Functional Characterization of Complement Proteases C1s/Mannan-binding Lectin-associated Serine Protease-2 (MASP-2) Chimeras Reveals the Higher C4 Recognition Efficacy of the MASP-2 Complement Control Protein Modules*

Received for publication, April 8, 2005, and in revised form, October 11, 2005. Published, JBC Papers in Press, October 14, 2005. DOI 10.1074/jbc.M503813200

Véronique Rossi, Florence Teillet, Nicole M. Thielen, Isabelle Bally, and Gérard J. Arlaud

From the Laboratoire d’Enzymologie Moléculaire, Institut de Biologie Structurale Jean-Pierre Ebel, 38027 Grenoble Cedex 1, France

C1s and mannan-binding lectin-associated serine protease-2 (MASP-2) are the proteases that trigger the classical and lectin pathways of complement, respectively. They have identical modular architectures and cleave the same substrates, C2 and C4, but show markedly different efficiencies toward C4. Multisite-directed mutagenesis was used to engineer hybrid C1s/MASP-2 molecules where either the complement control protein (CCP) modules or the serine protease (SP) domain of C1s were swapped for their MASP-2 counterparts. The resulting chimeras (C1s(MASP-2 CCP1/2) and C1s(MASP-2 SP) respectively) were expressed and characterized chemically and functionally. Whereas C1s(MASP-2 SP) was recovered as an active enzyme, C1s(MASP-2 CCP1/2) was produced in a proenzyme form and was susceptible to activation by C1r, indicating that the activation properties of the chimeras were dictated by the nature of their SP domain. Similarly, each activated chimera had an esterolytic activity characteristic of its own SP domain and cleaved C2 with an efficiency comparable with that of their parent C1s and MASP-2 proteases. Both chimeras cleaved C4, but whereas C1s(MASP-2 SP) and C1s had $K_m$ values in the micromolar range, C1s(MASP-2 CCP1/2) and MASP-2 had $K_m$ values in the nanomolar range, resulting in 21–27-fold higher $k_{cat}/K_m$ ratios. Thus, the higher C4 cleavage efficiency of MASP-2 arises from a higher substrate recognition efficacy of its CCP modules. Remarkably, C1s(MASP-2 CCP1/2) retained C1s ability to associate with C1r and C1q to form a pseudo-C1 complex and to undergo activation within this complex, indicating that the C1s-CCP modules have no direct implication in either function.

The classical and lectin pathways of complement activation, two major components of innate immunity against pathogenic microorganisms in vertebrates, elicit the same defense reactions in response to infection, and involve similar recognition and triggering mechanisms. C1, the complex that triggers the classical pathway, comprises a flower-like hexameric recognition subunit C1q and two copies of homologous, yet distinct modular serine proteases, C1r and C1s, which together form a Ca$^{2+}$-dependent tetramer C1r-C1s-C1r-C1s. C1r is responsible for internal C1 activation, a two-step process first involving autolytic C1r activation and then cleavage of proenzyme C1s. Activated C1s is a highly specific trypsin-like protease that mediates limited proteolysis of C4 and C2, the natural protein substrates of C1, thereby triggering activation of the pathway (1–3).

The lectin pathway can be triggered by three different oligomeric lectins (mannan-binding lectin, L-ficolin and H-ficolin), which exhibit an overall structure reminiscent of that of C1q and share the ability to form a complex with mannan-binding lectin-associated serine protease-2 (MASP-2) (4–6). MASP-2 (7) has a modular structure homologous to that of C1r and C1s, with an N-terminal CUB module (8), a Ca$^{2+}$-binding epidermal growth factor-like module (9), a second CUB module, two contiguous CCP modules (10), and a C-terminal chymotrypsin-like serine protease (SP) domain. The catalytic subunit of each activating complex is thought to be a MASP-2 homodimer, which thus combines C1r property to self-activate and C1s ability to cleave C4 and C2 (11–13).

Whereas C1s and MASP-2 have comparable C2 cleaving activities, MASP-2 cleaves C4 more efficiently than does C1s, because of a much lower $K_m$ for this substrate (12). Expression of recombinant modular segments from the C-terminal catalytic region of both proteases has revealed that, whereas C2 cleavage is mediated by their SP domain alone, C4 cleavage requires additional sites located in the CCP modules, likely involved in substrate recognition (14, 15). The present study was undertaken to assess the relative contribution of the SP domain and the CCP modules of C1s and MASP-2 to their C4 cleaving activity and investigate what determines the higher efficiency of MASP-2 toward this substrate. For this purpose, we took advantage of the fact that C1s and MASP-2 share homologous modular structures to engineer chimeric C1s molecules comprising either the CCP modules or the SP domain of MASP-2. This domain swapping strategy allows us to demonstrate that the higher C4-cleavage efficiency of MASP-2 arises from the higher C4 recognition efficacy of its CCP modules and reveals that the SP domain of each protease is able to cooperate with the CCP modules of the other.

