Baculovirus-mediated Expression of Truncated Modular Fragments from the Catalytic Region of Human Complement Serine Protease C1s

EVIDENCE FOR THE INVOLVEMENT OF BOTH COMPLEMENT CONTROL PROTEIN MODULES IN THE RECOGNITION OF THE C4 PROTEIN SUBSTRATE*

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C1s is the modular serine protease responsible for cleavage of C4 and C2, the protein substrates of the first component of complement. Its catalytic region (γ-B) comprises two complement control protein (CCP) modules, a short activation peptide (ap), and a serine protease domain (SP). A baculovirus-mediated expression system was used to produce recombinant truncated fragments from this region, deleted either from the first CCP module (CCP2-ap-SP) or from both CCP modules (ap-SP). The aglycosylated fragment CCP2-ap-SPag was also expressed by using tunicamycin. The fragments were produced at yields of 0.6–3 mg/liter of culture, isolated, and characterized chemically and then tested functionally by comparison with intact C1s and its proteolytic γ-B fragment. All recombinant fragments were expressed in a proenzyme form and cleaved by C1r to generate active enzymes expressing esterolytic activity and reactivity toward C1 inhibitor comparable to those of intact C1s. Likewise, the activated fragments γ-B, CCP2-ap-SP, and ap-SP retained C1s ability to cleave C2 in the fluid phase. In contrast, whereas fragment γ-B cleaved C4 as efficiently as C1s, the C4-cleaving activity of CCP2-ap-SP was greatly reduced (about 70-fold) and that of ap-SP was abolished. It is concluded that C4 cleavage involves substrate recognition sites located in both CCP modules of C1s, whereas C2 cleavage is affected mainly by the serine protease domain. Evidence is also provided that the carbohydrate moiety linked to the second CCP module of C1s has no significant effect on catalytic activity.

The first component of complement comprises two homologous, yet distinct modular serine proteases C1r and C1s as-

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1 The abbreviations used are: the nomenclature of complement proteins is that recommended by the World Health Organization; activated components are indicated by an overbar, e.g. C1s; the nomenclature of protein modules is that defined by Bork and Bairoch (1); CCP2-ap-SP, ap-SP, recombinant fragments from the C1s catalytic region lacking the sembedded in the form of a Ca²⁺-dependent tetramer C1s-C1r-C1r-C1s tetramer (2). C1r is responsible for intrinsic C1 activation, a two-step process first involving autolytic C1r activation and then C1r-mediated cleavage of proenzyme C1s. The active enzyme C1s is a highly specific protease with trypsin-like specificity that mediates limited proteolysis of C4 and C2, the two protein substrates of C1, thereby triggering activation of the classical pathway of complement in response to infection by various microorganisms (2–5). C4 cleavage occurs in the fluid phase and generates fragment C4b, which has the transient ability to bind covalently to the surface of the target. Cleavage of the second substrate C2, in contrast, takes place after prior formation of a C4b-C2 complex and yields C4b-C2a, the protease responsible for cleavage of complement protein C3 (5). This double proteolytic activity of C1s is controlled by C1 inhibitor, a member of the serine protease inhibitor (serpin) family, which reacts stoichiometrically with both C1r and C1s to form covalent protease-inhibitor complexes, resulting in disruption of the C1s-C1r-C1r-C1s tetramer (2).

Human C1s is synthesized as a 673-residue single chainzymogen which, upon activation by C1r, is cleaved between Arg422 and Ile423 to yield two disulfide-linked polypeptides, the N-terminal A chain comprising a series of five protein modules and the serine protease B domain (6–8). Limited proteolysis of C1s with plasmin yields a C-terminal fragment γ-B, comprising two contiguous complement control protein (CCP) modules, a 15-residue intermediary segment homologous to the activation peptide in chymotrypsinogen, and the serine protease domain (9). The CCP modules (originally known as short consensus repeats) are structural motifs of about 60 residues homologous to those mostly found in various complement regulatory proteins known to interact with components C3b and/or C4b (10). The C-terminal CCP module of C1s bears a heterogeneous, complex-type N-linked oligosaccharide with both biantennary and triantennary species (11). Based on chemical cross-linking and three-dimensional homology modeling, it was shown recently that this module closely interacts with the serine protease domain on the side opposite to both the active site and the Arg422-Ile423 bond cleaved upon activation (12).

