



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/12, C07K 14/47, 14/705, A61K 38/17		A2	(11) International Publication Number: WO 00/61752
			(43) International Publication Date: 19 October 2000 (19.10.00)
(21) International Application Number: PCT/US00/09288 (22) International Filing Date: 7 April 2000 (07.04.00) (30) Priority Data: 60/128,520 9 April 1999 (09.04.99) US (71) Applicant: WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US). (72) Inventors: ATKINSON, John, P.; 1030 N. Berry Street, St. Louis, MO 63122 (US). KRYCH, Malgorzata; 4225 West Pine #9, St. Louis, MO 63108 (US). HAUHART, Richard; 11878 Gold Leaf Drive, St. Louis, MO 63146 (US). (74) Agents: PABST, Patrea, L. et al.; Arnall Golden & Gregory, LLP, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: MODIFIED COMPLEMENT SYSTEM REGULATORS			
(57) Abstract			
Two functionally distinct but homologous sites in complement receptor type 1 (CR1)(CD35) were characterized by homologous substitution mutagenesis of two CR1 derivatives, each containing one site. In both sites, reducing negative and/or increasing positive charge augmented interaction with iC3/C3b and C4b, supporting a role of ionic forces in the binding reaction. In one case, substitution of Asp at the end of complement control protein repeat (CCP)2 with an Asn transformed the protein, with negligible cofactor activity and iC3 binding, into a mutant with activities similar to native CR1. Consequently, this protein, one-fourth the size of CR1, is a therapeutic candidate for a complement inhibitor. Another important observation is that the residues between two CCPs contribute to activity, probably because they influence positioning of one CCP relative to the next. The initial characterization of the third CCP of an active site led to identification of three peptides necessary for binding. In line with earlier findings for the first two CCPs, interactions with iC3/C3b are similar but not identical to those with C4b, implying overlapping but distinct binding domains. Moreover, changes in cofactor activity usually, but not always, parallel alterations in binding, indicating that these two activities are separable. Epitopes were mapped for a blocking and a function enhancing monoclonal antibody. Their effects can be explained by epitope location. The first antibody binds near functionally important residues. The second may shield inhibitory (negatively charged) residues. These results represent a comprehensive analysis of the active sites of CR1, which is built of modules found in more than 50 mammalian proteins.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

MODIFIED COMPLEMENT SYSTEM REGULATORS

Background of the Invention

The invention relates to modified forms of complement regulators derived from regulatory proteins of complement activation (RCA), especially CR1.

This application claims priority to U.S.S.N. 60/128,520 filed April 9, 1999.

The U.S. government has certain rights in this invention by virtue of National Institutes of Health Grant No. RO1 AI41592 to J. P. Atkinson.

The complement system serves to aid in the removal of foreign substances and of immune complexes from animal hosts. This system and its regulation is reviewed by Hourcade, D., et al., Advances in Immunol (1989) 45:381-416. Briefly, the complement system generates, either by a "classical pathway" or an "alternative pathway," C3b which binds to target immune complexes or foreign substances and marks them for destruction or clearance. C3b is generated from its precursor C3 by the proteolytic enzymes collectively designated "C3 convertase." One form of C3 convertase is generated in the classical pathway by the association of the proteins C4b and C2a. The other form is generated in the alternative pathway by association of C3b and Bb. Both C3 convertases can associate with an additional C3b subunit to form the C5 convertases, C3bBbC3b and C4bC2aC3b, both of which are active in the production of the C5-C9 membrane attack complex which can cause cell lysis, and the production of C5a, a major pro-inflammatory agent.

Both C3b, and less directly, C4b, are agonists in the complement system. The complement system is regulated via a number of interrelated mechanisms. There are two general mechanisms for inhibition of the destructive components of the complement system. The first mechanism is generally reversible, facilitating the dissociation of the C3 convertases--i.e., C3b from Bb and C4b from C2a. Facilitation of dissociation is sometimes known as decay acceleration. The dissociation may also involve reversible binding of the antagonist proteins to C3b or C4b components, thus preventing their reassociation. The other mechanism, which is an irreversible inactivation process, results from proteolytic cleavage of the C3 convertase components C3b or C4b by the serine protease factor I. This proteolytic cleavage occurs only in the presence of a cofactor. Both general regulatory mechanisms, the facilitation of dissociation of C3b and C4b and the inactivation of C3b and C4b through cleavage by factor I, also apply to the inhibition of the alternative pathway C5 convertase (C3bBbC3b) and the classical pathway C5 convertase (C4bC2aC3b).

The proteins encoded by a region of the genome which is designated the "regulators of complement activation" (RCA) gene cluster are involved in both of these pathways. Evaluation of the comparative sequences of CR1, CR2, DAF, MCP, C4bp, and factor H has established that the RCA proteins are organized into short consensus repeat ("SCR") containing and non-SCR-containing regions. The SCR repeats are composed of 60-70 amino acid and share a number of invariant or highly conserved amino acid residues with other SCRs in the same protein or SCRs in other family members. Those members of the family which are membrane bound also have at their C termini either transmembrane regions and intracellular regions or a glycolipid anchor. The SCRs form the extracellular portions of those members of the family which are membrane-bound and almost all of the protein structure in the secreted members.

These proteins are characterized by C4b-binding activity, C3b-binding activity, C4b cofactor activity, and C3b cofactor activity. In general, it takes two to three SCRs for each activity. Activities which are biologically important include decay acceleration or dissociation, C3b cofactor activity and C4b cofactor activity. Cofactor activity requires binding but binding alone may not be sufficient for cofactor activity.

Klickstein, L.B., et al., J. Exp. Med. (1988) 168:1699-1717, described the identification of distinct C3b and C4b recognition sites in CR1 using deletion mutagenesis. They concluded that a single primary C4b binding site is located in SCR 1-2, while two major C3b binding sites are located in SCR 8-9 and SCR 15-16. C3b cofactor activity was localized to SCR 8-9 and SCR 15-16. The CR1 active site containing SCR 8-9 extends to SCR 10, and by analogy, the active site that contains SCR 15-16 (which is only one amino acid different than SCR 8-9) must extend to SCR 17. (Kalli, et al., J. Exp. Med. 174, 1451-1460 (1991); Makrides, et al., J. Biol. Chem. 267, 24754-24761 (1992)). The CR1 active site containing SCR 1-2 extends to SCR 3 and/or 4, as reported by Makrides, et al., (1992). The murine C4bp binding site, and presumably the C4b cofactor and C4bC2a decay acceleration active sites, was reported to extend from SCRs 1-3 in the alpha chain by Ogata, et al., J. Immunology 150, 2273-2280 (1993). It is now generally accepted that CR1 C4b binding and cofactor activity requires SCRs 1, 2 and 3, 8, 9, and 10, or 15, 16, and 17, which are corresponding regions of the protein. C3b binding and cofactor activity requires SCRs 8, 9, and 10, or 15, 16, and 17, which are corresponding regions of the protein.

The multiple binding sites of CR1 can cooperate in their interactions with C3b-containing targets. *In vitro*, CR1 binds C3b-C3b dimers much more tightly than C3b

monomers because binding to dimers can occur simultaneously at two sites in the same CR1 molecule, as reported by Wong and Farrell, J. Immunol. 146:656 (1991); Ross and Medof Adv. Immunol. 37:217 (1985)). Deletion of one of the two primary C3b binding sites can reduce the binding of CR1 to C3b-C3b by a factor of ten, as reported by Wong and Farrell, J. Immunol. 146:656 (1991). It is likely that the primary C4b binding site also cooperates with the primary C3b-binding sites in interactions with targets that contain both C3b and C4b. These effects have an important consequence *in vivo*: CR1 has a higher affinity for targets densely coated with C3b and with targets densely coated with C3b plus C4b.

The C5 convertases, which are important in the stimulation of inflammation and in lysis of some target cells, are composed of multiple CR1 ligands: The classical C5 convertase contains C3b and C4b (C4bC3bC2a) while the alternative pathway C5 convertase contains two C3b proteins (C3bC3bBb). Inactivation of the C5 convertases by CR1 can also involve cooperation between more than one CR1 binding site. Wong and Farrell J. Immunol. 146:656 (1991) showed that more than one CR1 C3b binding site may be essential for effective inhibition of alternative pathway C3 and C5 convertases.

Human CD35 [complement receptor type 1 (CR1), C3b/C4b or immune adherence receptor] is a type 1 transmembrane glycoprotein. Four allotypes have been identified. The extracellular portion of the most common allotype A or F is composed of 30 complement control protein repeats (CCPs) or short consensus repeats (SCRs), each 60-71 amino acids long (Figure 1). All but the two carboxyl terminal CCPs are organized into four larger units, called long homologous repeats (LHR), each seven CCPs long, Hourcade D., et al. J Exp Med 168:1255-70 (1988) and Klickstein, L.B., et al. J Exp Med 165:1095-112 (1987). The number of LHRs varies from three to six among allotypes, leading to their size variation. The allotypes A or F (frequency of 0.82), B or S (0.16), C or F (S0.01) and D (<0.01) have an Mr of 220, 250, 190 and 280 k under reducing conditions on 5% SDS-PAGE, respectively, Holers, V.M., et al. Proc Natl Acad Sci USA 84:2459-63 (1987), Van Dyne S., et al. Clin Exp Immunol 68:570-9 (1987), and Wong, W.W., et al J Exp Med 169:847-63 (1989). A new nomenclature, along the lines of that utilized for other complement proteins, has been recommended (Table 1).

CD35 binds C3b and C4b with a high affinity and iC3b with a lower affinity. The number of C3b binding sites depends on the allotype and varies from one in type C to four in type D, Wong, W.W. J Invest Dermatol 94:64S-7S (1990). Each of these highly homologous interactive sites also binds C4b but with approximately a log lower affinity

than for C3b. Repeats 1-3 bind C4b with barely detectable C3b binding capability. The affinity for dimeric and polymeric C3b increases with the number of binding sites, Wong, W.W., et al. J Immunol 146:556-62 (1990). Each active site requires three contiguous CCPs and critical peptides and amino acids for ligand binding and regulatory activity have been identified, Kalli, K.R., et al. J Immunol 147:590-4 (1991), Krych, M., et al. J Biol Chem 269:13273-8 (1994), Krych, M., et al. J Biol Chem 273:8623-9 (1998), Krych, M., et al. Mol Immunol 35:403 (1998), and Krych, M., et al. Proc Natl Acad Sci USA 88:4353-7 (1991). Kalli et al. J. Exp. Med. 174, 1451-1460 (1991) reported that CR1/CR2 chimeric receptors in which various short consensus repeats (SCRs) of CR1 were attached to CR2 were transiently expressed on COS cells. 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 (Kd) 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 prepared by fusing SCRs 1-4 of LHR-B to the heavy chains of a murine F(ab')₂ monoclonal antibody 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. These studies showed that the function of SITE 2 was abolished if CCP 10 was replaced by CCP 3 of CR2.

PCT/US94/10820 by Washington University entitled "Modified Truncated Complement System Regulators" describes a number of site specific mutations of CR1 that alter activities. However, the effects of the different mutations have been determined to be unique and it is therefore still desirable to provide additional modified forms and information regarding changes that can be made to CR1 to alter its biochemical and biological properties.

It is therefore an object of the present invention to provide modified complement regulators.

It is a further object of the present invention to provide modified complement

regulators which can be administered to treat inflammatory disorders or to reduce an individual's ability to reject foreign materials.

