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J Immunol 2012; 189:2365-2373; Prepublished online 1 August 2012;
doi: 10.4049/jimmunol.1201085
http://www.jimmunol.org/content/189/5/2365

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/08/01/jimmunol.1201085.DC1

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Identification of a Catalytic Exosite for Complement Component C4 on the Serine Protease Domain of C1s


The classical pathway of complement is crucial to the immune system, but it also contributes to inflammatory diseases when dysregulated. Binding of the C1 complex to ligands activates the pathway by inducing autoactivation of associated C1r, after which C1r activates C1s. C1s cleaves complement component C4 and then C2 to cause full activation of the system. The interaction between C1s and C4 involves active site and exosite-mediated events, but the molecular details are unknown. In this study, we identified four positively charged amino acids on the serine protease domain that appear to form a catalytic exosite that is required for efficient cleavage of C4. These residues are coincidentally involved in coordinating a sulfate ion in the crystal structure of the protease. Together with other evidence, this pointed to the involvement of sulfate ions in the interaction with the C4 substrate, and we showed that the protease interacts with a peptide from C4 containing three sulfotyrosine residues. We present a molecular model for the interaction between C1s and C4 that provides support for the above data and poses questions for future research into this aspect of complement activation.


C omplement, an essential system for both innate and adaptive immunity, responds to the presence of a foreign pathogen within the vertebrate host. As well as directly affecting lysis of a pathogen, the complement cascade functions in inflammation and phagocytosis by acting as an opsonin, enhancing the migration of phagocytic cells to the infected area (1, 2), as well as initiating adaptive immune responses and regulating T and B cells (3, 4). The complement system generates a proteolytic cascade that initiates via one of three pathways of activation (5–7): classical, lectin, or alternative. Complement pathways have been associated with both unnecessary activation, causing inflammation in host tissues (8), and deficiencies, which contribute to autoimmunity and chronic infections (9, 10).

The classical pathway is initiated by the recognition molecule C1q, which is part of the 790-kDa C1 complex that assists in clearing infection and plays a role in immune tolerance and xenograft transplantation rejection (11, 12). The C1q molecule classically recognizes Ab–Ag complexes, but it also reacts with structurally different self and nonself targets, including C-reactive protein, bacterial porins, apoptotic cells, extracellular matrix proteins (13), polysaccharides, and prion–protein β-amyloid fibrils (14–16). C1q has an associated Ca2+-dependent tetramer composed of the serine proteases (SPs) C1s-C1r-C1r-C1s (17). Binding of C1 to a target ligand leads to autoactivation of C1r, after which the activated enzyme cleaves proenzyme C1s at an Arg-Ile scissile bond within the SP domain to form a two-chain, active enzyme (7, 18). The highly specific C1s binds and cleaves its substrates, C4 and C2, which subsequently form the C3 convertase (C4bC2a) (8).

C1s consists of six domains, of which the CUB1-EGF-CUB2 domains assist in binding to the C1 complex via Ca2+-binding sites (19), whereas the C-terminal CCP1-CCP2-SP fragment functions in the catalysis of C4 and C2. The exact mechanism by which C1s interacts with C4 has not been elucidated, although evidence suggests that the CCP domains contain an additional binding site (exosite) for C4, whereas the SP domain executes both the recognition and cleavage of C2 (20–22).

In this study, we show that the SP domain of C1s contains an exosite(s) that makes a major contribution to the binding and catalysis of C4. The residues making up an exosite on the C1s SP that contributes to the catalysis of C4 cleavage were identified. In parallel, evidence for the likely binding site on C4 for the C1s SP that contributes to the catalysis of C4 cleavage were identified. In parallel, evidence for the likely binding site on C4 for the C1s SP that contributes to the catalysis of C4 cleavage were identified.

Materials and Methods

Construction of recombinant plasmids for expression of the C1s fragments and mutagenesis

The cDNA for C1s was synthesized by GenScript (Piscataway, NJ). For all recombinant constructs, the pET17b expression vector was digested with NheI and EcoRI restriction endonucleases, and PCR products with identical sticky ends were ligated into the plasmid. Preceding the C1s sequences, the forward primer contained the codons for the three amino acids (Ala-Ser-Met) of the T7-Tag sequence, which increase the efficiency of recombinant protein expression. C1s fragments were amplified using the

Received for publication April 12, 2012. Accepted for publication June 21, 2012.

This work was supported by a National Health and Medical Research Council of Australia Program Grant (490900 to R.N.P.) and the Swedish Research Council (K2009-68X-14928-06-3 to A.B.).

