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The Cleavage of Two C1s Subunits by a Single Active C1r Reveals Substantial Flexibility of the C1s-C1r-C1r-C1s Tetramer in the C1 Complex

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The activation of the C1s-C1r-C1r-C1s tetramer in the C1 complex, which involves the cleavage of an Arg-Ile bond in the catalytic domains of the subcomponents, is a two-step process. First, the autolytic activation of C1r takes place, then activated C1r cleaves zymogen C1s. The Arg^{66}Gln mutant of C1r (C1r^{QI}) is stabilized in the zymogen form. This mutant was used to form a C1q-(C1s-C1r^{QI}-C1r-C1s) heteropentamer to study the relative position of the C1r and C1s subunits in the C1 complex. After triggering the C1 by IgG-Sepharose, both C1s subunits are cleaved by the single proteolytically active C1r subunit in the C1s-C1r^{QI}, C1r-C1s tetramer. This finding indicates that the tetramer is flexible enough to adopt different conformations within the C1 complex during the activation process, enabling the single active C1r to cleave both C1s, the neighboring and the sequentially distant one. The Journal of Immunology, 2000, 165: 2048–2051.

Materials and Methods

Cells and viruses

Spodoptera frugiperda 9 cells and wild-type (wt) Autographa californica nuclear polyhedrosis virus were provided by Max Summers (Texas A&M University, College Station, TX). Cells were grown at 27°C in Grace’s insect medium with 10% FCS (Serva, Heidelberg, Germany), 3.3 g/L lactalbumin hydrolysate (Sigma, St. Louis, MO), and 3.3 g/L yeastolate (Oxoid, Basingstoke, England). Recombinant viruses were produced as described by Summers and Smith (17).

Expression of the recombinant proteins

The expression was done essentially as described earlier (18). A total of $2 \times 10^7$ Spodoptera frugiperda 9 cells were infected in 175-cm$^2$ flask at a multiplicity of infection of 10. After incubation at 27°C for 1 h the medium was changed to 50 ml SF900 serum-free medium (Life Technologies, Grand Island, NY). The cell culture supernatant was harvested 72 h later and was concentrated 20-fold on a PM10 ultrafiltration membrane (Amicon, Beverly, MA).

Reconstitution of C1s-C1r^{QI}-C1r-C1s tetramer and the C1 complex

To reconstitute the C1 containing C1r-C1r^{QI} heterodimer, samples were prepared as described earlier by Dobó et al. (16). Fixed amounts of concentrated supernatant of insect cells that produce wt C1r (0.4 μg in 5 μl) were mixed with increasing amounts of concentrated supernatant containing C1r^{QI} (0.4, 0.8, 1.2, 1.6, and 2.4 μg in 6, 12, 18, 24, and 36 μl, respectively) to obtain heterodimer. One sample was prepared with 5 μl of wt C1r alone and one with 36 μl C1r^{QI} alone (Table I) to serve as 100 and 0% references, respectively. Samples were diluted to 65 μl with TBS buffer containing 1 mM final concentration of CaCl$_2$ and were incubated for 3 h.

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3 Abbreviations used in this paper: C1, the first component of complement; C1q, C1r, and C1s, subcomponents of C1; C1r^{QI}, Arg^{66}Gln mutant of C1r; wt, wild-type; CCP, complement control protein.

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at 4°C. Each sample was used for the reconstitution of C1 by adding recombinant C1s (3 μg in 20 μl concentrated supernatant) and 8.5 μg of C1q (Calbiochem, La Jolla, CA) in 15 μl, followed by incubation in the presence of 1 mM Ca²⁺ at 4°C for 20 min. Activation of C1s during this step was checked by immunoblotting, and no detectable activation was observed.

**Activation of C1 by IgG-Sepharose**

IgG-Sepharose was prepared from CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) and human IgG (Humán RT, Gödöllő, Hungary). For the coupling reaction, we followed the procedure recommended by the manufacturer. Small columns containing 40 μl IgG-Sepharose were equilibrated with TBS containing 10 mM CaCl₂. After binding of C1 samples for 10 min, the columns were washed twice with 80 μl TBS containing 10 mM CaCl₂. The columns were incubated at 37°C for 1 h to activate C1. C1r and C1s were eluted using 100 μl TBS containing 25 mM EDTA. Fifteen microliters of each sample was analyzed by SDS-PAGE-Western blot under reducing conditions.