Summary of the Invention

Two functionally distinct but homologous sites in complement receptor type 1 (CR1) (CD35) were characterized by homologous substitution mutagenesis of two CR1 derivatives, each containing one site specific mutation. In both sites, reducing negative and/or increasing positive charge augmented interaction with iC3/C3b and C4b, supporting a role of ionic forces in the binding reaction. In one case, substitution of Asp at the end of complement control protein repeat (CCP) 2 with an Asn transformed the protein with negligible cofactor activity and iC3 binding into a mutant with activities similar to native CR1. Consequently, this protein, one-fourth the size of CR1, is a therapeutic candidate for a complement inhibitor. Another important observation is that the residues between two CCPs contribute to activity, probably because they influence positioning of one CCP relative to the next. The initial characterization of the third CCP of an active site led to identification of three peptides necessary for binding. Interactions with iC3/C3b are similar but not identical to those with C4b, implying overlapping but distinct binding domains. Moreover, changes in cofactor activity usually, but not always, parallel alterations in binding, indicating that these two activities are separable. Epitopes were also mapped for a blocking and a function enhancing monoclonal antibody. Their effects can be explained by epitope location. The first antibody binds near functionally important residues. The second may shield inhibitory (negatively charged) residues. These results represent a comprehensive analysis of the active sites of CR1, which is built of modules found in more than 50 mammalian proteins.

These studies have resulted in the development of new CR1 analogs with site specific mutations which have modified CR1 activities and are therefore useful in regulation of the complement system, which can have applications in the treatment of autoimmune diseases, the suppression of rejection of transplants, in diagnosis and the reduction in tissue damage associated with myocardial infarctions and cerebral vascular injury, and prevention or treatment of infection with certain viruses and other pathogens. They may also play a role in the diagnosis of conditions associated with complement activation and immune complex formation.

Brief Description of the Drawings

Figure 1 is a schematic representation of CR1 derivatives and their binding domains. Extramembraneous part of CR1 is composed of 30 CCP (shown as boxes). Based on degree of homology, the first 28 CCP can be organized into LHR A, B, C and D, each seven CCP long, having arisen by duplication of a seven CCP unit. There are two distinct functional sites composed of three CCP. SITE 1 is located in LHR A. Two nearly identical copies of SITE 2 are present in LHR B and C. The first two CCP in SITE 1 (CCP 1 and 2) are distinct (about 40% different) from the first two CCP in SITE 2 (CCP 8 and 9) and they are marked by different shading. The last CCP in both sites varies by only one amino acid and is represented by a black box.

Figure 2 is a schematic of the mutations in CCP 1 and in CCP 2 of LHR A. Amino acids in CCP 1 are aligned with those in CCP 8 and the amino acids in CCP 2 are aligned with those in CCP 9. For CCP 8 and 9 only amino acids different from those in CCP 1 and 2 are shown. The four invariant cysteines per CCP are boxed. Key mutations T₁₄, N₂₉ and D₁₀₉ are in bold type. Multiple amino acid substitutions are identified by the numbers above the braces. The single amino acid substitutions 14a-j, 15a and b, and 10a-d, are indicated by the italicized letters below the alignment while those described in the prior art are in regular font. "C4b" above the alignment refers to the amino acid in SITE 1 necessary for interaction with C4b. "C3b" and "C3b/C4b" below the alignment indicate the amino acid in SITE 2 which, if transferred to SITE 1, increase its interaction with C3b or C3b and C4b, respectively. The subscript that precedes the first amino acid and the one that follows the last amino acid in a CCP indicate amino acid number in the mature CR1.

Figure 3 is a graph of the effect on iC3 binding (percent) of substitutions of D₁₀₉ in CCP 2 as a function of salt concentration (mM NaCl). open circle, soluble CR1; open diamond, Asn; triangle, LHR B; square, Gln; inverted triangle, valine; closed circle, threonine; closed diamond, LHR A.

Figure 4 is a schematic of mutations in CCP 10 of CR1. Amino acids in CCP 10 of CR1 are aligned with those of CCP 3 of CR2. The invariant cysteines are boxed. Amino acids in CCP 10 of CR1 were changed, initially a few and subsequently one at a time, into their counterparts in CCP 3 of CR2. Multiple amino acid substitutions are identified by the numbers above the braces and single amino acid changes by letters below the alignment. Because the numbering was initiated in earlier work in which mutants 1-15 were constructed (Krych, et al. 1991, 1994), the new mutants start with #16. In order not to introduce deletions, gaps in the CR2 sequence were filled with alanines in mutants 21, 23 and 25. To test the role of the amino

acid in the inter-CCP region, isoleucine and proline located between CCP 9 and 10 were changed into alanines in mutant 16. Amino acids important for C3b, C3b/C4b and mAb 3D9 binding are indicated by the arrows. CCP 17 differs from CCP 10 only in that in sequence 21 L₁₀₅₆ and R₁₀₅₉ replace P₆₀₆ and G₆₀₉, respectively, (as shown under the alignment). The subscript that precedes the first amino acid and the one that follows the last amino acid in a CCP indicate the amino acid number in the mature CR1.

Figures 5a and 5b are graphs of the effect of mutations in CCP 10 on binding to iC3 (Figure 5a) and C4b (Figure 5b).

Detailed Description of the Invention

The modified CR1 analogs can be used to modulate the complement system by altering the binding specificity of the protein in both membrane-bound and soluble forms.

Definitions

Members of the regulators of complement activation (RCA) protein family are composed entirely or mainly of independently folding complement control protein repeats (CCP), each 56-70 amino acids long (Hourcade, et al. Progress in Immunology 7, 171-177 (1989)). The smallest proteins of this multi-gene family, decay accelerating factor (DAF; CD55) and membrane cofactor protein (MCP; CD46) have four CCP which contain two overlapping active sites, one for C3b and one for C4b (Coyne, et al., J. Immunol. 149, 2906-2913 (1992); Adams, et al. J. Immunol. 147, 3005-3011 (1991)). Other proteins of this structurally and functionally related group contain sites multiplied in different ways. Factor H has three binding sites for C3b in a single polypeptide chain composed of 20 CCP (Sharma, et al. Proc. Natl. Acad. Sci. USA 93, 10996-11001 (1996)). Each site is different and only one has cofactor activity (CA). Human C4b binding protein contains seven copies of a single site as a result of joining seven identical protein chains by disulfide bonds (Chung, et al. Biochem. J. 230, 133 (1985)). Complement receptor type 1 (CR1; CD35) represents yet another way of increasing the number of active sites. In this case, as a result of internal duplication due to unequal crossing-over or gene conversion (Klickstein, et al., J. Exp. Med. 168, 1095-1112 (1988); Klickstein, et al. J. Exp. Med. 165, 1095-1112 (1987); Hourcade, et al., J. Exp. Med. 168, 1255-1270 (1988); Wong, et al., J. Exp. Med. 169, 847-863 (1989)), there are regions of high internal homology. Of 30 CCP present in the most common allelic form of CR1, all but the two carboxy-terminal CCP can be organized into four long homologous repeats (LHR), termed A, B, C and D, each seven CCP long.

RCA proteins interact with their ligands via CCP. In addition to six proteins of the

RCA family, over 40 other proteins possess CCP, which in many cases also participate in protein-protein interactions (Wiles, et al., *J. Mol. Biol.* 272, 253-265 (1997)). The functions of CR1, namely ligand binding, decay accelerating activity (DAA) and CA, result from the interaction of CCP with C3b and C4b. Thus, understanding CR1 function requires elucidation of structure-function relationships between CCP and their ligands.

Members of the regulators of complement activation protein family are composed entirely or mainly of independently folding complement control protein repeats (CCPs), each 56-70 aas long. The smallest proteins of this multi-gene family, decay-accelerating factor (CD55) and membrane cofactor protein (CD46) have four CCPs, which contain two overlapping active sites, one for C3b and one for C4b. Other proteins of this structurally and functionally related group contain sites multiplied in different ways. Factor H has three binding sites for C3b in a single polypeptide chain composed of 20 CCPs. Each site is different, and only one has cofactor activity. Human C4b-binding protein contains seven copies of a single site as a result of joining seven identical protein chains by disulfide bonds. Complement receptor type I (CR1)(CD35) represents yet another way of increasing the number of active sites. In this case, as a result of internal duplication due to unequal crossing-over or gene conversion, there are regions of high internal homology. Of 30 CCPs present in the most common allelic form of CR1, all but the two carboxyl-terminal CCPs can be organized into four long homologous repeats (LHRs), termed A, B, C, and D, each seven CCPs long.

The modified proteins described herein are collectively referred to as "modified CR1 analogs". "Truncated" proteins are typically modified to remove the C-terminal regions which effect membrane binding or secretion and sometimes modified further by deletion of one or more CCPS. "Hybrid" proteins are composed of portions, i.e., the CCPS, of one RCA protein combined with CCPs of one or more other RCA proteins. "Recombined" forms are those wherein the CCPs of an RCA protein are rearranged in a new order. "Modified RCA proteins" include proteins which result from combinations of these changes. In some embodiments, modifications are made using corresponding CCPs of the protein as sites for alteration. By "corresponding CCP" is meant the most highly homologous CCP as determined by comparison of the amino acid sequences of the protein. Exon structure can in some cases facilitate this assignment. CCPs 1-3 of CR1 correspond to CCPs 2-4 of DAF. CCPs 1-3 of factor H, CR1, C4bp and MCP correspond. CR1 is organized into a series of long homologous repeats (LHRS) containing 7 CCPs so that CR1

CCPs 1-7 correspond to CR1 CCPs 8-14; 15-21; and 22-28. CR2 is organized into a series of long homologous repeats of 4 CCPs in length. CCPs 1-2 of CR1 correspond to CCPs 3-4, CCPs 7-8, CCPs 11-12 and CCPs 15-16 of CR2.

Site Specific Mutations of CR1

There are two functionally distinct active sites in CR1. SITE 1 is located in CCP 1-3 of LHR A. SITE 2 is in CCP 8-10 of LHR B and its nearly identical [different by only three amino acids (aa)] copy is in CCP 15-17 of LHR C. SITE 1 binds mainly C4b (Klickstein, et al., (1988); Krych, et al., (1991); Krych, et al., (1994); Reilly, et al., *J. Biol. Chem.* 269, 7696-7701 (1994)). It has barely detectable cofactor ("CA") for cleavage of C4b and C3b. SITE 2 binds both C3b and C4b and possesses CA for both (Krych, et al., *J. Biol. Chem.* 269, 13273-13278 (1994); Kalli, et al., *J. Exp. Med.* 174, 1451-1460 (1991)). The amino acids sequences of the first two CCP of SITE 1 differ by approximately 40% from the first two CCP of SITE 2 while the third CCP in both sites differs by one amino acids. To analyze by site directed mutagenesis amino acids important for function of each site, two CR1 derivatives, LHR A, composed of CCP 1-7 which contains SITE 1 and LHR B, composed of CCP 8-14, which carries SITE 2 (Fig. 1) were used.