From a functional point of view, the fact that the proteolytic fragment γ-B retains a functional active site (9) and studies based on the use of monoclonal antibodies indicating that the γ segment may be involved in C4 binding (13, 14) together sug-
gest that γ-B may represent the catalytic region of C1s. How-
however, no precise information is available yet on the respective
role of the serine protease domain and CCP modules in the various
aspects of C1s catalytic activity. The present study provides identification of the domains of C1s involved in its
activation by C1r, proteolytic activity toward C4 and C2, and
reactivity toward C1 inhibitor, based on baculovirus-mediated
expression of truncated recombinant fragments from the γ-B
region.

EXPERIMENTAL PROCEDURES

Materials—Diisopropylphosphorofluoridate, neuraminidase (type X),
and the synthetic substrate N'-benzoyl-l-arginyl ethyl ester were from
Sigma. Human plasmin was obtained from Chromogenix AB
(Molndal, Sweden). Polyclonal anti-C1s antiserum was raised in rabbits
according to standard procedures. Restriction enzymes were from Boch-
ringer Mannheim. Vent polymerase was from New England Biolabs.
The pHC1s46 vector encoding human C1s (6) was kindly provided by
Dr. Mario Tosi (Laboratoire d'Immunogénétique, Institut Pasteur, Par-
is). The pVT-Bac vector was kindly provided by Dr. Thierry Vernet
(Institut de Biologie Structurale, Grenoble). Antibiotics and molecular
biology reagents were from Appligen Oncor.

Proteins—Proenzyme C1s and activated C1r and C1s were purified
from human plasma as described previously (15, 16). The C1s γ-B
fragment was obtained by limited proteolysis with plasmin (17) and
purified by ion-exchange chromatography on a Mono Q HR 5/5 column
(Pharmacia Biotech Inc.) as described previously (12). The two glyco-
forms of the C1s γ-B fragment, containing either a biantennary or a
triantennary oligosaccharide, were isolated by affinity chromatography
on concanavalin A-Sepharose (Pharmacia) as described previously (11).
The desialylated C1s γ-B fragment was obtained by treatment with
neuraminidase (10 units/mg protein) for 5 h at 25 °C. Complement
proteins C2, C4, and C1 inhibitor were isolated from human plasma by
means of published procedures (18–20). For the determination of pro-
etin concentrations the following absorption coefficients (A (1%, 1 cm) at
280 nm) and molecular weights were used: C1r, 12.4 and 86,300 (21);
C1s, 14.5 and 79,800 (21, 11); C1s γ-B, 18.3 and 47,550 (22, 11); C2, 10.0
and 100,000 (18); C4, 8.2 and 205,000 (23, 24); C1 inhibitor, 3.86 and
105,000 (25). The absorption coefficients (A (1%, 1 cm) at 280 nm) used
for the recombinant fragments ap-SP (17.0) and CCPγ-ap-SP (16.4)
were calculated from their amino acid composition by the method of
Edelhoff (26), and their molecular weights were determined by mass
spectrometry analysis (see “Results”).