The online version of this article contains supplemental material.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201085

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following primers: CCP1 5′ (GGGCTTAGCTGATAGGGCTGGTGGACAC-T TTGCATTATCAT), CCP2 5′ (GGGCTAGCTCCCTGAGACGGTGG), SP 5′ (GGGCTAGCCAGTGGTGGATGTC), CCP2 3′ (TACTGTTTCTTTCAAGGGTTCTTCGGG), and SP 3′ (CCGAAT-TCTTATGCTTCACCGGGGG). The proteins that were finally produced constituted Lys49 to Asp688 for the CCP1-CCP2-SP form and Pro532 to Asp688 for the SP form. Mutagenesis of the C1s fragments was carried out using the QuikChange Site-Directed Mutagenesis Kit, following the manufacturer’s methods and using splicing-overlap PCR.

Expression, refolding, and purification of the recombinant proteins

The expression plasmids were transformed into an Escherichia coli BL21Star(DE3) pLysS host strain, and the transformants were selected on Luria-Bertani medium plates containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). E. coli cultures were grown in 1 l 2YT at 37°C until an OD600 nm of 0.7 was reached, at which point a final concentration of 1 mM isopropyl-β-thiogalactoside was added. Following incubation, the culture was centrifuged (27,000 × g, 20 min, 4°C), the cells were collected in 30 ml 50 mM Tris-HCl, 20 mM EDTA (pH 7.4) and frozen at −80°C. The cells were thawed and sonicated on ice six times for 30 s, with ≥10 s between sonication cycles. The inclusion bodies were collected by centrifugation (27,000 × g, 20 min, 4°C), and the supernatant was discarded. The pellet was washed three times with 10 ml 50 mM Tris-HCl, 20 mM EDTA (pH 7.4) and frozen at −80°C in each wash. The inclusion bodies of the CCP1-CCP2-SP and SP constructs were solubilized in 8 M urea, 0.1 M Tris-HCl, 3 mM reduced glutathione, 1 mM oxidized glutathione, 5 mM EDTA, and 0.5 M arginine [pH 9]; 50 mM Tris-HCl, 3 mM reduced glutathione, 1 mM oxidized glutathione, 5 mM EDTA, and 0.5 M arginine [pH 10]). Following an overnight refolding procedure, the renatured protein solutions were dialyzed against 50 mM Tris-HCl (pH 9), and renatured proteins were purified on a 5-ml Q-Sepharose-Fast Flow column (GE Healthcare, Piscataway, NJ). The column was equilibrated with 50 mM Tris-HCl (pH 9) for six column volumes (CVs) before loading. The column was washed with 6 CVs 50 mM Tris-HCl, pH 9, and protein was eluted with a linear 0 to 120 min.

Preparation of the enzyme fragments and activity assay using synthetic peptides

Assays were carried out in fluorescence assay buffer [0.05 M Tris-HCl, 0.1 M NaCl, 1 mM DTT, 1 mM MgCl2, 2% (v/v) Tween 20, pH 7.5] using final substrate concentrations ranging from 0.5 to 20 μM. The C4 P4-P4′ fluorescence quenched substrate [2Abz-QLQRALE-lys(Dnp)NH2] (GL Biochem, Shanghai, China) was solubilized in 10% (v/v) N,N-dimethylformamide. The final concentration of the C1s protease fragments in the assays was 400 nM. The rate of increase of fluorescence for the assays was measured on a BMG Technologies FLUOstar Optima Plate Reader with excitation and emission wavelengths of 370 and 460 nm, respectively.

C1s protease fragments were incubated with H-Glu-Gly-Arg-chloromethylenketone (EGRk) (10 μM, Sigma, Sydney, Australia) for 4 h at 37°C to prepare inhibited fragments for ELISA tests. To check for complete inactivation, the activity of 5 μg/ml the preparation was tested against Leu-Gly-Arg-NHMec (Bachem, Bubendorf, Switzerland) substrate (50 μM) at 37°C using a FLUOstar Optima Plate Reader with excitation and emission wavelengths of 370 and 460 nm, respectively.

Measurement of the cleavage of C4 by protease variants

For the determination of EC50 values, C1s fragments were diluted to final concentrations of 1–1500 nM in assay buffer (20 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA [pH 7.4]). C4 (Complement Technologies) was diluted to 1 μM in assay buffer. The proteases and substrate were incubated separately at 37°C for 5 min and combined, and the cleavage reaction was allowed to proceed at 37°C for 1 h.

For time-course analyses, C1s single/cluster mutants were diluted to 1 nM, whereas the substrate C4 was diluted to 1 μM in assay buffer. The proteases and substrate were incubated separately at 37°C for 5 min and combined, and the cleavage reaction was allowed to proceed at 37°C for 0 to 120 min.

