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Functional Role of the Linker between the Complement Control Protein Modules of Complement Protease C1s

Isabelle Bally,* Véronique Rossi,* Nicole M. Thielens,* Christine Gaboriaud,† and Gérard J. Arlaud2*

C1s is the modular serine protease responsible for cleavage of C4 and C2, the protein substrates of the first component of C (C1). Its catalytic domain comprises two complement control protein (CCP) modules connected by a four-residue linker Gln340-ProVal-Asp343 and a serine protease domain. To assess the functional role of the linker, a series of mutations were performed at positions 340–343 of human C1s, and the resulting mutants were produced using a baculovirus-mediated expression system and characterized functionally. All mutants were secreted in a proenzyme form and had a mass of 77,203–77,716 Da comparable to that of wild-type C1s, except Q340E, which had a mass of 82,008 Da, due to overglycosylation at Asn391. None of the mutations significantly altered C1s ability to assemble with C1r and C1q within C1. Whereas the other mutations had no effect on C1s activation, the Q340E mutant was totally resistant to C1r-mediated activation, both in the fluid phase and within the C1 complex. Once activated, all mutants cleaved C2 with an efficiency comparable to that of wild-type C1s. In contrast, most of the mutations resulted in a decreased C4-cleaving activity, with particularly pronounced inhibitory effects for point mutants Q340K, P341I, V342K, and D343N. Comparable effects were observed when the C4-cleaving activity of the mutants was measured inside C1. Thus, flexibility of the C1s CCP1-CCP2 linker plays no significant role in C1 assembly or C1s activation by C1r inside C1 but plays a critical role in C4 cleavage by adjusting positioning of this substrate for optimal cleavage by the C1s active site. The Journal of Immunology, 2005, 175: 4536–4542.

The C1 complex is a 790-kDa multimolecular assembly comprised of three components, C1q, C1r, and C1s, which triggers the classical pathway of C, a major route of innate immuinity against pathogenic microorganisms. Recognition of the target microorganism is mediated by C1q and triggers autolytic activation of C1r, a modular serine protease (SP),3 which in turn activates a second protease C1s through cleavage of its Arg422-Ile423 bond (1–4). Active C1s then specifically cleaves a single arginyl peptide bond in C proteins C4 and C2, the natural substrates of C1, thereby triggering a cascade of proteolytic reactions that elicit various biological activities designed to provide a first line of defense against infection. Human C1s is synthesized as a 673-residue single-chainzymogen, which starting from the N terminus, comprises a CUB module (5), an epidermal growth factor (EGF) module, a second CUB module, two complement control protein (CCP) modules (6), and a chymotrypsin-like SP domain. This modular architecture is shared by C1r, and by the mannan-binding lectin-associated SPs (MASPs), a group of proteases involved in the lectin pathway of C (7). From a functional point of view, it is known that assembly of C1s-C1r-C1q-C1s, the tetrameric catalytic subunit of C1, involves Ca2+-dependent heterodimeric C1r-C1s interactions mediated by the N-terminal CUB1-EGF moiety of each protease (8–10). On the other hand, it is established that the enzymatic properties of C1r and C1s are mediated by their C-terminal CCP1-CCP2-SP regions (11, 12). In the case of C1s, expression of modular fragments has led to the conclusion that, whereas C2 cleavage only requires the SP domain, C4 cleavage involves substrate recognition sites located in the CCP modules (13). The structure of the CCP1-SP fragment of C1s has been solved by x-ray crystallography, revealing that the ellipsoidal CCP2 module is tightly anchored on the more globular SP domain by means of a rigid interface and may therefore act as a spacer and a handle (14). The structure of the C1s CCP1 module is not known yet, and the flexibility at the CCP1-CCP2 junction has not been characterized. Nevertheless, a number of structural studies have been performed on CCP module pairs, indicating that these exhibit varying degrees of flexibility at their intermodule interface (15–18). Based on these observations, it was hypothesized that the CCP1-CCP2 linker of C1s exhibits substantial flexibility, allowing the SP domain to switch from a position inside C1, allowing activation by C1r, to a more external location, required for cleavage of C4 and C2 (14). From a general standpoint, current knowledge of the C1 structure strongly suggests that its ability to mediate its finely tuned function relies for a large part on the flexibility of its individual subunits (4).

The objective of this study was to assess the particular role of the C1s CCP1-CCP2 linker in the various facets of the interaction and catalytic properties of this enzyme. Using site-directed mutagenesis, we show that this segment is not involved in the C1s activation mechanism but plays a key role in modulating its activity toward C4.