EXPERIMENTAL PROCEDURES

Materials—Diisopropyl phosphorofluoridate and the synthetic substrate Ac-Gly-Lys-OMe were from Sigma. Oligonucleotides were obtained from Oligoexpress, Paris. Restriction enzymes were from New...
England Biolabs, Beverly, MA. The C1s-Sepharose column was prepared by coupling activated C1s purified from human serum to CNBr-activated Sepharose 4B according to March et al. (16). Plasmids pBS-C1s, containing the full-length human C1s cDNA (17), and pBS-MASP-2, containing the full-length human MASP-2 cDNA (7), were kindly provided by Dr. Mario Tosi (University of Rouen, France) and Dr. Steffen Thiel (University of Aarhus, Denmark), respectively. Plasmid pFastBac1 was from Invitrogen.

Proteins—Complement proteins C2, C4, C1q, activated C1s, and proenzyme C1r were purified from human plasma according to published procedures (18–21). Wild-type C1s, the activated CCP2-SP fragment of C1r, and the CCP1/2-SP fragment of MASP-2 were produced in insect cells and purified as described previously (12, 22). C1 inhibitor was purified from human plasma essentially as described in Rossi et al. (12). The concentration of purified proteins was determined using the following absorption coefficients ($A_{1\%\text{,}1\text{cm}}$ at 280 nm) and the following molecular weights: C2, 10.0 and 100,000 (18); C4, 8.3 and 205,000 (19); C1q, 6.8 and 459,300 (20); C1r, 12.4 and 86,300 (23); wild-type C1s and C1s*, 14.5 and 77,400; C1r fragment CCP2-SP, 14.9 and 40,400 (22), MASP-2 CCP1/2-SP fragment, 18.3 and 42,700 (12), C1 inhibitor, 4.5 (24) and 104,000 (25). The absorption coefficients ($A_{1\%\text{,}1\text{cm}}$ at 280 nm) used for recombinant C1s$_{\text{MASP-2 CCP1/2}}$ and C1s$_{\text{MASP-2 SP}}$ (14.8 and 15.7, respectively) were calculated according to Edelhoch (26) using molecular weight values of 76,700 and 75,800, respectively, calculated from the amino acid sequence and the carbohydrate content. Because of the contaminating A chain fragment in the C1s$_{\text{MASP-2 SP}}$ preparation, the relative amount of the full-length, active enzyme was determined from SDS-PAGE and N-terminal sequence analysis.

Construction of Expression Plasmids—The parental plasmids used in this study, pFastBac C1s and pFastBac MASP-2, were derived from pBS-C1s and pBS-MASP-2 as described previously (12, 22). The plasmids encoding the chimeric proteins were generated by site-directed mutagenesis of the parental plasmids using the QuikChange™ multi site-directed mutagenesis kit from Stratagene (La Jolla, CA). Three single restriction sites, ClaI, AgeI, and NheI, were introduced into pFastBac C1s and pFastBac MASP-2 at the boundaries between modules CUB$_2$ and CCP$_1$, between CCP$_1$ and the SP domain, and after the stop codon, respectively (Fig. 1). The resulting mutated plasmids named pFastBac C1s* and pFastBac MASP-2* were subsequently used for domain swapping. The chimeric plasmid pFastBac C1s$_{\text{MASP-2 CCP1/2}}$ was obtained by inserting the ClaI (4969)/AgeI (5364) segment of pFastBac MASP-2* within the ClaI (4907)/AgeI (5293) sites of pFastBac C1s*. The chimeric plasmid pFastBac C1s$_{\text{MASP-2 SP}}$ was obtained in a similar way by insertion of the AgeI (5364)/NheI (6148) segment of pFastBac MASP-2* within the AgeI (5293)/NheI (6117) sites of pFastBac C1s*. All DNA constructs used in this study were checked by double-stranded DNA sequencing (Genome Express, Grenoble, France).

Cells and Viruses—The insect cells Spodoptera frugiperda (Ready-Plaque Sf9 cells from Novagen) and Trichoplusia ni (High Five™) were routinely grown and maintained as described previously (27). Recombinant baculoviruses were generated using the Bac-to-Bac™ system (Invitrogen). The bacmid DNA was purified using the Qiagen midiprep purification system (Qiagen S. A., Courtaboeuf, France) and used to transfect Sf9 insect cells utilizing cellfectin in Sf900 II SFM medium (Invitrogen) as recommended by the manufacturer. Recombinant virus particles were collected 4 days later and amplified as described by King and Possee (28).