Construction of C1s Fragments CCPγ-ap-SP and ap-SP-containing
Expression Plasmids—The following primers were obtained from Euro-
gentec (Seraing, Belgium) and used to amplify the desired human C1s
cDNA sequences in a polymerase chain reaction using Vent polymer-
ase, according to established procedures: GAGACAGCTGAGGAGCT-
ATCTGC (sense for CCPγ-ap-SP); GAGACAGCTGAGGAGCT-
AGCTGAGG (antisense for CCPγ-ap-SP and ap-SP); and GAAAGATC-
CTCCATTCCT (sense for CCPγ-ap-SP); GGAATTCTTAGTCCTCACGGGG-
GGT (antisense for CCPγ-ap-SP and ap-SP). The amplified
DNA fragments were excised with Bgl II and EcoRI and introduced into
the 39 site for in-frame cloning into the pNT-Bac expression vector or
at the 3′ end to create an EcoRI site. The pNT-Bac vector was con-
structed from the baculovirus expression vectors pVT-Bac (27) and
pFast Baci1TM (Life Technologies Inc.). Briefly, the segment comprised
between the EcoRV and KpnI sites was excised from plasmid pVT-Bac
and introduced between the SnaBI and KpnI sites in pFast Baci. The
resulting vector contains the polyhedrin promoter and the multiple
cloning site region of pVT-Bac (Fig. 1). The vector was used to
transfect insect cells, Sf900 II SFM medium containing 50 IU/ml penicillin and 50 μg/ml streptomycin (Sigma). Recombinant baculoviruses were generated by using the Bac-
to-Bac™ system (Life Technologies, Inc.) based on site-specific trans-
position of an expression cassette carried by the pNT-Bac-based recom-
binant plasmids into a baculovirus shuttle vector (bacmid) propagated
in Escherichia coli (28). Transposition of the pNT-Bac/CCPγ-ap-SP and
pNT-Bac/ap-SP plasmids into DH10Bac E. coli cells and selection of recombinant bacteria were performed as recommended by the manu-
facturer (Life Technologies, Inc.). The recombinant bacmids were puri-
ﬁed using the Qiagen midiprep DNA puriﬁcation system (Qiagen S.A.,
Courtaboeuf, France) and transfected into Sf21 cells with celfectin in
Sf900 II SFM medium (Life Technologies, Inc.) as recommended by
the manufacturer. The transfection supernatant was decanted 4 days later,
centrifuged, and stored at 4 °C until use. The recombinant viruses,
designated CCPγ-ap-SP and rap-SP, were titered by virus plaque assay
and ampliﬁed as described by King and Possee (29).

Protein Production and Purification—Sf21 cells (5 × 10^6 cells/ml in
spinner flasks or 2 × 10^7 cells/175-cm^2 tissue culture flask) were in-
fected with the recombinant viruses at a multiplicity of infection of 2–3
in Sf900 II SFM medium containing 50 IU/ml penicillin and 50 μg/ml streptomycin. Tunicamycin (5 μg/ml) was added for the production of the
glycosylated form of the CCPγ-ap-SP fragment. After 96 h at 28 °C, the
supernatant was collected by centrifugation, and diisopropylphenyl-
phosphorofluoridate was added to a ﬁnal concentration of 2 μM. The culture
supernatant was dialyzed against 5 mM EDTA, 20 mM sodium phos-
phate, pH 8.6, and loaded at 2 ml/min onto a Mono Q HR 10/10 column
(Pall Biotech) equilibrated in the same buffer containing 1 mM diisopro-

![Fig. 1. Schematic representation of the construction of the pNT-Bac vector. The pNT-Bac vector was constructed from the bacu-
loivirus expression vectors pVT-Bac and pFast Baci1TM, as described
under “Experimental Procedures.” Mel, melittin signal sequence; MCS,
multiple cloning site region; Ppol, polyhedrin promoter. Tn7L and
Tn7R, left and right hands of the bacterial transposon Tn7.](http://www.jbc.org/)
Recombinant Modular Fragments from Human C1s Protease

The nomenclature and symbols used for protein modules are those defined by Bork and Bairoch (1). The modular structures of human C1s and of the various truncated fragments used in this study are depicted in Fig. 2. Construction of the recombinant baculoviruses, transposition of the pNT-Bac/CCP2-ap-SP and pNT-Bac/ap-SP plasmids into DH10Bac E. coli cells, and transfection of the corresponding recombinant bacmids into SF21 cells were performed as described under “Experimental Procedures.” The virus titer of the transfection supernatants after amplification was about 10^7 plaque forming units/ml in both cases. Infection of SF21 cells by the supernatants was conducted for various periods at 28 °C, and the amount of recombinant proteins secreted into the culture medium was monitored by SDS-PAGE and Western blot analysis. Under normal culture conditions, protein bands with the expected apparent molecular weights (see below) became detectable at 48 h, their intensity increasing progressively to reach a plateau at 96 h. In contrast, secretion of the CCP2-ap-SP fragment in the presence of tunicamycin reached a maximum at 72 h and decreased considerably after 96 h. Analysis of protein production in the cell pellets showed kinetic profiles similar to those observed for the supernatants. Under optimal production conditions, the amounts of recombinant protein secreted into the culture medium were estimated to be approximately 0.6 μg/ml for ap-SP, 3 μg/ml for CCP2-ap-SP, and 1.5 μg/ml for the aglycosylated fragment CCP2-ap-SP (CCP2-ap-SPag) produced in the presence of tunicamycin.