Materials and Methods

Materials

Diisopropyl phosphorofluoridate was purchased from Sigma-Aldrich. The Pfu polymerase was obtained from Invitrogen Life Technologies. Restriction enzymes were purchased from New England Biolabs. The polyclonal
anti-C1s antiserum used for Western blot analysis was raised in rabbits according to standard procedures. The C1s-Sepharose column was prepared by coupling activated C1s purified from human serum (40 mg) to cyanogen bromide-activated Sepharose 4B (30 ml) according to the method of March et al. (19). The pBS-C1s plasmid containing the full-length human C1s cDNA (20) was kindly provided by Dr. M. Tosi (University of Rouen, Rouen, France). Oligonucleotides were purchased from MWG Biotec.

Proteins

C1q, proenzyme C1r, and activated C1s were purified from human plasma as described previously (21, 22). The activated CCP2-SP fragment of human C1r was expressed in a baculovirus/insect cells system and purified by ion-exchange and hydrophobic interaction chromatography as described previously (23). C proteins C2 and C4 were isolated from human plasma by means of published procedures (24, 25). The concentrations of purified proteins were determined using the following absorption coefficients (A280): C1q, 6.8 and 459,300; C1r, 12,416 and 86,300 (8); C1r fragment CCP2-SP, 14,900 and 40,400 (23); C2, 10,000 and 100,000 (24); and C4, 8,200 and 205,000 (26, 27).

Expression and purification of recombinant wild-type and mutant C1s

Recombinant wild-type C1s and its variants were expressed using a baculovirus/insect cells system. A DNA fragment encoding the C1s signal peptide plus the full-length mature protein (amino acid residues 1–673) was amplified by PCR using the Pfu polymerase and cloned into the pFastBac1 vector (Invitrogen Life Technologies), amplified, and titrated as described previously (28). The pBS-C1s plasmid containing the full-length human C1s cDNA (20) was kindly provided by Dr. M. Tosi (University of Rouen, Rouen, France). Oligonucleotides were purchased from MWG Biotec.

The recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen Life Technologies), amplified, and titrated as described previously (24). The pFastBac1/C1s expression plasmid containing the C1s full-length sequence was confirmed by dsDNA sequencing (Genome Express). These recombinant baculoviruses were used to transfect Sf9 cells using cellfectin in Sf900 II SFM medium (Invitrogen Life Technologies). The insect cells Spodoptera frugiperda (Ready-Plaque Sf9 cells obtained from Novagen) and Trichoplusia ni (High Five) were routinely grown and maintained as described previously (28). The bacmid DNA was used to transfect Sf9 cells using cellfectin in Sf900 II SFM medium (Invitrogen Life Technologies) as described in the manufacturer’s protocol. The recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen Life Technologies), amplified, and titrated as described previously (28). High Five cells (1.75 × 10^7 cells/175-cm² tissue culture flask) were infected with the recombinant viruses at a multiplicity of infection of two to three in Sf900 II SFM medium at 28°C for 48 h. The recombinant C1s proteins were purified from the cell culture supernatants using a one-step affinity chromatography on a Sepharose-C1s column. Briefly, each supernatant was dialyzed against 145 mM NaCl, 2 mM CaCl₂, and 50 mM triethanolamine hydrochloride (pH 7.4) containing 2 mM diisopropyl phosphorofluoridate and loaded onto the C1s-Sepharose column equilibrated in the same buffer. Elution was conducted with the same buffer containing 5 mM EDTA instead of CaCl₂. In between runs, the column was systematically washed with 50 mM triethanolamine hydrochloride and 0.5 M NaCl (pH 7.4). Each purified C1s variant was concentrated by ultrafiltration to 0.2–0.7 mg/ml, dialyzed against 145 mM NaCl, and 50 mM triethanolamine hydrochloride (pH 7.4) and stored at −20°C until use. The concentrations of the purified recombinant C1s variants were determined using an absorption coefficient (A_280) at 280 nm) of 1.45 (8) and the m.w. value determined in each case by mass spectrometry analysis (see Results).

Chemical characterization of the recombinant proteins

N-terminal sequence analysis was performed using an Applied Biosystems model 492 as described previously (11). Mass spectrometry analysis of the recombinant C1s molecules was performed using the MALDI technique on a Voyager Elite XL instrument (Applied Biosystems) under conditions described previously (29). The experimental error was approximately ±0.05% of the mass values, i.e., approximately ±40 Da for the values shown in Table I.

