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Synergy between Two Active Sites of Human Complement Receptor Type 1 (CD35) in Complement Regulation: Implications for the Structure of the Classical Pathway C3 Convertase and Generation of More Potent Inhibitors

Malgorzata Krych-Goldberg,* Richard E. Hauhart,* Tina Porzukowiak,* and John P. Atkinson2*

The extracellular domain of the complement receptor type 1 (CR1; CD35) consists entirely of 30 complement control protein repeats (CCPs). CR1 has two distinct functional sites, site 1 (CCPs 1–3) and two copies of site 2 (CCPs 8–10 and CCPs 15–17). In this report we further define the structural requirements for decay-accelerating activity (DAA) for the classical pathway (CP) C3 and C5 convertases and, using these results, generate more potent decay accelerators. Previously, we demonstrated that both sites 1 and 2, tandemly arranged, are required for efficient DAA for C5 convertases. We show that site 1 dissociates the CP C5 convertase, whereas the role of site 2 is to bind the C3b subunit. The intervening CCPs between two functional sites are required for optimal DAA, suggesting that a spatial orientation of the two sites is important. DAA for the CP C3 convertase is increased synergistically if two copies of site 1, particularly those carrying DAA-increasing mutations, are contained within one protein. DAA in such constructs may exceed that of long homologous repeat A (CCPs 1–7) by up to 58-fold. To explain this synergy, we propose a dimeric structure for the CP C3 convertase on cell surfaces. We also extended our previous studies of the amino acid requirements for DAA of site 1 and found that the CCP 1/CCP 2 junction is critical and that Phe82 may contact the C3 convertases. These observations increase our understanding of the mechanism of DAA. In addition, a more potent decay-accelerating form of CR1 was generated.


Introduction

The complement system is a major component of innate and adaptive immunities and is a mediator of the inflammatory response (1–3). Complement participates in autoimmune-mediated tissue damage (4). To avoid excessive damage to host cells and tissues, activation of the complement cascade is tightly regulated (5) through a set of membrane and plasma inhibitors. Reduced levels of complement inhibitors lead to tissue-damaging conditions such as hemolytic uremic syndrome, paroxysmal nocturnal hemoglobinuria, glomerulonephritis, and hereditary angioedema (6–10). Reduced complement regulation may also be a factor in ischemia reperfusion injury (11–14). In other situations, complement inhibition is undesirable. For example, the resistance of cancer cells to immune attack is due in part to their high expression of membrane inhibitors (15, 16). Many microbial pathogens hijack host surface inhibitors or synthesize their own mimics to evade destruction by complement (17–19). Elucidation of the mechanisms by which the system’s natural inhibitors work should facilitate a more rational design of therapeutic agents to modulate complement activity.

Human complement receptor type 1 (CR1; CD35; C3b/C4b receptor or immune adherence receptor), in addition to its main role in the binding and disposal of C3b/C4b-opsonized immune complexes, is an efficient inhibitor of complement. CR1’s versatile regulatory profile has led to it being used in clinical trials as a soluble form of CR1 (sCR1) (20). A truncated variant of sCR1, APT070, contains the three N-terminal modules of CR1 followed by a targeting peptide to permit insertion into a cell membrane (21). In another approach, CR1 was decorated with sialyl-Lewisα (sLex) tetrasaccharides so as to target E-selectin on endothelial cells at sites of inflammation (22–24). Progress in the development of CR1-based inhibitors could be enhanced by a better understanding of their interaction with C3 and C5 convertases.

The most common CR1 allotype has 30 extracellular modules or repeats, called complement control protein repeats (CCPs), short consensus repeats, or sushi domains. Each CCP, composed of ~60 aa, has a similar framework (25, 26). Based on the degree of homology, the first 28 CCPs are grouped into four long homologous repeats (LHRs), A–D, each composed of seven CCPs (Fig. 1) (27, 28). LHR A (CCPs 1–7) contains site 1 or CCPs 1–3. Site 1 binds C4b and, weakly, C3b (29, 30). It is an efficient inhibitor of the classical (CP) as well as alternative (AP) pathway C3 convertases (31). For efficient decay-accelerating activity (DAA) for C5 convertases, site 1 has to be followed by site 2 (31). Site 2 binds C3b and C4b (30), and there are two copies, one of which is in CCPs
In this report we present data supporting the hypothesis that in two-LHR proteins, site 1 provides DAA, whereas site 2 binds the C3b subunit of the CP C5 convertase. We demonstrate that two-LHR constructs are more efficient in the decay of C3 convertases up to 58-fold greater than that of LHR A and propose a dimeric structure for the CP C3 convertase.