To determine IC50 values for C4-derived peptides, all proteins and peptides were tested at a final molar ratio of 1:1 (peptide/protein/substrate). The sulfated tyrosine-containing peptide (Ac-[Nle]-EANEDYEDYEYDELPAKDDPD-NH2) was synthesized via fluorenylmethyl (Fmoc)-strategy solid-phase peptide synthesis. The sulfated tyrosine residues were introduced via coupling of the synthetic building block Fmoc-Tyr(OSu)-CH2(CH3)2 (which was prepared and introduced to the growing peptide chain using the method described previously (25, 26). The phosphorylated (Ac-[Nle]-EANEDYEDYEYDELPAKDDPD-NH2) and unmodified (Ac-[Nle]-EANEDYEDYEYDELPAKDDPD-NH2) C4 peptides were synthesized by GL Biochem using Fmoc-strategy solid-phase peptide synthesis. The modified and unmodified peptides were used at final concentrations of 3–500 μM. C1s CCP1-CCP2-SP and C1s SP were used at final concentrations of 25 and 370 nM, respectively. Protease fragments were incubated with individual peptide concentrations for 1 h at RT in assay buffer. C4 was diluted in assay buffer, incubated at 37°C for 5 min, and added to the protease/peptide mixture; the cleavage assay took place for 1 h at 37°C. The effect of the C4-derived peptides on the time course of C4 cleavage by C1s fragments was measured in a similar manner, using the same protease concentrations, whereas C4 was used at a final concentration of 500 μM. The cleavage reaction was allowed to proceed at 37°C for 0 to 120 min.

All reactions were stopped by the addition of reducing SDS-PAGE loading buffer, and samples were incubated at 90°C for 5 min, loaded onto 12.5% SDS-PAGE, and electrophoresed. Gels were stained with Coomassie blue R-250 stain and destained. The cleavage of C4 α band was analyzed using loading controls for the recombinant protein (48K, 532-, and 632-nm lasers) was used for densitometry analysis using IQTL ImageQuant software (1D Gel Analysis) (GE Healthcare).

EC50 values were derived by analyzing the data points by nonlinear regression using the following equation: Y = Ymin + (Ymax − Ymin)/(1 + ([0.5IC50] / X)2), where Ymin is the minimum Y value, Ymax is the maximum Y value, and b is the Hill slope.

Determination of equilibrium dissociation constants using ELISA

All incubation steps were carried out using 50-μl solutions for 1 h at RT, except where stated otherwise. Every step was followed by four washes with 100 μl 20 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5. Microtiter plates (Maxisorp; Nunc) were coated overnight at 4°C with C1s mutants (10 μg/ml) diluted in 75 mM Na2CO3 (pH 9.6). The wells were blocked for 2 h with 230 μl 1% (w/v) BSA in PBS (8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M NaCl, 0.01 M potassium chloride [pH 7.4]) (blocking solution). Dilutions of human C4, diluted in 50 mM HEPES, 100 mM NaCl, 2 mM CaCl2 (pH 7.4), were added to the plates and incubated for 2 h. After washing, plates were incubated with specific rabbit polyclonal Abs against C4-c (Dako) diluted 1:4000 in blocking solution. HRP-conjugated secondary Abs against rabbit (IgG) (Dako) were diluted 1:2000 in blocking solution and allowed to bind. Bound enzyme was quantified using the 3,3′,5,5′-tetramethylbenzidine/H2O2 colorimetric assay (Sigma), which was quenched with 2 M H2SO4, and A450 values were measured. The A450 values were corrected for the background absorbance of substrate alone. Data points were analyzed using GraphPad Prism Version 5.0, using a single-binding site equation: Y = Bmax*X/(Kd + X). This equation describes the binding of ligand to a receptor that follows the law of mass action. Bmax represents the maximal

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binding, and $K_D$ is the concentration of ligand required to reach one-half maximal binding.

**Complement deposition assay**

Unless stated otherwise, all incubations were carried out at RT in 50 μl solution, and each step was followed by washing, as described above for the ELISA binding assay. Aggregated IgG was diluted to 2.5 μg/ml in 75 mM Na₂CO₃ (pH 9.6) and coated onto MaxiSorp microtiter plates (Nunc) overnight at 4°C. The wells were blocked for 2 h with 200 μl 1% (w/v) BSA in PBS (blocking solution). Various concentrations of C1s constructs, diluted in Gelatin veronal buffer [2.5 mM Veronal buffer (pH 7.3) 150 mM NaCl, 0.1% (w/v) gelatin, 1 mM MgCl₂], were preincubated with 0.3% hum serum protein for 15 min. C1s-serum mixtures were added to the plates and incubated for 20 min at 37°C, followed by 1 h of incubation with specific rabbit polyclonal Abs against C4c (Dako), diluted 1:4000 in blocking solution. HRP-conjugated secondary Abs against rabbit (Dako) were diluted 1:2000 in blocking solution and allowed to bind for 30 min. Bound enzyme was quantified using the 1,2-phenylenediamine dihydrochloride/HzO₂ colorimetric assay (Dako), which was quenched with 0.5 M H₂SO₄, after which $A_{490}$ values were determined.

