Decay Accelerating Activity of Complement Receptor Type 1 (CD35)

TWO ACTIVE SITES ARE REQUIRED FOR DISSOCIATING C5 CONVERTASES*

(Received for publication, June 21, 1999, and in revised form, August 18, 1999)

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The goal of this study was to identify the site(s) in CR1 that mediate the dissociation of the C3 and C5 convertases. To that end, truncated derivatives of CR1 whose extracellular part is composed of 30 tandem repeating modules, termed complement control protein repeats (CCPs), were generated. Site 1 (CCPs 1–3) alone mediated the decay acceleration of the classical and alternative pathway C3 convertases. Site 2 (CCPs 8–10 or the nearly identical CCPs 15–17) had one-fifth the activity of site 1. In contrast, for the C5 convertase, site 1 had only 0.5% of the decay accelerating activity, while site 2 had no detectable activity. Efficient C5 decay accelerating activity was detected in recombinants that carried both site 1 and site 2. The activity was reduced if the intervening repeats between site 1 and site 2 were deleted. The results indicate that, for the C5 convertases, decay accelerating activity is mediated primarily by site 1. A properly spaced site 2 has an important auxiliary role, which may involve its C3b binding capacity. Moreover, using homologous substitution mutagenesis, residues important in site 1 for dissociating activity were identified. Based on these results, we generated proteins one-fourth the size of CR1 but with enhanced decay accelerating activity for the C3 convertases.

Complement receptor type 1 (CR1,1 or CD35, immune adherence or C3b/C4b receptor) is expressed by most peripheral blood cells including erythrocytes (1–3). On phagocytic cells, CR1 mediates adherence and ingestion of C3b/C4b-coated particles (4–6), while on B lymphocytes and follicular dendritic cells these activities promote antigen localization and processing (7). In this regard, the immune response, especially to T-dependent antigens, is impaired in mice lacking CR1 and complement receptor type 2 (2CD21) (8–10). On erythrocytes, CR1 binds C3b/C4b opsonized immune complexes (immune adherence) (11) and processes and transports them to the liver and spleen for clearance (12, 13). Microorganisms such as Leishmania, Mycobacteria, and human immunodeficiency virus become coated with C3b and use CR1 to enter host cells (14, 15).

Recently, CR1 was shown to be the receptor involved in rosette formation between malaria-infected and -uninfected erythrocytes (a phenomenon that correlates with cerebral malaria and a high mortality) (16). This interaction involves CR1 on the uninfected cells and varies with expression of CR1-related blood group antigens.

The complement cascade requires stringent control. The C3 and C5 convertases are inhibited by proteins encoded by the regulators of complement activation (RCA) gene family (17). Factor H and C4b binding protein are fluid phase regulators present in plasma, whereas membrane cofactor protein (CD46) and decay accelerating factor (DAF, CD55) are ubiquitously expressed membrane inhibitors. RCA proteins accelerate the dissociation of C3 and C5 convertases (decay accelerating activity (DAA)) and/or serve as cofactors for the factor I-mediated cleavage of C3b and C4b (cofactor activity (CA)). CR1 is the most versatile member of this group. It possesses DAA and CA and inhibits C3 and C5 convertases of both the classical and alternative pathways (CP and AP, respectively). Moreover, it is unique among the cofactor proteins in that through its cofactor activity it generates C3d, which serves as a ligand for complement receptor type 2. A recombinant, soluble form of CR1 (sCR1) inhibits self-destructive C activation in immune complex-mediated syndromes, ischemia/reperfusion injury (18), and hyperacute xenograft rejection (19). It is presently in clinical trials as a complement inhibitor (20).

Like all members of the RCA family, CR1 is composed of the ~60-amino acid-long repeating units called complement control protein repeats (CCPs) or short consensus repeats. Of the 30 CCPs in CR1, the first 28 can be organized, based on internal homology, into four long homologous repeats (LHRs), A–D, each composed of seven CCPs (21, 22). Analyses of CR1 derivatives carrying a single LHR established that the LHRs A, B, and C contain C3b/C4b binding sites (23, 24). LHR B and its structural and functional duplicate LHR C bind C3b and C4b and possess CA for their cleavage (25–27). LHR A binds C4b with a similar affinity as LHR B or C but binds C3b weakly and has barely detectable CA (27).

The initial three CCPs of LHRs A, B, and C form a functional unit, called site 1 in LHR A and site 2 in LHR B or C (21, 27). The amino acid sequences of CCPs 1 and 2 are 39% different from their counterparts in site 2 (CCPs 8 and 9 or CCPs 15 and 16). On the other hand, the amino acid sequences of the third CCP in each site (CCPs 3, 10, and 17) are nearly identical. Consequently, variation in the initial two CCPs determines the functional differences between the sites. Using homologous substitution mutagenesis, amino acids important for ligand binding and for CA in site 1 and in site 2 were identified (24, 27, 28). In this investigation, our goal was to localize site(s) for...
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DAA and to identify peptides and amino acids critical for this regulatory activity.