Previously, by interchanging amino acids in CCP 1 and 2 with their homologs in CCP 8 and 9 respectively, peptides and in some cases amino acids important for ligand binding and CA were identified (Krych, et al. 1991; Krych, et al. 1994). In particular, replacement of three short peptides in SITE 1 led to a marked increase in ligand binding. In the studies described herein the amino acids responsible for this increase are defined and the role of amino acids charge in the binding reaction further defined. Furthermore, because in CR1 three CCP are indispensable for the activity of each site, the studies of structure-function relationships to the third CCP were expanded. Amino acids from CCP 10 of CR1 were substituted with their homologs from CCP 3 of CR2. In both sites in CCP 10, reducing negative and/or increasing positive charge augmented interaction with iC3/C3b and C4b, supporting a role of ionic forces in the binding reaction. In one case, substitution of Asp at the end of complement control protein repeat (CCP) 2 with an Asn transformed the protein, with negligible cofactor activity and iC3 binding, into a mutant with activities similar to native CR1. Consequently, this protein, one-fourth the size of CR1, is a therapeutic candidate for a complement inhibitor. As it was observed that the residues between two CCPs contribute to activity, probably because they influence positioning of one CCP relative to the next. The initial characterization of the third CCP of an active site led to identification of three peptides necessary for binding. In line with earlier findings for the first two CCPs, interactions with iC3/C3b are similar but not identical to those with C4b, implying overlapping but distinct binding domains. Moreover, changes in cofactor activity usually, but not always, parallel

alterations in binding, indicating that these two activities are separable.

Epitopes were also mapped for a blocking and a function enhancing monoclonal antibody. Their effects can be explained by epitope location. The first antibody binds near functionally important residues. The second may shield inhibitory (negatively charged) residues.

These results represent a comprehensive analysis of the active sites of CR1, which is built of modules found in more than 50 mammalian proteins. This work is reported by Krych, M. et al. in "Structure-function analysis of the active sites of complement receptor type 1" *J. Biol. Chem.* 273(15):8623-8629 (April 10, 1998).

Regulators of complement activation interact with their ligands via CCPs. In addition to six proteins of the regulators of complement activation family, over 40 other proteins possess CCPs, which in many cases also participate in protein-protein interactions. The functions of CR1, namely ligand binding, decay-accelerating activity, and CA, result from the interaction of CCPs with C3b and C4b. Thus, understanding CR1 function requires elucidation of structure-function relationships between CCPs and their ligands.

Based on the discoveries described herein, it is possible to design a more potent soluble complement inhibitor by modifying corresponding regions to increase affinity for C4b and C3b or to design soluble complement inhibitors that specifically inhibit one part of the complement system. These modifications can be in the form of specific substitutions of amino acids that alter C3b or C4b binding within corresponding CCPs of CR1 or other RCA proteins, or substitution of CCPs from one protein into another.

Acquisition of function within an active site. Mutagenesis resulted in proteins with enhanced and/or new functional activities. Probably the most remarkable was the effect of the D₁₀₉→N change in mutant 10a which led to a modified SITE 1 in LHR A with enhanced activities. This protein acquired iC3 binding ability which was higher than that of LHR B and comparable to sCR1. C4b binding and CA for C4b and C3b were also increased and were similar to that observed with LHR B (SITE 2). These data suggest that D₁₀₉ may be located close to or be a part of a contact point with C3 and C4, being particularly critical for interaction with C3. Mutant 10a is a candidate for a complement inhibitor smaller than sCR1 but with a similar activity.

Another mutation which substantially augmented iC3 and C4b binding of SITE 1 was N₂₇→K (mutation 15b). Of interest, a short form (65 kDa) of baboon CR1 contains a modified SITE 1 which has K₂₇ and N₁₀₉ and each of these two amino acids confers properties of SITE 2

on SITE 1 (Birmingham, et al., *J. Immunol.* 157, 2586-2892 (1996)). This short form is the only CR1 expressed by baboon E and it contains just SITE 1 (CCP 1-8) and yet has activities of both human sites. Chimpanzee (Subramanian, et al., *J. Immunol.* 157, 2586-2892 (1996)) has a short CR1 has only SITE 1 (CCP 1-6). Further, the two amino acids by which it differs from human SITE 1 are present in the homologous position of human SITE 2. If they are placed in SITE 1, it acquires C3b binding. Thus, in the absence of SITE 2, a modified SITE 1 may serve as its functional equivalent (Subramanian, et al., 1996). Moreover, it only requires one or a few amino acid substitutions at homologous positions to acquire the necessary functional activity. Further, two distinct sets of substitutions can accomplish this end, either as per the 10a and baboon proteins or the one used by the chimpanzee protein.

The activity of SITE 1 and SITE 2 is increased if negative charge is reduced and/or positive charge enhanced at key positions. For example, activity of SITE 1 is increased by replacing T₁₄, N₂₉, D₁₀₉ or E₁₁₆ (mutant 11c in Ref 11) with their homolog in SITE 2, either K or N. In the case of D₁₀₉, additional substitutions indicated that its negative charge inhibits interaction of SITE 1 with iC3. Activity of SITE 2 was also increased as a result of higher positive charge. One example is the greater binding of iC3 and C4b by LHR C than by LHR B (instead of G₆₀₇ in CCP 10 of LHR B there is an R₁₀₅₉ in CCP 17 LHR C). These data complement well the findings of Isenman's group who demonstrated that several negatively charged amino acids at the N terminus of the alpha' chain of C3b, also conserved in C4b, are necessary for interaction with CR1 (Taniguchi-Sidle, et al., *J. Immunol.* 153, 5285-5302 (1994)).

Each of the sequences comprising mutants 10/11 and 14 in SITE 1 has three negatively charged amino acids while the corresponding peptides in SITE 2 have one or none. Interchanging these sequences increases activity of SITE 1. Moreover, the enhancing effect of mAb 8C9.1, which recognizes sequences 14 and 15 and confers iC3/C3b and increases C4b binding, might be explained by blocking negatively charged amino acids. These data suggest that the first half of CCP 1 may reduce ligand binding of SITE 1. Overall, the hypothesis is that CR1 binds C3 and C4 through ionic interactions with positive charges in the active sites of CR1 playing a key role.

Analysis of the third CCP required for an active site. This study is the initial analysis of functionally important amino acids in the third CCP which is indispensable for the activity of each site. The study begins by evaluating the role of CCP 10 of SITE 2 (as opposed to CCP 3 of SITE 1), because SITE 2 has higher affinity for ligand and is the main site for CA. The

mutational analysis points out that the interactions of CCP 10 with C3b and C4b are similar. To illustrate, of the single amino acids substitutions made in CCP 10, mutation of Y₅₉₆ (mutant 20c) had the greatest effect on binding and on CA for both iC3/C3b and C4b (Table I and II, Figure 5a and b). Therefore Y₅₉₆ may be a contact point with both C3b and C4b. This is consistent with absence of Y in the homologous, but presumably nonfunctional, CCP 24. Other contact point(s) for both ligands are likely to be in sequence 22 and/or 21 (Figure 5a and b, Table III and IV). Although as noted, interactions of CCP 10 with iC3/C3b and C4b are very similar, they are not identical. That the differences do exist is based, for example, on mutant 17 which has CA for C3b but no CA for C4b and on mutant 19 with C4b binding capability but very poor iC3 binding. Similar observations have been made for MCP (Adams, et al., *J. Immunol.* 147, 3005-3011 (1991)) and DAF (Coyne, et al., *J. Immunol.* 149, 2906-2913 (1992)), suggesting that the C3b and C4b sites, although overlapping, have distinctive features.

These experiments provide further evidence that binding and CA do not always change in parallel. The identification of peptides as important for one but not for the other function is the basis for this conclusion. For example, peptides 16 and 17 are important mainly for CA. On the other hand, peptide 19 has a major effect on iC3 binding but little on CA for C3b. Although it may seem a little puzzling at the first glance, high binding is not prerequisite for high cofactor activity. For example, MCP has much lower affinity for C3b than CR1 yet its CA is similar or higher than that of CR1 (Cole, et al., *Proc. Natl. Acad. Sci. USA* 82, 859-863 (1985); Seya, et al., *J. Biochem.* 107, 310-315 (1990)). Also, in the case of MCP, like in SITE 2 of CR1, the determinants of binding and CA are separable (Adams, et al., *J. Immunol.* 147, 3005-3011 (1991)).

Importance of amino acids between two CCP. Mutations of the amino acids between two CCP result in reduced activity. Thus, in mutant 16, in which I₅₇₃ and P₅₇₄ were both changed to A, CA was nearly abrogated (Tables III and IV). Another example is mutant 25 in which I₆₄₂ and I₆₄₃ between CCP 10 and 11 were both mutated to E. In this mutant, binding and, to a lesser degree CA, were reduced (Tables III and IV). This presumably demonstrates the importance of the correct positioning of one CCP relative to the other, consistent with the suggestions of Barlow et al. (Barlow, et al., *J. Mol. Biol.* 232, 268-284 (1993)).

Identification of epitopes for mAb 3D9 binding that inhibit function. The presence of 3D9 epitope in CCP 3, 10 and 17 provides an explanation for the ability of this mAb to block all activities of both sites. More precise mapping in CCP 10 demonstrated that the epitope is

located in peptides 20-22. Specifically, S₅₉₈ in sequence 20 is a part of the epitope because its mutation (#20d) resulted in a loss of recognition by 3D9. On the other hand, R₆₀₃ and P₆₂₀ in sequence 20 and 22, respectively, are probably not a part of the epitope but may be very close to it. This is because mutation of either one of them does not abrogate 3D9 binding but decreases it by about 50%. Importantly, peptides 20-22 were also shown by our mutagenesis experiments to be critical for interactions of SITE 2 with its ligands. These results are consistent with and therefore supportive of the inhibition of function by mAb 3D9.

In summary, an extensive mutational analysis of CCP 1 and 2, CCP 8 and 9 and now CCP 3/10 has been conducted. The results from these mutational analyses supplemented by epitope mapping of function altering mAb and evolutionary comparisons among primate forms of CR1 are mutually consistent. The data suggest that many of the key sequences within binding sites of CR1 have been identified. No other CCP containing protein has undergone such an extensive analysis of its active site(s). These results will provide helpful information not only for the structure of the active sites of CR1 but also for other CCP containing proteins.

Substitution of modifications to CCP regions of one regulatory protein into a second regulatory protein.

The identification of the amino acid sequences essential (or refractory) to binding to C4b and C3b and C4b and C3b cofactor activity permits transposition of similar sequences into corresponding regions of the same protein or corresponding regions of other family members or alteration of sequences which bind C3b and C4b so as to alter their affinities. Corresponding regions have been identified by degree of amino acid sequence homology.

In the case of CR1, four corresponding regions of interest are CCPs 1-3, CCPs 8-10, CCPs 15-17 and CCPs 22-24. The CCP portions labeled 2-4 in DAF correspond to those labeled 1-3, 8-10, 15-17 and 22-24 for CR1. Substitution of portions of DAF with homologous CR1 sequences provides forms of DAF with cofactor activity and/or binding activity, such as is exhibited by CR1. Similarly, substitutions of portions of MCP with homologous sequences provides forms of MCP with increased binding affinity and cofactor activity and/or increased dissociation activity.

Specific Amino Acid Substitutions

Addition of Binding Sites

Specific amino acids are selected for substitution based on studies that elucidate their roles in complement regulation in specific active sites. Substitution can be employed

in order to alter the activity of additional RCA active sites in the same or other proteins. In this manner, binding and cofactor sites can be added to CCPs not normally contributing directly to binding capacity.

For example, the C3b and C4b binding and cofactor sequence in CR1, N-A-A-H-W-S-T-K-P-P-I-C-Q, can be transferred to corresponding locations or to locations referenced to conserved amino acids in alternative CCPs to confer C3b binding. The C4b binding regions are shown to be associated with separate critical locations in the CCPs of CR1. Alterations in amino acid sequences of the corresponding CCPs in CR1 or in additional RCA family members or their truncated, hybrid, or recombined forms in these positions alter C4b binding and cofactor activities and in at least one case alter C3b binding and cofactor activities.