**Measurement of the inhibition of Ab binding to C4 in the presence of C1s**

ELISAs were carried out as described above, unless otherwise indicated. C1s CCP1-CCP2-SP S632A (10 μg/ml) was coated overnight. Following blocking, wells were incubated with either 7 or 70 mM C4 substrate, and the plates were washed four times and incubated with various concentrations of human IgG anti-sulfotyrosine, or the rabbit anti-C4c Ab described above, for 2 h at RT. Wells were diluted in blocking buffer, and concentrations of human IgG anti-sulfotyrosine were selected from a titration curve, showing low, medium, and high efficiency of binding to C4. Following four wash steps, goat anti-human IgG/HRP secondary Ab was incubated with wells containing human IgG anti-sulfotyrosine for 1 h at RT, wells containing the rabbit anti-C4c Ab were incubated as described previously.

**C4 phosphonylated C4 peptide column binding**

Phosphorylated C4 peptide was synthesized (GL Biochem) with a triglycine linker to a biotinylated C terminus (Ac-[Nle]-EANED[Pyr][Pyr][Pyr][Pyr]) DELPAKDDPD-GGGK[Biotin]). The peptide (1 mg) was solubilized in 10% (v/v) N,N-dimethylformamide, and the remaining 90% was solubilized in column-binding buffer (20 mM sodium phosphate, 0.15 M NaCl [pH 7.5]). The HiTrap Streptavidin column (1 ml) was equilibrated with 10 CV column-binding buffer, and the peptide was loaded onto the column at 0.1 ml/min. Both ends of the column were capped and incubated for 1 h at 4°C, after which the column was washed with 10 CV column-binding buffer. Approximately 0.3–1 mg purified protein was diluted in 5 ml buffer A (50 mM Tris-HCl [pH 7.4]) and then bound to the HiTrap Streptavidin column at 0.2 ml/min. The column was washed with 6 CV buffer A and then eluted with a linear NaCl gradient to buffer B (50 mM Tris-HCl, 1 M NaCl [pH 7.4]) over 7 ml at 0.2 ml/min. The $A_{280}$ (AU) and conductivity (mScm) values were plotted using GraphPad Prism Version 5.0.

**Docking model of C1s and C4 complex**

A model of the C1s–C4 complex was created to investigate the hypothesis that the sulfotyrosine residues of C4 interact with C1s. This model is based on the published C1s structure (1ELV), and we used the published C3 (2A73) structure as the template structure for C4, building in two missing loops, the 720 loop containing the scissile bond, and the 1350 loop, which is the large insert containing the three sulfotyrosine residues. The C1s was docked onto the 720 loop using the extended substrate interaction mode observed in crystal structures of serpins with thrombin (27–29). Docking of C1s on the 720 loop results in the alignment of N-terminal “stock” of the 1350 loop, containing the sulfotyrosine residues, with the region where a sulfate binds to the C1s structure. A small amount of structural regularization was undertaken to alleviate steric clashes. All molecular modeling and docking were carried out using the programs PyMol and Coot.

**Results**

**Production and characterization of recombinant C1s domain fragments**

Pure C1s domain fragments (Fig. 1) were obtained following refolding and purification at yields varying from 0.3 to 2.3 mg/ml per 1 l of E. coli culture, depending on the fragment being produced. All C1s domain mutants were purified to homogeneity using a two-step chromatography protocol, as indicated by SDS-PAGE analysis (Fig. 1) and reacted to Abs raised in chickens against CCP and SP domains (data not shown). C1s cannot autoactivate; therefore, we used C1r immobilized on an NHS-Sepharose column to activate the SP-containing fragments. All activated fragments were N-terminally sequenced to ensure that they had been correctly cleaved by C1r (data not shown). The activated fragments were able to react entirely with C1 inhibitor at a 1:1 ratio, indicating that they were 100% active (data not shown).

**Activity of recombinant C1s fragments against synthetic peptide substrates**

The kinetic parameters for cleavage of the fluorescent quenched C4 P4-P4’ (2Abz-GLQRALEI-Lys(Dnp)-NH₂) peptide substrate by the recombinant C1s fragments were compared with each other and plasma-derived C1s (Supplemental Table 1). Overall, the ki-
netic values were similar for all forms of the enzyme, indicating that there was little significant alteration to the active site upon deletion of the N-terminal domains of C1s. These results are analogous to previous studies (20). The results also indicate that all recombinant forms of the enzyme were efficiently activated by treatment with C1r.

**C4 deposition assay: the SP domain is required for high-affinity C4 binding**

The effect of each C1s domain mutant on C4 deposition via the classical pathway was tested. The classical pathway was activated using wells coated with aggregated IgG. The human serum used in the assay was preincubated in the absence or presence of increasing concentrations of the C1s domain mutants. The effect on C4b deposition is illustrated in Fig. 2, which demonstrates that the SP S632A (C1s numbering, S195A chymotrypsin numbering) form of C1s had the highest overall impact on C4b deposition, with complete inhibition achieved at 1 μM. The CCP1-CCP2-SP S632A form behaved similarly, with ~80% inhibition at a concentration of 1 μM.