EXPERIMENTAL PROCEDURES

General Design

All constructs were expressed as secreted proteins and quantified by enzyme-linked immunosorbent assay (27). They were then assessed for iC3b/C4b binding ability and for DAA.

Construction of CR1 Derivatives

Polymerase chain reaction (PCR) products were subcloned in vector pCR™II (Invitrogen, Carlsbad, CA), sequenced, and cut with the appropriate enzyme for cloning in the expression vector to generate the constructs shown in Fig. 1. Amino acid substitutions were made by site-directed mutagenesis (QuikChange™, Stratagene, La Jolla, CA) in the expression vector pSG5 (Stratagene) without subcloning.

Specific Constructs

LHR A contains CCPs 1–7 (i.e. amino acids 1–449 (27)). CCP 1–3 contains amino acids 1–194 (28). ΔCCP 1 and ΔCCP 2, lacking amino acids 1–60 and 61–122, respectively, were made from LHR A (24). ΔCCP 3, lacking amino acids 123–194, was produced from LHR A by oligonucleotide-directed mutagenesis using the Double Take™ mutagenesis Kit (Stratagene). LHR B—CR1(4,8,9), described earlier (27), was derived from CR1–4 (CCP 1–8 and one-half of CCP 9) by changing amino acids of CCPs 1 and 2 to those of CCPs 8 and 9, respectively. LHR B was then generated by converting CCP 3 to CCP 10 (mutation T132A) and by replacing Gly15 (the fourth position downstream of the last Cys in CCP 7) with a stop codon (28). Since CCPs 4–7 are identical to CCPs 11–14, the protein encoded by this cDNA is identical to CCPs 8–14 (identical to the homologous part of CCP 16). The latter contains a low value for ligation to CCP 8 (identical to CCP 15) and the first half of CCP 9, generating the amino-terminal portion of LHR AC and LHR BC, respectively. LHR C—CR1–4 (8,9), described earlier (27), was derived from CR1–4 (CCP 1–8 and one-half of CCP 9) by changing amino acids of CCPs 1 and 2 to those of CCPs 8 and 9, respectively. LHR B was then generated by converting CCP 3 to CCP 10 (mutation T132A) and by replacing Gly15 (the fourth position downstream of the last Cys in CCP 7) with a stop codon (28). Since CCPs 4–7 are identical to CCPs 11–14, the protein encoded by this cDNA is identical to CCPs 8–14 (identical to the homologous part of CCP 16). The latter contains a low value for ligation to CCP 8 (identical to CCP 15) and the first half of CCP 9, generating the amino-terminal portion of LHR AC and LHR BC, respectively.

Preparation of C3b Dimers

C3 was purified from plasma by anion exchange chromatography (29). To generate C3b dimers, C3 (2 mg) in 200 µl of phosphate-buffered saline at pH 7 was treated with 20 µg of trypsin (Sigma) for 3 min at 37 °C. The reaction was stopped with 200 µg of soybean trypsin inhibitor (Sigma). Cross-linking through the free sulphydryl group generated by breaking the thioester bond was then performed for 3 days at 4 °C using 15 µl of 0.34 mM bismaleimidohexane (Pierce) dissolved in methanol. With this procedure, the yield was over 50%. If, however, as recommended by the manufacturer, bismaleimidohexane was dissolved in Me₂SO, the yield of C3b dimers did not exceed 10%. The dimers were purified by size exclusion high performance liquid chromatography using a TSK 600SW column (TosoHaas, Montgomeryville, PA). The dimers were 95% pure based on a Coomassie Blue-stained gel.

Expression of CR1 Derivatives in COS 7 Cells

Plasmids containing cDNAs encoding CR1 derivatives were transfected into COS 7 cells using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. One day after transfection, the cells were washed and incubated in Opti-MEM I medium in the absence of serum to avoid possible DAA from the C4b binding protein and factor H in bovine serum. After a 2- or 3-day incubation, supernatants were collected, aliquoted, and stored at −70 °C. Protein levels were estimated by enzyme-linked immunosorbent assay (27) using two monoclonal anti-CR1 antibodies, 3D9 (4) and E11 (30). Because proteins CCP 1–3 and CCP 4–15–18 are not recognized by E11, a polyclonal antibody (25) was used for their quantification.

Ligand Binding

iC3 and C4b binding experiments were performed as described (27, 28), but the data are presented differently to be consistent with the decay accelerating results. Instead of reporting the fraction of a CR1 derivative that bound to iC3- or C4b-Sepharose, binding of the mutant peptides was expressed relative to the parental protein LHR A. A 100% value was assigned to the fraction of LHR A that bound to C4b- or iC3b-Sepharose. The results were expressed as the ratio of LHR A to mutant peptide. The decay accelerating results. Instead of reporting the fraction of a CR1 derivative that bound to iC3- or C4b-Sepharose, binding of the mutant peptides was expressed relative to the parental protein LHR A. A 100% value was assigned to the fraction of LHR A that bound to C4b- or iC3b-Sepharose.
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A hemolytic assay was employed. Cellular intermediates were prepared using purified C1, C4, and C2, and lytic sites were developed with C3-EDTA.