**Western blot analysis**

Protein samples were loaded onto a 10% SDS-polyacrylamide gel prepared as described by Laemmli (19). For the immunoblotting, the method of Towbin et al. (20) was followed. Proteins were transferred to a nitrocellulose sheet (0.45 μm; Schleicher & Schuell, Dassel, Germany) in a semidyry apparatus (Pharmacia Biotech). Nitrocellulose strips were incubated with goat anti-human C1s Ab (1:1000; Incstar, Stillwater, MN), then later with alkaline phosphatase-labeled rabbit anti-goat IgG conjugate as secondary Ab (1:3000; Sigma). Visualization was performed by the commercially available substrates of alkaline phosphatase: 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. For densitometry we used the GEL DOC 1000 instrument and Molecular Analyst Software (both obtained from Bio-Rad, Hercules, CA).

**Results and Discussion**

The aim of this study was to assess the flexibility of the C1r-C1s₂ and to test the accessibility of C1s by C1r in C1, a complex reconstituted with the composition (C1s-C1r₂-C1r-C1s-C1q) and activated on IgG-Sepharose. By adding increasing amounts of C1r₂ to fixed amounts of wt C1r, the proportion of wt C1r₂ homodimers was shifted from 100% to a negligible value, i.e., practically all enzymatically active C1r subunits were transferred into C1r₂-C1r₄ heterodimers as described earlier (16). The molar ratio of C1r₄-C1r varied from 0:1 to 6:1 (see Materials and Methods and Table I). After heterodimer formation, C1s and C1q were added at large molar excesses to consume all C1r, and C1 was reconstituted by incubating the mixtures for 20 min at 4°C. The C1 samples were absorbed onto IgG-Sepharose columns, and excess C1s was washed away. Samples were then incubated at 37°C for 1 h to trigger C1 activation. After activation, C1r and C1s were eluted using an EDTA-containing buffer and the components were separated by SDS-PAGE under reducing conditions. Control experiments verified that under the conditions of elution (concentration, temperature, time) no detectable activation of C1s by C1r occurs in the fluid phase. The samples were blotted to nitrocellulose membranes, and C1s was made visible using anti-C1s Abs (Fig. 1).

The relative amounts of uncleaved and cleaved C1s were assessed by densitometry of the membranes (see Figs. 2 and 3). Simple statistical considerations predict the composition of the C1 complex. A value n moles of wt C1r₂ homodimers bind and activate 2n moles of C1s in the complex. If large molar excesses of C1r₂, C1s, and C1q are added, 2n moles of C1 containing 2n moles of C1r₂-C1r₄ and 4n moles of C1s are formed; an excess of C1 containing inactive C1r₂ homodimers and C1s will also be present. The calculated amounts of the different tetramer variants in the C1 samples as a function of the initial concentration of the subcomponents are shown in Table I. Increasing total amounts of C1s are seen eluted by EDTA as the C1r₄-C1r ratio increases due to the increase in total amount of macromolecular C1 assembled. However, as the C1r₄ homodimers even in the assembled C1 do not activate C1s, the amount of uncleaved C1s (in the C1 bound to IgG-Sepharose) increases. The question to be answered was: are one or two C1s subunits cleaved in the C1 complex with C1s-C1r₄-C1r-C1s tetramer composition? If the single wt C1r cleaves two C1s subunits in C1, then we expect 2 moles of cleaved C1s per mole of wt C1r in the eluted samples, that is, the amount of activated C1s doubles as compared with the reference signal (sample 1 in Table I). As seen in Fig. 2, the signal for the uncleaved C1s

![FIGURE 1. Western blot detection of C1s activation. Samples were prepared using components as shown in Table I to reconstitute C1. After incubation at 4°C, the samples were applied to IgG-Sepharose columns. After binding, the columns were washed extensively with buffer containing Ca²⁺. IgG-Sepharose-bound C1 was activated at 37°C for 1 h. C1r and C1s were then eluted with buffer containing EDTA. The eluates were analyzed by Western blotting using anti-C1s Abs (more details in Materials and Methods).](http://www.jimmunol.org/)

Table 1. Calculated amounts of C1s-C1r₂-C1r-C1s, C1s-C1r₄-C1r-C1s, and C1s-C1r₄-C1r₂-C1s tetramer variants in the reconstituted C1 complex at equilibrium, as a function of the initial concentration of the subcomponents

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Initial Amount of</th>
<th>Calculated Amounts for Equilibrium in the C1 Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1r₁</td>
<td>C1r₄</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>0.14</td>
<td>1</td>
</tr>
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<td>5</td>
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</tr>
<tr>
<td>8</td>
<td>0.14</td>
<td>1</td>
</tr>
</tbody>
</table>