Protein Production and Purification—All chimeric proteins were produced and purified using the same procedure, except that diisopropyl phosphorofluoridate was omitted at all steps in the case of C1s$_{\text{MASP-2 SP}}$. Briefly, High Five cells (1.75 x 10$^7$ cells/175-cm$^2$ tissue culture flask) were infected with the recombinant viruses at a
multiplicity of infection of 2–3 in S900 II SFM medium at 28 °C for 72 h. Culture supernatants were collected by centrifugation, dialyzed against 50 mM triethanolamine hydrochloride, 145 mM NaCl, pH 7.4, and loaded onto the C1s-Sepharose column (3 × 5 cm) after the addition of 2 mM CaCl$_2$ and 2 mM diisopropyl phosphorofluoridate. Elution of the bound material was carried out with the same buffer containing 5 mM EDTA instead of CaCl$_2$. The eluate was concentrated to 0.3–0.5 mg/ml by centrifugation on a PM-30 membrane (Amicon, Millipore), dialyzed against 50 mM triethanolamine hydrochloride, 145 mM NaCl, pH 7.4, and stored at −20 °C until use.

**Activation of Wild-type C1s, C1s*, and C1s(MASP-2 CCP1/2)—**Wild-type C1s and its C1s* and C1s(MASP-2 CCP1/2) variants (3.6 μM each) were activated by incubation with a 2.2% molar ratio of the activated C1r CCP2-SP fragment for 100 min at 37 °C in 50 mM triethanolamine hydrochloride, 145 mM NaCl, pH 7.4. Activation was monitored by SDS-PAGE analysis under reducing conditions and subsequent quantification by gel scanning of the A and B chains characteristic of activated C1s.

**Esterolytic Assays—**The esterolytic activity of wild-type C1s, C1s*, the CCP1-2-SP fragment of MASP-2, and both C1s/MASP-2 chimeras was measured on the synthetic ester Ac-Gly-Lys-OMe using an assay described previously (29). Initial cleavage rates were determined at 30 °C at a substrate concentration of 1 mM and at protease concentrations ranging from 1 to 3 nM. The esterolytic activity of each variant (measured as mol of substrate cleaved/s mol of enzyme) was expressed relative to that of wild-type C1s.

**Proteolytic Assays—**The kinetic parameters of C4 and C2 cleavage were determined using the SDS-PAGE analysis-based method described previously (12, 14). For C4 cleavage by the CCP1-2-SP fragment of MASP-2 and the C1s(MASP-2 CCP1/2) chimera, the enzyme concentration was 0.5 nM, and substrate concentrations ranged from 50 to 500 nM. C2 cleavage by wild-type C1s, C1s*, and the C1s(MASP-2 SP) hybrid was performed at a 1 nM enzyme concentration, with substrate concentrations ranging from 0.5 to 4 μM. C2 cleavage was carried out under the same conditions in all instances, i.e. a 2 nM enzyme concentration and with C2 concentrations ranging from 1 to 4 μM. The kinetics constants were determined from initial rates analyses by the Lineweaver-Burk method using linear regression analysis and are based on four to five experiments at six different substrate concentrations.

**Titration with C1 Inhibitor—**Titration of the enzymatic activity of C1s and the C1s(MASP-2 CCP1/2) and C1s(MASP-2 SP) chimeras was performed essentially as described previously (12) by preincubating the proteases (0.25 μM each) with increasing concentrations of C1 inhibitor (50–500 nM) for 15 min at 37 °C in 145 mM NaCl, 50 mM triethanolamine-HCl, 1 mg/ml ovalbumin, pH 7.4. Residual C2 cleaving activity was then measured by incubating the enzymes (3.75 nM for C1s(MASP-2 SP) and 7.5 nM for C1s and C1s(MASP-2 CCP1/2)) with 2.25 μM C2 for 10 min at 37 °C followed by SDS-PAGE analysis of the cleavage as described above.

**Surface Plasmon Resonance Spectroscopy—**Analyses were performed using the BLAcore 3000 instrument (BLAcore AB, Uppsala, Sweden). C1q was immobilized on the surface of a CM5 sensor chip (BLAcore AB) using the amine-coupling chemistry as described previously (30). The C1q-C1r-C1r-C1s tetramer (10 nM) was reconstituted from proenzyme C1r and each C1s variant in 145 mM NaCl, 2 mM CaCl$_2$, 50 mM triethanolamine hydrochloride, pH 7.4, containing 0.005% surfactant P20 (BLAcore AB). Binding to immobilized C1q (10,000 resonance units) was measured at a flow rate of 20 μl/min in the buffer used for tetramer reconstitution. Equivalent volumes of each tetramer were injected on a surface with immobilized bovine serum albumin to serve as blank sensorsgrams. Regeneration of the surface was realized with injection of the same buffer containing 5 mM EDTA instead of 2 mM CaCl$_2$. Data were analyzed by global fitting to a 1:1 Langmuir binding model of the association and dissociation phases for several concentrations simultaneously using the BLAevaluation 3.1 software (BLAcore AB). The apparent equilibrium dissociation constants ($K_d$) were calculated from the ratio of the dissociation $k_{off}$ and association $k_{on}$ rate constants, and maximal binding capacities ($R_{max}$) were determined using the same model.

