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Analysis of C3b/C3d Binding Sites and Factor I Cofactor Regions Within Mouse Complement Receptors 1 and 2

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Human and murine CR1 and CR2 are defined as evolutionary homologues on the basis of their in vitro activities and a shared structural motif known as a short consensus repeat (SCR). To identify additional similarities between the two species, we analyzed the functional domains within the mouse receptors by constructing mouse-human chimeric cDNAs in which the C3 binding site of human CR2 has been replaced by different regions within the first eight SCRs of mouse CR1. Rosette analysis of cells expressing chimeric proteins, with erythrocytes bearing different mouse C3 fragments, coupled with rosette inhibition studies using specific anti-mouse CR1/CR2 mAbs reveal a weak C3b binding site within SCRs 1 and 2 of mouse CR1. There is no independent C3b interacting domain within SCRs 3 to 6, but their presence enhances C3 binding. A molecule that contains only the first six SCRs of mouse CR1 also binds C3b, but with less efficiency. There is no C3d binding area within the first six SCRs, but our data confirms previous studies indicating an additional C3b/C3d binding region within SCRs 7 and 8 of mouse CR1 (SCRs 1-2 of mouse CR2). The presence of SCRs 1 to 4 is required for C3 cofactor activity. 8C12, a mAb which blocks C3b erythrocyte rosette binding and the C3 cofactor activity of mouse CR1, binds only to chimeras containing SCRs 3 to 4. In summary, human and mouse CR1 contain two independent C3b binding sites and share a common framework consisting of SCRs involved in ligand binding and nonbinding SCRs that optimize activity, further supporting the hypothesis that these two molecules act as functional homologues.


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Abbreviations used in this paper: SCR, short consensus repeat; TM, transmembrane; IC, intracytoplasmic; E, erythrocyte; EA, Ab-sensitized SRBC.
CR2, but the former has six additional amino terminal SCRs that show higher homology to human CR1. This observation explains the reason that four of five mAbs raised against mouse CR1 cross-react with mouse CR2 (13). Only the mAb 8C12 is specific for mouse CR1 and has the property of inhibiting the C3b-binding and the C3 cofactor activity of the molecule. Two other mAbs, 7G6 and 4E3, completely inhibit C3d binding to both mouse CR1 and CR2 (14). Finally, CR2 is also expressed on thymocytes, T cells, and keratinocytes in the human, but not in the mouse (2).

Interestingly, the structural differences between the human and mouse CRs do not seem to affect greatly their activities. These additional amino terminal SCRs confer CR1-like properties to the mouse molecule. The major allele of human CR1 has two C3b binding sites. Two binding sites are also involved in mouse CR1 interaction with C3b, as defined by mAb inhibition studies; one is unique to mouse CR1 and is recognized by the mAb 8C12, and the other is shared with mouse CR2 and is recognized by mAbs 7G6 and 4E3. The second site is also involved in mouse CR1 and CR2 binding to C3d (15).

To further understand the similarities and differences between the human and murine CRs, we have investigated the details concerning mouse CR1/CR2 functional regions by constructing mouse CR1/human CR2 cDNA chimeras in which the C3 interacting domains of human CR2 have been substituted by different regions within the first eight SCRs of mouse CR1. Analysis of cells expressing these chimeric molecules has identified important functional areas within these proteins, including SCRs involved in ligand binding and in the C3 factor I cofactor activity. These studies define a series of new parallels between the two species and demonstrate how two structurally divergent molecules can maintain nearly identical activities and C3 interactions.

