune complexes.

Determination of the minimal structural requirements for a functional CR1 binding site would enable the construction of chimeric proteins having unique capabilities for the tissue-specific inhibition of complement activation. The present study demonstrates that, in addition to the NH₂-terminal two SCRs of LHRs-B and -C, the third and fourth SCRs are necessary to form a C3b-binding site that is functionally equivalent to that of the full-length receptor, perhaps accounting for these SCRs being the most conserved among those of the three ligand-binding LHRs. A (CR1)₂-F(ab')₂ chimeric protein that contains SCRs 1–4 of LHR-B is constructed and shown to be as effective an inhibitor of the activation of the alternative pathway as is sCR1, indicating the feasibility of this strategy for the targeting of complement inhibition.

Materials and Methods

Antibodies and Complement Proteins. HB-5 anti-CR2 IgG2a (16), YZ-1 anti-CR1 IgG1 (17), anti-C3 (Quidel Corporation, San Diego, CA), F(ab')₂ rabbit anti-CR1 (18), control RPC5.4 IgG2a (American Type Culture Collection, Rockville, MD), rabbit anti-sheep RBC stroma (hemolysin) (Sigma Chemical Co., St. Louis, MO), FITC goat anti-mouse IgG, and Texas Red goat anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA) were used. C3 was purified from fresh frozen plasma (19) (American Red Cross, Baltimore, MD) and converted to C3b with 0.8% (wt/wt) TPCK-trypsin digestion for 90 s at 37°C. Polymerized C3b (pC3b) was made by crosslinking concentrated C3b with an 80-fold M excess of glutaraldehyde (Sigma Chemical Co.) for 2 h at room temperature. Polymers were fractionated over gradients of 7.5–40% sucrose (wt/vol) in PBS by centrifugation at 200,000 g for 12 h at 4°C. After analysis on SDS-PAGE, fractions containing tetramers or higher order polymers were pooled, dialyzed, and stored at 0°C. The average molecular weight of pC3b was taken to be 10⁶. C3b dimers were generated by addition of a 1:4 molar ratio of bismaleimidohexane (BMH) (Pierce Chemical Co., Rockford, IL)/C3 in the initial trypsin reaction, followed by incubation 2–3 d at 0°C. BMH crosslinks C3b by reacting with the free sulfhydryls generated upon hydrolysis of the C3 thiolester. The mixture was fractionated on a Mono Q HR/10 column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), and fractions containing covalently associated dimers were pooled and further purified by gel filtration on a Superose 12 column (Pharmacia LKB Biotechnology, Inc.). The preparation contained primarily C3b dimers with ~5% C3b monomer contamination as assessed by Coomassie brilliant blue staining of proteins after SDS-PAGE.

Proteins were iodinated by the Iodo-gen (Pierce Chemical Co.) method (20). The specific activities ranged from 0.8 to 2 × 10⁶ cpm/μg. Free ¹²⁵I was removed by passing proteins over 5-ml Ex-cellulose desalting columns (Pierce Chemical Co.).

Construction and Expression of CR1 Chimeras. For the DNA encoding CR1/CR2 chimeras, various SCRs of CR1 were amplified by PCR using the BglI linearized full-length CR1 plasmid, piABCD (11), as template, and 25 cycles of 2 min of denaturation, annealing, and extension. Primers were as follows: 5' SCR 8 and 15: GCACCCGGGGTCTCTCGGGATTCTTGTC AAGCCCCAGATCAT; 5' SCR 9 and 16: GCACCCGGGGTCTCTCGGGATTCTTGTAAAACCTCTCCAGAT; 3' SCR 9 and 16: ACACCTCGAGACACTCGTTGACAAATTGGCCG; 3' CR 10 and 17: ACACCTCGAGAGACACAATGCACTGAGGGGCCGG; and 3' SCR 11 and 18: ACACCTCGAGAGACACCCTGGAGCAGCTTGGTAG. XmaI sites were included in the 5' primers and XhoI sites in 3' primers. After amplification, fragments were digested by XmaI and XhoI, and ligated into XhoI- and XmaI-digested piCR2.H. This plasmid was generated by oligonucleotide-directed mutagenesis of pi15, which is the eukaryotic expression vector containing the 15-SCR CR2 isoform in CDM8, to incorporate a silent G to C transversion in the third position of a codon encoding a proline residue within the CR2 leader peptide to create a unique XmaI site. The unique XhoI site in piCR2.H is between SCRs-2 and -3. Chimeras were sequenced on both strands throughout the CR1 insert by the dideoxynucleotide chain termination method (21) using the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH).

For construction of (CR1)₂-F(ab')₂, 25 cycles of PCR were used to amplify CR1 SCRs 8–11 using BglI-linearized piABCD as tem-