*1 unit = 2.41 x 10⁻¹² mol.*
increased with an increasing $C1r^Q/C1r$ molar ratio, while the signal of the activated $C1s$ (the $C1s$ A chain, Fig. 3) practically doubled when reaching the plateau level. This experiment clearly demonstrates that the wt $C1r$ in the $C1s$-$C1r^Q$-$C1r$-$C1s$ tetramer cleaves both $C1s$ subunits: the one binding directly to its N-terminal $\alpha$ region and the other one at the distant end of the tetramer, adjacent to the inactive $C1r^Q$. Two obvious explanations arise at first glance: 1) exchange among the subunits via dissociation and reassociation of the $C1$ complex or 2) conformational flexibility. Under our experimental conditions, the dissociation and reassociation of the $C1$ complex is negligible because the dissociation constant of unactivated $C1$ is in the picomolar range (21). It is plausible to assume that the $K_d$ of the half-activated $C1$ is not greater than that of the fully activated complex (i.e., 6.5 nM) (21), reflecting a fairly tight complex. If there were significant exchanges of subunits between the $C1$ complexes, the signal for activated $C1s$ would increase continuously instead of reaching a plateau of two activated $C1s$ per $C1r$.

There are two models for the structure of $C1$ that are consistent with the electron microscopic images; both models use symmetry and functional considerations. In both models, the $C1r_2$ portion of the tetramer is located inside the cone formed by the spreading $C1q$ arms, while the protruding arms of the tetramer (dominantly the two $C1s$ subunits) wrap around the collagenous arms of $C1q$. In the Los Angeles model (22), the entire $C1s$ is placed outside of the cone formed by the $C1q$ arms, having free access to the substrates (complement components $C2$, $C4$). This arrangement maximizes the number of identical contact surfaces with $C1q$, because the $C1s$-$C1r$-$C1r$-$C1s$ tetramer interacts with four collagen-like $C1q$ arms. The Grenoble model (4) assumes that the $C1s$ reenters the $C1q$ cone so that the catalytic domains of the $C1s$ zymogen can have access to the catalytic domains of $C1r$. In this model, the $C1s$-$C1r$-$C1r$-$C1s$ tetramer interacts with only two $C1q$ arms. Probing the structure of $C1$ with an anti-$C1s$ mAb led to the conclusion that these configurations are interconvertible and both can exist in solution (23). The conversion of the two forms of $C1$ requires considerable conformational flexibility of the pentameric

**FIGURE 4.** Eclectic model of $C1$. Illustration of the cleavage of two $C1s$ by one active $C1r$ in the complex, without dissociation. The flexibility of the necklace-like $C1s$-$C1r^Q$-$C1r$-$C1s$ tetramer provides access to both $C1s$ by both $C1r$. 

**FIGURE 3.** Densitometric analysis of the Western blot bands corresponding to activated $C1s$. The amount of activated $C1s$ reaches a plateau as increasing amounts of $C1r^Q$ are added to the samples. Experimental conditions were shown in Table I and treated as summarized in Fig. 1. Blots were scanned, and the intensity of the bands corresponding to uncleaved $C1s$ in each sample were normalized to sample 7. Each column represents the average of five experiments. SE bars are indicated.

**FIGURE 2.** Densitometric analysis of the Western blot bands corresponding to uncleaved $C1s$. The amounts of uncleaved $C1s$ increases along with the increasing amounts of $C1r^Q$ homodimers added to the different samples. The $C1$ complex was prepared as shown in Table I and treated as summarized in Fig. 1. Blots were scanned and the intensity of the bands corresponding to uncleaved $C1s$ in each sample were normalized to sample 7. Each column represents the average of five experiments. SE bars are indicated.
structure, in particular the flexibility of the tetramer inside the C1q arms (Fig. 4). Our results support this view by demonstrating that the C1s–C1r–C1r–C1s tetramer has indeed significant conformational flexibility within the C1 complex. In the case of the Grenoble model, this implies that the catalytic domains of C1r and C1s can change their relative position to each other inside the cone formed by the C1q arms during the activation process.

Models based on chemical cross-linking and homology modeling (9) suggest that the second complement control protein (CCP) module in C1r and C1s is attached to the serine protease domain while significant flexibility is assumed at the CCP1–CCP2 domain–domain interface. This assumption is in accord with experimentally (nuclear magnetic resonance) observed flexibility between two contiguous CCP modules in a related molecule, human factor H (24). The rotation of one CCP module about the longitudinal axis could provide sufficient conformational freedom to allow the suggested interconversion between the two forms of C1 complex together with some obvious flexibility (e.g., in the α region) of these extended multidomain serine protease subunits.

The finding that the C1 molecule is flexible enough to adopt a set of conformations allowing one of the two C1r subunits to cleave both C1s proenzymes is in accord with independent structural observations of C1r and C1s and points to the significance of conformational dynamics in the mechanism of activation of the first component of complement.

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