**Activation of C1s(MASP-2 CCP1/2) within the C1 Complex—**C1 (0.25 μM) was reconstituted from equimolar amounts of C1q and the tetramer containing proenzyme C1r and either wild-type C1s, C1s*, or C1s(MASP-2 CCP1/2) and incubated in 145 mM NaCl, 2 mM CaCl$_2$, 50 mM triethanolamine hydrochloride, pH 7.4, for various periods at 37 °C to allow spontaneous activation (31). The reaction mixtures were submitted to SDS-PAGE analysis under reducing conditions, and activation was measured by Western blot using a polyclonal anti-C1s antibody as described previously (14).

**Other Methods—**SDS-PAGE analysis was performed as described previously (23). Western blot analysis of the recombinant proteins was performed according to published procedures (14) using anti-C1s and anti-MASP-2 antibodies (12). N-terminal sequence analysis was performed as described previously (32).

**RESULTS**

The objective of this study was to evaluate the respective contribution of the SP domain and CCP modules of C1s and MASP-2 to their catalytic activity, notably with respect to their differential efficiency toward C4. For this purpose, two chimeric C1s molecules containing either the SP domain of MASP-2 (C1s(MASP-2 SP)) or its CCP1 and CCP2 modules (C1s(MASP-2 CCP1/2)) were engineered using multisite-directed mutagenesis. As detailed under “Experimental Procedures,” the domain swapping procedure used required introduction of new restriction sites at module boundaries in the C1s CDNA, resulting in slight amino acid changes (Fig. 2). Thus, at the CUB2-CCP1 junction, the Asp$^{273}$-Pro$^{274}$ segment of wild-type C1s was replaced by Ser-Met in C1s(MASP-2 CCP1/2), and by Ser-Ile in C1s(MASP-2 SP), and the intermediate species C1s* containing only the restriction sites (Fig. 2B). At the CCP2-SP boundary, Lys$^{405}$ of wild-type C1s was replaced by Val in C1s* and C1s(MASP-2 SP). As detailed later, the intermediate C1s* species was used as a control to evaluate the possible effects of these amino acid changes.

**Chemical Characterization of the C1s(MASP-2 Chimeras—**Wild-type C1s, C1s*, C1s(MASP-2 CCP1/2), and C1s(MASP-2 SP) were expressed in a baculovirus/insect cell system and secreted in the culture supernatant at concentrations of about 10, 6, 2, and 0.15 mg/liter, respectively. All C1s variants could be purified by affinity chromatography on a C1s-Sepharose column in the presence of Ca$^{2+}$ ions, indicating that the interaction properties characteristic of their N-terminal CUB-epidermal growth factor moiety (33) were unaltered. As detailed by SDS-PAGE analysis, C1s, C1s*, and C1s(MASP-2 CCP1/2) were essentially pure and exhibited a single-chain structure under reducing conditions, indicating that they were in the proenzyme state (Fig. 3A). Whereas C1s* and wild-type C1s had similar apparent molecular masses (83 kDa), that of C1s(MASP-2 CCP1/2) was significantly lower (78 kDa), consistent with the fact that the MASP-2 CCP2 module lacks N-linked glycosylation, contrary to its counterpart in C1s (Fig. 2A). As expected, C1s(MASP-2 CCP1/2) was reactive to both anti-C1s and anti-MASP-2 antibodies (data not shown).

SDS-PAGE analysis of the C1s(MASP-2 SP) chimera under nonreducing conditions (Fig. 3B) revealed two bands generally present in equivalent amounts, (i) a 79-kDa species corresponding to the full-length protease, yielding two sequences, Gly-Pro-Thr-Met-Tyr-Gly-Glu-Ile-Leu-Ser.
and Ile-Tyr-Gly-Gly-Gln-Lys-Ala-Lys-Pro-Gly, corresponding to the N-terminal ends of C1s and of the MASP-2 SP serine protease domain, respectively and (ii) a 50-kDa species yielding only the first sequence above and reacting only with anti-C1s antibodies, corresponding to a truncated fragment derived from the N-terminal end of the chimeric protein. Analysis under reducing conditions indicated that full-length C1s(MASP-2 SP) was totally activated, as it yielded two bands at 29 and 60 kDa corresponding to the serine protease domain and the N-terminal chain expected to be released upon activation (see Fig. 2). The former chain reacted only with anti-MASP-2 antibodies (data not shown), whereas the latter co-migrated with the truncated fragment mentioned above.