plate, and the following set of primers: 5' SCR 8, CAGCTG-CAGCTGGGTCAGTGTCAAGCC, and 3' SCR 11, GACCTG-CAGTTGGACCTGGCTCACCTGGAGCAGCTTGGTAG. PstI sites were present in both primers. The plasmid, pSV-V_{NP}- $\gamma\delta$, (provided by M. S. Neuberger, MRC Laboratory of Molecular Biology, Cambridge, UK) contains the VDJ, C_H1, and hinge regions of a mouse γ_2 gene, translation termination and polyadenylation sequences from the mouse C₃ gene (22), and encodes a secreted F(ab')₂-like molecule with specificity for the hapten 4-hydroxy-3-nitrophenacetyl (NP) when expressed in J558L cells (provided by S. L. Morrison, Molecular Biology Institute, UCLA, Los Angeles, CA), a line expressing an endogenous λ 1 chain and no heavy chain (23). The 2.2-kb EcoRI-BamHI fragment containing the VDJ region was removed and cloned into Bluescript (pBS) to yield pBS5'V_H. This plasmid was digested with PstI at a unique site immediately upstream of the VDJ region into which the 800-bp PstI-digested CR1 SCRs 8–11 was cloned to generate pBS5'V_H-CR1. The plasmid, pBSV_H.R1, which had been created by cloning the 4-kb EcoRI fragment from pSV-V_{NP}- $\gamma\delta$ into pBS, was completely digested with XhoI and partially digested with BamHI to remove a 2.2-kb fragment, which was replaced by the 3-kb EcoRI-BamHI fragment of pBS5'V_H-CR1 containing CR1 SCRs 8–11 upstream of the VDJ region, to create pBSV_H-CR1. The pBSV_H-CR1 was partially digested with EcoRI and the 4.8-kb fragment was cloned into EcoRI-digested pSV-V_{NP}- $\gamma\delta$ to yield pSV-V_{NP}- $\gamma\delta$ -CR1, the 13-kb expression vector used for transfections.

The plasmids piABCD, piBD, piCR1 (8–9)/CR2, piCR1(16–17)/CR2, piCR1(8–10)/CR2, piCR1(16–18)/CR2, and piCR1(8–11)/CR2 were transiently expressed in COS cells using the DEAE-dextran procedure (24). K562 cells were cotransfected by lipofection with piABCD, deletion mutants, and the piCR1/CR2 constructs, respectively, and p141, a plasmid conferring resistance to hygromycin. The cells were selected in hygromycin and sorted for highly expressing transfectants as described (25). J558L cells were cotransfected with pSVneo and pSV-V_{NP}- $\gamma\delta$ -CR1 by electroporation using 960 μ F and 350 V, and selected in 1 mg/ml G418. Cells were distributed in cloning chambers and those secreting high amounts of (CR1)₂-F(ab')₂ were identified by ELISA in which molecules binding NP were detected with anti- λ mAb conjugated to horseradish peroxidase (Fisher Scientific Co., Pittsburgh, PA). These transfectants were batch cultured, and (CR1)₂-F(ab')₂ was purified from culture supernatants by affinity chromatography on NP-sepharose (26, 27).

COS cells were maintained in DME (Gibco Laboratories, Grand Island, NY) with D-glucose at 4,500 mg/ml supplemented with 10% bovine calf serum (BCS) (HyClone Laboratories, Logan, UT) and 2 mM glutamine (Gibco Laboratories). K562 erythroleukemia and J558L cells were grown in RPMI 1640 (Gibco Laboratories) supplemented with 10% BCS (Hyclone Laboratories) and 2 mM glutamine and penicillin/streptomycin (Gibco Laboratories).

Assays for CR1 Function. Transiently transfected COS cells were assayed 2–3 d post-transfection for the presence of wild-type CR1 or deletion mutants of CR1 by incubation with YZ-1 followed by a 1:40 dilution of fluorescent goat anti-mouse IgG. CR1/CR2 chimeras, which lacked the YZ-1 epitope but contained the HB-5 epitope of CR2, were detected by substituting HB-5 for YZ-1. For C3b-binding activity of the transient transfectants, replicate samples of cells were sequentially incubated with incremental concentrations of pC3b, a 1:1,000 dilution of anti-C3, and the fluorescent mAb. Cells were held on ice in the dark until microscopic examination, and 280–500 cells from each slide were analyzed in a blinded manner.

Stably transfected K562 cells expressing various forms of CR1 were incubated on ice for 1 h in PBS containing 1% bovine serum albumin (PBSA) (Sigma Chemical Co.) and incremental concentrations of ¹²⁵I-pC3b in the presence or absence of rabbit anti-CR1, followed by centrifugation through 300 μ l of a 3:1 mixture of dibutyl/dinonyl phthalate for 45 s at 7K at room temperature. Tubes were cut above the cell pellets, and bound and free ligand were determined. Specific binding was defined as the amount bound of ¹²⁵I-pC3b in the absence of anti-CR1 minus that bound in the presence of anti-CR1, and was subjected to Scatchard analysis. The K_d values were determined by first order linear regression of Scatchard analyses.

Inhibition of ¹²⁵I-pC3b binding to E by (CR1)₂-F(ab')₂ or sCR1 was assayed in PBSA as above, except that cells were centrifuged through neat dibutyl phthalate.

To assay factor I-cofactor activity, K562 cells were lysed in buffer containing 1% NP-40, 10 mM triethanolamine, 150 mM NaCl, 1 mM EDTA, and the protease inhibitors leupeptin, pepstatin A, chymostatin, antipain, and diisopropyl fluorophosphate, and insoluble material was removed by centrifugation of the lysates. The lysates were precleared by sequential incubation with Trisacryl beads bearing protein A (Pierce Chemical Co.), and nonspecific RPC5.4 IgG2a and Trisacryl-protein A, and split into replicate samples which were subject to immunoprecipitation by 5 μ g/ml RPC5.4 IgG2a and HB-5, respectively, and Trisacryl-Protein A. The beads were washed five times in ice-cold lysis buffer, and cofactor activity was assessed by incubation with factor I and ¹²⁵I-labeled C3b dimers for 1 h at 37°C. Samples were reduced, boiled, and analyzed on 5–10% gradient SDS-PAGE. Gels were dried and exposed to X-OMAT-AR film (Eastman Kodak Co., Rochester, NY) with an enhancing screen for 16–48 h at –80°C. The cofactor activities of sCR1 and (CR1)₂-F(ab')₂ were similarly assayed, except that they were not immobilized on beads.