Materials and Methods

Construction of mutants

The cDNA constructs, made in vector pSG5 (Stratagene), are represented in Fig. 1. The construction of cDNAs for LHR A, LHR C, and LHR A/LHR C was previously reported (31), and that of the other cDNAs is described below. Site-directed mutagenesis was performed with the QuickChange kit (Stratagene). The sCR1 was a gift from H. Marsh (Avant Immunotherapeutics, Needham, MA).

Two-LHR constructs. To generate LHR A/LHR A, the pSG5 construct containing LHR A (aa residues 1–448 of the mature protein) was linearized with EcoRI at the 3′ end of CCP 7 after elimination of two other EcoRI sites by silent mutagenesis. PCR-amplified cDNA encoding LHR A was inserted into the EcoRI site. The resulting cDNA encodes a protein composed of two tandem copies of LHR A (Fig. 1). LHR A/LHR A (D109N) and LHR A(D109N,E116K) (LHR A(D109N) (an LHR followed by parentheses carries the mutation(s) specified in parentheses) were generated by a similar procedure, using appropriate LHR A mutants (29, 30). LHR A/LHR C (559DAAH.STKP...D571) was constructed like LHR A/LHR C, except that instead of LHR C, its mutant, LHR C (559DAAH.STKP...D571) was exchanged for CCP 1–7 in CR1–4 that encode eight and one-half CCPs 1–6/LHR C and CCP 1–5/LHR C.

To delete CCPs 4–7 from LHR A, this construct was described previously (31). CCP 1–6 were amplified by PCR and exchanged for CCPs 1–7 in CR1–4 that encode eight and one-half CCPs 1–6/LHR C and CCP 1–5/LHR C.

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CCP 1–4/LHR C. This construct was described previously (31). CCPs 1–3/LHR C. To delete CCPs 4–7 from LHR A, KpnI sites were placed at the CCP 3/CCP 4 and CCP 7/CCP 8 junctions of CR1–4 (29). CCPs 1–3 are therefore followed by CCP 1–6/LHR C and one half of CCP 9 (Fig. 1).

The remaining portion of LHR C was obtained by digestion of the LHR C-containing plasmid. The product of ligation codes for residues 1–193 of CCPs 1–3 followed by Gly and Thr, which are a result of cloning, and aa residues 899–1352 of LHR C (Fig. 1).

LHR A/4–7/LHR C. The CCPs 4–7 encoding fragment, obtained by digestion of CCP 4–7/LHR C, was inserted into CCP 1–6/LHR C. As described above. The resulting construct, LHR A/4–7/LHR C, contains DNA encoding aa 1–447 (LHR A); Gly and Thr, which are cloning products, aa residues 195–447 (LHR C); and aa 899–1352 (LHR C; Fig. 1).

Chimeric LHR A (CCP 22, followed by CCPs 2–7). To exchange CCP 1 for CCP 22 in LHR A, a PstI site was placed at the 3′ ends of CCP 1 in

FIGURE 1. A. Schematic representation of the structural and functional domains of sCR1 and its derivatives. The extramembranous portion of CR1 is composed of 30 CCPs (shown as boxes). Based on the degree of identity, the first 28 CCPs that form LHRs A, B, C, and D, arose through a duplication of a seven-CCP unit. There are two distinct functional sites, each composed of three CCPs. Site 1 is in LHR A, and site 2 in LHR B (Fig. 1).

To delete CCPs 4–7 from LHR A, this construct was described previously (31). CCPs 1–6 were amplified by PCR and exchanged for CCPs 1–7 in CR1–4 that encode eight and one-half CCPs 1–6/LHR C and CCP 1–5/LHR C.

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Chimeric LHR A (CCP 22, followed by CCPs 2–7). To exchange CCP 1 for CCP 22 in LHR A, a PstI site was placed at the 3′ ends of CCP 1 in
LHR A and of CCP 22 in LHR D (31). After cutting and pasting, CCP 22 (aa 1354–1415) is followed by CCP 2–7 (aa 63–449; Fig. 1).

Transfection
Transfection of 293T cells using Lipofectamine has been described previously (31).

iC3 (C3 with a broken thioester bond and with reactivity similar to that of C3b)/C4b binding assays

These assays have been previously described (30). Briefly, supernatants from transfected cells were incubated with iC3- or C4b-Sepharose for 30 min at room temperature. The bound proteins were eluted with 300 mM NaCl containing 1% Nonidet P-40 (Sigma-Aldrich). The protein level in the eluate was determined by ELISA, using mAb 3D9 as a capture Ab and 7G9 as a detection Ab. Binding of the mutant proteins was expressed relative to that of the parental protein LHR A. A 100% value was assigned to a fraction of LHR A that bound to iC3- or C4b-Sepharose. The binding data presented in the tables and figure insets represent the mean ± SD for three independent experiments.