Material and Methods
Preparation and expression of the mouse CR1/human CR2 cDNA chimeras and the mouse CR1 r1-6 TM/IC construct

Our previously described 15 SCR-containing human CR2 cDNA pBS-SCR2-15 (16) was cut with EagI/Xhol restriction enzymes, generating three fragments. One EagI/Xhol-flanked fragment essentially contains the pBSKS plasmid, and lacks any of the human CR2 sequence. The second fragment contains SCRs 3 to 15 plus the TMIC region of human CR2 flanked by Xhol sites. The third fragment contains the human CR2 signal peptide plus SCRs 1 and 2. The latter was not used in subsequent cloning. We also cut our CR2 M9 plasmid (17) with Xhol, and the fragment containing the pBSKS portion plus the human CR2 signal peptide was isolated. Then, by PCR-based methods using as template our mCR1 (18) cDNA, we amplified the signal peptide plus SCRs 1 and 2 (PCR 1–2), SCRs 1 to 4 (PCR 1–4), SCRs 1 to 6 (PCR 1–6), or SCRs 1 to 8 (PCR 1–8) of mouse CR1, flanking each segment with 5′ EagI/3′ Xhol restriction sites. These amplified cDNAs were cloned into the pBSKS-containing, EagI/Xhol-generated fragment of pBS-SCR2–15. We also amplified a SalI/Xhol-flanked cDNA containing SCRs 3 to 6 (PCR 3–6) of mouse CR1 and cloned it into the pBSKS-containing Xhol-generated fragment of CR2 M9. We then subcloned the Xhol-flanked fragment of pBS-SCR2–15 containing SCR 3 to 15 plus the transmembrane and intracytoplasmic regions of human CR2 into the Xhol site of the above constructs.

The mouse CR1 r1–6 TM/IC cDNA was prepared in the following manner. We used PCR-based methods to convert the sequence starting at nucleotide 1252 of mouse CR1 (11) from CATGATT to GTGCGA, introducing a SalI site three nucleotides downstream from the codon encoding the last cysteine of SCR 6. Because our mouse CR1 cDNA insert is in pBSKS, there is another SalI site within the multiple cloning site of the plasmid 20 nucleotides downstream from the 3′ end of the insert. We then cut this vector with SalI, removing a fragment containing SCRs 7 to 21 plus the TM/IC regions of mouse CR1, and instead inserted a PCR-amplified, SalI-flanked fragment containing only the TM/IC regions of mouse CR1 followed by a stop codon and an EcoRI site.

For eukaryotic expression, we subcloned each construct into the EcoRI site of the pSFFV-neo plasmid (19) in a sense or antisense orientation. Sequence analysis was made using specific oligonucleotide primers and double strand sequencing with Sequenase (United States Biochemical Corp., Cleveland, OH). The cDNAs were then transfected by electroporation into the human K562 erythroleukemia cell line using previously described techniques (17).

Abs and media

7E9, 7E6, 4E3, 8C12, and 8D9 are rat anti-mouse CR1 mAbs (13). 7E9, 7G6, 4E3, and 8D9 also react with mouse CR2. HBS (IgG2a) is a mouse anti-human CR2 mAb (17). UPCT-10 is a mouse IgG2a myeloma protein used as a control Ab (Sigma Chemical Co., St. Louis, MO). K562 transfected cells were grown in RPMI 1640 media supplemented with 2 mM glutamine, penicillin, and streptomycin, and 400 μg/ml of active G418 (Geneticin; Life Technologies, Inc., Grand Island, NY) as a selecting agent.

Flow cytometric analysis

One million K562 cells expressing the recombinant chimeric molecules, in the sense or antisense orientation, were incubated at 4°C with either 1 μg of the rat anti-mouse CR1 mAbs, 1 μg of HBS, or 1 μg of UPCT10 for 1 h in 100 μl of PBS/1% BSA. Cells were washed and FITC-conjugated goat anti-rat Ig (Cappel Laboratories, West Chester, PA) or FITC-conjugated goat anti-mouse IgG (Cappel Laboratories) was added. After 30 min incubation, cells were washed and analyzed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA).

E rosette formation

Sheep E bearing different mouse C3 components were prepared as previously described using standard techniques (14). In brief, Ab-sensitized sheep E (EA) bearing guinea pig C1, human C4b and C2a, and mouse C3b (EAC1423b) were prepared as described. EAC1423b were converted to EAC1423bi cells by incubation with mouse factor H (0.05 μg/ml) and mouse factor I (0.05 μg/ml) at 37°C for 30 min, and then washed with an isotonic Veronal buffer consisting of 2.47 mM sodium glutamate, penicillin, and streptomycin, and 400 μg/ml of active G418 (Geneticin; Life Technologies, Inc., Grand Island, NY) as a selecting agent.