The classical pathway inhibitory activities of (CR1)₂-F(ab')₂ and sCR1 were compared by incubating sheep E sensitized with rabbit IgM with incremental concentrations of the two recombinant proteins in normal human serum diluted 1:160 for 30 min at 37°C. Unlysed cells were pelleted by centrifugation and the supernatant was assessed for hemoglobin spectrophotometrically. The alternative pathway inhibitory activities of (CR1)₂-F(ab')₂ and sCR1 were compared by incubating normal human serum diluted 1:5 in veronal buffered saline containing 2 mM Mg²⁺ and 8 mM EGTA with 1 mg/ml zymosan and incremental concentrations of the two recombinant proteins for 20 min at 37°C, after which the generation of C3adesArg and C5adesArg was measured by radioimmunoassay (Amersham Corp., Arlington Heights, IL).

Results

Mapping of the C3b-binding Site of CR1. The C3b-binding capabilities of wild-type CR1, CR1 deletion mutants (15), and CR1/CR2 chimeras (Fig. 1) were compared to determine the requirements for a functional C3b-binding site. The CR1/CR2 chimeras were created by substituting cDNA encoding various PCR-amplified SCRs of CR1 for SCRs-1 and -2 of CR2 in an expression vector encoding the 15-SCR isoform of CR2. Transiently transfected COS cells were assayed for the ability to bind anti-CR1 or anti-CR2 mAb and pC3b by indirect immunofluorescence (Table 1). Essentially all COS

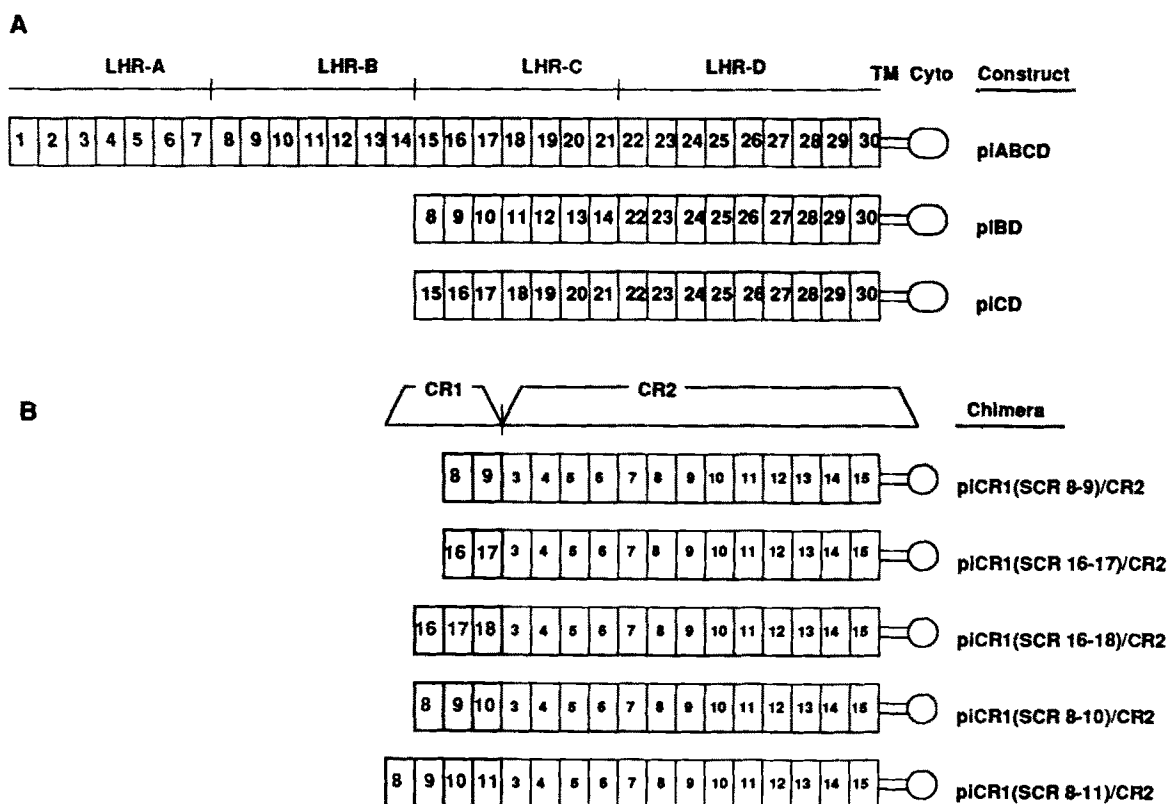


Figure 1. Schematic representations of (A) full-length CR1 and CR1 deletion mutants and (B) the CR1/CR2 chimeras created by ligating PCR-amplified SCRs of CR1 to CR2 lacking its ligand-binding SCR-1 and -2.