Substitution of Similar Amino Acids

Structurally, similar amino acids can be substituted in such transfers for some of the specified amino acids. Structurally similar amino acids include: (I,L,V); (F,Y); (K,R); (Q,N); (D,E); and (G,A).

Deletion of Amino Acids

It also may be advantageous to delete amino acids from specific active sites in order to alter or enhance complement regulatory activity.

Construction of Truncated Forms

In some embodiments, it will be advantageous to delete a specific activity by deletion of a region known to have a particular activity. It may also be desirable to delete the region of the protein which anchors the naturally occurring protein to the cell surface, for example, the transmembrane and cytoplasmic regions or the glycolipid anchor region.

Modifications which alter Cofactor Activity

In general, either C3b or C4b cofactor activity can be enhanced by substitutions which increase the binding activity of the other factor, i.e., to increase C4b cofactor activity, amino acids are substituted into the modified protein which increase C3b binding and *vice versa*.

Preparation of the Analogs

The modified proteins described herein are most conveniently prepared using recombinant techniques, although in some cases they can be prepared by enzymatic cleavage, for example, to yield truncated or soluble forms of the naturally occurring proteins. The genes encoding the various members of the RCA protein family are of known

sequence and are published.

cDNA encoding CR1 was described by Klickstein, L.B., et al., J. Exp. Med. (1987) 165:1095, Klickstein, L.B., et al., J. Exp. Med. (1988) 168:1699; Hourcade, D., et al., J. Exp. Med. (1988) 168:1255. See also accession numbers for human CR1: Swissprot: P17927; EMBL/GENBANK: Y00816 and X05309; PIR: S03843 and 28507. The availability of the cDNA sequence makes possible the preparation of genetic constructs encoding truncated forms and other modified forms of the proteins using standard site-directed mutagenesis techniques, such as those described by Kunkel, T.A., et al., Methods Enzymol (1987) 154:367-382.

After the gene encoding the analog is prepared, the modified gene is expressed using standard recombinant techniques. The gene sequence is ligated into a suitable expression vector under the control of sequences known to be appropriate to the desired host. Production of recombinant proteins in microbial systems such as *E. coli*, *B. subtilis*, various strains of yeasts, and other fungi, such as *Aspergillus*, is well known. It may be advantageous to produce the desired analogs in cells of higher organisms as well, such as the standard BPV/C127 system, the Baculovirus/insect cell system, CHO cells, COS cells, and other mammalian cells, or in transgenic animals. Standard expression systems in various cell lines are well known and standard in the art.

Transgenic animals can be constructed for several species. The gene is placed under the control of a suitable promoter, for example, the metallothionine promoter or a tissue specific promoter, and the gene microinjected into an embryo, which is then implanted into a surrogate mother. Production in transgenic animals is important in the context of preparing transplants for use in other species.

Construction of Transgenic Animals.

Animals suitable for transgenic experiments can be obtained from standard commercial sources. These include animals such as mice and rats for testing of genetic manipulation procedures, as well as larger animals such as pigs, cows, sheep, goats, and other animals that have been genetically engineered using techniques known to those skilled in the art. These techniques are briefly summarized below based principally on manipulation of mice and rats.

The procedures for manipulation of the embryo and for microinjection of DNA are described in detail in Hogan et al. Manipulating the mouse embryo, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1986), the teachings of which are incorporated

herein. These techniques are readily applicable to embryos of other animal species, and, although the success rate is lower, it is considered to be a routine practice to those skilled in this art.

Methods for the culturing of ES cells and the subsequent production of transgenic animals, the introduction of DNA into ES cells by a variety of methods such as electroporation, calcium phosphate/DNA precipitation, and direct injection are described in detail in Teratocarcinomas and embryonic stem cells, a practical approach, ed. E.J. Robertson, (IRL Press 1987), the teachings of which are incorporated herein. Transfection is carried out by one of several methods described in detail in Lovell-Badge, in Teratocarcinomas and embryonic stem cells, a practical approach, ed. E.J. Robertson, (IRL Press 1987) or in Potter et al., Proc. Natl. Acad. Sci. USA 81, 7161 (1984). Calcium phosphate/DNA precipitation, direct injection, and electroporation are the preferred methods. Direct injection results in a high efficiency of integration. Desired clones are identified through PCR of DNA prepared from pools of injected ES cells. Positive cells within the pools are identified by PCR subsequent to cell cloning (Zimmer and Gruss, Nature 338, 150-153 (1989)).

DNA is prepared and analyzed by both Southern blot and PCR to detect transgenic founder (F_0) animals and their progeny (F_1 and F_2). Once the transgenic animals are identified, lines are established by conventional breeding and used as the donors for tissue removal and implantation using standard techniques for implantation into humans.

Purification of Analogs

The analogs recombinantly produced in culture or animals can be purified from the cell culture using standard purification techniques such as chromatography, for example, immunoaffinity or ion-exchange chromatography, and electrophoresis, generally using the same procedures as have been published for use in purifying the naturally occurring material.

While recombinant production of the analogs is the most convenient and practical method of preparing the proteins, it may also be desirable to synthesize the analogs using protein synthesis techniques, such as standard solid-phase peptide synthesis technology. This approach may be suitable in particular in the case of truncated forms of the RCA protein family having modified amino acid sequences, especially in view of the discovery that proteins containing as few as three CCPs have useful biological activity.

Assay Systems

The analogs are tested for the desired biological activities among those characteristic of the RCA family using *in vitro* or *in vivo* assays. *In vitro* systems such as those described by Wong and Farrell (J. Immunol. (1991) 146:656) can be used to measure effects on the complement pathways. *In vivo* and general biological effects can be assessed as described by Weisman, et al. (Science (1990) 249:146); or Yeh, et al. (J. Immunol. (1991) 146:250), the teachings of which are incorporated by reference.

Binding Assays.

Affinity chromatography columns were prepared as described by Krych, et al., Proc. Natl. Acad. Sci. USA 88, 4353-4357. Binding assays were performed using 100 ml of iC3-Sepharose (iC3-S) or C4b-Sepharose (C4b-S) and 1.8 ml of medium containing of the mutant protein. Media were diluted to desired concentrations of NaCl. After 1 hr on a rotator at room temperature, samples were centrifuged, media removed and bound protein eluted from the Sepharose using 400 mM NaCl with 1% NP-40. Eluted proteins were quantitated by ELISA, using two monoclonal anti-CR1 antibodies, 3D9 and E11 (Hogg, et al., Eur. J. Immunol. 14, 236-240 (1984), O'Shea, et al., J. Immunol. 134, 2580-2587 (1985)). Since neither monoclonal antibody reacts with CR1 CCPs-1,2,8 or 9, all of the mutants derived from either CR1-4 or CR1-4(8,9) could be quantitated using this assay. For each mutant protein at least three binding assays from different transfections were performed.

Assay for Cofactor Activity

C3 and C4 were purified according to the method of Dykman, et al., Proc. Natl. Acad. Sci. USA 80, 1698-1702 (1983), Dykman, et al., J. Exp. Med. 157, 2160-2165 (1983) or purchased (Quidel, San Diego, CA), converted to C3b and C4b and labelled with ¹²⁵I using Iodogen coated beads (Pierce). Cofactor assays were performed using 200 ng of labelled C3b or C4b, 60 ng of factor I (Quidel) and media with mutant proteins. Amounts of the cofactor proteins were estimated in ELISA assay based on a standard curve of secreted CR1 (sCR1, Weisman, et al., Science 249, 146-151 (1990)). To test for cleavage of C3b, samples containing approximately 6 pg of the mutant proteins were incubated for 1 hr at 37°C. To test for cleavage of C4b, samples were incubated for up to 16 hrs at 37°C. After incubation, samples were reduced by boiling in the buffer containing 2% SDS and 5% beta-mercaptoethanol in 0.25% TRIS, pH 6.8 and electrophoresed on a 4-20% SDS-PAG (Integrated Separations) or on a 10% self-made gel. After drying the gels were autoradiographed at -70°C using an intensifying screen.

Preparation and Administration of Pharmaceutical Compositions

Complement activation can account for substantial tissue damage in a wide variety of autoimmune/immune complex mediated syndromes such as systemic lupus erythematosus, rheumatoid arthritis, hemolytic anemias, myasthenia gravis and others. Inhibition of the complement system is a desirable therapeutic intervention in these cases. In some instances, specific inhibition of the classical pathway alone by RCA analogs could be preferred since long-term inhibition of the alternative pathway could lead to side effects.

Inhibition of complement activation could also be desirable in cases that involve tissue damage brought about by vascular injury such as myocardial infarction, cerebral vascular accidents or acute shock lung syndrome. In these cases, the complement system may contribute to the destruction of partially damaged tissue as in reperfusion injury. Highly stringent inhibition of complement for relatively brief periods might be preferred in these instances and soluble RCA analogs designed for higher potency may prove especially useful.

The proteins encoded by the RCA gene cluster can be prepared recombinantly and used in diagnosis and therapy for the regulation of the complement system. The problems of transplantation of xenografts are reviewed by Platt, J.L., et al., in Immunology Today (1990) 11:450-457. Evidence has accumulated that the immediate hyperacute rejection of discordant xenografts is caused by recipient complement activity. Transgenic animals expressing human complement regulators (such as DAF or MCP) on cell surfaces could be an abundant source of organs that would be protected from hyperacute rejection in human recipients. A soluble complement inhibitor could also play a role in protecting xenografts from complement-mediated rejection.

Complement inhibition may also prove important in the prevention of xenograft rejection. Organs derived from animals transgenic for human DAF or MCP may be protected at least in part from complement-mediated hyperacute rejection by the expression of transgenic DAF or MCP on the cell surfaces of the xenograft. Animals transgenic for RCA analogs designed for higher potency may provide more successful xenografts. Soluble RCA analogs may also prove useful in protecting the transplant in the recipient.

Erythrocyte CD35 functions as an immune adherence receptor. It binds, processes and transports C3b/C4b bearing immune complexes to the fixed phagocyte system in liver and spleen, Herbert, L.A. *Am J Kidney Dis* 17:353-61 (1991), Nelson, R.A. Science 118:733-7 (1953), and Schifferli, J.A., et al. Kidney Int 35:993-1003 (1989). Some

microorganisms become coated with C3b, for example *Leishmania*, *Mycobacterium*, and HIV, and then use CD35 to enter host cells, Cooper, N.R. *Immunol Today* 1991; 12:327-31, and Thieblemont, N., et al. *Clin Exp Immunol* 1993; 92:106-13. In illnesses with circulating immune complexes like systemic lupus erythematosus, Atkinson, J.P., *LeRoy, E.C. Ed. M. Dekker*: New York, 1992; pp 525-46, and HIV infection, Jouvin, M.H., et al. *Aids* 1987; 1:89-94, and Munson, L.G. *Clin Immunol Immunopathol* 1995; 75:20-5, CD35 numbers on B-cells and erythrocytes are reduced in parallel with disease activity. CD35 mediates a rosette phenomenon between *Plasmodium falciparum* infected erythrocytes and normal erythrocytes, a marker of severe malaria, Rowe, J.A., et al. *Nature* 1997; 368:292-5. Accordingly, these CR1 analogs may be useful in treating autoimmune diseases or infections with viruses or parasitic infections.