Fluid-phase activation of the C1s mutants

To check their ability to undergo activation in the fluid phase, the C1s mutants (0.14 mg/ml) were incubated with the activated C1r CCP2-SP fragment (1.15 mg/ml) for 2 h at 37°C in 145 mM NaCl and 50 mM triethanolamine hydrochloride (pH 7.4), containing 1 mg/ml OVA. The activation extent was determined by SDS-PAGE analysis (30) under reducing conditions and measurement by gel scanning of the amount of the two chains characteristic of active C1s relative to the single-chain proenzymes.

Analysis by surface plasmon resonance spectroscopy of the ability of the C1s mutants to associate with C1r and C1q

Analysis by surface plasmon resonance spectroscopy of the ability of the C1s mutants to associate with C1r and C1q was performed using a BIAcore 3000 instrument (BIAcore AB). C1q was purified from human plasma using a reverse-phase HPLC. The C1s mutant was incubated with the activated C1r CCP2-SP fragment (1.15 mg/ml) for 2 h at 37°C in 145 mM NaCl and 50 mM triethanolamine hydrochloride (pH 7.4), containing 1 mg/ml OVA. The activation extent was determined by SDS-PAGE analysis (30) under reducing conditions and measurement by gel scanning of the amount of the two chains characteristic of active C1s relative to the single-chain proenzyme.

Table I. Mass spectrometry analysis of the C1s variants

<table>
<thead>
<tr>
<th>C1s Variant</th>
<th>Experimental Mass (Da)</th>
<th>Calculated Mass of the Polypeptide (Da)</th>
<th>Deduced Mass of the Carbohydrates (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>77,413</td>
<td>74,887</td>
<td>2,526</td>
</tr>
<tr>
<td>Q340A</td>
<td>77,338</td>
<td>74,830</td>
<td>2,508</td>
</tr>
<tr>
<td>Q340K</td>
<td>77,716</td>
<td>74,887</td>
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<tr>
<td>Q340R</td>
<td>77,317</td>
<td>74,915</td>
<td>2,402</td>
</tr>
<tr>
<td>Q340S</td>
<td>77,302</td>
<td>74,846</td>
<td>2,456</td>
</tr>
<tr>
<td>Q340D</td>
<td>77,366</td>
<td>74,874</td>
<td>2,492</td>
</tr>
<tr>
<td>Q340E</td>
<td>82,008</td>
<td>74,888</td>
<td>7,120</td>
</tr>
<tr>
<td>Q340E/N391Q</td>
<td>76,117</td>
<td>74,902</td>
<td>1,215</td>
</tr>
<tr>
<td>Q340E/D343N</td>
<td>77,203</td>
<td>74,887</td>
<td>2,316</td>
</tr>
<tr>
<td>Q340D/D343E</td>
<td>77,540</td>
<td>74,888</td>
<td>2,652</td>
</tr>
<tr>
<td>P341I</td>
<td>77,413</td>
<td>74,903</td>
<td>2,510</td>
</tr>
<tr>
<td>V342K</td>
<td>77,425</td>
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<td>D343N</td>
<td>77,231</td>
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<td>2,477</td>
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<tr>
<td>Q340K/P341I/V342K</td>
<td>77,493</td>
<td>74,932</td>
<td>2,561</td>
</tr>
</tbody>
</table>

a The experimental error is approximately ±0.4 Da for all values.
b These values account for the carbohydrates attached to Asn₁⁵⁹ and Asn₃⁰¹.
Activation of the C1s mutants in the C1 complex

The C1 complex (0.25 μM) was reconstituted by mixing C1q, proenzyme C1r, and each individual C1s variant, in relative molar amounts 1:2:2, respectively, and incubated for 90 min at 37°C in 145 mM NaCl, 1 mM CaCl₂, and 50 mM triethanolamine hydrochloride (pH 7.4). Samples were submitted to SDS-PAGE analysis (30) under reducing conditions using 10% acrylamide gels. C1s was revealed by Western blotting after electrophoresis onto a nitrocellulose membrane as described previously (13), and its extent of activation was determined by gel scanning from the relative proportion of the bands corresponding to the active and proenzyme forms.

Proteolytic assays

The proteolytic activity of the activated C1s variants toward C2 and C4 was measured by incubation of these protein substrates (2.25 μM each) at enzyme/protein molar ratios of 1:500 and 1:1125, respectively, for varying periods at 37°C. Enzyme dilutions were performed in 145 mM NaCl, 50 mM triethanolamine hydrochloride, and 1 mg/ml OVA (pH 7.4), containing 1 mM CaCl₂ in the case of the reconstituted C1s samples. The extent of reaction was determined after SDS-PAGE analysis under reducing conditions by gel scanning of the cleavage fragments, as described previously (13). For determination of the kinetic parameters of C4 cleavage, the C4 concentration ranged from 0.5 to 4 μM, and C1s was used at a fixed concentration of 2 nM. The kinetic constants were determined by the Lineweaver-Burk method using linear regression analysis and are based on triplicate measurements of initial rates at six different substrate concentrations.