Table 1. Indirect Fluorescence Analysis of the Binding of mAb and pC3b to COS Cells Transiently Transfected with CR1 Constructs

Construct	mAb*	pC3b ($\mu\text{g/ml}$)	
		40	10
piABCD [†]	8.1	9.2	6.1
piBD [†]	4.7	7.5	5.8
piCR1 (8-9)/CR2 [§]	10.3	0	ND
piCR1 (16-17)/CR2 [§]	15.6	0	ND
piCR1 (16-18)/CR2 [§]	16.1	3.7	0
piCR1 (8-10)/CR2 [§]	5.6	3.7	0.9
piCR1 (8-11)/CR2 [§]	7.7	7.4	8.1

* COS cells transfected with piABCD and piBD were assessed with YZ-1, and cells transfected with CR1/CR2 chimeras were stained with HB-5.

[†] The letter designations of these constructs refer to the LHRs that are encoded in the CR1 constructs.

[§] The numbers refer to the SCRs of CR1 that are encoded by the chimeric CR1 constructs.

^{||} Percent of transiently transfected cells staining positively.

cells expressing wild-type CR1, piBD, and piCR1 (8-11)/CR2 bound pC3b, whereas cells expressing piCR1 (8-10)/CR2 and piCR1 (16-18)/CR2, respectively, were less effective in binding pC3b, and neither chimera containing only two of the four NH₂-terminal SCR of LHR-B or -C bound pC3b.

To assess the affinities of various CR1 constructs for pC3b, replicate samples of stably transfected K562 erythroleukemia cells were incubated with incremental concentrations of ¹²⁵I-pC3b in the presence and absence of polyclonal anti-CR1, and cell-bound and free ligand were determined (Fig. 2). Wild-type K562 cells and K562 cells expressing piCR1 (16-17)/CR2 bound no ¹²⁵I-pC3b specifically. Scatchard analyses of the specific binding curves (Table 2) yielded K_d values in the range of 1.0–2.7 nM for wild-type CR1, the deletion mutants piBD and piCD, and the piCR1 (8-11)/CR2 chimera, a K_d of 6–9 nM for piCR1 (8-10)/CR2, and a K_d of 22–36 nM for piCR1 (16-18)/CR2. Therefore, the first four SCRs in LHR-B, and presumably of LHR-C, are sufficient and necessary to form a C3b-binding site with an affinity indistinguishable from that of the intact LHRs in wild-type CR1 and the deletion mutants. The first and fourth of these four SCRs contribute to but both are not required for C3b binding, as piCR1 (8-10)/CR2 binds pC3b with three- to fivefold lower affinity, and piCR1 (16-18)/CR2 with a 15- to 20-fold lower affinity than wild-type CR1.

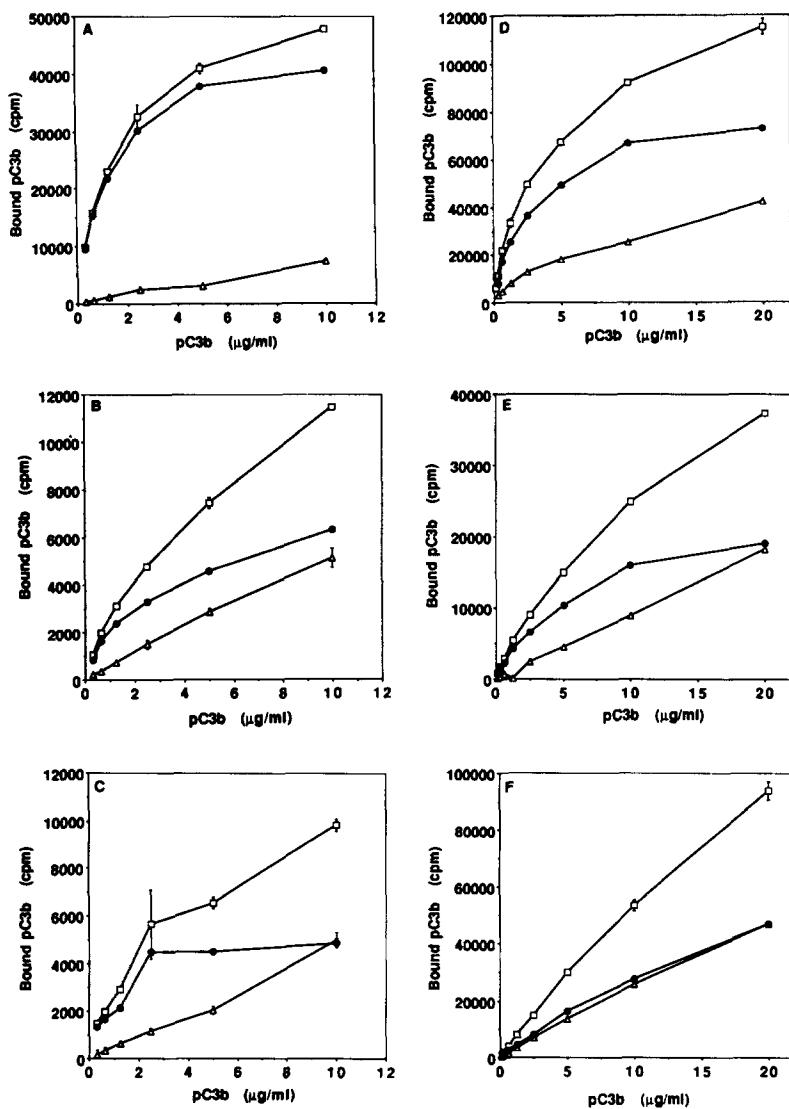


Figure 2. Saturation binding of ^{125}I -pC3b to K562 cells stably expressing piABCD (A), the piBD (B) and piCD (C) deletion mutants, and the CR1/CR2 chimeras piCR1(8-11)/CR2 (D), piCR1(8-10)/CR2 (E), and piCR1(16-18) (F). Specific binding (closed circles) was calculated by subtracting nonspecific binding occurring in the presence of rabbit anti-CR1 (triangles) from binding in the absence of antibody (squares).