Preparation of cell extracts

K562 cells expressing recombinant receptors were incubated at 10⁷ cells/ml in PBS containing 0.5% Nonidet P-40 (Sigma, St. Louis, MO) and 1 mM PMSF at room temperature for 15 min. The mixtures were centrifuged at 12,000 rpm for 10 min and the supernatants were stored frozen at −80°C until use.
Factor I cofactor activity

Mouse C3 (C3 mo) and factor H were purified to homogeneity from EDTA-plasma as described (20). Functionally pure mouse factor I was prepared from serum as described (21). SRBC EA were prepared as described (14). EAC142 cells were prepared by incubating EA cells sequentially with guinea pig C1, human C4, and human C2 in 1X Verona1 buffer with 60% dextrose and 0.1% w/v gelatin to generate 300 hemolytic sites/cell. C3 mo was labeled with $^{125}$I by using IODO-GEN (Pierce Chemical Co., Rockford, IL). $^{125}$I-labeled fluid phase mouse C3b was obtained in the supernatant by incubating $^{125}$I-C3 mo at 0.5 μg/ml with $5 \times 10^7$ EAC142 cells/ml in DGVB at 30°C for 15 min. A mixture of $^{125}$I-labeled C3b (100 ng/ml), factor I (200 ng/ml), and cell extracts (4 $\times$ 10^6 cell equivalents per ml) was incubated for 60 min at 37°C. Cleavage of the α'-chain of C3b was assessed by SDS-PAGE under reducing conditions followed by autoradiography (4).

Results

Expression of the mouse CR1/human CR2 cDNA chimeras and the mouse CR1 1-6 TM/IC construct

Figure 1 shows a schematic description of the cDNA constructs. PCR-amplified mouse CR1 fragments contain an EagI site in the 5' untranslated region of mouse CR1 four nucleotides upstream of the ATG methionine initiation codon and an XhoI site at the 3' end. We introduced these amplified mouse CR1 fragments into the EagI/XhoI pBSKS-containing portion of pBSCR2-15. In this way, the mouse CR1 sequence is followed by the human CR2 sequence, starting two amino acids upstream of the first cysteine of SCR 3 and extending through the TM/IC regions. The human CR2 SCR3-15 sequence does not contribute to the CR2 C3-binding capacity.

The above strategy applies to all of the chimeric constructs except for rCH 3-6. We used CR2 M9, in which the human CR2 signal peptide is followed by the codon encoding for the first cysteine of the human CR2 SCR 3. An Xhol site is found between the human CR2 signal peptide and SCR3. We inserted into this Xhol site a Sall/Xhol-flanked, PCR-amplified mouse CR1 fragment containing SCRs 3 to 6. In this way, the two amino acids immediately upstream of the mouse SCR 3 first cysteine were changed from Leu-Glu to Val-Asp, without any other change in the encoded protein.

Anti-mouse CR1 mAb reactivity of cells expressing chimeric cDNA constructs

We tested the ability of the five mAbs that recognize mouse CR1 to stain cells expressing the chimeric molecules. As noted in Figure 3, the monospecific mAb 8C12 recognizes both the rCH 1-4 and the rCH 3-6 chimeras, but not the rCH 1-2 chimera. In particular, it stains all of the chimeras containing SCRs 3 and 4. The mAb 4E3 only recognizes the chimera containing SCRs 7 and 8, in particular the rCH 1-8 chimera. The same applies to the mAb 7G6 (data not shown). The mAbs 7E9 and 8D9 do not stain any of the chimera-expressing cells. These results demonstrate that the 8C12 epitope is within mouse CR1 SCRs 3 and 4, and the 7G6 and 4E3 epitopes are within SCRs 7 and 8, which correspond to SCRs 1 and 2 of mouse CR2.
ANALYSIS OF MOUSE CR1 FUNCTIONAL DOMAINS

Figure 2. Flow cytometric analysis of cells transfected with the cDNA chimeric constructs stained with HB5 (solid line), or the control UPC10 (dashed line) Abs. x-axis is relative fluorescence on a log scale, y-axis is cell number.