**Analysis of the binding of recombinant C1s fragments to C4**

The affinity of each of the C1s forms for C4 was determined using an ELISA in which the enzyme fragments were coated to the plate. It was not possible to measure binding with C4 coated to the plate, possibly because of alterations to the substrate protein that precluded binding by the enzyme. Data for the binding of C4 to immobilized C1s fragments fitted well to a single-site binding equation to allow determination of $K_D$ values (Supplemental Fig. 1). Plasma-derived C1s and the CCP1-CCP2-SP forms of C1s exhibited similarly tight binding to C4, with $K_D$ values of 0.81 and 0.73 nM, respectively (Table I). The SP domain retained high affinity for C4, albeit with a 7-fold higher $K_D$ value than C1s CCP1-CCP2-SP. The active site S632A mutant of the CCP1-CCP2-SP form showed an ~7-fold increased $K_D$ value compared with the active form (Table I), suggesting that the active site has a role in the binding of the enzyme to C4. The C1s SP domain still exhibited binding to C4, despite the addition of the active site(s) or exosite(s) on the C4 domain.

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**Efficiency of cleavage of C4 by C1s domain mutants**

Previous reports demonstrated that the SP domain is unable to cleave C4 without the assistance of the CCP1-CCP2 domains (20); however, the results from both the C4-deposition assays and ELISA reported in this article showed significant interactions between this domain and C4. Therefore, the cleavage of native C4 by C1s CCP1-CCP2-SP and SP fragments was investigated. Various concentrations of each protease were incubated with C4 (1 μM) for 1 h at 37°C. Reactions were stopped with reducing SDS-PAGE loading buffer and subjected to electrophoresis using SDS-PAGE (Supplemental Fig. 2). The cleavage of C4 was calculated by quantifying the disappearance of the α-chain of the protein using densitometry; these values were plotted against enzyme concentration to determine EC₅₀ values of 1 and 141 nM for the CCP1-CCP2-SP and SP fragments, respectively (Table I). These results suggest that the SP domain of C1s is able to bind to C4 sufficiently to facilitate cleavage, albeit at a 140-fold lower efficiency than the CCP1-CCP2-SP form. It is likely that the results obtained showing that the SP domain retains the ability to cleave C4 are due to the higher concentrations of the SP domain used in the assays compared with the previous study (20). Therefore, we investigated whether the SP domain of C1s contained an exosite likely to form interactions with C4.

**Examination of the structure of C1s reveals a likely exosite location on the SP domain**

An electrostatic potential contour map of C1s CCP2-SP (PDB: 1ELV) showed that the majority of the protease surface was negatively charged, with only a few positively charged patches (Fig. 3). One positively charged patch at 90° to the active site binds a putative sulfate ion within the x-ray crystal structure of C1s (17). Four
positively charged amino acids from within this area in the SP domain surround the sulfate ion. These residues were chosen for mutagenesis, where conservative mutations were made, altering Lys or Arg residues to Gln, either singularly or in a cluster (Fig. 3).

Interaction of C1s mutants with synthetic peptide and physiological substrates

The first aim was to investigate the kinetics of cleavage of synthetic peptide substrates representing the cleavage sequences in C4 (P4-P4') (Supplemental Table I). Most single and cluster mutants showed modest changes in the individual k_{cat} and K_{0.5} values; however, generally these changes compensated for each other, resulting in little overall variation in their k_{cat}/K_{0.5} values compared with wild-type C1s. This suggests that the mutations had minor effects on the active site of C1s but little overall effect on the catalytic ability of the enzyme.

Interestingly, the mutations to the C1s SP had only little or no effect on the K_{D} values for the interaction of the enzyme catalytic fragment with C4 (Table II). The results indicate that the identified cluster of positively charged amino acids may not constitute a binding exosite for C4.

The cleavage of C4 was initially examined by determining EC_{50} values for cleavage of the substrate. This analysis demonstrated that three single mutants, R576Q, R581Q, and K583Q, were significantly altered in their efficiency of cleavage of the protein substrate (Table II). The mutant R576Q had an EC_{50} value that was 17-fold higher than the wild-type enzyme, whereas the R581Q and K583Q enzymes had even more significantly increased EC_{50} values (32–39-fold higher). Mutant K575Q showed little change and had an EC_{50} value that was increased 2-fold relative to wild-type.