Assays for DAA

We used a system in which there is a stepwise buildup of complement intermediates on E (31). Ab-sensitized sheep E (EA) and purified complement proteins were purchased from Advanced Research Technologies. EA sensitized by BSA or anti-A serum were prepared by incubation of sheep E with BSA or anti-A serum, respectively (31). Ab-sensitized sheep E (EA) and purified complement proteins were incubated with iC3- or C4b-Sepharose for 30 min at room temperature. The bound proteins were eluted with 300 mM NaCl, 0.15 mM CaCl2, 1 mM MgCl2, and 0.1% gelatin; pH 7.35). Cells were then coated sequentially with C1 (2 μg/ml) and C4 (6.7 μg/ml). To prepare the C3 convertase, these EAC14 cells were incubated for 4 min at room temperature with 0.5 μg/ml C2; for the CP C5 convertase, cells were incubated under the same conditions with C2 plus C3 (10 μg/ml). Fifty microliters of EAC142 or EAC1423 was incubated for 10 min at 30°C with 50 μl of inhibitor or buffer. At this point, 0.5 ml of guinea pig serum (Colorado Serum) diluted 20-fold in 40 mM EDTA-veronal buffered saline was added. After a 30-min incubation at 37°C, the mixture was centrifuged, and the OD of the supernatant was read at 414 nm in a spectrophotometer.

One representative experiment (of three) was chosen for the graphs showing the relationship between inhibition of hemolysis and inhibitor concentration. DAA results in the tables represent the mean ± SD for three independent experiments.

Results

DAA for the CP C5 convertase of two-LHR constructs

Effect of spacing between sites 1 and 2.

In CR1, four CCPs separate site 1 from site 2 (Fig. 1). Reducing the number of these intervening CCPs to one in CCP 1–4/LHR C led to reduced DAA (31). To further assess the role of spacer CCPs, a construct CCP 1–3/LHR C was produced in which site 1 is directly followed by site 2C. Its DAA is ∼10- and 100-fold less than the DAA of CCP 1–4/LHR C and LHR A/LHR C, respectively, and is similar to the DAA of LHR A alone (Fig. 2A). Next, we asked whether reducing the spacer from four CCPs in LHR A/LHR C to three or two CCPs affects DAA. When the constructs CCP 1–6/LHR C and CCP 1–5/LHR C were tested, no differences were detected compared with LHR A/LHR C (not shown). Then the distance between active sites was investigated using construct LHR A/CCP 4–7/LHR C, which has an eight-CCP-long spacer. It displayed DAA similar to that of the native form. These observations suggest that for optimal activity, at least two intervening CCPs are required.

Correlation with iC3 binding by the C-terminal LHR.

Previous experiments demonstrated that although LHR A is sufficient for efficient DAA for C3 convertases, for DAA for C5 convertases, LHR A has to be followed by site 2-containing LHR B or LHR C (31). Because C3b is the third component of the trimeric C5 convertases, we predicted that a role of LHR B or C in DAA is to bind to this subunit. To test this, constructs with C-terminal LHR that differ in iC3-binding ability were produced. In the construct LHR A/LHR C (559–571), iC3 binding of LHR C is reduced as a result of nine substitutions from the homologous positions of CCP C 2 (30). The DAA of this mutant protein was greatly diminished compared with that of the parental protein LHR A/LHR C (Fig. 2B). In another construct, LHR A/LHR A, the iC3-binding ability of the C-terminal LHR is similar to that of LHR C (559–571) (Fig. 2B, inset). DAA of LHR A/LHR A was reduced to a level similar to that of LHR A/LHR C (559–571), despite LHR A/LHR A consisting of two potent decay accelerators of C3 convertases. If iC3 binding of the C-terminal LHR in LHR A/LHR A was increased due to D109N substitution in CCP 2, DAA of the construct LHR A/LHR A(D109N) increased in parallel. These observations provide additional evidence that the C-terminal LHR serves to bind the C3b subunit of the CP C5 convertase.

Effect of mutations in the N-terminal LHR.

Binding to C3b of the C-terminal LHR in two-LHR constructs may facilitate access of the C4b2a part of the C5 convertase to the N-terminal LHR. Site 1 then would dissociate C2a. In this scenario, the role of site 1 in DAA for the C5 convertase would be similar to its role in DAA for the C3 convertase. In a previous report, we found that DAA of LHR A for C3 convertases is increased by two mutations in CCP 2, D109N and/or E116K (31). If the above reasoning is correct, these mutations may have a similar effect on DAA for C5 convertases. To test this, we placed them in the N-terminal LHR of LHR A/LHR A(D109N) to generate the construct LHR A(D109N,E116K)/LHR A(D109N). DAA for the CP C5 convertase was increased 3-fold compared with LHR A/LHR A(D109N) (not shown). This increase is similar to that observed...
for the DAA for C3 convertases with the same mutations in a single LHR A (31). Therefore, this observation is consistent with the N-terminal LHR being responsible for dissociation of the CP C5 convertase.