**Functional Characterization of the C1s/MASP-2 Chimeras**—Activation of C1s* and the C1s(MASP-2 CCP1/2) chimaera was readily achieved by incubation with the CCP2-SP fragment of C1r, as assessed by the release of two chains upon SDS-PAGE analysis under reducing conditions (data not shown). As expected, the light chains, corresponding to the SP domains (30 kDa), co-migrated with that of wild-type C1s. In contrast, the N-terminal heavy chain of C1s(MASP-2 CCP1/2) had an apparent mass of 58 kDa, significantly lower than those of C1s and C1s* (61 kDa). Compared with wild-type C1s, no significant difference was observed in the activation kinetics of C1s* and C1s(MASP-2 CCP1/2), and 95% activation was achieved in all cases upon incubation with C1r CCP2-SP under the conditions described under “Experimental Procedures.”

The esterolytic activity of the various activated C1s variants was next measured using Ac-Gly-Lys-OMe as a substrate and compared with those of wild-type C1s and of the CCP1/2-SP fragment of MASP-2, which retains the esterolytic and proteolytic activities of full-length MASP-2 (12). As illustrated in Fig. 4, C1s* and C1s(MASP-2 CCP1/2) cleaved this substrate with an efficiency very similar to that of wild-type C1s. In contrast, C1s(MASP-2 SP) had a significantly lower activity comparable with that of the CCP1/2-SP fragment of MASP-2. This clearly indicated that each activated variant was catalytically active and had an esterolytic activity characteristic of its own SP domain (12).

Both activated C1s/MASP-2 chimeras specifically cleaved C2, as assessed by their ability to split this protein into its characteristic C2a and C2b fragments (data not shown). In both cases, C2 cleavage was essentially complete after incubation for 20 min at 37 °C at an enzyme:substrate molar ratio of 1:1000. The kinetic parameters of C2 cleavage were determined for both chimeras and compared with those obtained...
with wild-type C1s, C1s*, and the CCP1/2 fragment of MASP-2. As listed in TABLE ONE, all enzymes showed comparable \( k_{cat} \) values. Although slight differences were observed in the \( K_m \) values, the C2 cleavage efficiency of each chimera, as measured by the \( k_{cat}/K_m \) ratio, was not significantly different from the values determined for wild-type C1s and the CCP1/2-SP fragment of MASP-2.

Both C1s/MASP-2 chimeras mediated specific cleavage of C4, as shown by their ability to cleave its C4\( \alpha \) chain into the smaller C4\( \alpha' \) fragment (data not shown). N-terminal sequence analysis of the reaction mixtures indicated that both enzymes yielded a C4\( \alpha' \) fragment with the sequence Ala-Leu-Glu-Ile-Leu-Gln-Glu-Glu-Asp-Leu, providing unambiguous evidence that cleavage occurred at the Arg\( ^{737} \)-Ala\( ^{738} \) bond normally recognized by wild-type C1s and MASP-2. Whereas C1s(MASP-2 CCP1/2) was readily active at C4 concentrations of 50–500 nM, efficient cleavage by C1s(MASP-2 SP) required substrate concentrations in the micromolar range. Determination of the kinetic parameters of C4 cleavage revealed that wild-type C1s, the CCP1/2-SP fragment of MASP-2, and both C1s/MASP-2 chimeras had comparable \( k_{cat} \) values (TABLE TWO). In contrast, whereas C1s(MASP-2 SP), wild-type C1s, and C1s* all showed \( K_m \) values in the micromolar range, C1s(MASP-2 CCP1/2) and the CCP1/2-SP fragment of MASP-2 both exhibited \( K_m \) values in the nanomolar range. As a result, the \( k_{cat}/K_m \) ratios for the latter two enzymes were 21–27-fold higher than those determined for the three proteases containing the C1s-CCP modules (TABLE TWO). Thus, introduction of the MASP-2 SP domain in C1s did not significantly modify the C4 cleaving activity of the latter, but introduction of the MASP-2 CCP modules resulted in a strong increase in C4 cleavage efficiency due to a dramatic decrease in \( K_m \).

To determine the active site concentration of the C1s/MASP-2 hybrids, these were titrated with C1 inhibitor. As observed for wild-type MASP-2 (12) and C1s (Fig. 5A), increasing the C1 inhibitor:protease ratio led to a linear decrease of the C2 cleaving activity of both chimeras, with complete inhibition at a molar ratio close to 1:1 (Fig. 5B and C). Thus, the protein concentration estimates determined for these enzymes (see “Experimental Procedures”) actually matched their active
site concentrations, yielding further indication that these chimeric molecules were properly folded and retained full enzymatic activity.

**Functional Behavior of C1s(MASP-2 CCP1/2) within the C1 Complex**—The ability of the C1s(MASP-2) hybrids to associate with C1r and C1q to form a pseudo-C1 complex was measured by incubating each variant with an equimolar amount of proenzyme C1r and then monitoring the interaction of the resulting pseudo-C1s-C1r-C1r-C1s tetramer with immobilized C1q using surface plasmon resonance spectroscopy. Interaction was observed in the case of C1s(MASP-2 SP), but likely due to the presence of the N-terminal fragment, the binding curves did not fit a simple 1:1 Langmuir interaction model, and therefore, the kinetic constants could not be determined. The other chimera C1s(MASP-2 CCP1/2) yielded binding curves strikingly similar to those obtained in the case of wild-type C1s (Fig. 6) and C1s* (not shown). Determination of the kinetic parameters yielded comparable \( k_{\text{on}} \) values (TABLE THREE). Only a slight increase in \( k_{\text{off}} \) and in the resulting \( K_D \) value was observed in the case of C1s(MASP-2 CCP1/2), indicating that this chimeric molecule essentially retained the C1s ability to associate with C1r and C1q within C1.