The effect of the single mutations on the time course of cleavage of C4 was examined. The time course analysis revealed that the K575Q mutant cleaves C4 at a slower rate than does the wild-type enzyme, but it has a similar end point. The remaining three mutants (R576Q, R581Q, and K583Q) all displayed considerably slower C4 catalysis, achieving ∼40% C4 cleavage after 120 min, compared with the wild-type enzyme (Fig. 4A). Mutants of the entire cluster of positively charged residues were then made, with the residues changed to either Gln or Ala. Preliminary determination of the EC_{50} values demonstrated that large concentrations of the cluster mutant enzymes were required for 50% cleavage of substrates (data not shown); therefore, the effects on the catalysis of C4 were tested using a time course analysis. Mutants were incubated with a standard concentration of C4 (1 μM), and the reaction was stopped at time points ranging from 0 to 120 min. As seen in Fig. 4B, the Gln cluster mutant only cleaved C4 to ∼25% of the extent of wild-type C1s, whereas the Ala cluster mutant had <5% cleavage of the substrate compared with the wild-type enzyme, even after 16 h.

Inhibition of anti-sulfotyrosine Ab binding to C4 in the presence of C1s

Because we had established that the residues on the surface of the SP domain of C1s making contact with a sulfate ion in the crystals used for determination of the enzyme’s structure were important for catalysis of the C4 substrate, we next wished to investigate whether sulfotyrosine residues on the surface of C4 were an important point of contact for C1s in its interaction with the substrate protein. C4 contains three sulfotyrosine residues on the α-chain contained within a 20-aa stretch of mostly negatively charged residues. To provide initial evidence that the area of interest on C4 may serve as a binding site for C1s, an experiment was conducted to investigate whether sulfotyrosine Abs could still bind to C4 in the presence of C1s. This was performed using two concentrations of C4 and three concentrations of the Ab to overcome any possible equilibrium-binding constraints. The anti-sulfotyrosine Ab concentration used was derived from titration curves, yielding concentrations showing low-, medium-, and high-efficiency binding to C4. Binding of the anti-sulfotyrosine Ab to C4 alone was used as a control to reveal 100% binding at each concentration of the Ab. Additionally, the binding of C4 to C1s was demonstrated using an anti-C4 Ab, which showed that C4 was equivalently present in these cases and should have been available for binding to the anti-sulfotyrosine Ab if the sulfotyrosine residues were available for interactions with the Ab. The results indicated that C1s strongly inhibited the binding of the anti-sulfotyrosine Ab, suggesting that the sulfotyrosine-containing area on C4 is indeed involved in the interaction with C1s (Fig. 5).

Analysis of the effect of a C4-derived peptide on cleavage of C4 by C1s

Three C4 peptides were synthesized with three sulfotyrosine (Ac-[Nle]-EANED[sY]ED[sY]E[sY]DELPAKDDPD-NH2), three phosphorylated tyrosine (Ac-[Nle]-EANED[pY]ED[pY]E[pY]DELPAKDDPD-NH2), and three undervatized tyrosine (Ac-[Nle]-
EANEDYEDYEYDELPAKDDPD-NH2) residues to assess the effect of the peptide alone, as well as the modifications of the tyrosine residues by sulfate or phosphate groups. The relatively difficult synthesis of the sulfotyrosine-containing peptide resulted in a low yield of the material. It was shown previously that the substitution of sulfate with phosphate groups on tyrosine residues had minimal effects on the binding interaction of certain proteins and ligands (31); therefore, the effects of the phosphate modification on the interaction were also investigated. The replacement of the sulfate groups with phosphate greatly improved the yield and stability of the peptide, which was beneficial in later experiments.

Initial experiments demonstrated that a concentration of 500 μM of the C4 peptides showed the most significant impact on C4 cleavage for both C1s domain fragments; therefore, this concentration was chosen to investigate their effects on the time course of C4 cleavage (data not shown). Proteases were incubated either alone or with C4 peptide and then allowed to cleave C4 over a 2-h time course. Preincubation of sulfated peptide with both C1s fragments reduced their C4 cleavage, but the most significant results were once again seen for C1s SP (Fig. 6A, 6B). Typically, the percentage of C4 cleavage was half of that for the enzyme in the absence of the peptide at each time point when the protease was preincubated with the sulfated C4 peptide. Preincubation of the enzymes with the phosphorylated C4 peptide resulted in small reductions in C4 cleavage, and both C1s fragments had cleaved the same percentage of C4 by the end of the assay in the presence or absence of the phosphorylated peptide (Fig. 6C, 6D). Finally, preincubation with the nonsulfated C4 peptide had minimal effects on the cleavage of C4 by the C1s SP fragment, and it decreased the C4 cleavage activity of C1s CCP1-CCP2-SP by ∼20% (Fig. 6E, 6F). This latter effect may be due to relatively nonspecific binding of the peptide to the CCP domains of the protease.