The CR1/CR2 chimeras containing SCRs 8-11, 8-10, and 16-18 were assessed for their ability to serve as cofactors for factor I-mediated cleavage of C3b. Cell lysates of K562 transfectants were adjusted to contain amounts of CR1/CR2 chimeras that were equivalent with respect to C3b-binding function. Thus, fivefold more CR1 (8-10)/CR2 and 20-fold more CR1 (16-18)/CR2 were present than were CR1 (8-11)/CR2. The lysates were immunoadsorbed with immobilized HB-5 or control RPC5.4 IgG2a, washed, and incubated with factor I and ^{125}I -labeled C3b dimers for 1 h at 37°C, after which the samples were reduced, boiled, and analyzed by 5-10% SDS-PAGE autoradiography (Fig. 3). Dimeric C3b was used in this assay to permit higher affinity bivalent interactions with the immobilized CR1/CR2 chimeras. The BMH crosslinking reagent used for generating covalent dimers is not reducible, and undigested α' chains appear as 220-kD bands. Cleavage by factor I of the α' chain releases a 43-kD protein from monomeric or dimeric α' chains, making a band

of this molecular mass the most clearly detectable signal of cofactor activity. No cleavage of C3b occurred in reactions containing beads bearing nonspecifically adsorbed proteins (lanes 7-11). The CR1 (8-11)/CR2 (lane 4) and CR1 (8-10)/CR2 (lane 5) chimeras had cofactor activities that were nearly equivalent when adjusted for their relative binding functions, whereas that of CR1 (16-18)/CR2 (lane 6) was less, although detectable. Therefore, the SCRs mediating the cofactor function of CR1 cannot be distinguished clearly from those that bind C3b.

A (CR1)₂-F(ab')₂ Chimera with Inhibitory Function in the Alternative Complement Pathway. To determine whether the C3b-binding site of CR1 could be functionally expressed on another protein lacking SCRs, the cDNA encoding CR1 SCRs 8-11 was ligated into a vector containing the truncated heavy chain of a murine F(ab')₂ mAb specific for NP (Fig. 4). The expression vector was cotransfected with pSVneo into J558L cells, which express an appropriate λ 1 light chain

Table 2. Summary of the Affinities of the CR1 Constructs for pC3b

Construct	K_d	r^*
	<i>nM</i>	
piABCD	1.0	-0.97
piBD	2.7	-0.94
	2.1	-0.91
piCD	1.4	-0.87
	2.6	-0.82
piCR1 (8-11)/CR2	2.4	-0.87
	1.8	-0.97
piCR1 (8-10)/CR2	6.2	-0.94
	7.3	-0.88
	8.7	-0.82
piCR1 (16-18)/CR2	36	-0.82
	22	-0.52

* Correlation coefficient of the first order linear regression of the Scatchard analysis.

for the NP-specificity of the recombinant F(ab')₂, and the resulting (CR1)₂-F(ab')₂ was purified from culture supernatants of transfected cells by adsorption to and elution from NP-sepharose, indicating that the hapten-binding site was intact. The capacities of (CR1)₂-F(ab')₂ and sCR1 to bind C3b were compared by incubating human E with ¹²⁵I-pC3b in the absence or presence of incremental concentrations of the two forms of soluble CR1, and assaying cell-bound radiolabeled ligand (Fig. 5). The (CR1)₂-F(ab')₂ was equivalent to sCR1 in its capacity to inhibit competitively the binding of

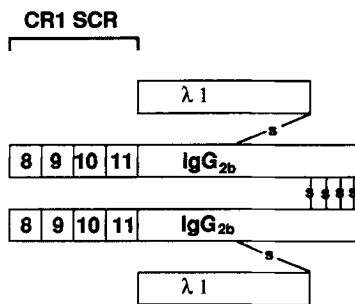


Figure 4. Schematic representation of (CR1)₂-F(ab')₂. SCR 8-11 of CR1 were amplified by PCR and ligated to a vector encoding a truncated murine γ_{2b} gene which, when transfected into J558L cells expressing endogenous $\lambda 1$ chain, led to the secretion of a (CR1)₂-F(ab')₂ chimera. The recombinant protein was purified from culture supernatant on NP-sepharose.

¹²⁵I-pC3b to cellular CR1, with 50% inhibition occurring at 20-30 nM for each.

The factor I-cofactor function of (CR1)₂-F(ab')₂ was compared with that of sCR1 by incubating incremental concentrations of each with radiolabeled C3b dimers and factor I for 1 h at 37°C, followed by SDS-PAGE and autoradiography (Fig. 6). The chimera served to promote the cleavage of all dimeric C3b at 9.76×10^{-10} M, with complete conversion to dimeric C3dg at 2.50×10^{-7} M. sCR1 promoted those reactions at approximately fourfold lower concentrations.