Soluble analogs having decreased activity may also be useful as competitive inhibitors of the natural inhibitors, in cases where an increased complement mediated response is desirable or where an individual has a disorder in which their immunity is compromised by overproduction of the natural inhibitors.

The ability of a recombinant soluble form of CR1 to inhibit inflammation in the reversed passive Arthus reaction in rats was described by Yeh, C.G., et al., *J. Immunol* (1991) 146:250-256. This soluble CR1 was obtained from Chinese hamster ovary (CHO) cells expressing a CR1 genetic construct which had been mutated to remove the transmembrane and cytoplasmic domains. The ability of a similar soluble CR1, produced recombinantly in CHO cells, to inhibit post-ischemic myocardial inflammation and necrosis in rats was reported by Weissman, H.F., et al., *Science* (1990) 249:146-151.

Recombinant soluble (stop codon placed before transmembrane domain) CR1 inhibits unwanted complement activation in autoimmune diseases, Piddlesden, S.J., et al. *J Immunol* 1994; 152:5477-84, and Piddlesden, S.J., et al. *J Neuroimmunology* 1996; 71:173-7. It also reduces complement induced tissue damage in conditions such as ischemia/reperfusion injury and prevents hyperacute xenograft rejection, Mulligan, M.S., et al. *J Immunol* 1992; 148:1479-85, Pruitt, S.K., et al. *Transplantation* 1991; 52:868-73, and Weisman, H.F., et al. *Science* 1990; 249:146-51. Because of these animal studies and successful proof of concept investigations in man, Liszewski, M.K., et al. *Exp Opin Invest Drugs* 1998; 7:323-32, and Liszewski, M.K., et al. *Clin Immunol Newsletter* 1998; 15:291-4, sCR is in clinical trials.

The most desirable analogs based on the *in vitro* assays are tested *in vivo*. In

general, the *in vitro* assays are accepted as highly correlated with the corresponding *in vivo* activity. The appropriate dosages are determined by comparing the *in vitro* activity of the naturally occurring protein with that of the analog, comparing the *in vitro* activity of the naturally occurring protein with the *in vivo* activity of the naturally occurring protein, then calculating the expected *in vivo* activity of the analog, adjusting for any measured differences in half-life.

The analogs can be administered locally or systemically in pharmaceutically acceptable carriers such as saline, phosphate buffered saline, or a controlled release formulation. The dosage level and mode of administration of the analogs depend on the nature of the analog, the nature of the condition to be treated, and the history of the individual patient. Systemic administration is generally required, which may be by injection or by transmucosal or transdermal delivery. Administration by injection may be intravenous, intramuscular, intraperitoneal or subcutaneous. Formulations for injection are generally biocompatible solutions of the active ingredient such as Hank's solution or Ringer's solution. Formulations for transdermal or transmucosal administration generally include penetrants such as fusidic acid or bile salts in combination with detergents or surface-active agents. The formulations can then be manufactured as aerosols, suppositories, or patches. Oral administration is generally not favored for protein or peptide active ingredients; however, if suitably formulated so as to be protected from the digestive enzymes, oral administration can also be employed.

Suitable formulations for a desired mode of administration can be found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA. The dosage levels and precise formulations are obtainable by routine optimization procedures as is generally known in the art.

Diagnostic Applications

The analogs which are capable of binding C3b and/or C4b are useful as diagnostic tools in assessing the presence, absence or amount of C3b or C4b or C3b/C4b-bearing immune complexes in biological fluids. Such assays take advantage of the ability of the analog specifically to bind C3b and/or C4b and can be conducted in a variety of formats as is generally known. Formats for specific binding partner assays include direct and competitive formats, sandwich assays, and agglutination assays. Complexation between members of the specific binding pair can be conducted in solution or on a solid phase and can be detected using a variety of labeling techniques including

fluorescence, radioisotopes, chromophores, and visible particles.

Typical reagent kits useful in assays for C3b and/or C4b and/or C3b/C4b-bearing immune complexes include the analog specifically binding to the analyte, optionally coupled to a solid support and additional labeling reagents useful in the assay. For example, one of many formats for the assay might include treating the sample to be tested with a solid support to which is coupled the analog as a specific binding partner, washing the support which has been treated with sample suspected of containing analyte, and then treating the washed support with anti-C3b or anti-C4b antibody labeled with an enzyme such as horseradish peroxidase. The presence of labeled enzyme on a support is detected by addition of a substrate solution which results in the development of a color in the presence of the enzyme.

Example 1: Determination of Critical Sites for Modification of Activity(ies) of CR1.

EXPERIMENTAL PROCEDURES

Construction of mutants. Substitution mutants in the plasmid pSG5 (Stratagene, La Jolla, CA) were made by oligonucleotide directed mutagenesis using Muta-Gene™ M13 *in vitro* Mutagenesis Kit (Bio-Rad, Hercules, CA), Morph™ Plasmid DNA Mutagenesis (5 Prime→3 Prime, Boulder, CO) or QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Only the first method requires a single stranded vector and a subcloning step. In the other methods, mutagenesis was performed directly in the double stranded plasmid without subcloning. LHR B was obtained from LHR A by changing amino acids in CCP 1-3 into those present in CCP 8-10 (Krych, et al., *J. Biol. Chem.* 269, 13273-13278 (1994)). ΔCCP 1, lacking amino acids 1-60, and ΔCCP 2, lacking amino acids 61-122, were described by Krych, et al., *Proc. Natl. Acad. Sci. USA* 88, 4353-4357 (1991). The construct CCP 1-3 was made by replacing 195th amino acid (the fourth position after the last C of CCP 3) in the protein LHR A with a STOP codon. The construct 8-10 was made by replacing 645th amino acid (the fourth position after the last C of CCP 10) in the protein LHR B with a STOP codon. The strategy for homologous substitution mutagenesis of CCP 1 and 2 in LHR A was described by Krych, et al., (1991) and (1994) and is shown in Figure 2.

The strategy for mutagenesis of CCP 10 was based on the observation that binding and CA of SITE 2 were abrogated when CCP 10 was replaced with CCP 3 of CR2 (Kalli, et al., *J. Exp. Med.* 174, 1451-1460 (1991)). Therefore, to identify functionally important residues in

CCP 10 amino acids were mutated, a few at a time, into their homologs in CCP 3 of CR2 (Figure 4).

Construction of LHR C. CR1 cDNA in Ap^rM8, obtained from Lloyd Klickstein (Harvard Medical School, Boston, MA), served as a template for PCR amplification of the signal peptide and of CCP 15-21 (LHR C). 5' primer for amplification of the signal peptide, ATA TAC AGA TCT ATG GGA GCC TCT TCT CCA AGA, introduces an upstream BglII site (underlined) for cloning to the expression vector pSG5 (Stratagene). The 3' primer ACA GTG ACC TAG GCC CCA TGC AAC TGG TAG CGC AAG CA introduces five silent mutations to generate AvrII site (underlined) for ligation to LHR C and to disrupt GC rich stretches. For amplification of LHR C, the 5' primer, ATA TCT ATC ATC CTA GGT CAC TGT CAA GCA CCA, was used. It includes codons for four amino acids between the last cysteine of CCP 14 and the first cysteine of CCP 15. Due to three silent substitutions, it creates an AvrII site (underlined) and interrupts sequence of four C nucleotides. The 3' primer for LHR C, AAT ATT ATA GAT CTT TAA CCA GCA CGA ACA GAA AGT TC, includes codons for five out of six amino acids between the last cysteine of CCP 21 and the first cysteine of CCP 22 followed by translation STOP codon and BglII site (underlined). Each PCR product was subcloned into TA cloning vector (Invitrogen, San Diego, CA), sequenced and cut out with AvrII and BglII. The signal peptide and LHR C fragments were then ligated through AvrII site and the ligation product cloned into BglII site of pSG5.

Expression of CR1 derivatives in COS 7 cells. Plasmids with cDNA encoding CR1 derivatives were transfected into COS 7 cells using Lipofectamine (Life Technologies, Grand Island, NY) according to manufacturer's procedure. Twenty four h after the transfection, cells were washed and OPTI-MEM I medium without serum was added to avoid possible CA in bovine serum. After 48 to 72 h incubation supernatants were collected, aliquoted and stored at -70° C until use.

Binding assays. A modification of the previously described procedure was used (Krych, et al. 1991). iC3 and C4b, isolated as described (Dykman, et al., *Proc. Natl. Acad. Sci USA* 80, 1698-1702 (1983); Dykman, et al., *J. Exp. Med.* 157, 2160-2165 (1983)), were coupled at 1 mg/ml to cyanogen-bromide activated SepharoseTM (SepharoseTM 6B, Pharmacia LKB Biotechnology Inc., Piscataway, NJ). iC3, C3 with a disrupted thioester bond, has the same reactivity with CR1 as does C3b. To 200 µl of iC3- or C4b-SepharoseTM (iC3-S or C4b-S), medium (1 ml) from transfected cells containing a CR1 derivative was added after adjusting the salt and protein concentration. Following a 30 min incubation at room temperature with

occasional mixing, the columns were washed with 850 μ l of appropriately diluted PBS containing 1% NonidetTM P-40 and the proteins bound to iC3-SepharoseTM or C4b-SepharoseTM were eluted with 1 ml of 300 mM NaCl containing 1% NonidetTM P-40. The levels of CR1 derivatives in the eluates were determined by sandwich ELISA using mAb 3D9 (O'Shea, et al., *J. Immunol.* 134, 2580-2587 (1985)) coated wells and mAb E11 (a gift from Ronald Taylor, University of Virginia School of Medicine, Charlottesville, VA) (Hogg, et al., *Eur. J. Immunol.* 143, 236-243 (1984)), conjugated to horse radish peroxidase. For quantification of the mutants not recognized by 3D9, a rabbit polyclonal Ab to human CR1 (Makrides, et al., *J. Biol. Chem.* 267, 24754-24761 (1992)) was used. Results are expressed as percent of CR1 derivative bound to iC3-S or C4b-S of that initially offered to the SepharoseTM.

Effect of mAb 8C9.1 (a gift from Henry Marsh, T Cell Sciences, Inc., Needham, MA) on ligand binding was analyzed in the binding assay described above. The medium containing the CR1 derivative and the mAb (1 μ g/ml) was added to the SepharoseTM affinity columns.

Mapping 8C9.1 and 3D9 epitopes. The 8C9.1 epitope was mapped by ELISA in which wells were coated with 3D9. Bound LHR A derivatives were then detected with biotinylated 8C9.1 followed by horse radish peroxidase conjugated to extravidin (Sigma Chemical Co., St. Louis, MO). The reduced binding (to mutants 14e and 15a) was assessed by comparing the results of this assay with the standard ELISA which uses E11 as a detection Ab.

The 3D9 epitope was mapped by standard ELISA in which wells were coated with 3D9 and E11 was used to detect bound proteins. The reduced binding (to mutants 20d and 22a) was measured by comparing these results with those in which wells were coated with polyclonal Ab and E11 served as a detection Ab.