Analysis of the binding of C1s fragments to a phosphorylated C4-derived peptide

The binding efficiency of each C1s fragment for the C4 peptide was then investigated. Ideally, a sulfated C4 peptide would have been preferred for such assays because it had the strongest effects on C4 cleavage by C1s enzyme fragments, but such assays were difficult because of the very low amounts of peptide available. The results above show that the phosphorylated C4 peptide was able to interact with the proteases enough to inhibit their ability to cleave C4; therefore, a biotinylated phosphorylated C4 peptide was synthesized. A triglycine linker was attached to the C-terminal end of the peptide, followed by a biotin molecule. The biotin-labeled peptide was bound to a streptavidin column, and proteins, in turn, bound to the peptide were eluted using a linear NaCl gradient (0–1 M); the proteins with high binding efficiency for the C4 peptide eluted

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Time course analysis of C4 cleavage by single and quadruple mutants of C1s. Cleavage of C4 (1 μM) was carried out at 37°C, and the final enzyme concentration used in each case was 1 nM. (A) Single mutants: K575Q, R576Q, R581Q, K583Q. Reactions were stopped at 0, 1, 2, 5, 15, 30, 60, or 120 min. (B) Gln (K575Q, R576Q, R581Q, K583Q) and Ala (K575A, R576A, R581A, K583A) quadruple mutants: reactions were stopped at 0, 0.5, 1, 2, 4, 8, or 16 h. In both cases, the loss of the α-chain was quantified using densitometry, and the γ-chain was used as a loading control. Results are representative of three replicate experiments.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Effect of C1s on binding of anti-sulfotyrosine Abs to C4. C1s inhibition of binding of human anti-sulfotyrosine IgG (1–50 nM) to C4 at 7 nM (black bars) or 70 nM (diagonal black line bars). Values were normalized using the binding of anti-sulfotyrosine IgG to C4 alone at 7 nM (gray bars) or 70 nM (white bars) as 100%. The presence of C4 binding to C1s at 7 nM (horizontal lined bar) or 70 nM (cross-hatched bar) was confirmed in each case by measuring the binding to an anti-C4 Ab. The ability of anti-sulfotyrosine Ab to bind to C1s was also measured (stippled white bar). Results are representative of three replicate experiments. ***p < 0.0001, two-way ANOVA.
from the column at higher NaCl concentrations. Activated C1s SP once again had the highest affinity for the peptide (Fig. 7), followed closely by activated C1s CCP1-CCP2-SP. The four C1s CCP1-CCP2-SP single mutants of interest (K575Q, R576Q, R581Q, K583Q) and both the quadruple Ala and Gln mutants demonstrated no binding to the C4 peptide. The interaction between the exosite on the SP of C1s and the sulfated stretch of amino acids on C4 was modeled (Fig. 8), indicating that the exosite on the SP could engage the loop containing the sulfotyrosine residues in C4 simultaneously to the engagement of the active site of the enzyme with the cleavage site on C4.

Discussion

The cleavage of C4 by the C1s protease of the C1 complex is a key point in the activation of the classical pathway of the complement system. Because the unregulated activation of complement underlies the pathogenesis of many inflammatory diseases (8), understanding the mechanisms of its initiation and regulation is vital to potentially develop therapeutic agents targeting the system. The modular nature of C1s has given rise to some debate as to the molecular mechanism by which it interacts with C4 and cleaves it to form C4b (20–22). In this article, we provided considerable insight into the interaction between the enzyme and its primary substrate.

Previous data in the field indicated a key role for the CCP domains of C1s in the cleavage reaction for C4, and some inferences have been made about the binding of the enzyme to the substrate based on kinetic constants for the catalytic reaction (20, 22). Such inferences assume a linear relationship between the $K_m$ values for the catalytic reaction and equilibrium dissociation constants for the enzyme–substrate reaction that rarely exist. In this study, we probed the specific role of the SP domain of the catalytic region of C1s using a combination of binding and catalytic reactions. The data from our experiments are in agreement with previous literature in showing that the CCP domains of the enzyme play a role in binding and catalysis by the enzyme (20–22). However, our studies showed that the SP domain of the enzyme played a greater role in binding to C4 than previously thought and, in particular, they showed that region(s) on the SP outside of the active site of the enzyme are important for binding C4, consistent with the C1s SP housing an exosite(s) for C4. This is somewhat different from the situation with the MASP-2 protease of the lectin pathway, which also has C4 as its primary substrate. For MASP-2, it appears that the CCP2 domain of the protease is the primary location of an exosite for C4 (32).
In our efforts to identify likely regions of the C1s SP that might constitute the exosite for C4, we noted that the protein contains sulfate ions in the form of three sulfotyrosine residues incorporated into the α-chain of C4 in close proximity to each other among a stretch of negatively charged residues \(1412^{\text{NED}}-^{1420^{\text{YE}}}\). Interestingly, nonsulfated C4 was shown to have 50% less hemolytic activity in complement activation assays, and a 10-fold higher concentration of C1s is required for cleavage of C4 (36). We also noted that the crystallized CCP2-SP enzyme had a sulfate ion bound to its surface in the midst of a cluster of positively charged residues: K575, R576, R581, and K583 (Fig. 3). The presence and location of the sulfate ions in the crystal structure of C1s and the potential for sulfate groups to be involved in the interaction between C1s and C4 assisted in selecting positively charged residues of C1s for mutagenesis to elucidate their role in interactions with C4.