The recombinant fragments were essentially pure as judged by fractionation of the culture supernatants by ion-exchange chromatography on a Mono Q column (Pharmacia). Both fragment CCP2-ap-SP and CCP2-ap-SPag yielded visible peaks at 280 nm eluting during the ascending salt gradient, slightly later than the C1s γ-B fragment produced by limited proteolysis. Fragment ap-SP, in contrast, was not retained on the column and eluted in the large flow-through fraction. Final purification was achieved by hydrophobic interaction chromatography. As illustrated in Fig. 3, fragment CCP2-ap-SP eluted as a single peak at the end of the descending ammonium sulfate gradient, slightly later than the whole C1s γ-B fragment. Its aglycosylated form eluted essentially at the same position, whereas the shorter fragment ap-SP eluted at the middle of the gradient and was therefore significantly more hydrophilic. The amounts of purified proteins produced were typically about 0.4, 1.5, and 0.5 mg per liter of culture for fragments ap-SP, CCP2-ap-SP, and CCP2-ap-SPag, respectively.

The recombinant fragments were essentially pure as judged from SDS-PAGE analysis (Fig. 4). Each yielded a single band under both non-reducing and reducing conditions, indicating that they were in the proenzyme form, with apparent molecular weights of about 30,000 (ap-SP), 40,000 (CCP2-ap-SP), and 38,000 (CCP2-ap-SPag), consistent with the predicted values. No sign of proteolytic degradation was observed on the gels upon storage of the recombinant proteins for several months at −20 °C.

Chemical Characterization of Recombinant Proteins—Edman degradation of the purified fragments CCP2-ap-SP and HCl, 145 mM NaCl, pH 7.4, followed by SDS-PAGE analysis under reducing conditions of the formation of the covalent C1 inhibitor-serine protease B chain complexes (20).

Other Methods—SDS-PAGE analysis was performed as described previously (12). Western blot analysis of the recombinant proteins after SDS-PAGE was performed with transfer to a nitrocellulose membrane and blocking of unoccupied sites with 5% non-fat dry milk in 10 mM Tris-HCl, 200 mM NaCl, pH 7.2. The immobilized proteins were visualized by using a rabbit polyclonal anti-C1s antiserum, followed by a goat anti-rabbit immunoglobulin fraction conjugated to alkaline phosphatase (Sigma) and staining according to the manufacturer’s instructions.

RESULTS

Expression and Purification of Recombinant Proteins—The modular structures of human C1s and of the various truncated fragments used in the present study are depicted in Fig. 2. The esterolytic activity of C1s and its various activated fragments was measured against a benzoyl-L-arginine ethyl ester, using an alcaloh dehydrogenase/NAD-coupled spectrophotometric test, as described previously. The reaction of the activated proteases toward C1 inhibitor was assessed by incubation in the presence of equimolar amounts of C1 inhibitor for 15 min at 37 °C in 50 mM triethanolamine

![Fig. 2. Comparative representation of the modular structures of human C1s and of the truncated fragments used in this study. The nomenclature and symbols used for protein modules are those defined by Bork and Bairoch (1). CUB, module found in complement C1r/C1s, Uegf, and bone morphogenetic protein-1; EGF, epidermal growth factor-like module; Ser Pr, serine protease domain; the unla-beled segment corresponds to the activation peptide. The arrow indicates the Arg^{469}-Ile^{472} bond cleaved on activation. The only disulfide bridge shown is that connecting the activation peptide to the serine protease domain.](http://www.jbc.org/)
CCP2-ap-SP gave a single sequence Asp-Leu-Asp-(Cys)-Gly-Ile-Pro-Glu-Ser-Ile... in both cases, corresponding to the segment Asp343-Ile350 of human C1s preceded by the two residues Asp-Leu expected to be added at the N terminus, due to the introduction of bases at the 5' end of the cDNA to create a BglII site (see “Experimental Procedures”). In the same way, fragment ap-SP gave a single sequence Asp-Leu-Pro-Val-(Cys)-Gly-Val-Pro-Arg-Glu... derived from the segment Pro408-Glu415 of C1s, corresponding to the N-terminal end of the activation peptide.