To check its ability to undergo activation inside C1, C1s(MASP-2 CCP1/2) was mixed with appropriate amounts of C1q and proenzyme C1r, and the resulting pseudo C1 complex was allowed to activate as described under “Experimental Procedures.” As illustrated in Fig. 7, C1s(MASP-2 CCP1/2) yielded an activation curve very similar to those obtained with wild-type C1s and C1s*, demonstrating that the C1s-CCP1 segment of C1s by its MASP-2 counterpart did not significantly alter its ability to undergo activation in C1.

**DISCUSSION**

The major aim of this study was to investigate why MASP-2 and C1s, the homologous modular proteases that trigger the lectin and classical pathways of complement, respectively, exhibit differential catalytic efficiencies toward their common substrate C4. For this purpose, chimeric C1s molecules containing either the CCP1-CCP2 module pair or the SP domain of MASP-2 were engineered by domain swapping and expressed in insect cells. In this respect, it is noteworthy to note that, whereas wild-type C1s and the control molecule C1s* were produced at comparable yields (6–10 mg/liter), the expression level of the C1s(MASP-2 SP) chimera was much lower (0.15 mg/liter) and similar to those observed for full-length MASP-2 (12) or its CCP1-SP segment. In contrast, the other chimera C1s(MASP-2 CCP1/2) was consistently produced at a higher yield (2 mg/liter). As judged from these figures, proteins harboring the SP domain of MASP-2 were produced in significantly smaller amounts, suggesting that the presence of this particular domain is a limiting factor for expression in a baculovirus/insect cells system. Indeed, comparative analysis of the cDNA sequences of the various MASP-2 segments indicates that the SP domain contains a significantly higher proportion of unfavorable codons, as defined for *Drosophila melanogaster* (34), suggesting that the observed low expression level may be linked to codon usage.

Another conclusion arising from this study is that the activation characteristics of the C1s(MASP-2) chimeras are dictated by the nature of their SP domain. Thus, as also observed for wild-type C1s, the C1s(MASP-2 CCP1/2) chimera containing the C1s SP domain was secreted in a proenzyme form and did not undergo activation upon prolonged storage. Conversely, the chimeric molecule containing the MASP-2-SP domain had a behavior strikingly similar to that of wild-type MASP-2 expressed in a baculovirus/insect cells system (12). Thus, C1s(MASP-2 SP) was recovered as a two-chain, active form, indicating that it underwent spontaneous activation during or after the synthesis and secretion processes. In addition, as described previously for MASP-2 (12), C1s(MASP-2 SP) contained a significant amount of a 50-kDa N-terminal fragment, suggesting autolytic cleavage at a site located at the junction between the CCP2 module and the SP domain. It cannot be excluded, however, that this fragment resulted from other processes, such as abortive synthesis due to particular features of the cDNA coding for the MASP-2 SP domain.
With respect to activation, it should also be emphasized that the variants containing the C1s SP domain (C1s(MASP-2 CCP1/2) and C1s*) were both susceptible to activation by C1r in a manner comparable with wild-type C1s. The fact that this property is retained by C1s(MASP-2 CCP1/2) chimera provides further indication that recognition by C1r is restricted to the C1s SP domain and does not involve determinants in the upstream part of the protein, in full agreement with previous findings (14).

Each C1s/MASP-2 chimera retained an esterolytic activity characteristic of its own SP domain, providing a first indication that the functionality of this domain was not significantly altered by the swapping process. The fact that each chimera reacted with C1 inhibitor in a 1:1 stoichiometry fully supported this conclusion. With respect to C2 cleavage, both chimeras had an efficiency comparable with that of their parent proteases C1s and MASP-2. This was expected considering that C2 cleavage by C1s and MASP-2 only requires their SP domain (14, 15) and that both proteases have comparable efficiencies toward C2 (12). Thus, introduction of the MASP-2 CCP modules in C1s was not expected to modify its C2 cleaving activity, whereas introduction of the MASP-2 SP domain was expected to have little effect.