Cofactor assays. C3b and C4b (Advanced Research Technologies, San Diego, CA) were biotinylated by incubating 125 μ g of C3b or C4b with 3.4 μ g of NHS-LC biotin (Pierce, Rockford, IL) for 1 h at room temperature followed by removal of unincorporated biotin on MicroconTM 30 microconcentrators (Amicon, Beverly, MA). Biotinylated C3b or C4b (250 ng) were mixed with 50 ng of factor I (Advanced Research Technologies) and with medium from transfected cells containing 0.002 pmoles of a CR1 derivative in 25 mM NaCl. The cofactor protein was limiting reagent in this assay system. A negative control containing all components except factor I was always included. After incubation at 37° C, for 1 hr for C3b and 16 hr for C4b, the samples were separated on 10% polyacrylamide gel (Novex, San Diego, CA) following by a transfer of the proteins to Immun-Lite membranes using a Bio-Rad semidry transfer apparatus. The biotinylated proteins were incubated with extravidin-HRP and

visualized using the Supersignal CL-HRP substrate system* (Pierce).

RESULTS

The three initial CCP of LHR A, B and C are functional if attached to irrelevant CCP of CR1 or to an unrelated protein (Klickstein, et al., J. Exp. Med. 168, 1699-1717 (1988); Kalli, et al., 1991)). Proteins composed only of CCP 1-3 or CCP 8-10 were tested. The specificity of binding was identical to the parental proteins in that CCP 1-3 bound C4b and CCP 8-10 bound C4b and C3b. Ligand binding was reduced by 10-30%² as compared to their parental proteins LHR A and LHR B. For example, at 25 mM NaCl a 30% decrease was observed as 80% of LHR B versus 50% of CCP 8-10 bound to the iC3-S ligand. This result is consistent with the data of Kalli et al. (1991) who showed that the presence of CCP 11 (i.e., binding of a construct bearing CCP 8-10 versus one with CCP 8-11) increased the affinity of SITE 2 for C3b.

Mutations in CCP 1 and 2

Effect on binding and Cofactor Activity (CA). Binding and CA of mutants 14 and 15 (See Fig. 2) were substantially increased relative to the parental protein LHR A. To identify residues responsible for this increase, mutants with single amino acid changes were constructed within the context of LHR A (Fig. 2 and Table I). The 20-47% binding of the mutants represented a modest (3-30%) increase over 17% binding of LHR A. None of them, however, approached the 86% binding of mutant 14. The effect on C4b binding was similar in that there was a 6-23% increase in binding for individual mutants versus a 42% for mutant 14 (Table I). Of the single amino acids substitutions, T₁₄→K (mutant 14c) augmented binding of iC3 and C4b the most. CA for C3b and C4b was increased in mutant 14a but not detectably changed in mutants 14b-j. For mutant 15, the change of N₂₉→K (mutation 15b) accounted for all of the enhanced iC3 and C4b binding (Table I) and CA for C3b and C4b observed in mutant 15. The other mutation, Y₂₇→S (mutant 15a), had no effect (Table I).

Individual residues were also altered. Substitution of D₁₀₉ with N within the context of LHR A (mutant 10a) caused a marked enhancement of iC3 and C4b binding, above that of LHR B and to a level similar to that of soluble CR1 (sCR1) (Fig. 3). Also, CA for C4b and C3b was similar to LHR B. The other three single amino acids mutations in region 10 produced minimal or no change in binding or CA. That changing D to N caused a major effect suggested a role for the amide group. To test this further, D₁₀₉ was changed to Q because, like N, Q has the amide group but is larger. Two other replacements of D₁₀₉ were made with amino acids of a similar size, namely nonpolar V and polar T. The iC3 binding capability of all three

mutants at 25 mM NaCl was intermediate (41-49%) between binding of LHR A (12%) and 10a (91%) (Fig. 3). The greater effect of N as compared to V or T is consistent with the critical role of the amide. Since N leads to higher binding than Q, the size of the amino acids may play a role as well. Finally, the low binding of the wild type protein (LHR A) suggests that negative charge inhibits the interaction with iC3.

For C4b binding, each of the four substitutions for D₁₀₉ led to an increase above the level of LHR A but none had as dramatic an effect as did N on iC3 binding.

mAb 8C9.1 recognizes CCP 1 and enhances iC3 and C4b binding by LHR A. Of 20 mAb to CR1 tested (Nickells, et al. *Clin. Exp. Immunol.* 1998), 8C9.1 was the only one specific for LHR A, suggesting that its epitope is in CCP 1 or 2. By testing LHR A deleted of CCP 1 or 2, the 8C9.1 epitope was localized to CCP 1 (Table II). To map the epitope more precisely, the substitution mutants in CCP 1 were mapped and it was found that sequences 14 and 15 are necessary for 8C9.1 binding (Table II). Further mapping, using single amino acids mutants in sequences 14 and 15, demonstrated that mutations 14b, c, d and h and 15b abrogate 8C9.1 binding while mutations 14e and 15a significantly reduce it (to less than 20% relative to E11, Table II). An unusual feature of this mAb is that it enhances binding by LHR A. At 25 mM the mAb caused an increase in iC3 binding from 4 to 30% while C4b binding was augmented from 27 to 60%.

Mutations in CCP 10

The mutants constructed are shown in Fig. 4.

Effect on binding and CA. The results of iC3 and C4b binding and CA for C3b and C4b of mutants in CCP 10 are summarized in Tables III and IV and Fig. 5. Mutants 20 and 22 had barely detectable iC3 and C4b binding ability while mutant 19 had markedly reduced iC3 binding (Fig. 5). A comparison of binding and CA results (Tables IV and V) indicate that they do not always coincide. For example, mutants 16, 17 and 19 bound C4b similarly to parental protein LHR B but lacked (mutants 16 and 17) or had minimal (mutant 19) CA for C4b. In mutant 16, C3b binding was largely preserved but CA was considerably diminished.

Individual substitutions were next made in sequence 20 because it is critical for binding of both ligands and in sequence 19 because it is important for iC3 binding only (mutations 20 a-f and 19 a-d in Tables III and IV and in Fig. 4). Within sequence 20, mutation 20c, Y₅₉₆→P, produced a marked (about 60-70% at 50 mM) reduction of both iC3 and C4b binding. Mutation R₆₀₃→S (20f) reduced only iC3 binding by 30% at 25 mM and V₅₉₉→S (20e) reduced only C4b binding by 50% at 25 mM. Within region 19, mutations T₅₈₉→E (19a) and

R₅₉₁→V (19b) reduced iC3 binding (Table III).

The presence of P in position 22a (Fig. 4) in LHR A, B and C but not in LHR D suggested it might be important. However, mutation P₆₂₀→K had a minimal influence on binding or CA for C3b but did lead to a marked reduction in CA for C4b (Table IV).

Effect on 3D9 binding. mAb 3D9 blocks C3b and C4b binding (Krych, et al., 1994; O'Shea, et al., 1985), CA and DAA. Previously, it was reported that 3D9 recognizes LHR A as well as LHR B (Krych, et al. 1991; Krych, et al. 1994). The epitope is in CCP 3 and 10 because deletion of CCP 3 or CCP 10 from LHR A or LHR B, respectively, abrogates 3D9 binding (Table V). To map the epitope more precisely, mutants in CCP 10 were tested. Mutants 20, 21 and 22 did not bind 3D9 (Table V). Within sequence 20, only mutation R₆₀₃→S (20f) abrogated 3D9 binding. Mutation S₅₉₈→L (20a) and P₆₂₀→K (22a) reduced binding by approximately 50%.

Comparison of LHR B with LHR C. The copy of SITE 2 present in LHR C differs by three amino acids from the one in LHR B. One difference is in CCP 9 and 16 and it was demonstrated earlier that it has no effect on binding or CA (see mutant 10b(r) in Krych, et al. 1994). The other two amino acids differences are between CCP 10 and 17, specifically within peptide 21 (Fig. 4). In CCP 17, L₁₀₅₆ and R₁₀₅₉ are present instead of P₆₀₆ and G₆₀₉, respectively, in CCP 10. To test the effect of these differences on binding, LHR B and C were compared. LHR C binds iC3 and C4b 10-40% more efficiently at 12.5 - 100 mM NaCl. These observations provide additional support for a role of sequence 21 in ligand binding.

The present invention will be further understood by reference to the following tables VI-VIII.

TABLE I
Amino acids responsible for increase in ligand
binding by mutants 14 and 15

Construct	Binding ^a	
	iC3	C4b
	%	
LHR A	17	31
LHR B	80	53
Mutants in CCP 1		
14	86	73
a (R12K)	31	39
b (P13L)	25	38
c (T14K)	47	54
d (N15T)	39	46
e (L16Q)	20	37
g (D18N)	36	46
h (E19A)	36	49
i (F20S)	35	41
j (E21D)	30	42
15	66	65
a (Y27S)	9	27
b (N29K)	63	62

^a iC3 binding was performed at 12.5, 25, and 100 mM NaCl. C4b binding was performed at 12.5, 25, and 50 mM NaCl. Protein quantification was by ELISA. Results at 25 mM NaCl are shown. In all tables, % binding refers to the fraction of a CR1 derivative offered to iC3-S (or C4b-S) that bound to it.

TABLE II
Localization of 8C9.1 epitope in CCP 1

Construct	8C9.1 binding ^a
LHR A	+
Δ CCP 2	+
Δ CCP 1	-
Mutants in CCP1	
1	+
2	+
3	+
4	+
12	+
13	+
14	-
a	+
b	-
c	-
d	-
e	+/-
g	+
h	-
i	+
j	+
15	-
a	+/-
b	-

^a +, binding equivalent to E11; +/-, <20% of E11 binding; -, no detectable binding. Binding assay was performed twice with identical results.

TABLE III
Summary of iC3 binding and CA for mutants in CCP 10

Construct	iC3 binding ^a	CSb CA ^b
LHR B	++++	++++
Mutants in CCP 10		
16	+++	+
17	+++	+++
18	+++	+++
19	+	+++
a (T589E)	++	++++
b (R591V)	++	+++
c (E592G)	++++	+++
d (N593S)	++++	++++
20	+	-
a (F594I)	++++	++++
b (H595A)	++++	++++
c (Y596P)	+++	+
d (S598L)	++++	++++
e (V599S)	++++	++++
f (R603S)	+++	++++
21	++	+++
22	+	-
a (P620K)	++++	++++
23	++	+++
24	++++	++++

^a Experiments were performed twice at 12.5, 25, 50, and 100 mM NaCl. Protein quantification was by ELISA. Results of binding at 25 mM NaCl are shown. +++++, more than 80% bound; +++, 40-80%; ++, 20-40%; +, less than 20%.

^b Each experiment was done twice at 25 mM NaCl. Cofactor activity of LHR B was considered 100%, or +++++. Cofactor activities of other mutants are expressed as the percentage of LHR B activity: +++, 60-90%; ++, 30-60%; +, 10-30%; -, no activity detected.

TABLE IV
Summary of C4b binding and CA for mutants in CCP 10^a

Construct	C4b binding ^a	C4b CA ^b
LHR B	++++	++++
Mutants in CCP 10		
16	+++	-
17	+++	-
18	++++	++++
19	+++	+
a	++++	++++
b	++++	+++
c	++++	+++
d	++++	++++
20	+	+
a	++++	++++
b	++++	+++
c	++	++
d	++++	+++
e	+++	+++
f	+++	++++
21	++	+++
22	+	+
a	+++	+
23	+++	+++
24	+++	++++
25	++	+++

^a Experiments were performed twice at 12.5, 25, and 50 mM NaCl. Protein quantification was by ELISA. Results of binding at 25 mM NaCl are shown. +++++, 40-60% bound; +++, 30-45%; ++, 15-30%; +, less than 15%.

^b Each experiment was done twice at 25 mM NaCl. Cofactor activity of LHR B was considered 100%, or +++++. Cofactor activities of other mutants are expressed as the percentage of LHR B activity: +++, 60-90%; ++, 30-60%; +, 10-30%; -, no activity detected.