Analysis of fragment ap-SP by MALDI mass spectrometry gave a molecular mass of 29,627 ± 15 Da, consistent with a sequence comprising residues Asp-Leu followed by the two residues Asp-Leu expected to be added at the N terminus, due to the introduction of bases at the 5' end of the cDNA to create a BglII site (see “Experimental Procedures”). In the same way, fragment ap-SP gave a single sequence Asp-Leu-Pro-Val-(Cys)-Gly-Val-Pro-Arg-Glu... derived from the segment Pro408-Glu415 of C1s, corresponding to the N-terminal end of the activation peptide.

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As judged from SDS-PAGE analysis under reducing conditions, fragments CCP2-ap-SP, CCP2-ap-SPag, and ap-SP retained a monochain structure upon prolonged incubation at 37 °C, indicating that the preparations were free of contaminating proteases. To test their activation by C1r, i.e., their ability to be split at the Arg422-Ile423 bond cleaved upon activation of intact C1s, each fragment was incubated for various periods at 37 °C in the presence of C1r. SDS-PAGE analysis under reducing conditions indicated that all three fragments were cleaved by high mannose oligosaccharides attached to Asn391 of fragment CCP2-ap-SP.

**Functional Characterization of Recombinant Proteins**—As judged from SDS-PAGE analysis under reducing conditions, fragments CCP2-ap-SP, CCP2-ap-SPag, and ap-SP retained a monochain structure upon prolonged incubation at 37 °C, indicating that the preparations were free of contaminating proteases. To test their activation by C1r, i.e., their ability to be split at the Arg422-Ile423 bond cleaved upon activation of intact C1s, each fragment was incubated for various periods at 37 °C in the presence of C1r. SDS-PAGE analysis under reducing conditions indicated that all three fragments were cleaved by high mannose oligosaccharides attached to Asn391 of fragment CCP2-ap-SP.
activity was tested against 

CCP2-ap-SP and ap-SP by C1r showed that all three proteins 

displayed proteolytic activity, their ability to cleave the C1s 

and abolished when both CCP modules were removed, indicat-

ing that both modules participate in C4 recognition by C1r.

The shorter fragment ap-SP also cleaved C2, although with a $k_{\text{cat}}$ value slightly less (about 50%) than those determined for the other fragments. Nevertheless, all of the fragments 

cleaved C2 sufficiently, indicating that the first CCP module of C1s and very likely also the second CCP module do not play a major role in C2 cleavage.

In the next step, we compared the ability of the various C1s fragments to cleave C4. Kinetic analyses as a function of enzyme concentration clearly showed that all C1s fragments had the ability to cleave C2 and yielded the C2a and C2b fragments characteristic of cleavage by intact C1s (not shown). As summarized in Table I, the larger fragment γ-B and both fragments CCP2-ap-SP (either glycosylated or aglycosylated) displayed kinetic constants very similar to those of intact C1s. That the carbohydrate linked to the second CCP module had no significant influence on C2 cleavage was confirmed by the fact that the two glycoforms of C1s γ-B, as well as the desialylated protein, all exhibited comparable kinetic constants (not shown). The shorter fragment ap-SP also cleaved C2, although with a $k_{\text{cat}}$ value slightly less (about 50%) than those determined for the other fragments. Nevertheless, all of the fragments cleaved C2 efficiently, indicating that the first CCP module of C1s and very likely also the second CCP module do not play a major role in C2 cleavage.

