Structure-Function Analysis of the Active Sites of Complement Receptor Type 1*

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Two functionally distinct but homologous sites in complement receptor type 1 (CR1) (CD35) were further 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. We also mapped epitopes 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.

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 (1). 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 (2, 3). 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 (4). 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 (5). 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 (6–9), 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.

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 (10). 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.

There are two functionally distinct active sites in CR1. Site 1 is located in CCPs 1–3 of LHR A. Site 2 is in CCPs 8–10 of LHR B, and its nearly identical copy (different by only three amino acids (aas)) is in CCPs 15–17 of LHR C. Site 1 binds mainly C4b (6, 11–13). It has barely detectable CA for cleavage of C4b and C3b. Site 2 binds both C3b and C4b and possesses CA for both (12, 14). The aa sequences of the first two CCPs of site 1 differ by ~40% from the first two CCPs of site 2, whereas the third CCP in both sites differs by one aa. To analyze by site-directed mutagenesis aas important for function of each site, we used two CR1 derivatives, LHR A, composed of CCPs 1–7, which contain site 1 and LHR B, composed of CCPs 8–14, which carries site 2 (Fig. 1). Previously, by interchanging aas in CCPs 1 and 2 with their homologs in CCPs 8 and 9, respectively, we identified peptides and in some cases aas important for ligand binding and CA (11, 12). In particular, replacement of three short peptides in site 1 led to a marked increase in ligand binding (11, 12). In the present report, we determine the aas responsible for this increase and further define the role of aa charge in the binding reaction. Furthermore, because in CR1, three CCPs are indispensable for the activity of each site, we expanded our studies of structure-function relationships to the third CCP. The approach to mutagenesis in this case was based on the observation of Kalli et al. (14) that the function of site 2 was abolished if CCP 10 was replaced by CCP 3 of CR2. Therefore, we substituted aas from CCP 10 of CR1 with their homologs from CCP 3 of CR2. Lastly, we mapped epitopes for two mAbs, one of which abrogates activity of both sites and one of which enhances activity of LHR A (site 1).
CR1 Functional Sites

Fig. 1. Schematic representation of CR1 derivatives and their binding domains. The extramembranous part of CR1 is composed of 30 CCPs (shown as boxes). Based on degree of homology, the first 28 CCPs can be organized into LHRs A, B, C, and D, each seven CCPs long, which arose through duplication of a seven-CCP unit. There are two distinct functional sites composed of three CCPs. Site 1 is located in LHR A. Two nearly identical copies of site 2 are present in LHRs B and C. The first two CCPs in site 1 (CCPs 1 and 2) are distinct (about 40% different) from the first two CCPs in site 2 (CCPs 8 and 9), and they are marked by different shading. There is only one aa difference between the third CCP (represented by a black box) in each site.

EXPERIMENTAL PROCEDURES

Construction of Mutants—Substitution mutants in the plasmid pSG5 (Stratagene, La Jolla, CA) were made by oligonucleotide-directed mutagenesis using Mutu-Gen M13 in vitro mutagenesis kit (Bio-Rad). Morph™ plasmid DNA mutagenesis kit (5 Prime — 3 Prime, Inc., 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 aa in CCPs 1–3 into those present in CCPs 8–10 (12). ACCP 1, lacking aa 1–60, and ACCP 2, lacking aa 61–122, were described earlier (11). The construct consisting of CCPs 1–3 was made by replacing the 195th aa (the fourth position after the last Cys of CCP 3) in the protein LHR A with a stop codon. The construct composed of CCPs 8–10 was made by replacing the 645th aa (the fourth position after the last Cys of CCP 10) in the protein LHR B with a stop codon. The strategy for homologous substitution mutagenesis of CCPs 1 and 2 in LHR A was described previously (11, 12) and is shown in Fig. 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 (14). Therefore, to identify functionally important residues in CCP 10, as were mutated, a few at a time, into CCP 3 of CR2 (14). This strategy was repeated with the second CCP of site 2 (CCPs 11 and 12). Replacement of CCP 10 with CCP 3 of CR2 was then repeated with the second CCP of site 1 (CCPs 21 and 22).

Expression of CR1 Derivatives in COS 7 Cells—Plasmids with cDNA encoding CR1 derivatives were transfected into COS 7 cells using LipofectAMINE (Life Technologies, Inc.) according to the 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 48–72 h of incubation, supernatants were collected, aliquoted, and stored at –70 °C until use.