E rosette formation

We tested the ability of the chimeric-expressing cells to form rosettes with E coated with either mouse C3b or C3d and then tested the ability of informative mAbs to interfere with this. As previously reported (18), 7G6 and 8C12 partially inhibit C3b rosettes to full length mouse CR1 (7G6 greater than 8C12) or CR2 (7G6 only) expressed in this form as recombinant membrane proteins. First, no rosettes are formed by using EAC14 (data not shown), in which C4b is derived from human C4. As shown in Table I, however, rCH 1–2 forms a low but specific and reproducible number of rosettes with E bearing C3b. The rosette percentage increases if additional SCRs are added to the molecule. rCH 3–6 does not form rosettes with C3b-coated E. Cells expressing the r1–6 TM/IC construct also form rosettes with E bearing C3b, although with less efficiency as compared with the mouse CR1 control. There is no C3d-dependent rosette formation, however, with these cells expressing the first six SCRs of mouse CR1 only. Only rCH 1–8 is able to form rosettes with C3d-coated E. In fact, the rosette percentage is comparable with the mouse CR2 control.

We then analyzed the C3b-dependent rosette inhibition by using the anti-mouse CR1/CR2 mAbs (Table II). As expected, 8C12 has no effect on rosette formation between rCH 1–2 and EAC1423b, but completely abolished the rosetting capacity of rCH 1–4 and rCH 1–6. It partially inhibited the rosetting capacity of rCH 1–8, comparable with the mouse CR1 control, suggesting the presence of an additional C3b-interacting site within SCRs 7 and 8. 7G6 partially inhibited C3b binding to the rCH 1–8 and to the mouse CR1 molecule. Only a combination of both mAbs completely abolished C3b interaction with rCH 1–8. These observations are identical with those we have previously reported for the nonchimeric receptors (18), and

Table I. Percentage of specific E rosettes

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>EAC1423b</th>
<th>EAC1423d</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCH 1–2 antisense</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rCH 1–2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>rCH 1–4</td>
<td>17</td>
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<tr>
<td>rCH 1–6</td>
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</tr>
<tr>
<td>rCH 1–8</td>
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<td>56</td>
</tr>
<tr>
<td>rCH 3–6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>r1–6 TM/IC</td>
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<td>0</td>
</tr>
<tr>
<td>rMCR1</td>
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<td>20</td>
</tr>
<tr>
<td>rMCR2</td>
<td>20</td>
<td>66</td>
</tr>
</tbody>
</table>

* Three hundred cells were counted and the results are expressed as a percentage of positive rosettes minus nonspecific binding detected in cells transfected with the antisense construct. Positive controls include cells expressing recombinant mouse CR1 (rMCR1) or CR2 (rMCR2). Results are means of at least three determinations.
Table II. Rosette inhibition by anti-mouse CR1/CR2 mAbs

<table>
<thead>
<tr>
<th></th>
<th>7E9</th>
<th>8C12</th>
<th>7G6</th>
<th>8C12 + 7G6</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCH 1–2</td>
<td>4</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
</tr>
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<td>17</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rCH 1–6</td>
<td>51</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rCH 1–8</td>
<td>60</td>
<td>41</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>rMCR1</td>
<td>65</td>
<td>42</td>
<td>18</td>
<td>ND</td>
</tr>
<tr>
<td>rMCR2</td>
<td>31</td>
<td>29</td>
<td>1</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a Results are expressed as a percentage of positive rosettes minus nonspecific binding detected in cells transfected with the antisense construct. Positive controls include cells expressing recombinant mouse CR1 (MCR1) or CR2 (MCR2). Results are means of at least three determinations. ND, not determined.