In the next step, we compared the ability of the various C1s fragments to cleave C4. Kinetic analyses as a function of enzyme concentration were performed initially, indicating that the larger fragment γ-B cleaved C4 to yield the expected C4α'-chain, with an efficiency comparable to that of intact C1s (not shown). In contrast, as illustrated in Fig. 7, a dramatic decrease in cleaving efficiency was observed for fragments CCP2-ap-SP and CCP2-ap-SPag, whereas C4-cleaving activity was abolished in the case of the shorter fragment ap-SP. No proteolytic activity toward C4 was detectable after incubation for 30 min at 37 °C using ap-SP/C4 molar ratios up to 0.07 (not shown). Determination of the kinetic constants for C4 cleavage confirmed the above findings, indicating that C1s and its γ-B fragment had comparable $k_{\text{cat}}$ and $K_m$ values (Table II). The two glycoforms and the asialylated form of C1s γ-B all exhibited similar catalytic constants (not shown). In the case of both fragments CCP2-ap-SP, in contrast, the $k_{\text{cat}}$ decreased about 15–20-fold, and the $K_m$ increased about 4-fold, leading to a mean decrease of the $k_{\text{cat}}/K_m$ value of about 70, in agreement with the data shown in Fig. 7. Thus, C4-cleaving activity was strongly reduced upon deletion of the N-terminal CCP module and abolished when both CCP modules were removed, indicating that both modules participate in C4 recognition by C1s.

Finally, experiments aimed at checking the ability of the activated recombinant C1s fragments to react with the physiological protein inhibitor of C1s, C1 inhibitor, were performed. After incubation with equimolar amounts of C1 inhibitor followed by SDS-PAGE analysis under reducing conditions (not shown), C1s and all three fragments CCP2-ap-SP, CCP2-ap-SPag, and ap-SP yielded a band of apparent molecular weight $>120,000$, corresponding to the covalent complex formed between C1 inhibitor and the serine protease domain. In all cases, including C1s, formation of the covalent complex was not complete under the conditions used, as shown by the presence of significant amounts of C1 inhibitor and the free form of the serine protease domain. However, the relative proportion of complex formed was comparable in all cases. As judged from these experiments, all fragments exhibited a reactivity toward C1 inhibitor comparable to that of intact C1s, indicating that the C-terminal end of the protease, comprising the activation
peptide and the serine protease domain, contains the structural determinants required for reaction with this inhibitor.

**DISCUSSION**

The baculovirus/insect cells expression system has been used successfully to produce a number of intact multidomain proteins, including human complement proteases C1r and C1s (35, 36). In the present study, the recently developed Bac-to-Bac™ system (28) was used to express recombinant modular fragments from the catalytic region of human C1s. Although the secretion yields (0.6–3.0 mg/liter cell culture) were low compared with some of those reported for intact proteins, they were sufficient to produce the purified fragments for chemical and functional characterization. In this respect, the significantly lower level of secretion of fragment ap-SP is probably related, at least in part, to a decreased stability and/or solubility compared with the larger fragment CCP2-ap-SP. Indeed, we have provided evidence that the second CCP module of C1s is closely associated with the serine protease domain (12). Removal of this module may therefore be expected to unmask hydrophobic areas of the serine protease domain and thereby to decrease its solubility. In contrast, the observed slight decrease in the secretion yield of the aglycosylated fragment CCP2-ap-SPag is probably not linked to a reduced stability of the protein in solution due to the lack of the oligosaccharide chain but rather is an indirect consequence of the deleterious effect of tunicamycin on insect cells, which is known to affect secretion of some proteins (37). As shown by Edman degradation and mass spectrometry analyses, the recombinant C1s fragments produced in this study were homogeneous at the polypeptide level and had the expected N-terminal sequences and amino acid compositions. In contrast, the analyses performed on the glycosylated fragment CCP2-ap-SP are consistent with the occurrence at Asn391 of heterogeneous high mannose type oligosaccharides containing a varying number of mannose residues ranging from 4 to 8, depending on the preparation, and even within a given preparation (see Fig. 5). Such a heterogeneity of the carbohydrate moiety of the glycoproteins expressed in baculovirus/insect cells systems could be a major disadvantage if these are produced for crystallographic purposes.