Binding Assays—A modification of the previously described procedure was used (12). iC3 and C4b, isolated as described (15, 16), were coupled at 1 mg/ml to cyanogen bromide-activated Sepharose (Sepharose 6B, Amersham Pharmacia Biotech). iC3 (C9) with a disrupted thioester bond) has the same reactivity with CR1 as does C3b. To 200 µl of iC3-Sepharose (iC3-S) or C4b-Sepharose (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 µl of appropriately diluted PBS containing 1% Nonidet P-40, and the proteins bound to iC3-S or C4b-S were eluted with 1 ml of 300 mM NaCl containing 1% Nonidet P-40. The levels of CR1 derivatives in the eluates were determined by sandwich ELISA using mAb 3D9 (17), coated wells and mAb E11 (a gift from Ronald Taylor, University of Virginia School of Medicine, Charlottesville, VA) (18), conjugated to horseradish peroxidase. For quantification of the mutants not recognized by 3D9, a rabbit polyclonal antibody to human CR1 (19) was used. Results are expressed as a percentage of CR1 derivative bound to iC3-S or C4b-S of that initially offered to the Sepharose.

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 µg/ml) was added to the Sepharose 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 horseradish peroxidase conjugated to extravidin (Sigma). The reduced binding (to mutants 14e and 15a) was assessed by comparing the results of this assay with our standard ELISA, which uses E11 as a detection antibody.

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 antibody and E11 served as a detection antibody.

Cofactor Assays—C3b and C4b (Advanced Research Technologies, San Diego, CA) were biotinylated by incubating 125 µg of C3b or C4b with 3.4 µg of NHS-LC biotin (Pierce) for 1 h at room temperature followed by removal of unincorporated biotin on Microcon 30 microcon-
The three initial CCPs of LHRs A, B, and C are functional if attached to irrelevant CCPs of CR1 or to an unrelated protein (6, 14). We tested proteins composed only of CCPs 1–3 or CCPs 8–10. The specificity of binding was identical to the parental proteins LHR A and LHR B. For example, at 25 mM NaCl, a 30% decrease was observed because 50% of CCPs 8–10 bound to the iC3-S in the case of C4b and for 16 h in the case of C4b, the samples were separated on a 10% polyacrylamide gel (Novex, San Diego, CA) followed by a transfer of the proteins to Immun-Lite membranes using a Bio-Rad semidry transfer apparatus. The biotinylated proteins were incubated with extravidin-horseradish peroxidase and visualized using the Super-signal chemiluminescent-horseradish peroxidase substrate system (Pierce).

**RESULTS**

The specificity of binding was identical to the parental proteins in that CCPs 1–3 bound C4b and CCPs 8–10 bound C4b and C3b. Ligand binding was reduced by 10–30% as compared with their parental proteins LHR A and LHR B. For example, at 25 mM NaCl, a 30% decrease was observed because 50% of LHR B versus 50% of CCPs 8–10 bound to the iC3-S. This result is consistent with the data of Kalli et al. (14), who showed that the presence of CCP 11 (i.e. binding of a construct bearing CCPs 8–10 versus one with CCPs 8–11) increased the affinity of site 2 for C3b.

**Mutations in CCPs 1 and 2**

**Effect on Binding and CA—** Previously, we reported that binding and CA of mutants 14 and 15 (see Fig. 2) were substantially increased relative to the parental protein LHR A and were similar to those of LHR B (12). 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% increase in binding of mutants represented a modest (3–30%) increase over the 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% increase for mutant 14 (Table I). Of the single aa substitutions, two (Asp109 and T14K) 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 (not shown). For mutant 15, the change of N29K (mutation 15b) accounted for all of the enhanced iC3 and C4b binding (Table I) and CA for C3b and C4b (not shown) observed in mutant 15. The other mutation, Y27S (mutant 15a), had no effect (Table I).

