A Common Neoepitope Is Created When the Reactive Center of C1-Inhibitor Is Cleaved by Plasma Kallikrein, Activated Factor XII Fragment, C1 Esterase, or Neutrophil Elastase

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Abstract

The reactive center of C1-inhibitor, a plasma protease inhibitor that belongs to the serpin superfamily, is located on a peptide loop which is highly susceptible to proteolytic cleavage. With plasma kallikrein, C1s and β -Factor XIIa, this cleavage occurs at the reactive site residue P₁ (Arg⁴⁴⁴); with neutrophil elastase, it takes place near P₁, probably at residue P₃ (Val⁴⁴²). After these cleavages, C1-inhibitor is inactivated and its conformation is modified. Moreover, in vivo, cleaved C1-inhibitor is removed from the blood stream more rapidly than the intact serpin, which suggests that proteolysis unmasks sites responsible for cellular recognition and the uptake of the cleaved inhibitor. In the study reported here, we show, using an MAb, that an identical neoepitope is created on C1-inhibitor after the cleavage of its exposed loop by plasma kallikrein, C1s, β -Factor XIIa, and by neutrophil elastase.

Introduction

Serine proteases are controlled in the plasma milieu by protease inhibitors that belong to the serpin (serine protease inhibitor) superfamily, a recently identified family of structurally related glycoproteins (1, 2). Serpin deficiency or dysfunction is a key pathogenic factor for the development of several clinical syndromes. For example, emphysema is observed with a deficiency of α 1-antitrypsin (3), thrombosis with a deficiency of antithrombin (4), and angioedema with a deficiency of C1-inhibitor (5). The mechanism by which serpins inactivate serine proteases has been extensively studied (1, 2). Inhibitors react with their target proteolytic enzymes to form stable, inactive and covalent equimolar complexes. During enzyme inactivation, the inhibitor reactive center, which is located on a protein loop exquisitely susceptible to proteolysis, is cleaved between adjacent amino acid residues termed P_1 and P'_1 . This process irreversibly modifies the conformation of the serpin molecule,

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creating sites responsible for the rapid metabolic clearance of enzyme-serpin complexes and for the regulation of serpin biosynthesis (6-11). The following observations support the notion that serpins undergo major conformational changes when they are cleaved at or next to their reactive center: (a) complex formation between thrombin and antithrombin reduces the β -sheet structure of antithrombin from 6–8 to 1–2% (12); (b) Met³⁵⁸ residue of α 1-antitrypsin (residue P₁) is found 67 Å apart from Ser^{359} (residue P'₁) when the inhibitor is cleaved at P_1 - P'_1 , whereas the distance between C and N in an intact peptide bond is 1.3 Å (6, 13); (c) proteolytic cleavage of the exposed loops of α 1-antitrypsin, antithrombin, or C1-inhibitor strikingly increases the molecular stability of these proteins (7, 14, 15); and (d) C1-inhibitor that has reacted with plasma kallikrein expresses an epitope that is undetectable on the native serpin molecule (16). In the study reported here, we have examined further the consequences for the conformation of C1-inhibitor of proteolytic cleavage of its exposed loop. We have found that an identical site is created when C1-inhibitor reacts with its target enzymes kallikrein, C1s, and β -Factor XIIa or when it is catalytically inactivated by neutrophil elastase. These observations have important implications for the understanding of the mechanism responsible for the recognition of inactive serpin molecules by cellular receptors and for their catabolism.

Methods

Proteins. C1-inhibitor (17), kallikrein (18), β -Factor XIIa (19), C1s (20), neutrophil elastase (21), and recombinant α 1-antitrypsin Met³⁵⁸ (22) were prepared using published procedures. High molecular weight protein standards (myosin, β -galactosidase, phosphorylase b, BSA, and ovalbumin) were obtained from Bio-Rad Laboratories, Richmond, CA.

Electrophoretic studies. SDS-PAGE was performed using vertical slab gels and a Bio-Rad Protean II system (23). Before electrophoresis, the samples were incubated for 5 min in a boiling water bath. For reduced SDS-PAGE, the samples were boiled in the presence of 0.64 M β -mercaptoethanol.