Modeling of the C1s catalytic domain

A three-dimensional model of the CCP₁-CCP₂-SP fragment of C1s was assembled from the C1r CCP₁ module, as seen in the x-ray structure of the C1r CCP₁-CCP₂-SP fragment (12), and the C1s CCP₂-SP domain, as seen in the x-ray structure of the corresponding fragment (14). Relative positioning of CCP₂ with respect to CCP₁ was achieved by superimposing the C1r CCP₁-CCP₂ module pair onto the C1s CCP₂ module. The root mean square deviation between the two CCP₂ modules was 0.67 Å, based on 56 positions. In CCP₁, Lys355 (equivalent to Gln 340 of C1s) was replaced by Gln.

Results

The objective of this study was to perform point mutations in the sequence stretch 340–343 located between the CCP₁ and CCP₂ modules of human C1s and to determine the functional properties of the resulting mutants to assess the role of this region in the function of C1s, particularly in the context of the C1 complex. Our strategy was based initially on a comparative analysis of the sequence of this segment in C1s and in other proteases of the same family (C1r, MASP-1, MASP-2, and MASP-3) (Fig. 1). The wild-type and mutant C1s molecules were expressed in an insect cells system and secreted in the culture supernatant, at concentrations from 5 to 10 mg/L culture, except for one of the mutants (Q340S/P341I), which showed a significantly decreased production yield (1 mg/L). All recombinant proteins could be successfully purified by affinity chromatography on a C₁s-Sepharose column in the presence of Ca²⁺ ions, providing strong indication that, as expected, the interaction properties characteristic of their N-terminal CUB-EGF moiety (10) were unaltered. All variants were essentially pure, as assessed by SDS-PAGE analysis, and exhibited a single-chain structure under reducing conditions, indicating that they were in the proenzyme state (Fig. 2).

The Q340E mutation enhances glycosylation at Asn³⁹¹

A series of point mutations were performed initially at position 340, where the Gln residue was replaced by Lys (as in C1r and MASP-1/3), Ser (as in MASP-2), Ala, Arg, Asp, or Glu. As judged by SDS-PAGE analysis, all of the mutants had an electrophoretic behavior similar to that of the wild-type protein, except mutant Q340E, which surprisingly exhibited a much higher apparent m.w. (Fig. 2). This observation was confirmed by mass spectrometry analysis, which revealed that, whereas the other point mutants at position 340 had mass values of 77,302–77,716 Da comparable to that of wild-type C1s (77,413 Da), the Q340E mutant had a mass of 82,008 Da (Table I). The sequence of the plasmid corresponding to the Q340E mutant was confirmed by dsDNA sequencing, whereas N-terminal sequence analysis of the recombinant protein yielded the expected Glu-Pro-Thr-Met-Tyr... sequence. Therefore, it appeared likely that the observed increase in molecular mass was due to an increase in the size of one or both of the N-linked oligosaccharides attached to C1s, at positions 159 and 391 (32). Preliminary characterization of mutant Q340E based on treatment with peptide/N-glycosidase F and fragmentation with plasmin (data not shown) provided indication that the observed modification likely occurred at the carbohydrate linked to Asn³⁹¹. To confirm this hypothesis, a double mutant was expressed, carrying both the Q340E mutation and an Asn to Glu mutation at position 391. Analysis by mass spectrometry of the resulting Q340E/N391Q mutant yielded a mass of 76,117 Da, much lower than the value determined for Q340E, which is consistent with the presence at position 159 of a carbohydrate with a mass of 1,215 Da compared to that of wild-type C1s (77,413 Da), the Q340E/N391Q mutant had a mass of 82,008 Da (Table I). The sequence of the plasmid corresponding to the Q340E mutant was confirmed by dsDNA sequencing, whereas N-terminal sequence analysis of the recombinant protein yielded the expected Glu-Pro-Thr-Met-Tyr... sequence. Therefore, it appeared likely that the observed increase in molecular mass was due to an increase in the size of one or both of the N-linked oligosaccharides attached to C1s, at positions 159 and 391 (32). Preliminary characterization of mutant Q340E based on treatment with peptide/N-glycosidase F and fragmentation with plasmin (data not shown) provided indication that the observed modification likely occurred at the carbohydrate linked to Asn³⁹¹. To confirm this hypothesis, a double mutant was expressed, carrying both the Q340E mutation and an Asn to Glu mutation at position 391. Analysis by mass spectrometry of the resulting Q340E/N391Q mutant yielded a mass of 76,117 Da, much lower than the value determined for Q340E, which is consistent with the presence at position 159 of a carbohydrate with a mass of 1,215 ± 40 Da (Table I). Therefore, it became clear that the extra mass of mutant Q340E was due to an increase in the size of the oligosaccharide chain linked to Asn³⁹¹, which, as deduced from the values determined for the Q340E and Q340E/N391Q mutants, had an estimated mass of ~5905 Da. This value is ~5-fold that of the oligosaccharide chains attached to the other C1s variants expressed in this study (Table I).