**Decay accelerating activity for the classical pathway C3 convertase**

A dose-response curve was performed with sCR1 and with each derivative. 100% inhibition was assigned to sCR1. Percentage of inhibition is expressed as a ratio of concentration of sCR1, which reduced hemolysis by 50%, to the concentration of a derivative, which reduced hemolysis by the same amount. Data represent mean ± S.D. of three experiments.

**Decay accelerating activity for the alternative pathway convertase in a hemolytic assay**

Cellular intermediates were prepared using purified components and lytic sites developed with C3-EDTA.

Hemolytic Assay for the Alternative Pathway DAA

The convertase was assembled using purified components (32). EAC14 cells (0.5 ml), prepared as above but at a concentration of 1.5 × 10^9/ml, were incubated with 0.225 μg of C2 and 50 μg of C3 for 30 min at 30 °C. After washing and resuspending in veronal-buffered saline containing 128 mM NaCl, 4.5 mM veronal (pH 7.35), 0.1% gelatin, and 10 mM Na_2-EDTA, cells were incubated for 2 h at 37 °C to allow dissociation of C1 and C2. The resulting EAC43 cells were washed twice with the above buffer; washed twice with buffer (Mg_2^+-EDTA) containing 128 mM NaCl, 4.5 mM veronal (pH 7.35), 0.1% gelatin, and 10 mM Na_2-EDTA; washed twice with the above buffer; washed twice with buffer (Mg_2^+-EGTA) containing 10 mM Na_2-EGTA, 7 mM MgCl_2, 115 mM dextrose, 0.83% gelatin, 59 mM NaCl, and 4.2 mM veronal (pH 7.35); and then resuspended in 6 ml of the same buffer.

EAC43 cells (100 μl) were incubated for 30 min at 30 °C with 5 ng of factor D, 45 ng of properdin, 1.5 ng of factor B, and 75 μl of inhibitor or Mg_2^+-EGTA. After adding 0.3 ml of C3-EDTA, hemolysis was estimated as for the classical pathway. In the modified assay, C3-EDTA was replaced with 300 μl of buffer containing 300 ng of each terminal component (C5–C9) with or without C3.

Microtitre Plate Assay for the Alternative Pathway DAA

For the C3 convertase decay accelerating assay (33), microtitre plates were coated overnight with 5 μg/ml C3b (Advanced Research Technologies) in phosphate-buffered saline. Plates were blocked for 2 h at 37 °C with phosphate-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20. Plates were then incubated for 15 min at 37 °C with 10 ng of factor B, 1 ng of factor D, and 0.8 mM NiCl_2 in 2.5 mM veronal buffer, pH 7.4, containing 71 mM NaCl and 0.05% Tween 20. Using this same buffer, sequential 1-h incubations were performed with 1–10 ng of a CR1 derivative, 0.129 μg of goat anti-human factor B antibody (Incstar, Stillwater, MN), and 100 μl of a 1:15,000 dilution of anti-goat antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PAI). Color was developed with O-phenylenediamine. In this assay, DAF and factor H behave as expected as mediators of decay accelerating activity (see Ref. 33), and we have detected C3a release using the Amersham Pharmacia Biotech C3a

**TABLE I**

| CR1 derivative | Inhibition (%) |
|----------------|---------------|
| sCR1           | 100           |
| LHR A          | 60 ± 8        |
| LHR B          | 12 ± 3        |
| LHR C          | 8 ± 5         |
| LHR D           | 0             |

**TABLE II**

| CR1 derivative | Inhibition (%) |
|----------------|---------------|
| sCR1           | 100           |
| LHR A          | 0.5 ± 0.08    |
| LHR B          | 0             |
| LHR C          | 0             |
| LHR D          | 0             |
| LHR A mixed with LHR B | 0.6 ± 0.08 |
| LHR A mixed with LHR D | 0.4 ± 0.09 |

**FIG. 1. Schematic representation of the structural and functional domains of CR1 and its derivatives.** The extramembranous part of CR1 (sCR1) is composed of 30 CCPs, which are shown as boxes.

Based on the degree of identity, the first 28 CCPs form LHRs A, B, C, and D, which arose through duplication of a seven-CCPs unit. There are two distinct functional units, each composed of three CCPs. Site 1 was localized to LHR A, and two nearly identical copies of site 2 were localized to LHRs B and C (23–28). The first two CCPs in site 1 (CCPs 1 and 2) are 30% different from the first two CCPs in site 2 (CCPs 8 and 9) as well as from CCPs 15 and 16, and they are marked by different shading. The third CCP in site 1 is nearly identical to the third CCP (10 or 17) in site 2. CCPs 3, 10, and 17 are represented by boxes with horizontal lines. In addition to LHR D, LHR D^+ includes CCPs 29 and 30.