Because our previous study (12) showed that iC3 and C4b binding of mutant 10 was also substantially increased, individual residues were altered. Substitution of Asp109 with Asn 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 (Fig. 3). Also, CA for C4b and C3b was similar to that for LHR B (not shown). The other three single aa mutations in region 10 produced minimal or no change in binding or CA (not shown). That changing Asp to Asn caused a major effect suggested a role for the amide group. To test this further, Asp109 was changed to Gln because,
like Asn, Gln has the amide group, but Gln is larger. Two other replacements of Asp were made with aas of a similar size, namely nonpolar Val and polar Thr. 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). A greater effect of Asn as compared with Val or Thr is consistent with the critical role of the amide. Because Asn leads to higher binding than does Gln, the size of the aa 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 Asp led to an increase above the level of LHR A but none had as dramatic an effect as did Asn on iC3 binding (not shown).

mAb 8C9.1 Recognizes CCP 1 and Enhances iC3 and C4b Binding by LHR A—Of 20 mAbs to CR1 tested (20), 8C9.1 was the only one specific for LHR A, suggesting that its epitope is in CCP 1 or 2. By testing LHR A with CCP 1 or 2 deleted, we localized the 8C9.1 epitope to CCP 1 (Table II). To map the epitope more precisely, we employed the substitution mutants in CCP 1 and found that sequences 14 and 15 are necessary for 8C9.1 binding (Table II). Further mapping, using single aa mutants in sequences 14 and 15, demonstrated that mutations 14b, c, d, and h and 15b abrogate 8C9.1 binding, whereas mutations 14d 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%, whereas 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, whereas mutant 19 had markedly reduced iC3 binding (Fig. 5). A comparison of binding and CA results (Tables IV and V) indicates 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 binding of both ligands and in sequence 19.

Effect on 3D9 Binding—mAb 3D9 blocks C3b and C4b binding (12, 17), CA, and decay-accelerating activity. Previously, we reported that 3D9 recognizes LHR A as well as LHR B (11, 12). Here, we establish that the epitope is in CCPs 3 and 10 because deletion of CCP 3 and CCP 10 from LHR A and 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 R603S (20d) reduced iC3 binding by 30% at 25 mM and V599S (20e) reduced only C4b binding by 50% at 25 mM. Within region 19, mutations T589E (19a) and R591V (19b) reduced iC3 binding (Table III).

The presence of Pro in position 22a (Fig. 4) in LHRs A, B, and C but not in LHR D suggested that it might be important. However, mutation P620K had a minimal influence on binding or CA for C3b but did lead to a marked reduction in CA for C4b (Table IV).

Comparison of LHR B with LHR C—The copy of site 2 present in LHR C differs by three aas from the one in LHR B. One difference is between CCPs 9 and 16, and we demonstrated earlier that it has no effect on binding or CA (see mutant 10b(r) in Ref. (12)). The other two aa differences are between CCPs 10 and 17, specifically within peptide 21 (Fig. 4). In CCP 17, Leu1056 and Arg1059 are present instead of Pro1066 and Gly1069, 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 (not shown). These observations provide additional support for a role of sequence 21 in ligand binding.

DISCUSSION

Acquisition of Function within an Active Site—Mutagenesis resulted in proteins with enhanced and/or new functional ac-

3 M. Krych, R. Hauhart, and J. P. Atkinson, unpublished observations.
activities. Probably the most remarkable was the effect of the D109N change in mutant 10a, which led to a modified site 1 in LHR A with enhanced activities. This protein acquired iC3 binding ability that was higher than that of LHR B and comparable to soluble CR1. 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 Asp109 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 soluble CR1 but with a similar activity.

Another mutation that substantially augmented iC3 and C4b binding of site 1 was N27K (mutation 15b). Of interest, a short form (65 kDa) of baboon CR1 contains a modified site 1 that has Lys27 and Asn109, and each of these two aas confers properties of site 2 on site 1 (21). This short form is the only CR1 expressed by baboon E, and it contains just site 1 (CCPs 1–8) and yet has activities of both human sites. Earlier, we observed in chimpanzee (22) that its short CR1 has only site 1 (CCPs 1–6). Furthermore, the two aas 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 (22). Moreover, it only requires one or a few amino acid substitutions at homologous positions to acquire the necessary functional activity. Furthermore, two distinct sets of substitutions can accomplish this end, as per either the 10a and baboon proteins or the one used by the chimpanzee protein.

In this and in our previous reports (11, 12), the activity of site

FIG. 4. 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 aa substitutions are identified by the numbers above the braces, and single aa changes are identified by letters below the alignment. Because the numbering was initiated in our earlier work in which mutants 1–15 were constructed (11, 12), 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 aa in the inter-CCP region, isoleucine and proline located between CCP 9 and CCP 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, Leu1056 and Arg1059 replace Pro606 and Gly609, respectively, (as shown under the alignment). The subscript numbers that precede the first aa and follow the last aa in a CCP indicate the aa number in the mature CR1.