To understand why the Q340E mutation had an effect on the extent of glycosylation at Asn³⁹¹, a three-dimensional model of the C1s catalytic domain was constructed as described under Materials and Methods. Based on this model, it appeared plausible, considering the location of residues Glu³⁴⁰ and Asp³⁴³ on either side of the CCP₁-CCP₂ interface, that the Q340E mutation would generate repulsion between the carboxyl groups and thereby possibly modify the relative positioning of the CCP modules (see Discussion for more details). Strong support to this hypothesis came from

![FIGURE 1. Sequence alignment of the segments at the junction between the CCP₁ and CCP₂ modules of proteases of the C1r/C1s/MASP family.](image)

![FIGURE 2. SDS-PAGE analysis of the recombinant C1s variants. Lanes 1–17 correspond to wild-type C1s and mutants Q340A, Q340K, Q340R, Q340S, Q340D, Q340E, Q340E/N391Q, Q340E/D343N, Q340D/D343E, P341I, V342K, D343N, Q340K/P341I, Q340S/P341I, and Q340K/P341I/V342K, respectively. All samples were analyzed under reducing conditions. Molecular masses of marker proteins (expressed in kDa) are indicated.](image)
the observation that the double mutant Q340E/D343N had a mass of 77,203 Da, indicating that removal of the negative charge at position 343 restored a normal glycosylation pattern at position 391 (Table I). The double mutant Q340D/D343E also exhibited normal glycosylation at Asn391, suggesting that the presence of Glu at position 340, and Asp at position 343 was an absolute prerequisite for modification of the carbohydrate size. As listed in Table I, a series of other mutations were performed within the C1s sequence stretch 340–343, and none of these other mutations had an effect on the size of the oligosaccharide attached to Asn391.

The Q340E mutation impairs C1s activation

To check whether any of the mutations at position 340 could interfere with C1s ability to undergo activation in the fluid phase, the corresponding variants were incubated with the activated C1r CCP2-SP fragment, and the extent of activation was measured by SDS-PAGE analysis as described under Materials and Methods. As shown in Fig. 3, replacement of Glu by Ala, Lys, Arg, Ser, or Asp had no significant effect on the activation process, but the Q340E mutant was virtually totally resistant to C1r-mediated cleavage. Activation was fully restored in the Q340E/N391Q mutant lacking glycosylation at Asn391 and in Q340E/D343N exhibiting a normal glycosylation pattern (Fig. 3B), demonstrating that the lack of activation of Q340E was due to the increased size of the oligosaccharide attached to Asn391. Again, none of the other mutations performed at positions 340–343 had a significant effect on C1r-mediated activation of C1s (data not shown).

![Figure 3](image)

**FIGURE 3.** Fluid-phase C1s activation by C1r is impaired in the Q340E mutant. A, Different C1s variants comprising a mutation at position 340 were submitted to fluid-phase activation by the CCP2-SP fragment of C1r as described under Materials and Methods, and the extent of activation was compared with that of wild-type C1s. Error bars correspond to the SD of duplicate experiments. B, SDS-PAGE analysis of C1r-mediated activation of selected C1s mutants. Lane 1, untreated wild-type C1s; lanes 2–5, wild-type C1s and mutants Q340E, Q340E/N391Q, and Q340E/D343N incubated with C1r CCP2-SP for 2 h at 37°C. All samples were analyzed under reducing conditions. OVA was not incorporated in the samples for the sake of clarity. Molecular masses of marker proteins (expressed in kDa) are indicated.