TABLE V
Localization of mAb 3D9 epitope in CCP 10

Construct	3D9 binding ^a
LHR A	+
Δ CCP 3	-
LHR B	+
Δ CCP 10	-
Mutants in CCP 10	
17	+
18	+
19	+
20	-
a	+
b	+
c	+
d	+/-
e	+
f	-
21	-
22	-
a	+/-
23	+
24	+
25	+

^a +, binding equal to that of LHR B; +/-, binding reduced by about 50%; -, no binding. Experiment was performed twice with identical results.

TABLE VIa. Functional assessment of the initial set of mutants derived from CR1-4 (CCP 1-8 1/2).

Mutant	iC3 binding	CA C3b	DAA for AP C3 convertase	C4b binding	CA C4b	DAA for CP C3 convertase
AA 1-543 (CR1-4)	+	+	+++	+++	++	+++
AA 1-60 deleted (Δ CCP 1)	-	-	-	-	-	+
AA 61-122 deleted (Δ CCP 2)	-	-	-	-	-	+
AA 123-194 deleted (Δ CCP 3)	-	-	-	-	-	+
AA 1-254 (CCP 1-4)	-	+	+++	+++	+	+++
AA 1-194 (CCP 1-3)	-	+	+++	+++	+	+++
35:G→E; 37:S→Y	+	++	+++	+	-	-
35:G→E	+	+	+	+	+	+
37:S→Y	++	+	+++	+++	++	+++
44,47,49:I...K...S→T...D...L	+	+	+++	+++	++	+++
52-54:T-G-A→S-S-P	+	+	+++	+++	++	+++
57,59:R...R→V...K	+	+	+++	+++	++	+++
64,65:R-N→K-T	+	+	++	+	-	++
64:R→K	+	+	+++	++	++	++
65:N→T	+	+	++	++	++	++
78,79:K-G→T-D	+	+	++	+++	++	++
85,87:Q...K→R...N	+	+	+++	+++	++	+++
92,94:K...Y→T...H	+	+	+++	+	-	++
94:Y→H	+	+	+++	+	++	+
99,103:S...T→H...E	+	+	++	+++	++	+
109-112:D-T-V-I→N-A-A-H	++	++	++	+++	+++	+++
114-117,121:D-N-E-T...D→S- T-K-P...Q	++	+	+++	+++	++	+++
114:D→S	+	+	+++	+++	++	+++
115:N→T	+	+	+++	+++	++	+++
116:E→K	++	+++	+++	+++	+++	+++
117:T→P	+	+	+++	+++	++	++
116,117:E-T→K-P	++	ND	ND	+++	ND	ND
121:D→Q	+	+	++	++	++	++

TABLE VIb. Functional assessment of the second set of mutants derived from LHR A (CCP 1-7).

Mutant	iC3 binding	CA C3b	DAA for AP C3 convertase	C4b binding	CA C4b	DAA for CP C3 convertase
AA 1-449 (CCP 1-7)	+	+	+++	+++	++	+++
1,3:Q...N→H...Q	+	+	+++	+++	++	+++
6-9:E-W-L-P→D-H-F-L	+	+	+	+++	+	++
6:E→D	+	+	+++	+++	+	+++
7:W→H	+	+	+	+++	++	+++
8:L→F	+	+	+++	+++	++	+++
9:P→L	+	+	+++	+++	++	+++
12-16,18-21:R-P-T-N-L...D-E-F-E→K-L-K-T-Q...N-A-S-D	+++	++	ND	++++	+++	ND
12:R→K	+	+	++	++	++	++
13:P→L	+	+	++	+++	++	++
14:T→K	+	+	++	+++	++	++
15:N→T	+	+	++	+++	++	++
16:L→Q	+	+	+	++	++	++
18:D→N	+	+	++	+++	++	++
19:E→A	+	+	++	+++	++	++
20:F→S	+	+	++	+++	++	++
21:E→D	+	+	++	+++	++	++
27,29:Y...N→S...K	++	++	ND	++++	+++	ND
27:Y→S	+	+	+++	+++	++	+++
29:N→K	++	++	+++	++++	+++	+++
79:G→D	+	+	+	+++	++	+
82:F→V	+	+	+	+++	+++	+
92:K→T	+	++	++	+++	++	++
99:S→H	+	+	+++	+++	++	++
103:T→E	+	+	++	+++	++	+

TABLE VIb. (continued)

Mutant	iC3 binding	CA C3b	DAA for AP C3 convertase	C4b binding	CA C4b	DAA for CP C3 convertase
109: D→N	+++++	+++	+++	+++	+++	+++
109: D→T	+++	ND	ND	+++	ND	ND
109: D→Q	+++	ND	ND	+++	ND	ND
109: D→V	+++	ND	ND	+++	ND	ND
110: T→A	+	+	+++	+++	++	++
111: V→A	+	+	+++	+++	++	+
112: I→H	+	+	+++	+++	++	+++
109-112,114-117,121: D-T-V-I...D-N- E-T...D→N-A-A-H ...S-T-K-P...Q	+++	+++	++	+++	+++	++
109,116:D...E→N...K	+++++	+++	++++	+++	+++	+++

TABLE VIIc. Functional assessment of LHR AC and its derivatives.

Mutant	iC3 binding	CA C3b	DAA for AP convertases		C4b binding	CA C4b	DAA for CP convertases	
			C3	C5			C3	C5
AA 1-449 followed by AA 899-1353 (LHR AC)	+++++	++++	+++	+++	++++	+++++	++++	+++
AA 1-254 followed by AA 899-1153 (CCP 1-4, 15-18)	ND	ND	++++	++	ND	ND	++++	++
AA 1-254 followed by AA 899-1353 (CCP 1-4, 15-21)								
AA 1-193 followed by AA 899-1353 (CCP 1-3, 15-21)								
AA 1-448 followed by AA 1-447 (CCP 1-7, 1-7 or LHR A, LHR A)								

TABLE VIIa. Functional assessment of the mutants derived from LHR B (CCP 8-14).

Mutant	iC3 binding	CA C3b	C4b binding	CA C4b
AA 451-899 (CCP 8-14)	+++	+++	+++	+++
AA 573-644 (Δ CCP 10) deleted	-	-	-	-
AA 451-704 (CCP 8-11)	ND	++	ND	++
AA 451-642 (CCP 8-10)	++	++	++	++
451,453:H...Q→Q...N	++++	++++	++++	++++
456-459:D-H-F-L→E-W- L-P	++++	++++	++++	++++
462-466,468-471:K-L-K- T-Q...N-A-S- D→R-P-T- N-L...D-E-F-E	++	+++	++	+++
477,479:S...K→Y...N	+++	+++	+++	+++
487:Y→S	+++	+++	+++	+++
494,497,499:T...D...L→ I...K...S	+++	+++	+++	+++
502-504,507,509:S-S- P...V...K→T-G- A...R...R	++++	++++	++++	++++
485,514-515,544:E...K- T...H→G...R-N...Y	+++	+++	+++	+++
528-529,532:T-D...V→K- G...F	+++	+++	+++	+++
535,537:R...N→Q...K	+++	+++	+++	+++
542,544:T...H→K...Y	++++	++++	++++	++++
549,553:H...E→S...T	+++	+++	+++	+++
559-562:N-A-A-H→D-T-V-I 564-567,571:S-T-K- P...Q→D-N-E-T...D	++	++	++	++
559-562,564-567,571:N-A- A-H...S-T-K-P...Q→D-T- V-I... D-N-E-T...D	++	+	++	++
	+	-	+	+

TABLE VIIa. (continued).

Mutant	iC3 binding	CA C3b	C4b binding	CA C4b
559:N→D	++	++	++	+
560:A→T	++++	++++	++++	++++
561:A→V	++++	++++	++++	++++
562:H→I	++++	++++	++++	++++
564:S→D	++++	++++	++++	++++
565:T→N	++++	++++	++++	++++
566:K→E	+++	+	+++	+
567:P→T	++++	++++	++++	++++
571:Q→D	++++	++++	++++	++++
573-574:I-P→A-A	+++	+	+++	-
576-578,580,582: G-L-P...T...A→P-A- L...M...H	+++	+++	+++	-
585-587:D-F-I→H-H-T	++++	+++	+++	+++
589,591-593: T...R-E-N→E...V-G-S	+	+++	+++	+
589:T→E	+	+++	+++	+++
591:R→V	++	++	++	++
592:E→G	+++	+++	+++	+++
593:N→S	++++	++++	++++	++++
594-596,598-599,603: F-H-Y...S-V...R→I-A- P...L-S...S	+	-	+	+
594:F→I	+++	+++	+++	+++
595:H→A	+++	+++	+++	+++
596:Y→P	++	+	++	++
598:S→L	+++	+++	+++	+++
599:V→S	++++	++++	++++	++++
603:R→S	++	++	++	++

TABLE VIIa. (continued).

Mutant	iC3 binding	CA C3b	C4b binding	CA C4b
605-607,610-615; N-P-G...G-R-K-V-F-	++	+++	++	+++
E→A-A-E...A-A-A-A- Y-L				
620-621,623:P-S...Y→K- I...N	+	-	+	+
620:P→K	+++	+++	+++	+
625,627-631,633:T...N-	++	++	+++	+++
D-D-Q-V...I→L...A-A- A-A-S...K				
636-638,640:G-P-	+++	+++	+++	+++
A...Q→A-V-P...T				
642-645:I-I-P-N→E-E-A-A	++	++	++	+++

TABLE VIIb Functional assessment of LHR BC and its derivatives.

Mutant	iC3 binding	CA C3b	DAA for AP C3 convertase	C4b binding	CA C4b	DAA for CP C3 convertase -
AA 451-1353 (LHR BC)	++++	++++	+	++++	++++	+
AA 451-704 followed by 899-1154 (CCP 8-11, CCP 15-18)	ND	ND	ND	ND	ND	ND

TABLE VIII. Functional assessment of mutants derived from LHR C (CCP 15-21).

Mutant	iC3 binding	CA C3b	C4b binding	CA C4b
AA 898-1353 (CCP 15-21)	++++	++++	++++	+++
AA 898-1353 Nglyc ⁻	++++	++++	++++	ND
(CCP 15-21 Nglyc ⁻)				
AA 901-1095 (CCP 15-17)	+++	++++	++++	+++
AA 901-1095 Nglyc ⁻	++++	++++	++++	ND
(CCP 15-17 Nglyc ⁻)				

We claim:

1. An analog of a protein regulating complement activation selected from the group of CR1 analogs consisting of
 - modifications of CR1 LHR A (CCP1-7), 6:E D; 7:W H; 8:L F; 9:P L; 82:F V; 99:S H; 103:T E; and 109,116:D... E N...K;
 - modifications of CR1 LHR AC amino acids 1-449 followed by 899-1353;
 - CCP 1 4, 15-18 amino acids 1-254 followed by amino acids 899-1153;
 - CCP 1-4, 15-21 amino acids 1-254 followed by amino acids 899-1353; CCP 1-3, 15-21 amino acids 1-193 followed by amino acids 899-1353; CCP 1--7, 1-7 or LHR A, LHR A amino acids 1-448 followed by amino acids 1-447;
 - LHR BC amino acids 451-1353; CCP 8-11, CCP 15-18 amino acids 451-704 followed by 899-1154, and truncated or hybrid forms thereof.
2. The analogs of claim 1 in soluble form.
3. The analogs of claim 1 further comprising a pharamaceutically acceptable carrier.
4. A nucleic acid molecule encoding a protein regulating complement activation selected from the group of CR1 analogs consisting of
 - modifications of CR1 LHR A (CCP1-7), 6:E D; 7:W H; 8:L F; 9:P L; 82:F V; 99:S H; 103:T E; and 109,116:D... E N...K;
 - modifications of CR1 LHR AC amino acids 1-449 followed by 899-1353;
 - CCP 1 4, 15-18 amino acids 1-254 followed by amino acids 899-1153;
 - CCP 1-4, 15-21 amino acids 1-254 followed by amino acids 899-1353; CCP 1-3, 15-21 amino acids 1-193 followed by amino acids 899-1353; CCP 1--7, 1-7 or LHR A, LHR A amino acids 1-448 followed by amino acids 1-447;
 - LHR BC amino acids 451-1353; CCP 8-11, CCP 15-18 amino acids 451-704 followed by 899-1154, and truncated or hybrid forms thereof.
5. The nucleic acid molecule of claim 4 further comprising an expression vector.
6. The nucleic acid molecule of claim 4 in a construct for making a transgenic animal.
7. The nucleic acid molecule of claim 4 in an expression system which is capable, when transformed into a compatible recombinant host cell, of expressing a DNA encoding the analog; the expression system comprising

a DNA encoding the analog operably linked to control sequences compatible with the host.