**DAA for the CP C3 convertase**

**Synergy between active sites in two-LHR constructs.** We next asked whether the presence of two copies of site 1 in a single protein affects DAA for the CP C3 convertase. The protein LHR A/LHR A was 5.8-fold more active than LHR A (Fig. 3 and Table I). Thus, the increase in DAA of LHR A/LHR A was greater than the 2-fold increase predicted for two independently acting copies of site 1. If DAA for the C3 convertase of at least one copy of site 1 was increased, DAA of a two-LHR construct was greater than that of LHR A/LHR A. Similarly to LHR A/LHR A, in other two-LHR constructs, DAA exceeded values expected if the two sites acted independently. For example, for independently acting sites, the relative DAA of the construct LHR A/LHR A (D109N) would be the sum of the relative activities of two sites, 1 + 3.7, and would result in 4.7-fold higher DAA than that of LHR A. Instead, DAA is 25-fold higher than that of LHR A. The highest DAA was observed for LHR A(D109N,E116K)/LHR A(D109N). Instead of the anticipated ~8-fold increase over LHR A, its DAA was 58-fold higher.

Interestingly, the predicted DAA for two-LHR constructs containing one copy each of sites 1 and 2 was also lower than experimentally determined. For example, DAA of LHR A/LHR C was expected to be similar to LHR A, because site 2 has ~10% of site 1’s DAA. Instead, DAA of LHR A/LHR C was 2.3 times greater than DAA of LHR A. DAA of LHR A(D109N,E116K)/LHR C was also underestimated. These results demonstrate that rather than acting independently, functional sites in two-LHR constructs work synergistically in decaying the CP C3 convertase.

**The role of Gly35**. Gly35 is a critical residue, as demonstrated by a loss of DAA for the CP C3 convertase and of C4b binding in mutant G35E (Table II) (29, 31). To determine whether this loss of function was due to a negative charge, substitutions with neutral Ala/Ile or positively charged Lys were introduced. Mutation G35K had the most profound effect on C4b binding and DAA for the CP C3 convertase. However, the other mutants also had reduced binding and DAA compared with the wild-type LHR A (Table II). Mutation G35A had the smallest effect.

**CP1/CP2 junction.** To assess the role of positively charged amino acids at CP1/CP2 junction, a double mutant was generated in which the peptide 59ArgArgLys61 was substituted with three Ile. An Ile, like Arg and Lys, has a bulky side chain, but no charge. Simultaneous mutation of all three amino acids resulted in a low DAA for the CP C3 convertase and undetectable C4b binding (Table III). Similar results were obtained for single amino acid substitutions, R59I and K61I. Mutant R60H had almost unchanged DAA despite an approximately two-thirds decrease in C4b binding.

**Role of CCP 1.** Although, as noted above, CCP 1 is indispensable for the functionality of site 1, the only amino acid previously identified as critical is Gly35, which is unlikely to be contact point because it lacks a side chain (29). Two explanations could account for the apparent lack of contact points in CCP 1. One is that it may play a structural role; for example, it may be required for the spatial organization of site 1. Alternatively, the contact points could be present among the amino acids that are conserved between sites 1 and 2 and therefore would not have been identified by our prior mutagenesis in which nonconserved residues were interchanged between sites 1 and 2 (29). If CCP 1 played only a structural role, another CCP might substitute without a major loss of DAA. To test this, CCP 22, the homologous CCP from LHR D (which does not interact with iC3/C4b) (32), was substituted for CCP 1 in LHR A. CCP 22 is 60% identical with CCP 1 and residues equivalent to Gly35 in CCP 1 and to 59ArgArgLys61 in the linker are present.