FIGURE 4. Cofactor activity of the chimeric proteins and the r1–6 TM/IC construct. 125I-labeled C3b (100 ng/ml) was incubated with buffer alone (lane 1), factor I alone (lane 2), or factor I plus extracts of cells (4 × 10⁶ cells equivalents per ml) expressing the recombinant receptors: r1–6 TM/IC (lane 4), rCH 1–2 (lane 5), rCH 1–4 (lane 6), rCH 1–6 (lane 7), rCH 1–8 (lane 8), and rCH 3–6 (lane 9). After incubation for 60 min at 37°C, cleavage at the α'-chain of C3b into the α'-70 and α'-43 fragments was assessed by SDS-PAGE on a 7.5% gel under reducing conditions and autoradiograph. Lane 3 contains factor I plus the antisense r1–6 TM/IC construct as a negative control.

FIGURE 5. Schematic representation of mouse CRs 1 and 2 showing important functional domains and areas of Ab reactivity.

**Discussion**

Previous observations have suggested the presence of two C3b binding regions within mouse CR1 (15). In this report, we confirm these original conclusions and also further define the specific SCRs involved in such interactions. We have shown the presence of a weak C3b binding site within SCRs 1 and 2 of mouse CR1. Although no C3b binding was detected between SCRs 3 to 6, their presence substantially increases the binding efficiency. This enhancing effect of SCRs 3 to 6 is supported by the observation that 8C12, a mAb which does not directly recognize the interaction site within SCRs 1 and 2 but instead recognizes an epitope within SCRs 3 and 4, is able to inhibit C3b-dependent rosette formation to cells expressing rCH 1–4 and rCH 1–6. An additional C3b interaction site is present between SCRs 7 and 8 of mouse CR1, corresponding to SCRs 1 and 2 of mouse CR2. These SCRs are also involved in C3d binding to both mouse CR1 and CR2, and they contain epitopes for mAbs 7G6 and 4E3. No C3d binding region is found within the first six SCRs of mouse CR1. Figure 5 is a schematic summary of our findings.

Although our data are in general agreement with that of Pramoonjago et al. (15), some minor differences exist between our findings and theirs. In their experiments, they found that C3b-bearing thiol-Sepharose beads bind equally to...
detergent-solubilized mouse CR1 and CR2. In our analysis, we consistently have seen a decrease in rosette formation between C3d-coated E and cells expressing mouse CR1 as compared with mouse CR2. One possible explanation for these contrasting results is the different technique used in the studies. The soluble receptor could have slightly different properties compared with the membrane-bound receptor, thus affecting the efficiency of ligand interactions. In the human system, CR2 forms molecular complexes with other membrane proteins (22), a property that can be altered when the receptor is detergent-solubilized. On the other hand, we have found that rCH 1–8 forms C3d-dependent rosettes comparable with the mouse CR2 molecule. We do not know whether the human portion of the chimeric protein in some way affects the C3d binding characteristics by making the ligand-interacting site more accessible, by facilitating the collaboration of the receptor with associated modifying human cell membrane molecules, or both.

Pramoonjago et al. (15) also reported that the C3b binding to mouse CR1 was not affected by mAbs 7G6 and 4E3. We have consistently found a partial C3b-dependent rosette inhibition to mouse CR1 and rCH 1–8 using mAb 7G6 (18; this paper). Again, the different techniques used are likely to be the explanation for the different results. Interestingly, in our experiments, 7G6 inhibits C3b-dependent rosette formation more efficiently than 8C12. It is possible that 7G6 has not only an effect on the C3b/C3d interaction, but also on the other interacting site or whether the presence or absence of additional amino terminal SCRs modifies the ligand binding to SCRs 7 and 8.