Analysis of C4 cleavage by the C1s/MASP-2 chimeras yields precise information about the relative contribution of the SP domain and CCP modules of C1s and MASP-2 to their activity toward this substrate and provides an explanation for the observed higher efficiency of MASP-2. Swapping the C1s-CCP modules for those of MASP-2 had no significant effect on $k_{cat}$ but resulted in a considerable increase in C4 cleavage efficiency by decreasing $K_m$ to a value similar to that determined for MASP-2 (12) and its CCP1-2-SP fragment. This observation is fully consistent with previous data indicating that C4 cleavage by C1s and MASP-2 requires substrate recognition by sites located in their CCP modules (12, 15) and clearly demonstrates that the CCP modules of MASP-2 are much more efficient in terms of C4 recognition than those of C1s. On the other hand, the fact that replacing the C1s SP domain by its MASP-2 counterpart had no significant impact on $k_{cat}$ provides strong indication that both domains are equally catalytically active toward this substrate. It may be concluded from these observations that the higher C4 cleavage efficiency of MASP-2 compared with C1s arises mainly, if not only, from a better efficacy of its CCP modules in terms of C4 recognition. In this respect it should be noted that, whereas C4 binding is considered to involve the CCP1 module of C1s (12, 35), the CCP2 module has been implicated in the case of MASP-2 (15). In light of these observations, it appears likely that the markedly different ability of these proteases to recognize C4 arises from the fact that they make use of distinct binding sites rather than homologous sites with differential binding properties.

It is remarkable that the C1s(MASP-2 CCP1/2) chimera is virtually as efficient toward C4 as is MASP-2 itself despite the unnatural junction between the MASP-2 CCP2 module and the C1s SP domain. The structure of the CCP2-SP interface has been determined for both C1s and MASP-2 (36, 37), indicating that the domains interact in a similar fashion in each case, through a network of interactions involving hydrogen bonds and van der Waals contacts. Nevertheless, although many of the interacting residues are conserved in both proteins, there are significant differences (37). It appears likely, therefore, that despite these local differences, each domain retains the ability to accommodate its partner in such a way that the stability of the interface is maintained, at least to a certain extent. Indeed, the intrinsic flexibility of the CCP2-SP interface, as observed in the case of MASP-2 (37), possibly allows this type of adaptation. From a general standpoint, the fact that both C1s(MASP-2 CCP1/2) and C1s(MASP-2 SP) are readily active on C4 demonstrates that the SP domain of each protease is able to cooperate efficiently with the CCP modules of the other, implying that the overall C4 cleavage mechanism does not involve stringent structural constraints between catalytic and recognition elements. An alternative possibility is that both chimeras retain flexibility at their CCP2-SP junction, allowing them to retain efficient C4 cleaving ability.

As shown by surface plasmon resonance spectroscopy and activation measurements, the C1s(MASP-2 CCP1/2) chimera also retains C1s ability to associate with C1q and C1r and to undergo activation in the context of the resulting pseudo C1 complex, indicating that, remarkably, swapping the C1s-CCP modules for those of MASP-2 has no significant effect on either function. In full agreement with recent studies based on site-directed mutagenesis (35), this clearly demonstrates that, in terms of interaction or recognition, the CCP1-CCP2 segment of C1s plays no direct role in C1 assembly and in the C1s activation process but is probably only used as a linker in both cases. In contrast, the flexibility at the CCP1-CCP2 interface (35) and the recognition properties of the CCP modules are key factors of the C4 cleavage reaction.

Acknowledgement—We thank J.-P. Andrieu for performing N-terminal sequence analyses.