Antibodies. MAbs 4C3 and 3C7 were obtained as described earlier (16). In brief, a BALB/c mouse was immunized with 50 μ g of the protein mixture that resulted from the inactivation for 60 min at 37°C of 1.5 μ M kallikrein by 1.7 μ M C1-inhibitor (1.7 μ M). The animal was boosted with 75 μ g of this mixture at 4 and 8 wk. 3 d after the last injection, mouse spleen cells were removed, fused with NS2 myeloma cells, and distributed in five 96-well plates. Wells exhibiting hybrid cell growth were examined for the production of MAbs. Two hybrids were cloned by limiting dilution; one produced an antibody that reacted with the kallikrein-C1-inhibitor (MAb 4C3), and the other detected an

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epitope that was located on native C1-inhibitor, and on C1-inhibitor complexed to and cleaved by plasma kallikrein (MAb 3C7). The clones producing 4C3 and 3C7 were grown as ascites tumors in pristaneprimed BALB/c mice. The epitopes recognized by 4C3 and 3C7 are of sequential nature, since their expression is not modified by incubating relevant C1-inhibitor samples for 5 min in a boiling water bath.

Antigen preparation. Native C1-inhibitor $(M_r, 105,000; Fig. 1, lane)$ B) was incubated with plasma kallikrein (M_r , 88,000 and 85,000; Fig. 1, lane C), C1s (M_r , 85,000, Fig. 1, lane D) or β -Factor XIIa (M_r , 28,000; Fig. 1, lane E). This led to the formation of 1:1 stoichiometric enzyme-inhibitor complexes involving C1-inhibitor and kallikrein (M_r, 195,000; Fig. 1, lane F), C1-inhibitor and C1s (M_r, 190,000; Fig. 1, lane G), or C1-inhibitor and β -Factor XIIa (M_r , 135,000; Fig. 1, lane H). Modified C1-inhibitor was also produced $(M_r, 95,000; Fig. 1, lanes$ F-H). Two protocols were employed for obtaining elastase fragments: 6.9 µM C1-inhibitor was incubated with neutrophil elastase with final concentrations of 0.026 µM (protocol A) or 1.26 µM (protocol B) at 37°C in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. After 30 min, the reactions were stopped by incubating the protein mixtures with 2 mM diisopropylfluorophosphate for 10 min at 37°C. Protocol A resulted in the formation of a cleavage fragment with an M_r of 88,000, but part of C1-inhibitor remained uncleaved; after protocol B, C1-inhibitor was completely converted into fragments with $M_{\rm r}$ of 77,000 or lower (not illustrated).

Immunochemical assays. For solid-phase RIAs, polyvinyl chloride microtiter plates (96 wells; Dynatech Laboratories, Inc., Alexandria VA) were coated with the various antigens under investigation (0.1 ml/well). The coating solution contained protein at 0.1 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4, supplemented with 1.5 M



Figure 1. SDS-PAGE analysis (7.5–12% gradient gel, nonreduced) of the inactivation of plasma kallikrein, C1s, and β -Factor XIIa by C1inhibitor. Lane A, molecular weight standards (myosin, 200,000; β galactosidase, 116,000; phosphorylase b, 97,400; BSA, 66,200; ovalbumin, 42,700; carbonic anhydrase, 31,000); lane B, C1-inhibitor; lane C, plasma kallikrein; lane D, C1s; lane E, β -Factor XIIa; lane F, protein mixture resulting from a 60-min incubation at 37°C of plasma kallikrein with a molar excess of C1-inhibitor; lane G, protein mixture resulting from a 60-min incubation at 37°C of C1s with a molar excess of C1-inhibitor; lane H, protein mixture resulting from a 60-min incubation at 37°C of β -Factor XIIa with a molar excess of C1-inhibitor. Each lane contained 30 μ g of protein, which was stained using Coomassie Blue. Right margin numbers are $M_r \times 10^{-3}$.