### Table I. Synergy between active sites in two-LHR constructs for decay accelerating activity for the CP C3 convertase

<table>
<thead>
<tr>
<th>Constructs</th>
<th>DAA for C3 Convertase (relative to LHR A)</th>
<th>Predicted for Two-LHR Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single LHRs</td>
<td>DAA Detected&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Predicted&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LHR A</td>
<td>1.0</td>
<td>N/A</td>
</tr>
<tr>
<td>LHR A(D109N)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.7 ± 0.3</td>
<td>N/A</td>
</tr>
<tr>
<td>LHR A(D109N, E116K)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.2 ± 0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>LHR C</td>
<td>0.1 ± 0.2</td>
<td>N/A</td>
</tr>
<tr>
<td>Two-LHR constructs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LHR A/LHR A</td>
<td>5.8 ± 0.3</td>
<td>2.0</td>
</tr>
<tr>
<td>LHR A/LHR A(D109N)</td>
<td>25.0 ± 0.2</td>
<td>4.7</td>
</tr>
<tr>
<td>LHR A(D109N, E116K)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>58.0 ± 0.2</td>
<td>7.8</td>
</tr>
<tr>
<td>LHR A/LHR C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3 ± 0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>LHR A(D109N, E116K)/LHR C</td>
<td>11.4 ± 0.3</td>
<td>4.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Value of 1 was assigned to DAA of LHR A.
<sup>b</sup> Predicted if DAA of the two sites was additive.
<sup>c</sup> N/A, not applicable to single LHRs.
<sup>d</sup> For comparison, DAA of several previously reported (31) constructs is shown.

### Table II. Effect of Gly35 substitutions on decay accelerating activity for the CP C3 convertase and on C4b binding

<table>
<thead>
<tr>
<th>Construct</th>
<th>DAA (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C4b Binding (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHR A</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>G35E</td>
<td>29 ± 8</td>
<td>18 ± 7</td>
</tr>
<tr>
<td>G35I</td>
<td>14 ± 6</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>G35A</td>
<td>42 ± 8</td>
<td>24 ± 8</td>
</tr>
<tr>
<td>G35K</td>
<td>nd&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7 ± 6</td>
</tr>
</tbody>
</table>

<sup>a</sup> DAA is for inhibitors at a concentration of 4 nM, DAA of LHR A is considered 100% while that of LHR A mutants is expressed as a percentage of LHR A. Means of three experiments ± SD are shown.
<sup>b</sup> Fraction of LHR A that bound to C4b-Sepharose was considered 100%. Binding level of mutant proteins is expressed as percentage of the binding of LHR A. Means of three experiments ± SD are shown.
<sup>c</sup> nd, Not detectable.
Despite this, an LHR A derivative in which CCP 22 replaced CCP 1 demonstrated no activity for the CP C3 convertase (not shown).

Role of Phe82 in CCP 2. Homologous substitution mutagenesis of site 1 led to the identification of only one amino acid, Phe82, that is critical for DAA for both C3 convertases, but is not required for ligand binding (31). To further evaluate the role of Phe82, the effects of mutations F82Y, F82H, and F82A were assessed (Table IV). Mutation F82A abrogated DAA for the CP convertase, whereas F82H and even the conservative F82Y substantially reduced DAA. C4b binding by these mutants was not changed, indicating that Phe82 is required primarily for DAA.

Discussion

C5 convertase

Previously, we reported that reducing the spacer between active sites from four CCPs in LHR A/LHR C to one CCP in the protein CCP 1–4/LHR C (31) reduced DAA for C5 convertases ~10-fold (31). In this study we show that eliminating the spacer altogether leads to an additional ~10-fold decrease in DAA. It is noteworthy that DAA of CCPs 1–3/LHR C is similar to DAA of LHR A (Fig. 2A). This suggests that in the absence of the spacer between sites 1 and 2C, site 2C is not capable of participating in DAA. Reducing the distance between the two sites from four CCPs in LHR A/LHR C to three and two in CCPs 1–6/LHR C and CCPs 1–5/LHR C, respectively, had no effect on DAA, nor did extending this distance to eight CCPs. This indicates that for optimal DAA, two intervening CCPs are required and sufficient. The requirement for a minimum of two spacer CCPs for DAA for the CP C5 convertase is consistent with our earlier hypothesis that one role of CCPs between active sites is to facilitate an interaction with a dimeric ligand of a defined structure (31). Such a structure is present in the CP C5 convertase, in which C3b is preferentially attached to a specific residue in the C4d fragment (33, 34). A covalent linkage between two C3b molecules was found in the AP C5 convertase, in which C3b is preferentially attached to IgG, the thioester of the second C3b reacts with an as yet to be identified residue within the C3d portion of the first C3b (36).

To explore the mechanism of the DAA for the CP C5 convertase, two-LHR constructs that differ in C3b-binding ability of the C-terminal LHR were studied. The results are consistent with the C-terminal LHR’s role being binding to C3b, the third component of the C5 convertases. This binding may be required to orient C4b2a relative to site 1 for the dissociation of C2a. Alternatively, binding may weaken the interaction of C3b-C4b with C2a. This could occur through a conformational change in C3b-C4b due to binding of an inhibitor. Alternatively, site 2C of a two-LHR inhibitor could bind to C3b/C4b molecules that are located nearby and are not a part of the same convertase complex. In any case, the N-terminal LHR probably dissociates C2a, as it does in the C3 convertases. This line of reasoning is supported by the observation that mutations D109N and E116K in the N-terminal LHR of the protein LHR A(D109N,E116K)/LHR A(D109N) increase DAA for the CP C5 convertase ~4-fold, similar to their effect on the C3 convertase.