The cofactor activity of mouse CR1 maps to SCRs 1 to 4, mAb 8C12, which blocks cofactor activity of mouse CR1, also maps to SCRs 3 and 4. Our data suggest that cofactor activity originates from two distinct sites, with SCRs 1 and 2 providing the initial C3b-binding region and SCRs 3 and 4 contributing the cofactor activity. However, we cannot rule out that these four SCRs combine to form a single functional unit. This second situation has been noted in other studies in which the SCRs mediating the cofactor activity of human CR1 could not be separated from those that bind C3b (23). On the other hand, the former state has been suggested in another human SCR-containing C regulatory molecule, membrane cofactor protein (MCP). In this system, ligand binding did not always parallel the cofactor activity. This implied that there was another interaction site, in addition to the C3 ligand binding site, which directed cofactor activity (24).

It is of particular significance to analyze the ligand-interacting regions within these receptors to determine how the activities of these molecules are preserved between species despite divergence in their structure and genomic organization. This information also provides insights into details concerning the specific properties intrinsic to the SCR motif. In humans, Krickstein et al. determined by E rosette analysis that a C3b-binding domain of CR1 was localized in SCR 8 and/or SCR 9, and in an additional region encompassing SCR 15 and/or SCR 16 (25). Krych et al. (26) found that substitution of five amino acids at the end of SCR 2, in a multi-SCR site which does not bind C3b, with residues of a homologous region of SCR 9 resulted in the acquisition of C3b binding by this site. These data demonstrated that a contact site between C3b and human CR1 is present in these two SCRs. In other studies, Kalli et al. have shown that additional SCRs, beyond the two previously demonstrated to provide a contact site with the ligand, are important to optimize binding (23). On the basis of these findings, Kalli et al. suggested that the anatomy of a long homologue repeat within human CR1 could be separated into SCRs involved in direct C3b and C4b binding, followed by SCRs that support that binding, and SCRs that provide adequate spacing to allow multiple interactions between CR1 and C-activating complexes containing C3b and C4b (23). In their experiments, chimeras containing SCR 8 and 9 of human CR1 did not bind C3b if SCR 10 and 11 of human CR1 were substituted by SCR 3 and 4 of human CR2. It is not known from the studies, however, whether these additional human CR1 SCRs provide an additional contact site or whether they just change the spatial conformation of SCR 8 and 9 to improve binding.

Our data are in accordance with this general human CR1 C3 ligand-binding sites model. It thus provides more evidence for the previously described functional homologies between the human and mouse CR1 molecules and the hypothesis that these two proteins are evolutionary counterparts. In the mouse, SCRs 1 and 2 include a C3b binding region. This is followed by supporting SCRs (SCRs 3–6) which improve C3b binding. SCRs 7 and 8 provide a second C3b binding site which, interestingly, also includes a C3d binding site. The observation that the r1–6 TM/IC construct has a decreased rosette formation capacity as compared with the parent molecule suggests that spacing and nonbinding SCRs are also needed for optimal activity of mouse CR1. Finally, like human CR1, the mouse molecule CR1 is unique in its capacity for efficient cofactor activity of C3.

Because of these similarities, we have also searched for a consensus sequence in human CR1 and murine CR1 or CR2 that might be responsible for C3b binding. There is no obvious site. This is not unexpected, however, as published data suggest that the C3b binding site in CR1 may be different from the sites in other related proteins, factor H and factor B. A similar sequence motif is not obvious in the homologous positions in their SCRs (26). In addition, the dispersal of amino acids involved in C3b ligand binding across many SCRs in CR1 likely obscures the ability to detect important sites by sequence comparison alone. Finally, it is important to point out that there still are some major differences and unresolved points comparing the mouse and human.
CR1 molecules (2). First, mouse CR1 does not seem to be the immune complex receptor. In mouse, platelets are involved in the trafficking of immune complexes in the circulation, but mouse platelets do not express CR1 (13). Mouse CR1 also has a CR2-like IC tail. In addition, whether mouse CR1 binds mouse C4b is not yet known. Mouse CR1 does not bind human C4b (2), but this could be secondary to species-related restriction. Until mouse C4b is isolated in the quantity and purity needed to perform these experiments, this question will remain unresolved.

References