Several lines of evidence indicate that the carbohydrate borne by the second CCP module of C1s is not significantly involved in the functional activities of this protease. Thus, the two glycoforms and the asialylated form of fragment C1s γ-B all display comparable proteolytic activities toward C2 and C4. Moreover, the aglycosylated form of fragment CCP2-ap-SP exhibits proteolytic kinetic constants similar to those of its glycosylated counterpart. It is very likely therefore that the two naturally occurring glycoforms of human C1s display identical catalytic properties in vivo. The fact that complete removal of the carbohydrate from fragment CCP2-ap-SP does not impair its functional activity also suggests that this does not significantly affect the folding of the protein. Thus, despite the location of Asn391 in the vicinity of the CCP module/serine protease domain interface (12), the oligosaccharide chain itself is probably not directly involved in the interaction between the two domains. This observation lends further support to the above-mentioned hypothesis that the decreased secretion of fragment CCP2-ap-SPag results from an indirect effect of tunicamycin on insect cells.

All of the recombinant fragments were produced and remained stable in a preenzyme form, indicating that, like the parent C1s molecule, none of them undergoes self-activation. In contrast, both fragments CCP2-ap-SP and ap-SP were readily activated in solution by C1r, with an efficiency comparable to intact C1s. The shorter C1s fragment ap-SP therefore contains the structural information required for C1r-mediated cleavage. Indeed, this appears consistent with the fact that C1s cleavage by C1r normally does not occur in the fluid phase, but inside the C1 complex, in which the activation site of the former protease and the active site of the latter are expected to be pre-positioned with respect to each other (4). It is likely, therefore, that C1s recognition by C1r mainly involves a restricted number of residues on either side of the C1s Arg422-Ile423 bond cleaved upon activation. In any case, it is clear that efficient cleavage does not require accessory binding sites outside the serine protease domain of C1s. It is noteworthy that C1r-mediated cleavage of fragments CCP2-ap-SP and ap-SP leads to the development of esterolytic activity in both cases, which again implies that the latter is self-sufficient for the formation of a fully active site, in agreement with current knowledge on active site formation in other serine proteases (38).

Our observation that the short C1s fragment ap-SP retains the ability to form a stable complex with C1 inhibitor indicates that this contains the structural elements required for reaction with C1 inhibitor. It should be mentioned, however, that the reaction of C1s with C1 inhibitor is a complex and still not fully understood process. Like other members of the serpin family, C1 inhibitor acts as a pseudosubstrate: C1s cleaves C1 inhibitor at the Arg444-Thr445 “reactive center,” then the cleavage products do not dissociate, but a tight, SDS-stable enzyme-inhibitor complex is formed (25). It has been recently proposed

**FIG. 7.** Comparative analysis of C4 cleavage by increasing amounts of activated C1s and its recombinant fragments CCP2-ap-SP, CCP2-ap-SPag, and ap-SP. C4 (2.25 μM) was incubated at 37 °C in 20 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA, pH 7.4, in the presence of increasing concentrations of C1s (●) or the activated recombinant fragments CCP2-ap-SP (○), CCP2-ap-SPag (□), and ap-SP (△). The initial rate of C4 cleavage V0 was measured by kinetic analysis by SDS-PAGE of the production of the C4α chain of fragment C4b (see “Experimental Procedures”).

**Table II**

Kinetic constants for proteolytic cleavage of C4 by intact C1s and various C1s fragments

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>kcat</th>
<th>Km μM</th>
<th>kcat/Km μM⁻¹·s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1s</td>
<td>7.6</td>
<td>5.0</td>
<td>1,520,000</td>
</tr>
<tr>
<td>γ-B</td>
<td>7.6</td>
<td>5.7</td>
<td>1,330,000</td>
</tr>
<tr>
<td>CCP2-ap-SP</td>
<td>0.4</td>
<td>20.0</td>
<td>20,000</td>
</tr>
<tr>
<td>CCP2-ap-SPag</td>
<td>0.5</td>
<td>22.0</td>
<td>23,000</td>
</tr>
<tr>
<td>ap-SP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
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*Values not measurable due to the lack of C4 cleaving activity.*
that formation of this stable complex involves a secondary interaction site on C1 inhibitor (39). Obviously, a precise kinetic analysis of the reaction of C1 inhibitor with native C1s and its ap-SP fragment would be necessary to check whether the latter behaves exactly as the parent protein at the different steps of the reaction. Nevertheless, our observation that C1s and its fragment ap-SP yield comparable amounts of stable complexes with C1 inhibitor suggests that the proposed secondary interaction site on C1 inhibitor binds to the serine protease domain of C1s.