The interaction site on C4 for C1s was investigated using a range of techniques and representative sequences from the substrate. The kinetic values for cleavage of the synthetic substrates based on the cleavage sequences in C4 were determined for all mutants. Mutation of the four residues surrounding the sulfate ion on the C1s SP domain had little effect on the kinetics of cleavage of peptide substrates by the mutant enzymes, indicating that these residues were not involved in direct interactions between the enzyme and the cleavage sequences of the substrates. However, mutagenesis of the four residues resulted in enzymes that were significantly altered with respect to their cleavage of the protein substrate C4 compared with wild-type C1s. The K575Q mutant appeared to be the least affected of the mutants, showing a moderate change in \(K_{\text{cat}}\) and rate of cleavage of C4 in a time course assay. The other three mutants, R576Q, R581Q, and K583Q, showed markedly increased \(K_{\text{cat}}\) values for cleavage of C4 in comparison with wild-type C1s. All of these mutants were also substantially affected in time course analyses of C4 cleavage. It is interesting to note that these are the three residues that are in close vicinity to the trilobed sulfate ion, with K575 slightly further away. The data and characteristics fit with the four residues making up an exosite for the C4 substrate. Therefore, these residues were targeted to create two quadruple mutants, altering the Lys or Arg residues to either Gln or Ala residues. Cleavage of C4 was significantly reduced for both the Gln and Ala cluster mutants, confirming that the four charged residues together most likely form an exosite that mediates efficient cleavage of C4. The single mutants or quadruple mutants of C1s showed little alteration in their equilibrium dissociation constants for C4. This may indicate that there are still yet-to-be-discovered binding residues on the C1s SP for C4 or that further investigation of the individual rate constants of the overall reaction is required to better understand the kinetic mechanism of C4 binding. In any event, it is clear that the cluster of positively charged residues on the C1s SP at the very least constitutes an exosite required for efficient catalysis of the substrate by C1s. This implies that the cluster of positive charges ideally positions the protease on the substrate for efficient cleavage.

As noted previously, the major potential interaction site for a positively charged region on C1s would be the highly negatively charged region on C4 containing three sulfated tyrosine residues. The inability of the anti-sulfotyrosine Ab to bind C4 in the presence of C1s, as well as the ability of peptides with the sequence of the sulfotyrosine-containing stretch of C4 to interfere with cleavage of the protein by C1s, provided strong indications that this region of C4 is important for interactions with C1s, particularly when coupled with previous literature indicating the importance of sulfotyrosines for interaction with the enzyme (35). The ability of the CCP1-CCP2-SP and SP forms of C1s to bind to the immobilized phospho-tyrosine peptide provided further evidence that a site on the SP was able to interact with the peptide, whereas the inability of any of the mutants of C1s to bind under physiological salt conditions strongly suggests that the identified positively charged exosite on the C1s SP is the site of interaction of this C4 sequence with C1s. A model of C4 containing the large loop on which the sulfotyrosine residues are located was developed, and this was docked to C1s without any prior assumptions. Interestingly, the model shows only the SP domain interacting with the substrate C4. The structure demonstrates that the middle and C-
terminal sulfotyrosine residues (Tyr1420, Tyr1422) interact with the C1s binding pocket containing the K575, R576, R581, and K583 residues identified to be important for our study (Fig. 8). Importantly, binding of the sulfotyrosine loop with the exosite of C1s occurred simultaneously with binding of the C4 cleavage sequence AGLQRKALEL into the active site.

The data presented in this article provide the most detailed elucidation of the molecular details of the mechanism of interaction between C1s and C4. We identified a critical role for exosite(s) on the SP domain of the enzyme in the binding and catalysis of C4 and defined the location of one such positively charged exosite on the C1s SP domain necessary for efficient cleavage of C4 by C1s. We also provided strong evidence to suggest that this exosite of C1s binds to a stretch of negatively charged residues on C4 containing three sulfotyrosine residues and provided preliminary validation for this interaction using molecular modeling. The studies provide a firm basis to guide a full elucidation of the molecular details underpinning the kinetic mechanism by which C1s interacts with C4. It is possible that the identified exosite also plays a role in the binding of highly sulfated heparin by C1s, an interaction that is vital for full regulation of the protease by the serpin, C1 inhibitor (37, 38). Because unregulated complement activation underlies many inflammatory diseases, the data revealed in this study provide a basis for the design of therapeutic molecules for the targeted prevention of activation of complement via the classical pathway.

Acknowledgments

We thank Dr. Andrew Bradbury of Los Alamos Laboratory, U.S. Department of Energy (Los Alamos, NM) for the gift of the human anti-sulfotyrosine Ab.

Disclosures

The authors have no financial conflicts of interest.

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