Mutations in the CCP1-CCP2 linker alter C4 cleavage but not C2 cleavage

We next investigated the ability of the activated C1s mutants to cleave C4 and C2. For this purpose, all mutants were submitted to fluid-phase activation by the C1r CCP2-SP fragment, and a comparative analysis of their proteolytic activity was performed using the assays described under Materials and Methods. As shown in Fig. 5, all mutants cleaved C2 with a relative efficiency comparable to that of wild-type C1s, indicating that none of the mutations had a significant effect on the recognition and/or cleavage of this substrate. In contrast, most of the mutations significantly altered C4 cleavage (Fig. 6). Thus, with the exception of Q340A and Q340E/P341I, all mutations resulted in a decreased C4-cleaving activity, with particularly pronounced inhibitory effects for point mutants Q340K, P341I, V342K, and D343N. Interestingly, the multiple mutations Q340K/P341I, Q340S/P341I, and Q340K/P341I/V342K, yielding CCP1-CCP2 linkers identical to those of human C1s, did not result in a loss of C4-cleaving activity. The ability of the C1s mutants to associate with C1r and C1q to form the C1 complex was measured by incubating each variant with an equimolar amount of proenzyme C1r and then monitoring interaction of the resulting Ca2+-dependent C1s-C1r-C1r-C1s tetramer with immobilized C1q using surface plasmon resonance spectroscopy. As illustrated by the representative examples shown in Fig. 4, most of the samples yielded binding curves very similar to those obtained in the case of wild-type C1s, although slight and likely not significant variations in the binding intensity were observed for some mutants, such as Q340E, P341I, or V342K. The kinetic parameters of the interaction were determined in the case of the tetramer containing wild-type C1s, yielding $k_{on}$ and $k_{off}$ values of $1.0 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ and $1.8 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$, respectively, and a resulting $K_D$ of $1.8 \pm 0.5 \times 10^{-7} \text{ M}$, indicative of high affinity.

To check whether the C1s mutants retained the ability to activate inside C1, each variant was mixed with appropriate amounts of C1q and proenzyme C1r, and the resulting C1 complex was allowed to activate as described under Materials and Methods. As previously observed in the fluid phase, the Q340E mutant was totally resistant to activation, whereas none of the other mutations had a significant effect on the activation process (data not shown). Again, activation of the Q340E/N391Q, Q340E/D343N, and Q340D/D343E mutants was similar to that of wild-type C1s.

**FIGURE 4.** Analysis by surface plasmon resonance spectroscopy of the interaction between immobilized C1q and the C1s-C1r-C1r-C1s tetramer reconstituted from various C1s mutants. Wild-type C1s and its mutants were incubated individually with proenzyme C1r in the presence of Ca2+ ions, and the resulting C1s-C1r-C1r-C1s tetramers (50 nM) were allowed to activate as described under Materials and Methods. From top to bottom, the binding curves shown correspond to P341I, Q340E, D343N, Q340R, wild-type C1s, and V342K.
As listed in Table II, all mutants tested had reaction were determined for some of the mutants in their isolated terted the C4-cleaving activity of C1s, the kinetic constants of the

Materials and Methods

FIGURE 5. Comparative analysis of C2 cleavage by the C1s variants. Wild-type C1s and its mutants were activated in the fluid phase by the C1r CCP2-SP fragment and tested for their ability to cleave C2, as described under Materials and Methods. Error bars correspond to the SD of triplicate experiments.

MASP-1/3, MASP-2, and C1r, respectively (see Fig. 1), all resulted in C4-cleaving activities < 20% compared with that of wild-type C1s.

Additional experiments were aimed at measuring the C4-cleaving activity of the activated C1s mutants in the context of the macromolecular C1 complex. For this purpose, representative mutants were incorporated in C1, allowed to activate, and their C4-cleaving activity was measured in the complex. As shown in Fig. 6, although differences were observed in the case of mutants Q340A and Q340K, most of the mutants tested (P341I, D343N, Q340K/D343N, Q340S/P341I, and Q340E/N391Q) exhibited a cleavage pattern similar to that obtained with isolated C1s.

To investigate why the mutations in the CCP1-CCP2 linker altered the C4-cleaving activity of C1s, the kinetic constants of the reaction were determined for some of the mutants in their isolated form. As listed in Table II, all mutants tested had $K_m$ values similar to that determined for wild-type C1s. Consistent with the data shown in Fig. 6, mutants Q340A and Q340E/N391Q had $k_{cat}$ values close to that of wild-type C1s. In contrast, all mutants showing decreased C4-cleaving activity in Fig. 6 (Q340K, P341I, D343N, and Q340S/P341I) had decreased $k_{cat}$ values.