One ml of these EAC14 cells was centrifuged, washed, resuspended in 1 ml for 4 min at room temperature. To prepare the C5 convertase, EAC14 cells were incubated with 0.5 ml of buffer containing 500 ng of each terminal component (C5–C9), with or without C3. After a 30-min incubation at 37 °C and centrifugation, the OD of the supernatant was read at 414 nm.

Hemolytic assay

Dose-response curve was performed with sCR1 and with each derivative. 100% inhibition was assigned to sCR1. Percentage of inhibition is expressed as a ratio of concentration of sCR1, which reduced hemolysis by 50%, to the concentration of a derivative, which reduced hemolysis by the same amount. Data represent mean ± S.D. of three experiments.
Characterization of the structural requirements for DAA proceeded in three steps: first, identification of the required LHRs; second, localization of the necessary CCPs within an LHR; and third, determination of critical peptides and individual amino acids within CCPs. DAA was analyzed for the CP and AP C3 and C5 convertases, and the activity of the constructs (illustrated in Fig. 1) was compared.

**LHRs Required for DAA—A hemolytic assay** was employed in which the CP C3 convertase was formed using purified components, and the lytic sites were developed with C5–C9 or EDTA. The main site of DAA for the CP C3 convertase was in LHR A (Table I), which had 60% of the activity of sCR1. LHR B or C had approximately 1/10 the activity of sCR1. LHR D had no detectable activity. In contrast, if DAA for the AP was assessed by a similar hemolytic assay, LHR A had only 0.5% of the activity of sCR1 (Table II), while LHRs B, C, and D had no detectable activity (even if the molar concentration of these proteins was 100-fold higher than sCR1). Mixing LHR A with LHR B or D did not result in activity above that of LHR A alone. These results were surprising because it was expected that, as for the CP C3 convertase, a single LHR would be capable of dissociating the AP C3 convertase. To further assess this issue, constructs bearing more than one LHR were evaluated (Table III). While LHR BC had barely detectable activity, LHR AC had DAA equal to 50% of the DAA of sCR1.

These data suggested that LHR A was sufficient for DAA of the CP C3 convertase, while LHR AC was required for the AP C3 convertase. Because the two C3 convertases are so similar, a second assay for the AP C3 convertase was established. To test this possibility, the hemolytic assay was modified such that instead of developing lytic sites in the appropriate C3 convertase, a single LHR would be capable of dissociating the AP C3 convertase. To further assess this issue, constructs bearing more than one LHR were evaluated (Table III). While LHR BC had barely detectable activity, LHR AC had DAA equivalent to that of sCR1, whereas LHR B or C had 12 and 14%, respectively (Table IV). These results, consistent with the data obtained in the CP C3 convertase assay, indicate that LHR A contains the major site for CP and AP DAA.

To account for the requirement that LHR A be linked to LHR C for efficient decay of the AP convertase on EA, we hypothesized that during the convertase assembly step both the C3 convertase (C3bBb) and the C5 convertase ((C3b)_2Bb) had been generated. This might occur if C3b dimers were produced by the classical pathway C3 convertase employed to deposit C3b. If so, LHR A would be sufficient for DAA for the C3 convertase, whereas LHR AC might be necessary for efficient dissociation of the C5 convertase. To test this possibility, the hemolytic assay was modified such that instead of developing lytic sites with C5–C9, they were developed using purified C5–C9, with or without the addition of C3. The rationale was that, if a C5

### Table III

| CRI derivative | Inhibition |
|---------------|------------|
| sCR1          | 100        |
| LHR A         | 0.5 ± 0.08 |
| LHR B         | 0          |
| LHR C         | 0          |
| LHR AC        | 50 ± 9     |
| LHR BC        | 0.6 ± 0.3  |

* A dose-response curve was performed with sCR1 and with each derivative. 100% inhibition was assigned to sCR1. Percentage of inhibition is expressed as a ratio of concentration of sCR1, which reduced hemolysis by 50%, to the concentration of a derivative, which reduced hemolysis by the same amount. Data represent mean ± S.D. of three experiments.

### Table IV

| CRI derivative | Inhibition |
|---------------|------------|
| sCR1          | 100        |
| LHR A         | 95 ± 5     |
| LHR B         | 12 ± 2     |
| LHR C         | 14 ± 4     |

* A dose-response curve was performed with sCR1 and with each derivative. 100% inhibition was assigned to sCR1. Percentage of inhibition is expressed as a ratio of concentration of sCR1, which reduced hemolysis by 50%, to the concentration of a derivative, which reduced hemolysis by the same amount. Data represent mean ± S.D. of three experiments.