Synergy between active sites in two-LHR constructs. Because DAA of LHR A is 10-fold higher compared with that of LHR B (or C), site 1 is the main site of DAA for the C3 convertase (31). In the present report, DAA of several two-LHR constructs was tested. DAA of LHR A/LHR A was greater than that of LHR A/LHR C. DAA was further increased if one and especially if both copies of site 1 in LHR A/LHR A carried mutations that enhance DAA in single-LHR constructs. The protein LHR A(D109N,E116K)/LHR A(D109N) was the most potent decay accelerator. It was 58-fold more active than LHR A (Table I). D109N and D109N,E116K increased C4b binding (and C3b) binding >2-fold and DAA for the CP C3 convertase ~3–to 4-fold. Augmented C4b binding of each LHR may lead by itself to an overall increase in DAA of the construct LHR A(D109N,E116K)/LHR A(D109N). In addition, increased C4b binding by one site may favorably change the conformation of another site to enhance its DAA (31). Previously, we (31) showed that DAA of sCR1 is equivalent to that of LHR A and is ~2-fold greater compared with that of LHR A. Thus, LHR A(D109N,E116K)/LHR A(D109N) is ~30-fold more potent deactivator than sCR1.

In all two-LHR constructs tested, the detected DAA exceeded DAA that was predicted for two sites acting independently (Table I). We propose that this synergy in two-LHR constructs has implications for the structure of the CP C3 convertase (Fig. 4). A second C4b molecule may preferentially bind to the first C4b molecule, which is, in turn, covalently attached to E. In support of dimeric C3 convertase, C4b dimers were found on the E surface

Table III. Effect of mutations in the CCP1/2 linker on decay accelerating activity for the CP C3 convertase and on C4b binding

<table>
<thead>
<tr>
<th>Construct</th>
<th>DAA (%)</th>
<th>C4b Binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHR A</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>59RRKK61→59III61</td>
<td>20 ± 7</td>
<td>ND</td>
</tr>
<tr>
<td>R59I</td>
<td>32 ± 9</td>
<td>ND</td>
</tr>
<tr>
<td>R60I</td>
<td>90 ± 14</td>
<td>34 ± 12</td>
</tr>
<tr>
<td>R61II</td>
<td>36 ± 13</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> See legend for Table II for calculation of DAA and ligand binding.

<sup>b</sup> ND, not detectable; detection level was >3%.

Table IV. Effect of Phe<sup>82</sup> substitutions on decay accelerating activity for the CP C3 convertase and on C4b binding

<table>
<thead>
<tr>
<th>Construct</th>
<th>DAA (%)</th>
<th>C4b binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHR A</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>F82V</td>
<td>4 ± 2</td>
<td>95 ± 12</td>
</tr>
<tr>
<td>F82Y</td>
<td>34 ± 6</td>
<td>89 ± 2</td>
</tr>
<tr>
<td>F82A</td>
<td>&lt;5</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>F82H</td>
<td>23 ± 5</td>
<td>87 ± 6</td>
</tr>
</tbody>
</table>

<sup>a</sup> See legend for Table II for calculation of DAA and ligand binding.

C3 convertase

To explore the mechanism of the DAA for the CP C5 convertase, two-LHR constructs that differ in C3b-binding ability of the C-terminal LHR were studied. The results are consistent with the C-terminal LHR’s role being binding to C3b, the third component of the C5 convertases. This binding may be required to orient C4b2a relative to site 1 for the dissociation of C2a. Alternatively, binding may weaken the interaction of C3b-C4b with C2a. This could occur through a conformational change in C3b-C4b due to binding of an inhibitor. Alternatively, site 2C of a two-LHR inhibitor could bind to C3b/C4b molecules that are located nearby and are not a part of the same convertase complex. In any case, the N-terminal LHR probably dissociates C2a, as it does in the C3 convertases. This line of reasoning is supported by the observation that mutations D109N and E116K in the N-terminal LHR of the protein LHR A(D109N,E116K)/LHR A(D109N) increase DAA for the CP C5 convertase ~4-fold, similar to their effect on the C3 convertase.