8. The nucleic acid molecule of claim 4 stably incorporated into the genome of a transgenic animal.

9. The use of an analog of a protein regulating complement activation selected from the group of CR1 analogs consisting of

modifications of CR1 LHR A (CCP1-7), 6:E D; 7:W H;

8:L F; 9:P L; 82:F V; 99:S H; 103:T E; and 109,116:D... E N...K;

1,3:Q...N H...Q; 6-9:E-W-L-P D-H-F-L; 12:R K; 13P L; 14:T K;

15:N T; 16:L Q; 18:D N; 19:E A; 20:F S; 21:E D; 109:D N;

109:D T; 109:D Q; 109:D V; 110:T A; 111:V A; 112:I H;

modifications of CR1 LHR AC amino acids 1-449 followed by 899-1353; CCP 1 4, 15-18 amino acids 1-254 followed by amino acids 899-1153; CCP 1-4, 15-21 amino acids 1-254 followed by amino acids 899-1353; CCP 1-3, 15-21 amino acids 1-193 followed by amino acids 899-1353; CCP 1--7, 1-7 LHR A, LHR A amino acids 1-448 followed by amino acids 1-447;

modifications of LHR BC amino acids 451-1353; CCP 8-11, CCP 15-18 amino acids 451-704 followed by 899-1154, CCP 8-11 amino acids 451-704; 573-574:I-P A-A; 576-578,580,582:G-L-P...T... A P-A-L...M...H; 585-587:D-F-I H-H-T; 589,591-593:T...R-E- N E...V-G-S; 589:T E; 591:R V; 592:E G; 593:N S; 594-596,598-599,603:F-H-Y...S-V...R I-A-P...L-S...S; 594:F I; 595:H A; 596:Y P; 598:S L; 599:V S; 603:R S; 605-607,610-615:N-P-G...G-R-K-V-F- E A-A-E...A-A-A-A-Y-L; 620-621,623:P-S...Y K-I...N; 620:P K; 625,627-631,633:T...N-D-D-Q-V...I L...A-A-A-A-S...K; 636-638,640:G-P-A...Q A-V-P...T; 642-645:I-I-P-N E-E-A-A;

modifications of CCP 1-4, amino acids 1-254, CCP 1-3, amino acids 1-194;

modifications of LHR C, CCP 15-21 amino acids 898-1353; CCP 15-21 Nglyc-, amino acids 898-1353 Nglyc-; CCP 15-17, amino acids 901-1095; CCP 15-17 Nglyc-, amino acids 901-1095;

and truncated or hybrid forms thereof, in medicine or

diagnostics.

10. The use of claim 9 wherein the analog is administered to a patient in need thereof in an amount effective to treat an inflammatory or complement mediated disorder.

11. The use of claim 9 wherein the analog is administered to a patient in need thereof in an amount effective to inhibit a viral or parasitic infection.

12. The use of claim 9 wherein the analog is administered to a patient in need thereof in an amount effective to decrease organ or tissue rejection.

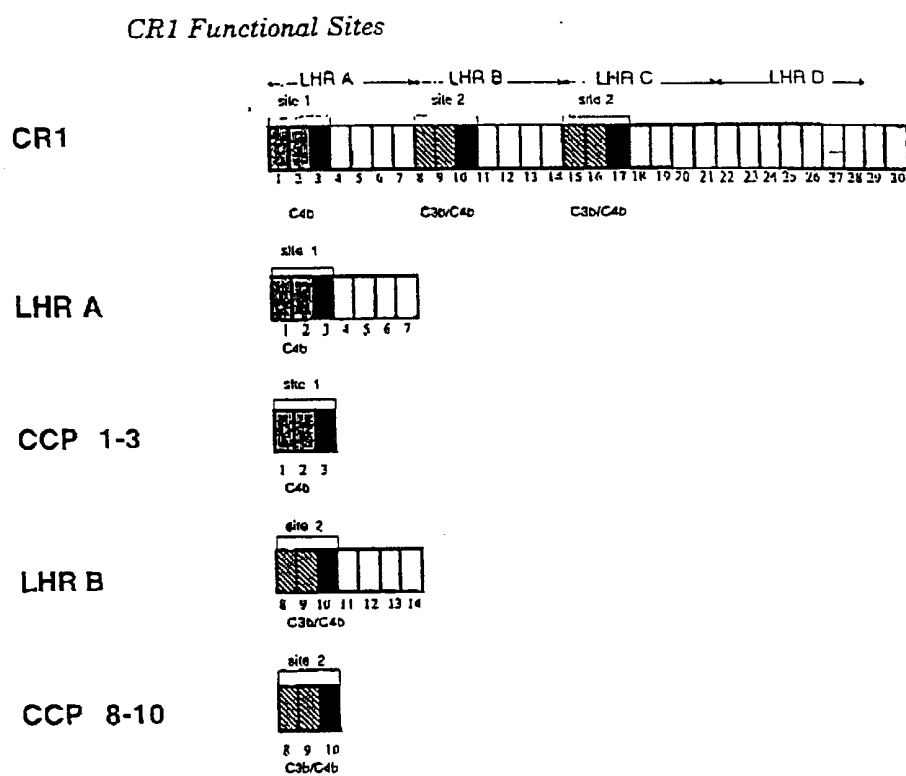


FIGURE 1

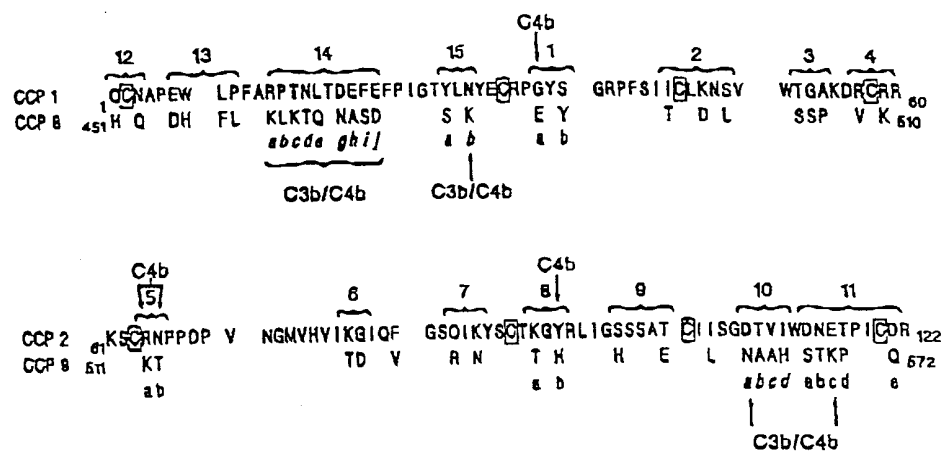


FIGURE 2

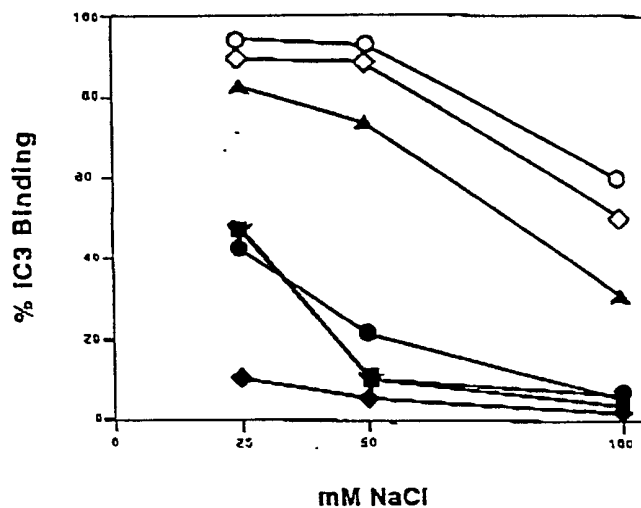


FIGURE 3

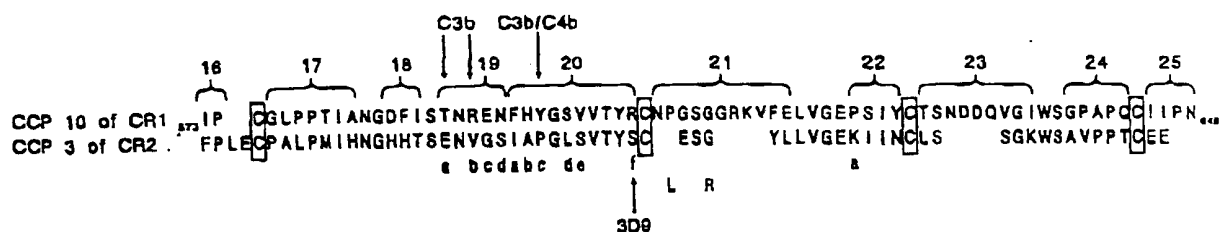


FIGURE 4

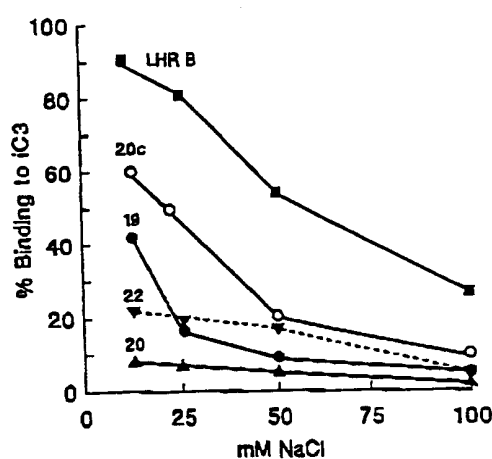


FIGURE 5a

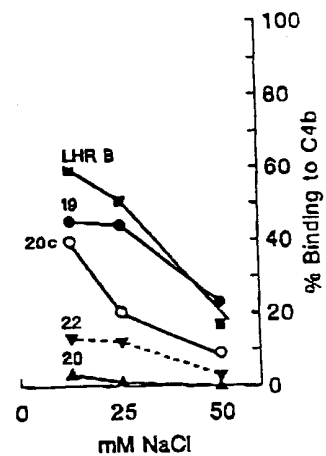


FIGURE 5b