**FIG. 2. LHR AC is more efficient than LHR A in decaying the alternative pathway C5 convertase in the micromolar assay.**

**TABLE V**

| CRI derivative | C3 convertase | C5 convertase |
|----------------|---------------|---------------|
| sCR1           | 100           | 100           |
| LHR A          | 60 ± 8        | 0.5 ± 0.3     |
| LHR AC         | 100 ± 9       | 95 ± 8        |
| CCP 1–4,15–18   | 78 ± 10       | 14 ± 2        |
| LHR BC         | 8 ± 9         | 3 ± 1         |

* Concentration of the inhibitors was a log lower in the C5 convertase assay than in the C3 convertase assay. A dose-response curve was performed with sCR1 and with each derivative. 100% inhibition was assigned to sCR1. Percentage of inhibition is expressed as a ratio of concentration of sCR1, which reduced hemolysis by 50%, to the concentration of a derivative, which reduced hemolysis by the same amount. Data represent mean ± S.D. of three experiments.

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convertase were generated during the initial steps of the assay, hemolysis would be independent of additional C3. 60% of erythrocytes were lysed with the addition of C5–C9, and 80% were lysed if C3 was also added. The 60 ± 7% value is similar to the 56 ± 8% lysis if C3-EDTA was used to develop lytic sites. Because hemolysis was largely independent of the added C3, we concluded that the AP C5 convertase had been generated on erythrocytes, probably as a result of C3b dimer formation (34).

To further address the question, microtiter wells were precoated with purified C3b dimers and incubated with Ni²⁺ and factors B and D. The resulting complexes, (C3b)₂BbNi²⁺, were treated with LHR A or with LHR AC. LHR AC was 10 times more effective than LHR A in dissociating the C5 convertase (Fig. 2). This finding was consistent with the results obtained in the hemolytic assay (Table III) in which a C5 convertase was also present.

We next asked if LHR AC is required for efficient DAA of the CP C5 convertase as well. An assay was employed in which a C5 convertase was formed on EA and lytic sites developed by the addition of purified C5–C9. DAA of the C5 convertase by LHR AC was similar to that of sCR1, while LHR A was approximately 200-fold less effective (Table V). As in the case of the AP C5 convertase, LHR BC had little DAA for the CP C5 convertase (Table V). These results establish that optimal DAA of C5 convertases requires two functional sites.

### Table VI

| CR1 derivative[^a] | Inhibition[^b] | Classical | Alternative |
|-------------------|---------------|-----------|-------------|
| sCR1              | 100           | 100       |             |
| LHR A             | 60 ± 8        | 95 ± 5    |             |
| CCP 1–4           | 71 ± 6        | 90 ± 8    |             |
| CCP 1–3           | 69 ± 5        | 97 ± 7    |             |
| ΔCCP 1            | 12 ± 4        | 3 ± 2     |             |
| ΔCCP 2            | 10 ± 5        | 10 ± 3    |             |
| ΔCCP 3            | 16 ± 4        | 0         |             |

[^a]: DAA for the classical pathway was tested in a hemolytic assay and DAA for the alternative pathway was tested in a microtiter plate assay.  
[^b]: A dose-response curve was performed with sCR1 and with each derivative. 100% inhibition was assigned to sCR1. Percentage of inhibition is expressed as a ratio of concentration of sCR1, which reduced hemolysis by 50%, to the concentration of a derivative, which reduced hemolysis by the same amount. Data represent mean ± S.D. of three experiments.

**Fig. 3.** LHR AC is required for efficient decay of the C5 convertases in the hemolytic assays. A, classical pathway; B, alternative pathway.
Identification of CCPs Required for DAA—To determine which CCPs within LHR A are responsible for DAA of the CP and AP C3 convertases, truncated LHR A derivatives, containing a full or partial site 1, were analyzed (Table VI). DAA of CCP 1–3 (site 1) was comparable with that of LHR A or CCP 1–4, indicating that CCPs 4–7 do not contribute to this regulatory function. We next constructed LHR A derivatives deleted of CCP 1, 2, or 3 (Table VI). DAA was substantially reduced, indicating that each CCP is necessary for DAA as they are for ligand binding (24, 28).

Since LHR A linked to LHR C was required for DAA for the C5 convertases, we asked if CCPs 5–7, located between the two active sites, play a role. The construct CCP 1–4,15–18, lacking CCPs 5–7, had 7- and 10-fold lower activity for the CP and AP, respectively, than did LHR AC but much higher activity than LHR A (Fig. 3, Table V). Construct CCP 1–4,15–21 was very similar to CCP 1–4,15–18 (not shown). Because CCPs 5–7, located between the active sites, possess no activity on their own, a requirement for proper spacing between sites 1 and 2 in the decay acceleration of C5 convertases is strongly suggested.