FIGURE 6. Comparative analysis of C4 cleavage by the C1s variants. Wild-type C1s and all mutants were activated in the fluid phase by the C1r CCP2-SP fragment and tested for their ability to cleave C4, as described under Materials and Methods. The resulting C4-cleaving activity of each mutant is expressed relative to that of wild-type C1s and shown as light gray bars. In a second series of experiments, wild-type C1s and selected mutants (Q340A, Q340K, Q340E/N391Q, P341I, D343N, Q340K/D343N, and Q340S/P341I) were incorporated into C1, allowed to activate, and then tested for their ability to cleave C4 in the context of the C1 complex. The relative C4-cleaving activity of these mutants is shown as dark gray bars. Error bars correspond to the SD of triplicate experiments.

Discussion

The aim of this study was to explore the functional role of the segment 340–343 at the interface between the CCP1 and CCP2 modules of C1s. For this purpose, a series of mutants were generated by site-directed mutagenesis, expressed using a baculovirus/insect cells system, and tested for their ability to mediate the various interaction and catalytic properties of C1s, as measured outside and within the C1 complex.

A totally unexpected finding of these investigations is that substitution of Glu for Gln at position 340 results in a marked increase in the mass of the oligosaccharide chain attached to Asn391 from a value of ~1250 Da, corresponding to the average size of the highmannose N-linked carbohydrates usually produced by insect cells (13), to a value of ~5905 Da. The presence of Glu at position 340 was found to be a specific requirement, as substitution with other residues, including Asp, was ineffective. In addition, none of the other mutants produced in this study had abnormal glycosylation at Asn391. The fact that the double mutant Q340E/D343N exhibited a normal glycosylation pattern rules out the possibility of a specific recognition of Glu by one of the enzymes involved in the glycosylation process. On the other hand, this observation provides strong support to the hypothesis that introducing a Glu at position 340 generates repulsion between its negative charge and that of residue Asp343. Thus, despite the fact that the model of the C1s catalytic domain at the origin of this hypothesis (Fig. 7) is partly based on the CCP1-CCP2 interface as seen in C1r (12), it is probably valid to some extent, at least with regard to the relative positioning of residues 340 and 343 on either side of the linker. Based on these observations, we suggest that repulsion between the carboxyl groups of Glu340 and Asp343 modifies the structure and/or flexibility of the CCP1-CCP2 linker of C1s in such a way that it facilitates access of the glycosylation enzymes to Asn391.

![Figure 7](https://www.jimmunol.org/)

**FIGURE 7.** Three-dimensional space-filling model of the C1s catalytic domain. The model was built as described under Materials and Methods. The CCP1 module is colored blue, whereas CCP2 and the SP domain are colored red. The side chains of residues Gln340 and Asp343 are shown in yellow and gray, respectively. The position of the active site is shown by residue Ser617 (light blue), and the approximate location of the Arg422/Ile423 activation site is indicated by residue Phe147 (black). The structure of the carbohydrate moiety attached to Asn391, shown in green, is as defined in the x-ray structure of the C1s CCP2-SP fragment (14).

### Table II. Kinetic constants for proteolytic cleavage of C4 by different C1s variants

<table>
<thead>
<tr>
<th>C1s Variant</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ ($\mu$M)</th>
<th>$k_{cat}/K_m$ ($M^{-1} \times s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6.7 ± 1.0</td>
<td>2.3 ± 0.9</td>
<td>2.95 × 10^6</td>
</tr>
<tr>
<td>Q340A</td>
<td>7.7 ± 0.7</td>
<td>2.6 ± 0.1</td>
<td>3.00 × 10^6</td>
</tr>
<tr>
<td>Q340E/N391Q</td>
<td>7.3 ± 0.5</td>
<td>2.0 ± 0.5</td>
<td>3.65 × 10^6</td>
</tr>
<tr>
<td>Q340K</td>
<td>2.5 ± 0.9</td>
<td>3.2 ± 1.1</td>
<td>0.80 × 10^6</td>
</tr>
<tr>
<td>P341I</td>
<td>1.8 ± 0.9</td>
<td>3.2 ± 0.7</td>
<td>0.60 × 10^6</td>
</tr>
<tr>
<td>D343N</td>
<td>2.6 ± 1.0</td>
<td>1.9 ± 0.5</td>
<td>1.40 × 10^6</td>
</tr>
<tr>
<td>Q340S/P341I</td>
<td>1.3 ± 1.0</td>
<td>2.9 ± 0.6</td>
<td>0.45 × 10^6</td>
</tr>
</tbody>
</table>

*Mean values determined from three separate experiments.*
hypothesis appears plausible in light of the location of the oligosaccharide chain relative to the CCP1-CCP2 linker (Fig. 7). It is interesting to note that, whereas Asn391 of C1s purified from human serum is linked to a homogeneous biantennary oligosaccharide, Asn991 is occupied by either a biantennary oligosaccharide, or a triantennary, triasialylated species, or a fucosylated, triasialylated species, in relative proportions of ~1:1:1 (32). It appears likely from our data that this natural heterogeneity arises from an intrinsic variability in the conformation of the C1s CCP1-CCP2 linker.