Synergy between active sites in two-LHR constructs. Because DAA of LHR A is 10-fold higher compared with that of LHR B (or C), site 1 is the main site of DAA for the C3 convertase (31). In the present report, DAA of several two-LHR constructs was tested. DAA of LHR A/LHR A was greater than that of LHR A/LHR C. DAA was further increased if one and especially if both copies of site 1 in LHR A/LHR A carried mutations that enhance DAA in single-LHR constructs. The protein LHR A(D109N,E116K)/LHR A(D109N) was the most potent decay accelerator. It was 58-fold more active than LHR A (Table I). D109N and D109N,E116K increased C4b binding (and C3b) binding >2-fold and DAA for the CP C3 convertase ~3–to 4-fold. Augmented C4b binding of each LHR may lead by itself to an overall increase in DAA of the construct LHR A(D109N,E116K)/LHR A(D109N). In addition, increased C4b binding by one site may favorably change the conformation of another site to enhance its DAA (31). Previously, we (31) showed that DAA of sCR1 is equivalent to that of LHR A and is ~2-fold greater compared with that of LHR A. Thus, LHR A(D109N,E116K)/LHR A(D109N) is ~30-fold more potent deactivator than sCR1.

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FIGURE 4. Model of a dimeric C3 CP convertase. Some complexes of the enzyme convertases may form by binding of the second C4b molecule to the first one, which is already deposited on the E surface. This results in dimeric CP C3 convertases. Thioester bond is represented as a short line.
A C4b dimer was also a predominant C4b form during the CP C3 convertase assembly on the sensitized liposome surface (38). Formation of C4b dimers could also explain why site 2, which by itself has low DAA, becomes much more efficient if combined with site 1 in the protein LHR A/LHR C. Higher avidity of CR1 to C3b or C4b dimers compared with monomers is well documented (39–42). Moreover, in CHO, CR1’s cofactor activity for C4b is higher if it forms C4b/C3b dimers (43). Although our data are consistent with a dimeric C3 convertase, the possibility that the C3 convertase is monomeric and CR1 inhibitors bind to two separate C4b molecules located next to each other (i.e., clustered around the C1 attachment point) cannot be excluded.

**Toward definition of the binding surface**

The second part of this work focused on further defining surface in site 1 that is in contact with C3 convertases. Each of three CCPs of site 1 is required for activity (31). A mutagenesis strategy, in which amino acids in CCPs 1 and 2 were changed into their homologues in CCPs 8 and 9, led to the identification of CCP 2 of one residue, Phe82 (Fig. 5), critical for DAA for C3 convertases, but not for ligand binding (31). In those experiments Phe82 was mutated to Val. To further probe the role of Phe82, we constructed additional Phe82 mutants. F82A practically abrogated DAA, and F82H and F82Y substantially reduced DAA. The decrease in DAA caused by F82Y is especially informative, because the only difference is a hydroxyl on the phenolic ring. The effect of F82H was similar to that of F82Y. Other substitutions with nonconserved amino acids, Val and Ala, practically abrogated DAA. Thus, even the most conservative replacement of Phe82 results in a loss of function of LHR A, consistent with the hypothesis that Phe82 is a contact point.

Several regulators of complement activation have two or three positively charged amino acid residues between the first two CCPs of an active site. These residues are required for the activity of C4-binding protein and decay-accelerating factor (DAF) (44, 45). The functional importance of these amino acids in site 1 was assessed by analysis of mutants in which three linker residues, individually or combined, were mutated into Ile. Ile, like Lys and Arg, has a long aliphatic chain, but lacks a charge. Mutation of all three amino acid residues led to a loss of DAA as well as C4b binding. Individual mutations of Arg50 or Lys64 reduced DAA for the CP C3 convertase by approximately two-thirds, whereas C4b binding was undetectable (Table III). Thus, a weak interaction with the ligand may be sufficient for the dissociation of the convertases. It could also be that the interaction with the convertases may be stronger than the interaction with their subunits, as is the case for DAF (46). The positively charged linker residues may also participate in the intermodular junctions, as has been shown for the CCP 15/CCP 16 junction (47). Alternatively, junctional amino acids may be contact points. This latter scenario is more likely if the junction is poorly defined, in which case linker residues may not be critical to the structure of the junction. A poorly defined junction was observed in the solution structure of biologically active CCPs 1 and 3 of DAF (48). Few, if any, interactions between modules CCPs 2 and/or 3 and linker residues 125LysLysLys127 were detected. The structure of site 1 with its ligands is required to definitively determine whether junctional amino acid residues are also contact points.