Identification of Peptides and Amino Acids Important for DAA—Modified forms of LHR A, carrying amino acid substitutions in CCPs 1 and 2 derived from the homologous positions of CCPs 8 and 9, respectively (Fig. 4), were assessed for DAA for C3 convertases. For these experiments, a hemolytic assay for the CP and a microtiter assay for the AP were employed. All mutants shown in Fig. 4 were tested, but only mutations that produced a \( \pm 20\% \) change are listed in Table VII. The effects of the mutations on DAA and on ligand binding (Refs. 24 and 28; present report) defined the three groups.

Group I represents substitutions, which decrease DAA for one or both convertases but have no effect on ligand binding. Hence, these mutations identify residues likely to be specifically required for DAA. In this group, the most marked effect was caused by F82V, which nearly abrogated DAA for both C3 convertases. To a lesser extent, mutations T103E, T110A, and V111A also reduced DAA for both C3 convertases. Other mutations reduced DAA for one pathway only. For example, W7H almost abrogated DAA for the AP, while mutations K92T and S99H reduced DAA for the CP.

Groups II and III are mutations that caused parallel changes in DAA and in ligand binding. Group II mutations produced a decrease in DAA and binding. For example, mutant G35E had reduced C4b and C3b binding and DAA for the AP and CP C3 convertases. Two substitutions, R64K and Y94H, produced a reduction in C4b binding and DAA for the CP C3 convertase. N65T reduced C3b binding and DAA for the AP C3 convertase only.

Group III mutations increased ligand binding and DAA. Mutant D109N had enhanced DAA for the C3 convertases and ligand binding (Table VII). This mutant and the double mutant D109N,E116K are of special interest because of the marked enhancement in DAA, which is further illustrated in the kinetic analysis shown in Fig. 5. Two other mutations, E6D and N29K, increased DAA for the CP C3 convertase, while mutation S37Y increased DAA for the AP convertase. In addition, mutant 14, with nine amino acid substitutions, showed increased DAA for the AP. No single mutation was responsible for this effect.

**DISCUSSION**

**DAA for C3 Convertases**—LHR A was identified as the main site of DAA for the CP and AP C3 convertases (Refs. 35 and 36; this report). Within LHR A, CCPs 1–3 account for all DAA, being equivalent to CCPs 1–4 or LHR A itself. If CCP 1, 2, or 3 was deleted from LHR A, DAA was markedly reduced. Thus, CCPs 1–3 (site 1) are necessary and sufficient for the dissociating activity. That site 1 was the major region for DAA for both convertases was unexpected because C3b binding by CCPs 1–3 is very low. However, this seemingly paradoxical observation is consistent with the functional profile of DAF, which has a low affinity for C3b or C4b but efficiently dissociates both C3 convertases (37). Transient interaction of an inhibitor with a convertase appears sufficient and probably optimal for deactivation. Like DAF, site 1 of CR1 may have a higher affinity for the convertases than for C3b or C4b alone (37). Previously, we found that most of the cofactor activity for C3b and C4b resides in site 2, which is located in CCPs 8–10 and duplicated in the nearly identical CCPs 15–17 (27). Here we show that this site has inefficient DAA in its own right. Therefore, site 1 is DAF-like, whereas site 2 is membrane cofactor protein-like.

While the major site of DAA for the C3 convertases is in LHR A, several lines of evidence indicate a contribution from site 2. First, LHRs B and C have detectable DAA. Second, compared with sCR1, LHR A has 60% of DAA for the CP C3 convertase, while LHR AC has 100%. These results suggest that site 2 participates in regulating the CP C3 convertase. Regarding the AP C3 convertase, in the microtiter assay system, site 1 accounts for all of the DAA detected with sCR1. In the hemolytic assay, the contribution of site 2 to the decay of the C3 conver-
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TABLE VII
Effect of amino acid substitutions in site 1 on decay accelerating activity for C3 convertases