NaCl. After the plates were washed three times with the same buffer, they were incubated for 30 min with BSA (Fraction V, 1% in buffer, 0.2 ml/well) (Sigma Chemical Co., St. Louis, MO), washed again, and incubated with various concentration of ascites fluid. After washing the plates thoroughly, they were incubated for 3 h with ¹²⁵I-labeled F(ab')2 fragment of sheep anti-mouse Ig (New England Nuclear, Boston, MA) and washed again. Thereafter, the wells were cut and counted for radiactivity. All incubations were performed at room temperature. C1-inhibitor concentration in experiments examining protein stability (see below) was determined by rocket immunoelectrophoresis. Glass plates $(7.2 \times 10 \text{ cm})$ were coated with 12 ml of 1% agarose (agarose A; Pharmacia Fine Chemicals, Inc., Piscataway, NJ) in a buffer containing 25 mM Tricine and 81 mM Tris-base, pH 8.6, supplemented with monospecific rabbit antiserum against C1-inhibitor (1.7%, vol/vol). 5-µl samples were electrophoresed at 3 V/cm for 15 h. A linear doseresponse relationship (rocket height vs. log antigen concentration) was observed with C1-inhibitor concentrations ranging from 40 to 320 μ g/ml. For immunoblotting analyses (24), antigens were separated by SDS-PAGE (7.5% gel, unreduced) and transferred onto nitrocellulose sheets (Millipore Continental Water Systems, Bedford, MA) at 1 V/cm for 15 h using a Bio-Rad Trans-Blot cell. The transfer efficiency was established by staining both the gels and the nitrocellulose sheets, respectively, with Coomassie Brilliant Blue G-250 and naphthol blue black B. The blots were soaked for 2 h in 0.1 M Tris-HCl buffer, pH 7.4. containing 10 mM EDTA and 20% normal rabbit serum. Thereafter, the blots were incubated for 2 h with a 1:10,000 dilution of ascites fluids, washed three times, incubated for 3 h with ¹²⁵I-labeled F(ab')2 fragment of sheep anti-mouse Ig (New England Nuclear), and finally washed four times. All the above described incubation steps were performed at room temperature under rotative agitation. For autoradiography, the blots were exposed 15-48 h at -70°C to Typox-RP-L-Film NIF films (Typon, Burgdorf, Switzerland) using intensifying screens.

Protein stability. The fraction of C1-inhibitor remaining soluble after a 1-h incubation at 70°C was employed as the criterion for C1-inhibitor stability (7). The following protein mixtures were evaluated by this method: (a) native C1-inhibitor; (b) C1-inhibitor cleaved by a 1.2-fold molar excess of kallikrein; and (c) C1-inhibitor heated at 60°C for 1 h. The concentration of C1-inhibitor present in supernatants obtained after centrifuging these mixtures at 12,000 g for 5 min was determined by rocket immunoelectrophoresis. With each treatment, the concentration of C1-inhibitor was determined before and after the 1-h incubation at 70°C. To minimize the influence of solvent evaporation on antigen concentration, all incubations were performed in sealed test tubes, which were allowed to cool to room temperature before centrifugation.

Results

Reaction of C1-inhibitor with its target enzymes. Previous studies have shown that the enzymatic activity of kallikrein, C1s, or β -Factor XIIa is inhibited by C1-inhibitor (25, 26), a reaction that is accompanied by the formation of 1:1 stoichiometric enzyme-inhibitor complexes and of cleaved C1-inhibitor (16, 27-31). To probe the structure of the products of the reaction of C1-inhibitor with C1s or β -Factor XIIa, we have employed MAb 4C3, which identifies a neoepitope created on C1-inhibitor after the inactivation of plasma kallikrein (16). By solid-phase RIA, the binding site for 4C3 was expressed by C1-inhibitor that had reacted with C1s (Fig. 2, *circles*) or β -Factor XIIa (Fig. 2, diamonds). The binding of 4C3 to the protein mixture that resulted from the incubation of C1-inhibitor with kallikrein is also illustrated (Fig. 2, dotted line). The various molecular forms of C1-inhibitor that are detectable after C1s or β -Factor XIIa inactivation were then analyzed by



Figure 2. Binding of MAb 4C3 to the protein mixture that resulted from the incubation of C1-inhibitor with C1s (circles) or β -Factor XIIa (diamonds). The ordinate indicates specific binding of ¹²⁵I-labeled F(ab)2 fragment of sheep anti-mouse Ig to ascites fluid. Specific binding is equal to total binding minus nonspecific binding (binding to native C1 inhibitor). Results are expressed as means±SD (n = 3). Solid lines, least-square fit of the ex-

perimental points; and *dotted line*, binding of 4C3 to the protein mixture resulting from the inactivation of plasma kallikrein by C1-inhibitor. The binding of 4C3 to native C1-inhibitor (nonspecific binding, mean \pm SD) at a 1:10,000 dilution was 130 \pm 64 cpm.