REFERENCES

1. Cooper, N. R. (1985) Adv. Immunol. 37, 151–216
2. Schumaker, V. N., Zavodszky, P., and Poon, P. H. (1987) Annu. Rev. Immunol. 5, 71–132
3. Gaboriaud, C., Thielens, N. M., Gregory, L. A., Rossi, V., Fontecilla-Camps, J. C., and Arlaud, G. J. (2004) Trends Immunol. 25, 368–373
4. Matsushita, M., and Fujita, T. (1992) J. Exp. Med. 176, 1497–1502
5. Matsushita, M., Endo, Y., and Fujita, T. (2000) J. Immunol. 164, 2281–2284
6. Matsushita, M., Kuraya, M., Hamasaki, N., Tsujiura, M., Shiraki, H., and Fujita, T. (2002) J. Immunol. 168, 3502–3506
7. Thié, S., Vorup-Jensen, T., Stover, C. M., Schmehle, W., Larsen, S. B., Poulsen, K., Willis, A. C., Eggleton, P., Hansen, S., Holmskov, U., Reid, K. B. M., and Jensenius, J. C. (1997) Nature 386, 506–510
8. Bork, P., and Beckmann, G. (1993) J. Mol. Biol. 231, 539–545
9. Gregory, L. A., Thielens, N. M., Matsushita, M., Sorensen, R., Arlaud, G. J., Fontecilla-Camps, J. C., and Gaboriaud, C. (2004) J. Biol. Chem. 279, 23931–23937
10. Reid, K. B., Bentley, D. R., Campbell, R. D., Chung, L. P., Sim, R. B., Kristensen, T., and Tack, B. F. (1986) Immunol. Today 7, 230–234
11. Vorup-Jensen, T., Petersen, S. V., Hansen, A. G., Poulsen, K., Schmehle, W., Sim, R. B., Reid, K. B. M., Davis, S. J., Thié, S., and Jensenius, J. C. (2000) J. Immunol. 165, 2093–2100
12. Rossi, V., Cieh, S., Bally, I., Thielens, N. M., Jensenius, J. C., and Arlaud, G. J. (2001) J. Biol. Chem. 276, 40880–40887
13. Thié, S., Víug-Camp, J., Steiner, I., and Chevalier, C. (2004) J. Immunol. 174, 2870–2877
14. Rossi, V., Bally, I., Thielens, N. M., Esser, A. F., and Arlaud, G. J. (1998) J. Biol. Chem. 273, 1232–1239
15. Ambrus, G., Gal, P., Kojima, M., Szilagyi, K., Balazs, J., Antal, J., Graf, L., Laich, A., Moffatt, B. E., Schmehle, W., Sim, R. B., and Zavodszky, P. (2003) J. Immunol. 170, 1374–1382
16. March, S. C., Parilk, I., and Cruceanus, P. (1974) Anal. Biochem. 60, 149–152
17. Luo, C., Thielens, N. M., Gagnon, J., Gal, P., Sarvari, M., Tseng, Y., Tosi, M., Zavodszky, P., Arlaud, G. J., and Schumaker, V. N. (1992) Biochemistry 31, 4254–4262
18. Thielens, N. M., Villiers, M. B., Rebolu, A., Villiers, C. L., and Colomb, M. G. (1982) FEBS Lett. 141, 19–24
19. Dodds, A. W. (1993) Methods Enzymol. 223, 46–61
20. Arlaud, G. J., Sim, R. B., Dupla, A. M., and Colomb, M. G. (1979) Mol. Immunol. 16, 445–450
21. Arlaud, G. J., Villiers, C. L., Chesne, S., and Colomb, M. G. (1980) Biochim. Biophys. Acta 616, 116–129
22. Lacroix, M., Ebel, C., Kardos, J., Dobo, J., Gal, P., Zavodszky, P., Arlaud, G. J., and Thielens, N. M. (2001) J. Biol. Chem. 276, 36233–36240
23. Thielen, N. M., Aude, C. A., Lacroix, M. B., Gagnon, J., and Arlaud, G. J. (1990) J. Biol. Chem. 265, 14469–14475
24. Harpel, P. C. (1976) Methods Enzymol. 45, 751–760
25. Bock, S. C., Skriver, K., Nielsen, E., Thogersen, H. C., Wiiman, B., Donaldson, V. H., Eddy, R. L., Marrinan, J., Radziejewska, E., Huber, R., Shows, T. B., and Magnusson, S. (1986) Biochemistry 25, 4292–4301
Complement Proteases C1s/MASP-2 Chimeras

26. Edelhoch, H. (1967) Biochemistry 6, 1948–1954
27. Thielens, N. M., Cseh, S., Thiel, S., Vorup-Jensen, T., Rossi, V., Jensenius, J. C., and Arlaud, G. J. (2001) J. Immunol. 166, 5068–5077
28. King, L. A., and Possee, R. D. (1992) The Baculovirus Expression System: A Laboratory Guide, pp. 111–114, Chapman and Hall, Ltd., London
29. Arlaud, G. J., and Thielens, N. M. (1993) Methods Enzymol. 223, 61–82
30. Zandel, S., Cseh, S., Lacroix, M., Dahl, M. R., Matsushita, M., Andrieu, J. P., Schwaeble, W. J., Jensenius, J. C., Fujita, T., Arlaud, G. J., and Thielens, N. M. (2004) J. Immunol. 172, 4342–4350
31. Tacnet-Delorme, P., Chevallier, S., and Arlaud, G. J. (2001) J. Immunol. 167, 6374–6381
32. Rossi, V., Gaboriaud, C., Lacroix, M., Ulrich, J., Fontecilla-Camps, J. C., Gagnon, J., and Arlaud, G. J. (1995) Biochemistry 34, 7311–7321
33. Gregory, L. A., Thielens, N. M., Arlaud, G. J., Fontecilla-Camps, J. C., and Gaboriaud, C. (2003) J. Biol. Chem. 278, 32157–32164
34. Duret, L., and Mouchiroud, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4482–4487
35. Bally, I., Rossi, V., Thielens, N. M., Gaboriaud, C., and Arlaud, G. J. (2005) J. Immunol. 174, 4536–4542
36. Gaboriaud, C., Rossi, V., Bally, I., Arlaud, G. J., and Fontecilla-Camps, J. C. (2000) EMBO J. 19, 1755–1765
37. Harmat, V., Gal, P., Kardos, J., Szilagyi, K., Ambrus, G., Végó, B., Naray-Szabó, G., and Zavodszy, P. (2004) J. Mol. Biol. 342, 1533–1546