| Mutant | CR1 derivative | Inhibition of C3 convertase | C4b binding | iC3 binding |
|--------|----------------|-----------------------------|-------------|-------------|
| Group I |                | CP  | AP       | %           |             |
| LHR A  | None           | 100 | 100      | 100         | 100         |
| 6c     | F82V           | 8 ± 6| 5 ± 5    | 87 ± 8      | 85 ± 10     |
| 13b    | W7H            | 91 ± 6| 4 ± 3    | 94 ± 12     | 82 ± 8      |
| 9      | S99H,T103E     | 26 ± 2| 36 ± 4   | 80 ± 8      | 84 ± 9      |
| 9a     | S99H           | 71 ± 8| 99 ± 8   | 90 ± 9      | 87 ± 7      |
| 9b     | T103E          | 26 ± 4| 65 ± 6   | 81 ± 13     | 90 ± 10     |
| 10b    | T110A          | 60 ± 6| 55 ± 8   | 84 ± 11     | 115 ± 6     |
| 10c    | V111A          | 50 ± 5| 36 ± 7   | 81 ± 10     | 90 ± 18     |
| 10d    | I112H          | 95 ± 9| 70 ± 6   | 109 ± 13    | 118 ± 5     |
| 8a     | K92T           | 68 ± 6| 96 ± 9   | 112 ± 10    | 95 ± 12     |
| Group II |                |     |         |             |             |
| 1a     | G35E           | 20 ± 6| 38 ± 6   | 18 ± 6      | 64 ± 4      |
| 5a     | R64K           | 50 ± 4| 98 ± 8   | 60 ± 10     | 112 ± 10    |
| 5b     | N65T           | 97 ± 9| 69 ± 7   | 35 ± 4      | 70 ± 6      |
| 8b     | Y94H           | 27 ± 3| 95 ± 8   | 22 ± 3      | 92 ± 8      |
| Group III |              |    |         |             |             |
| 10a    | D109N,E116K    | 416 ± 10| 242 ± 8 | 216 ± 20    | 212 ± 15    |
| 10a    | D109N          | 365 ± 15| 233 ± 12| 258 ± 13    | 233 ± 12    |
| 13a    | E6D            | 127 ± 8| 108 ± 9  | 177 ± 10    | 74 ± 9      |
| 15b    | N29K           | 200 ± 16| 98 ± 8   | 241 ± 15    | 192 ± 15    |
| 1b     | S37Y           | 95 ± 8| 140 ± 12 | 148 ± 16    | 161 ± 13    |
| 14     | RPTNLDEFE→KLK | 95 ± 8| 172 ± 10 | 255 ± 18    | 220 ± 20    |

a A dose-response curve was performed with sCR1 and with each derivative. 100% inhibition was assigned to sCR1. Percentage of inhibition is expressed as a ratio of concentration of sCR1, which reduced hemolysis by 50%, to the concentration of a derivative, which reduced hemolysis by the same amount. Data represent mean ± S.D. of three experiments.
b A 100% value was assigned to the fraction of LHR A, which bound to C4b- or iC3-Sepharose and served as a reference for the assessment of the binding of the mutant proteins.
c DAA decreased, binding unchanged.
d DAA and binding decreased.
e DAA and binding increased.
f Amino acids 12–16 and 18–21 in CCP 1 were mutated.

CCPs 5–7, which are not required for ligand binding and are not formally part of a functional site, are necessary for full CP and AP convertase DAA. This suggests that a function of CCPs 5–7 is to serve as a spacer; i.e. this arrangement of active sites may optimize an interaction with C4b/C3b heterodimers and C3b/C3b homodimers. These studies therefore provide an explanation for the seven-CCP-long unit, which is repeated four times in CR1. In addition to facilitating the decay of the C5 convertases, binding to dimers and other multiplicatives of ligands is probably optimized by this arrangement. Indeed, efficiency of C3b dimer binding by the four polymorphic size variants of CR1 is enhanced as the copy number of site 2 increases from one to three (38). A cooperative interaction between the two functional sites of CR1 was suggested previously based on greater cofactor activity for C4b if C3b was adjacent (39).

The different requirements for decay of the CP C3 versus the CP C5 convertase were readily demonstrable. In contrast, the interpretation of results for the AP convertases was initially complicated by the simultaneous presence of both C3 and C5 convertases in a hemolytic assay designed to assess decay of the C3 convertase. The presence of the AP C5 convertase in this system was conclusively shown by developing the lytic sites with purified C5–C9 rather than with C’-EDTA. Because lysis occurred without the addition of C3, C5 convertases must have been present. Thus, besides monomers, C3b dimers were deposited in this assay system, and the subsequent addition of factors B, D, and P resulted in the formation of the AP C5 convertase. That AP C5 convertase can be generated without an intermediate stage of the C3 convertase has been reported.

Effects of amino acid substitutions in site 1 on decay accelerating activity for C3 convertases

- LHR A
  - 6c: F82V, 8 ± 6, 5 ± 5, 87 ± 8, 85 ± 10
  - 13b: W7H, 91 ± 6, 4 ± 3, 94 ± 12, 82 ± 8
  - 9: S99H,T103E, 26 ± 2, 36 ± 4, 80 ± 8, 84 ± 9
  - 9a: S99H, 71 ± 8, 99 ± 8, 90 ± 9, 87 ± 7
  - 9b: T103E, 26 ± 4, 65 ± 6, 81 ± 13, 90 ± 10
  - 10b: T110A, 60 ± 6, 55 ± 8, 84 ± 11, 115 ± 6
  - 10c: V111A, 50 ± 5, 36 ± 7, 81 ± 10, 90 ± 18
  - 10d: I112H, 95 ± 9, 70 ± 6, 109 ± 13, 118 ± 5
  - 8a: K92T, 68 ± 6, 96 ± 9, 112 ± 10, 95 ± 12