The alternative pathway inhibitory functions of (CR1)₂-F(ab')₂ and sCR1 were compared by incubating human serum containing Mg²⁺ and EGTA with zymosan in the absence or presence of incremental concentrations of the two recombinant proteins for 20 min at 37°C, and assaying for the generation of the C3 and C5 activation fragments C3adesArg and C5adesArg (Fig. 7). The (CR1)₂-F(ab')₂ was as effective as sCR1 in suppressing the alternative pathway C5 convertase, and approximately one-half as effective in-

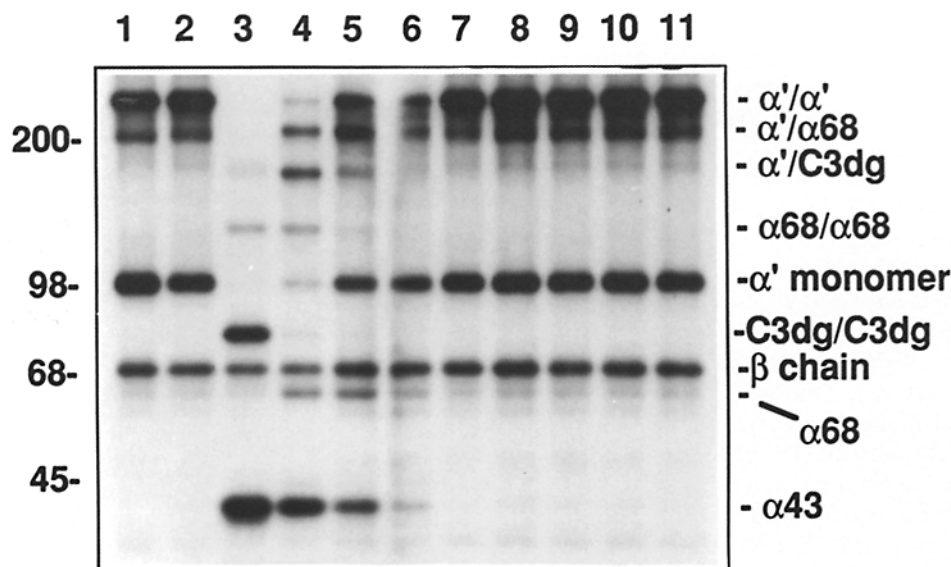


Figure 3. Factor I-cofactor activity of immunoprecipitated CR1/CR2 chimeras. K562 stable transfectants expressing piCR1(8-11)/CR2, piCR1(8-10)/CR2, piCR1(16-18)/CR2, and wild-type K562 cells, respectively, were lysed in NP-40 containing buffer. The lysates were immunoadsorbed with immobilized HB-5 (lanes 4-7) or irrelevant IgG2a (lanes 8-11), and the immobilized complexes were incubated with factor I and ¹²⁵I-C3b dimers for 1 h at 37°C. Cleavage of the ¹²⁵I-C3b dimer was assessed by SDS-PAGE followed by autoradiography. The ¹²⁵I-C3b dimer was also incubated with (CR1)₂-F(ab')₂ alone (lane 1), with factor I alone (lane 2), and with (CR1)₂-F(ab')₂ and factor I (lane 3).

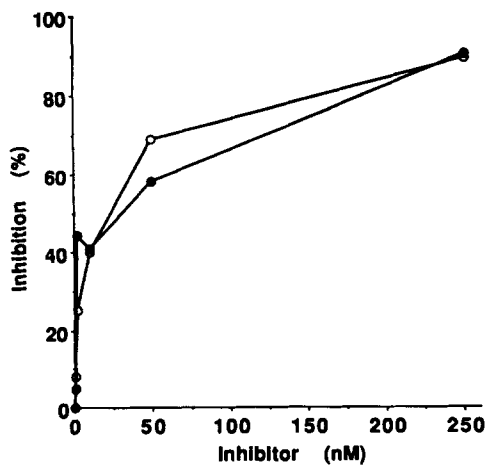


Figure 5. Competitive inhibition of specific ^{125}I -pC3b binding to human E by incremental concentrations of $(\text{CR1})_2\text{-F(ab')}_2$ (closed circles) and sCR1 (open circles).

hibiting C3 convertase function. In contrast to these effects of $(\text{CR1})_2\text{-F(ab')}_2$ on the C3 and C5 convertases of the alternative pathway, the chimera had no inhibitory activity in an assay of classical pathway activation, the lysis of IgM-sensitized sheep E in human serum (Fig. 8), consistent with its lacking the C4b-binding site of CR1.

Discussion

SCRs are present in 12 complement proteins that interact with at least one or more of the homologous proteins, C3, C4, and C5 (28, 29), suggesting a unique role for this structural motif in these interactions. The presence of SCRs only and no other structural elements in some of these proteins, such as factor H and the extracytoplasmic regions of CR1, CR2, and DAF, was proof of this role, but it has not been possible to define a "standard" C3/C4/C5-binding site having a set number of SCRs because of the wide variation of the occurrence of these structural units in each protein which ranges from as many as 30 for the most common allotype of CR1 to as few as two for C1r and C1s.