Gly35 was the only amino acid in CCP 1 identified by homologous substitution mutagenesis to impact strongly on function (29, 31). The original mutation, G35E, reduced all activity of site 1. Additional substitutions of Gly35 demonstrated that this loss of functionality is not due to introduction of a negative charge. For example, a neutral mutation, G35A, also caused a major decrease in functionality, as did the bulky neutral Ile. Moreover, mutation G35K, which increases the positive charge of site 1, nearly abrogated the activities of site 1. These observations are consistent with the hypothesis that Gly35 is involved in a functionally important turn. If CCPs 1 and 2 are modeled ([www.bru.ed.ac.uk/~dinesh/ccp-db.html]) using the solution structure of CCPs 15 and 16, Gly35 sits at the top of a turn adjacent to CCP 2 (Fig. 5). Two other residues required for DAA, Thr102 and Thr110, are also close to the CCP 1/CCP 2 junction (Fig. 5). Thus, the CCP 1/CCP 2 junction and the nearby CCP 2 surface, defined by Thr103 and Thr110 as well as by Arg64 and Asn65, appear to be important for binding and DAA.

Although CCP 1 is indispensable for the function of site 1, as noted above, Gly35 was the only amino acid identified as critical for all activities (29, 31). Because it is unlikely that Gly35 is a contact point, CCP 1 may play primarily a structural role, e.g., it may hold CCP 2 in the correct position. This hypothesis was examined with an LHR A construct in which CCP 1 was replaced by CCP 22. Although CCP 22 is 60% identical with CCP 1 and possesses both the Gly35 homologue and the ArgArgLysSer linker (identical with the CCP 1/2 linker), this chimeric LHR A had no detectable DAA. Because interchanging homologous amino acids between CCPs 1 and 8 did not identify potential contact points in CCP 1, they may be among residues conserved between CCPs 1 and 8, but not conserved between CCPs 1 and 22. The amino acids that fulfill these two conditions are Ala5, Arg12, Thr17, Ile24, Arg30,
Pro\(^{40}\), and Lys\(^{55}\). In DAF and C4-binding protein, amino acids equivalent to Arg\(^{39}\) are functionally important (48, 49).

Although only experiments with CP convertases were described in this study, all constructs were also tested for the AP convertases. Overall, the results for the AP were similar to those obtained for the CP. However, as we have previously reported (31), a mixture of C3 and C5 convertases is generated on E which limits the ability to assess a specific inhibitory effect on either the C3 or C5 AP convertase.

Comparison with other DAA sites

Comparing mutagenesis data of two sites, CCPs 1–3 of CR1 and CCPs 2–4 of DAF, reveals potentially important similarities in the requirements for decaying the CP C3 convertase (45). As noted above, the three positively charged amino acids between the first and second CCPs are critical in both sites as is Phe\(^{82}\) in CR1 and its counterpart Phe\(^{148}\) in DAF. Of additional interest, residues in the homologous positions of active sites may be functionally important, but not conserved, reflecting general similarity of a surface interacting with ligands. In one case, Trp\(^{7}\) in CR1 and Arg\(^{66}\) in DAF, and in another case, Thr\(^{103}\) in CR1 and Phe\(^{169}\) in DAF, were important for DAA. Yet another example is Asn\(^{65}\) in site 1 in CR1 and His\(^{67}\) in the corresponding position of the active site in C4-binding protein. Although not conserved, both amino acid residues are required for C4b binding, possibly because they occupy homologous positions (44, 50).

The emerging picture of the surface that interacts with the CP C3 convertases centers around the CCP 1/CCP 2 junction and possibly includes the surface in CCP 2 defined by Phe\(^{7}\), Arg\(^{4}\), Asn\(^{65}\), Thr\(^{103}\), Thr\(^{110}\), and Val\(^{111}\) (29, 31). Asp\(^{109}\), whose substitution with Asn results in a gain of function (31), also lies nearby, adding support for the importance of this area in site 1’s functionality. Importantly, these amino acid residues are not limited to one face of CCP 2, suggesting that the ligand wraps around this CCP.

In conclusion, this report provides insight into the mechanism of DAA by CR1. Both sites 1 and 2 of CR1 were required for the dissociation of the CP C5 convertases. In two-LHR constructs, site 1 in the N-terminal LHR removes the catalytic subunit C2a, as it does in the decay of the CP C3 convertase. Site 2 in the C-terminal LHR is necessary for binding C3b. To function optimally in DAA for the C5 convertases, sites 1 and 2 need to be separated by at least two CCPs. Importantly, evidence for synergy between active sites in dissociating the CP C3 convertase was also generated. In addition, modifications were introduced in site 1 that resulted in enhanced DAA. Based on these data, inhibitors were constructed with DAA for the CP C3 convertase many-fold greater than that of sCR1. These results raise interesting points, including how the synergy between sites is mediated and the possibility that the CP C3 convertase is composed of a covalently linked C4b dimer.

Disclosures

The authors have no financial conflict of interest.

References