- Group II
  - 1a: G35E, 20 ± 6, 38 ± 6, 18 ± 6, 64 ± 4
  - 5a: R64K, 50 ± 4, 98 ± 8, 60 ± 10, 112 ± 10
  - 5b: N65T, 97 ± 9, 69 ± 7, 35 ± 4, 70 ± 6
  - 8b: Y94H, 27 ± 3, 95 ± 8, 22 ± 3, 92 ± 8

- Group III
  - 10a: D109N,E116K, 416 ± 10, 242 ± 8, 216 ± 20, 212 ± 15
  - 10a: D109N, 365 ± 15, 233 ± 12, 258 ± 13, 233 ± 12
  - 13a: E6D, 127 ± 8, 108 ± 9, 177 ± 10, 74 ± 9
  - 15b: N29K, 200 ± 16, 98 ± 8, 241 ± 15, 192 ± 15
  - 1b: S37Y, 95 ± 8, 140 ± 12, 148 ± 16, 161 ± 13
  - 14: RPTNLDEFE→KLK, 95 ± 8, 172 ± 10, 255 ± 18, 220 ± 20

Fig. 5. Increased DAA of mutants D109N (10a) and D109N, E116K (10a11c) for the classical pathway C3 convertase.
FIG. 6. Amino acids important for DAA in CR1 and conserved in DAF.

Alignment of the amino acids of CCP 1 of CR1 with those of CCP 2 of DAF and of the amino acids of CCP 2 of CR1 with those of CCP 3 of DAF. The numbered amino acids represent residues in site 1 of CR1 important for DAA that are identical or conserved in the active site of DAF but not in site 2 of CR1.

The effects of several other mutations indicate that DAA is also a function of ligand binding. For example, substitution of Gly35 caused a parallel reduction in DAA for both C3 convertases and for C3b and C4b binding. If Arg64 or Tyr94 were altered, C4b binding was reduced, as was DAA for the CP C3 convertase. Additional evidence that ligand binding is important for DAA comes from the mutant D109N, in which increased C3b and C4b binding is associated with enhanced DAA for both C3 convertases. Another example is mutant 14 with increased C3b binding and DAA for the AP C3 convertase.

In a previous report (27), the peptide sequence in site 2 homologous to regions 10 and 11 in site 1 was shown to be critical for ligand binding and cofactor activity. Here we demonstrate that peptides 10 and 11 are important for the DAA of site 1. Reduced DAA was observed after replacement of Thr110 and Val111. The importance of the region 10 and 11 is further underscored by the increase in DAA for C5 convertases, above the level of sCR1, as a result of the substitution of one amino acid, D109N, or two amino acids, D109N and E116K. In addition to increased DAA, these proteins have increased C3b and C4b binding and cofactor activity (28). They are candidates for a new generation of complement inhibitors because 1) their DAA for C3 convertases not only exceeds DAA of their parental protein LHR A but also that of sCR1, 2) they are one-fourth the size of sCR1, and 3) they can be further truncated to include only CCPs 1–3. The potential also exists for improving the efficiency of DAA for the C5 convertases by modification of LHR A in LHR AC. Last, as previously suggested (24) and further emphasized by these data, homologous peptides in regions 10 and 11 are likely to be functionally important in DAF, membrane cofactor protein, C4b binding protein, and factor H.

Some of the amino acids important for DAA in site 1 are present in the homologous positions of the active site (CCPs 2–4 of DAF) (Fig. 6). One example is Phe82, whose counterpart in DAF, Phe146, is especially noteworthy because its substitution, along with the preceding Leu437, caused a substantial decrease in DAA by DAF for convertases of both pathways (46). These residues were predicted to be involved in DAA of DAF, based on a computer model in which they are a part of the groove between CCP 2 and 3 (47). Other residues important for DAA in site 1, Gly35, Asn65, Tyr94, Ser99, and Val111, are also found in the homologous positions of the active site of DAF, and one other, Thr110, is replaced with a conservative Ser. The prediction is that these amino acids are also essential for the function of DAF, serving as convertase contact points or as essential structural elements.

In conclusion, DAA for the CP and AP C3 convertases is mediated primarily by CCPs 1–3 (site 1) of LHR A. Optimal DAA for C5 convertases requires two contiguous LHRs, one carrying site 1 for DAA and the other carrying site 2 for C3b binding. The spacing between these two sites is important. Within site 1, amino acids were identified that are important solely for DAA, and others were identified that alter DAA and ligand binding in parallel. Proteins were generated in which
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DAA for only one or the other C3 convertase is reduced as well as proteins with increased DAA for C3 convertases but one-fourth the size of CR1. These results increase our understanding of the structure-function relationships in CR1 including a reason for its unusual highly homologous repetitive structure. They provide insights into the structural requirements for DAA of CR1 as well as of related proteins. Together with our prior analysis of ligand binding and cofactor activity in CR1, these data should facilitate the analysis of the three-dimensional structure of active sites of proteins bearing CCPs.

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