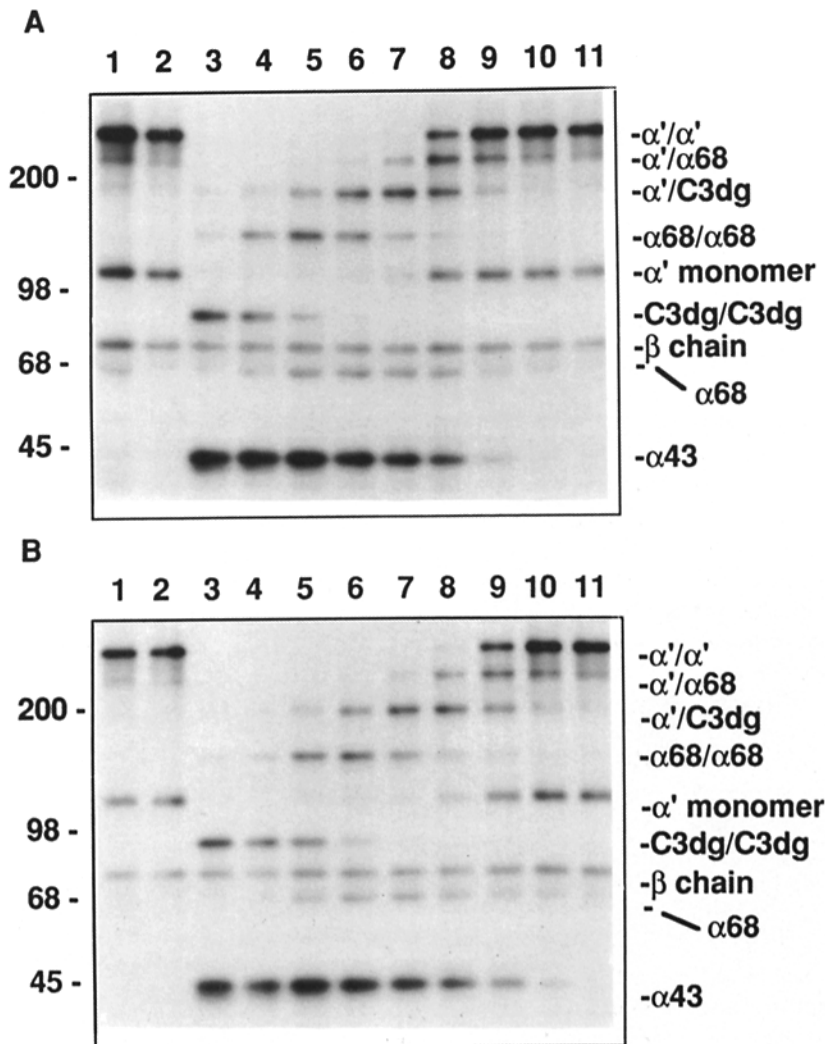


Figure 6. Factor I-cofactor activity of $(\text{CR1})_2\text{-F(ab')}_2$ and sCR1. ^{125}I -C3b dimers were incubated for 1 h at 37°C with recombinant CR1 alone (lane 1, A and B), with factor I (lane 2, A and B), or with factor I and incremental concentrations of $(\text{CR1})_2\text{-F(ab')}_2$ and sCR1 (A and B, respectively; lane 3, 2.50×10^{-7} M; lane 4, 6.25×10^{-8} M; lane 5, 1.56×10^{-8} M; lane 6, 3.90×10^{-9} M; lane 7, 9.76×10^{-10} M; lane 8, 2.44×10^{-10} M; lane 9, 6.10×10^{-11} M; lane 10, 1.52×10^{-11} M; and lane 11, 3.81×10^{-12} M).

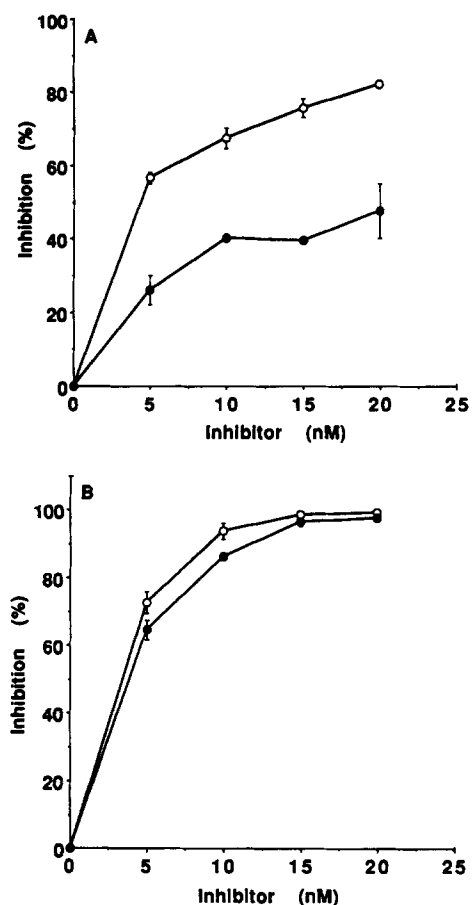


Figure 7. Inhibition of zymosan-induced activation of the alternative pathway in human serum by incremental concentrations of (CR1)₂-F(ab')₂ (closed circles) and sCR1 (open circles), respectively. Activation was assayed by radioimmunoassay of the concentrations of C3adesArg (A) and C5adesArg (B).