Another interesting observation is that the increased size of the carbohydrate attached to Asn991 totally prevents C1s activation by C1r, both in the fluid phase and in the context of the C1 complex. Therefore, it may be concluded that due to its abnormal size the carbohydrate chain linked to Asn991 extends over the C1s SP domain on the side opposite to the active site, hence creating steric hindrance and preventing access of the C1r SP domain to the C1s activation site (Fig. 7).

In contrast, overglycosylation at Asn991 has no effect on C1r reconstitution, as judged from the ability of the Q340E mutant to assemble with C1r and C1q (Fig. 4). In this respect, it is remarkable that none of the mutations at the CCP1-CCP2 linker had any significant effect on the ability of C1s to incorporate in C1 as shown by surface plasmon resonance spectroscopy, implying that these have no impact on the ability of C1s to associate with C1r, and of the resulting C1s-C1r-C1q tetramer to associate with C1q. In addition, once incorporated in C1, all mutants, except Q340E, retained activation properties similar to that of wild-type C1s. It may be concluded from these observations that flexibility of the C1s CCP1-CCP2 linker plays no significant role in C1 assembly or in the mechanism involved in C1s activation by C1r inside the C1 complex. Thus, contrary to what was initially hypothesized (14), it appears unlikely that C1s activation by C1r requires a movement of its SP domain toward the interior of the C1 complex to allow cleavage of its susceptible Arg-Ile bond by the C1r active site. As proposed in a recent model (4), a more likely hypothesis is that C1s SP domain is located at the periphery of C1 and that the C1r SP domain moves toward the outside to mediate cleavage.

Our observation that none of the mutations in the CCP1-CCP2 linker had a significant effect on the C2-cleaving activity of C1s is consistent with previous findings (13) indicating that the CCP2-SP and SP fragments of C1s retain the ability of full-length C1s to cleave C2 and provide additional evidence that C2 recognition and cleavage by C1s only involve structural motifs located within its SP domain. In contrast, our data indicate that most of the mutations in the CCP1-CCP2 linker result in a significant decrease of the C4-cleaving activity of C1s. Again, these findings are in line with previous studies indicating that removal of the CCP1 module of C1s greatly reduces (~70-fold) its ability to cleave C4 (13). Taken together, these observations strongly support the hypothesis that C4 recognition by C1s involves a major binding site located in CCP1, allowing positioning of the protein substrate in such a way that its cleavage site fits properly into the C1s active site. In this hypothesis, flexibility at the CCP1-CCP2 junction appears as a critical factor because it would be used to finely adjust positioning of C4 for optimal cleavage by the C1s active site. This hypothesis is strongly supported by the observation that, for all mutants tested, the decreased C4-cleaving ability arises from a decreased $k_{cat}$ value, the $k_{\text{cat}}$ of the reaction being unchanged. Indeed, modification at the CCP1-CCP2 junction would not significantly modify the affinity of the interaction between C4 and the CCP1 module but would be expected to alter the flexibility of the linker and thereby, in most cases, would not allow optimal positioning of C4 with respect to C1s active site, hence the observed decreased $k_{cat}$ values.

In this respect, it is interesting to note that, whereas the Q340K mutant is poorly active on C4, the double mutant Q340E/N391Q shows optimal activity. A tempting hypothesis is that due to the repulsion effect discussed above the CCP1-CCP2 linker shows increased flexibility in the latter case, allowing optimal cleavage. In contrast, the linker could be “locked” in a more rigid configuration in the Q340K mutant, hence its decreased efficiency. It is also noteworthy that replacement of the C1s CCP1-CCP2 linker by that of other members of the C1r/C1s/MASP family results in all cases in a decreased C4-cleaving activity. The fact that this also applies to MASP-2, a protease that shares the ability to cleave C4, provides strong indication that C1s and MASP-2 use different mechanisms to recognize this substrate.

Finally, it is interesting to note that most of the mutations at the CCP1-CCP2 interface have very similar effects on the C4-cleaving activity of C1s, whether this is measured on the isolated protease or in the context of the C1 complex. In this respect, it appears unlikely that the discrepancies observed in the case of Q340A and Q340K reflect a particular behavior of these mutants. Thus, in line with other data discussed above, it may be deduced from these observations that the C1s catalytic domain is readily accessible in C1, in full agreement with the hypothesis that it is located at the periphery of the complex (4).

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Disclosures

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References