FIG. 5. Effect of mutations in CCP 10 on binding to iC3 and C4b.

### Table III

Summary of iC3 binding and CA for mutants in CCP 10

| Construct | iC3 bindinga | C3b CAa |
|-----------|--------------|---------|
| 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.
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 Thr14, Asn29, Asp109, or Lys or Asn. In the case of Asp109, 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 Gly1067 in CCP 10 of LHR B, there is an Arg1059 in CCP 17 LHR C). These data complement well the findings of Taniguchi-Sidle and Isenman (23), who demonstrated that several negatively charged aas at the amino terminus of the α’ chain of C3b, also conserved in C4b, are necessary for interaction with CR1.

Another observation in line with the above discussion is that the sequences comprising mutations 10+11 and 14 in site 1 have four and three negatively charged aas, respectively, whereas 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, confers iC3/C3b binding, and increases C4b binding, might be explained by blocking negatively charged aas. These data suggest that the first half of CCP 1 may reduce ligand binding of site 1. Overall, our hypothesis is that CR1 binds C3 and C4 through ionic interactions; positive charges in the active sites of CR1 play a key role.

Analysis of the Third CCP Required for an Active Site—This study is the initial analysis of functionally important aas in the third CCP, which is indispensable for the activity of each site. We chose to begin 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 binding and is the main site for CA. The mutational binding analysis points out that the interactions of CCP 10 with C3b and C4b are similar. To illustrate, of the single aa substitutions made in CCP 10, mutation of Tyr596 (mutant 20c) had the greatest effect on binding and on CA for both iC3/C3b and C4b (Tables I and II, Fig. 5). Therefore, Tyr596 may be a contact point with both C3b and C4b. This is consistent with absence of Tyr in the homologous, but presumably nonfunctional, CCP 24. Other contact points for both ligands are likely to be in sequence 22 and/or 21 (Fig. 5, Tables 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, which has C4b binding capability but very poor iC3 binding. Similar observations have been made for membrane cofactor protein (3) and decay-accelerating factor (2), 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 a prerequisite for high cofactor activity. For example, membrane cofactor protein has much lower affinity for C3b than CR1, yet its CA is similar to or higher than that of CR1 (24, 25). Also, in the case of membrane cofactor protein, as in site 2 of CR1, the determinants of binding and CA are separable (3).

Importance of Amino Acids between Two CCPs—Mutations of the amino acids between two CCPs result in reduced activity. Thus, in mutant 16, in which Ile573 and Pro574 were both changed to Ala, CA was nearly abrogated (Tables III and IV). Another example is mutant 25, in which Ile642 and Ile643, between CCPs 10 and 11, were both mutated to Glu. 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. (26).

Identification of the Epitope for Function Blocking mAb 3D9—The presence of the 3D9 epitope in CCPs 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, Ser596 in sequence 20 is a part of the epitope because its mutation (20d) resulted in a loss of recognition by 3D9. On the

| Construct | C4b binding | C4b CA |
|-----------|-------------|--------|
| LHR B     | ++++        | ++++   |
| Mutants in CCP 10 |           |        |
| 16        | +++         | -      |
| 17        | +++         | -      |
| 18        | ++++        | ++++   |
| 19        | +           | +      |
| a         | ++++        | ++++   |
| b         | +           | +      |
| c         | +           | +      |
| d         | +           | +      |
| e         | +           | +      |
| f         | +           | +      |
| 20        | +           | +      |
| 21        | +           | +      |
| 22        | +           | +      |
| 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.
other hand, Arg603 and Pro620 in sequences 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.

**Future Directions**—In summary, we have undertaken an extensive mutational analysis of CCPs 1 and 2, CCPs 8 and 9, and now CCPs 3 and 10 as well. The results from these mutational analyses (11, 12) supplemented by epitope mapping of function-altering mAbs and evolutionary comparisons among primate forms of CR1 are mutually consistent (21, 22). Our 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. The finding that three CCPs of each site are necessary and sufficient for activity has implications for structural studies. It suggests that crystallography or NMR studies of active sites should be carried out with three CCPs. To this end, we are preparing CCPs 1–3 and CCPs 8–10 for such structural studies.

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