CR2 was the first complement protein in which the number of SCRs required for a ligand binding site was determined (30, 31). Chimeric proteins containing the NH₂-terminal two SCRs of CR2 attached to either CR1 or a murine mAb were capable of binding the CR2 ligands, iC3b (18), C3dg, and the EBV (30), with affinities that were indistinguishable from those of the full-length 15-SCR or 16-SCR isoforms of the receptor. Constructs containing only the first or second SCR were inactive. When these observations were considered with earlier work demonstrating that both SCRs in a noncomplement protein, the IL-2 receptor α chain, were required for binding IL-2 (32), and with the findings that no SCR-containing protein had fewer than two SCRs, it seemed possible that a general principle might be that the ligand binding sites of this family of proteins would be formed by two SCRs. Analogous studies of the C3b- and C4b-binding sites of CR1 had not been performed, but the demonstration that the specificities of LHR-A and of LHRs-B and -C for C4b and C3b, respectively, were dependent on their respective NH₂-terminal two SCRs (11), and that substituting amino acids of SCR-1 and SCR-2 of LHR-A with residues

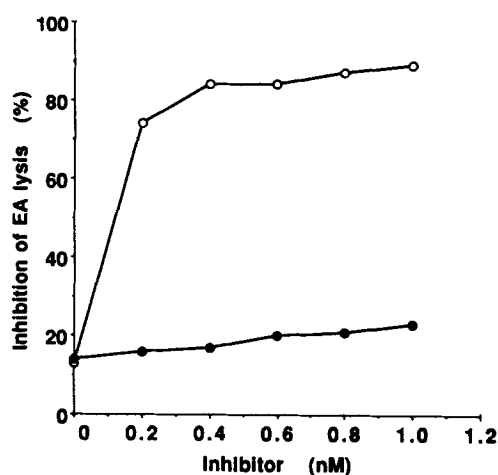


Figure 8. Inhibition of classical complement pathway-mediated lysis of IgM-sensitized sheep E in human serum by incremental concentrations of (CR1)₂-F(ab')₂ (closed circles) and sCR1 (open circles).

from corresponding positions in SCR-8 and -9 of LHR-B led either to altered C4b-binding or to the acquisition of C3b binding (13), were interpreted as indicating that each binding site in CR1, like the single site in CR2, also would be formed by two SCRs.

The original study (11) of the ligand-binding activities of the deletion mutants of CR1 had concluded that the specificity of the LHRs for C3b or C4b was determined by the first and second SCRs, and did not exclude possible contributions of other SCRs. In fact, its analysis of the structure of CR1 was consistent with the possibility that the third and fourth SCRs of LHRs-A, -B, and -C had a role in ligand binding. They were the most highly conserved among all SCRs in the LHRs having this function, and they were not conserved in LHR-D, which had no demonstrable ligand binding activity.

The present study now provides the functional correlate of this original structural analysis by demonstrating that only the CR1/CR2 chimera containing the NH₂-terminal four SCRs of LHR-B or -C had an affinity for C3b and factor I-cofactor activity that was comparable to full-length CR1. The chimera having only the first two SCRs had no detectable C3b-binding activity (Table 1), addition of the third SCR formed a binding site having an affinity that was approximately one-fourth that of the four SCR chimeras (Table 2; Fig. 2), and deletion of the first SCR from the four SCR chimeras severely impaired ligand-binding function (Tables 1 and 2; Fig. 2). In addition, the factor I-cofactor function did not clearly segregate to one of the two chimeras having only three SCRs (Fig. 3). Thus, the C3b-binding site and, by inference, the C4b-binding site of CR1 differ from the iC3b/C3dg/EBV-binding site of CR2 by being constituted of four rather than of two SCRs, although binding activity could be demonstrated with three SCR forms. It is of interest that the two other membrane-associated RCA proteins with inhibitory activities, DAF and membrane cofactor protein (MCP), contain four SCRs (29). The anatomy of an LHR can now be dissected into two SCRs that differentiate be-

tween C3b and C4b (11, 13), followed by two SCRs that support binding of both C3b and C4b, and three SCRs that probably provide adequate spacing between the ligand-binding SCRs to allow multivalent interaction of CR1 with complement-activating complexes containing multiple C3b and C4b molecules.

A potential practical application of the mapping of the functional domains of CR1 to specific SCRs is the capacity to generate by molecular engineering chimeric proteins combining the complement inhibitory function of CR1 with unique capabilities of the fusion partner. The Ig molecule is especially suitable for carrying the active sites of CR1. It effectively dimerizes these sites, thereby increasing the avidity of the recombinant protein for oligomeric or surface-bound C3b. The Ig molecule can target complement inhibition to

specific tissues through its antigenic specificity, and, if a nonspecific antibody is used, it can extend temporally complement inhibition because of its long plasma half-life. We have demonstrated the feasibility of this approach by finding that the (CR1)₂-F(ab')₂ chimera (Fig. 4) containing two copies of the SCRs 8–11, the C3b-binding unit of CR1, was capable of binding both to antigen and pC3b (Fig. 5), serving as a cofactor for cleavage of C3b by factor I (Fig. 6), and inhibiting activation of the alternative pathway (Fig. 7). The absence of classical pathway inhibition by the chimera (Fig. 8) emphasizes the important role that the C4b-binding site in sCR1 has for this function, and indicates that a chimeric CR1/Ig construct having the full inhibitory range of sCR1 (9) must carry this site in addition to the C3b-binding domain.

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