With regard to C2 cleaving activity, all C1s fragments were found to cleave this substrate with an efficiency comparable with that of the intact protease. The fact that the shorter fragment ap-SP exhibits a slightly decreased \( k_{cat} \) value can be rationalized by the following observations: (i) as discussed above, removal of the second CCP module may be expected to uncover hydrophobic patches of the serine protease domain and possibly to induce local denaturation resulting in partial loss of substrate binding site(s) at the surface of the domain; (ii) due to its lower solubility, the uncertainty on concentration measurements and hence on \( k_{cat} \) was probably higher in the case of fragment ap-SP. Indeed, the observation that ap-SP exhibits a \( K_M \) value for C2 comparable with that of the other fragments tends to favor the latter explanation. Therefore, our conclusion is that C2 recognition and cleavage by C1s only involve structural motifs located within the ap-SP fragment and probably confined to the serine protease domain. In any case, our data provide no indication of a significant involvement of the remainder of the C1s molecule, particularly the CCP modules.

In contrast, our data clearly show that, while fragment \( \gamma \)-B exhibits a C4 cleaving activity comparable with that of intact C1s, this activity is greatly reduced upon removal of the first CCP module and abolished when both CCP modules are deleted. Therefore, we conclude that both of these modules contribute accessory recognition sites for C4, which likely mediate binding and positioning of the substrate in such a way that the scissile bond fits into the C1s active site, thereby allowing efficient cleavage of the protein. This hypothesis is consistent with the observation that fragment CCP1-ap-SP shows both an increased \( K_M \) for C4 and a decreased \( k_{cat} \) value (see Table II). Thus, removal of the first CCP module would result in partial loss of the C4 binding site, resulting in improper positioning of the protein, and thereby in decreased cleavage efficiency. Our finding that both CCP modules of C1s participate in C4 binding is in full agreement with previous studies (13, 14) indicating that monoclonal antibodies directed to the \( \gamma \) segment of the protease inhibit its ability to cleave C4. From a more general point of view, it should be emphasized that all complement regulatory proteins known to bind the C4b fragment of C4 (including complement receptor type 1 (CR1), membrane cofactor protein, decay accelerating factor, and C4b-binding protein) are composed mainly or entirely of CCP modules (10). In the case of CR1, it has been shown by deletion experiments and site-directed mutagenesis that both the first and second CCP modules are required for C4b binding (40). By analogy, the most likely hypothesis is therefore that the two CCP modules of C1s bind to the C4b moiety of C4. This does not preclude, however, that other binding sites may be involved in the C1s-C4 interaction. In this respect, it should be mentioned that plasmid degradation of the serine protease domain of C1s was reported to result in the loss of C4 binding ability (14). In the same way, it has been suggested that C1s may interact with the C4a moiety of C4 through acidic residues close to the active site (41). It may be hypothesized, therefore, that efficient positioning of C4 with respect to C1s requires two types of interactions: (i) between the C4b moiety and the CCP modules and (ii) between the C4a moiety and the serine protease domain.

It is not surprising that C4 and C2 recognition by C1s involves distinct structural requirements, since these protein substrates are quite different both in terms of size and modular organization. Also, it is well established that, in vivo, C4 cleavage occurs in the fluid phase, whereas C2 is cleaved within the C2-C4b complex that forms after covalent attachment of C4b in the vicinity of the C1 complex (5). It may be expected, therefore, that appropriate “presentation” of C2 with respect to the C1s active site is effected, at least in part, by C4b. In this respect, it is noteworthy that initial binding of C2 to C4b has recently been shown to involve the CCP modules of C2 (42). Thus, the C4b moiety of native C4 would bind first to the CCP modules of C1s, then the C4b fragment released upon C1s cleavage would acquire preferential affinity for the homologous modules of C2.

The present study provides the first precise identification of the domains of C1s involved in the various aspects of its catalytic activity, and this study also shows that its very restricted specificity and its ability to discriminate its two protein substrates arise for a large part from the occurrence of accessory substrate binding sites located outside the serine protease domain. Further studies are in progress to identify the residues of the CCP modules of C1s that participate in C4 binding.

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