immunoblotting. Control experiments with MAb 3C7 indicated that the transfer of the C1-inhibitor-C1s complex (M_r , 185,000), β -Factor XIIa-C1-inhibitor complex (M_r , 135,000), native C1-inhibitor (M_r , 105,000), and modified C1-inhibitor (M_r , 95,000) onto the nitrocellulose sheets was adequate (Fig. 3, lanes A and C). Further studies employing 4C3 demonstrated that the neoepitope detected by this antibody was located on the C1-inhibitor-C1s complex (M_r , 190,000; Fig. 3, lane B), on the C1-inhibitor- β -Factor XIIa complex (M_r ,



Figure 3. Immunoblotting of the protein mixture resulting from the incubation of C1-inhibitor with C1s (lanes A and B) or β -Factor XIIa (lanes C and D). The blots were incubated with MAbs 3C7 (lanes A and C) or 4C3 (lanes B and D). The binding of ¹²⁵I-labeled F(ab')2 fragment of sheep anti-mouse Ig was revealed by autoradiography. Left margin numbers ($M_r \times 10^{-3}$) indicate the migration of the C1s-C1-inhibitor complex (M_r , 190,000), native C1-inhibitor (M_r , 105,000), and modified C1-inhibitor (M_r , 95,000). Right margin numbers indicate the migration of the β -Factor XIIa-C1-inhibitor complex (M_r , 135,000), native C1-inhibitor (M_r , 105,000), and modified C1-inhibitor (M_r , 105,000).



Figure 4. SDS-PAGE analysis (7.5% gel, nonreduced) of the effect of temperature on C1-inhibitor structure and function. Lane A. plasma kallikrein; lane B. C1inhibitor; lane C, protein mixture resulting from a 5-min incubation at 37°C of plasma kallikrein with a molar excess of C1-inhibitor; lane D, protein mixture resulting from a 30-min incubation at 37°C of plasma kallikrein with a molar excess of C1-inhibitor that had been exposed for 2 h at 65°C. Each lane contained 15 µg of protein, which was stained using Coomassie Blue. Right margin numbers ($M_{\rm r} \times 10^{-3}$) indicate the migration of the kallikrein-C1inhibitor complex (M_r , 195,000), native C1-inhibitor (Mr, 105,000), modified C1-inhibitor $(M_r,$ 95,000), and unreacted plasma kallikrein (Mr, 88,000).

135,000; Fig. 3, lane D), and on the modified C1-inhibitor $(M_r, 95,000; \text{ lanes } B \text{ and } D)$, but was undetectable on the native inhibitor $(M_r, 105,000)$.

Thermic denaturation of C1-inhibitor. Heating native C1inhibitor at 60°C for 1 h, or cleaving and inactivating it with kallikrein increased the inhibitor solubility. By rocket immunoelectrophoresis, 90% of the initial C1-inhibitor concentration was recovered when heat-treated C1-inhibitor was employed as the starting material, whereas only 59% was recovered when native C1-inhibitor was substituted for the heat-treated protein. Recovery after exposure to kallikrein was 66%. SDS-PAGE analysis indicated that heat-treated C1-inhibitor, which did not form a bimolecular complex with kallikrein and was not cleaved by the enzyme, had an intact covalent structure (Fig. 4, lane D). Moreover, the epitope for MAb 4C3 was not created when heat-inactivated C1-inhibitor was exposed to catalytically active kallikrein (Table I). However, once formed, this epitope was not altered by a 1-h incubation at 70°C (Table I).

Studies with fragments produced by limited proteolysis of C1-inhibitor by neutrophil elastase. Neutrophil elastase cleaves and inactivates C1-inhibitor (32). When the products of the reaction of neutrophil elastase (1.8 pmol) with C1-inhibitor (140 pmol) were analyzed by SDS-PAGE, two large fragments were observed, with M_r values of 88,000 and 77,000, respectively (Fig. 5, lane C). These fragments were termed I'¹ (M_r , 88,000) and I'' (M_r , 77,000). To assess whether these fragments had retained inhibitory activity, the above described protein mixture was incubated with 190 pmol β -Factor XIIa. This treatment resulted in the disappearance of I' and in the formation of a new component having an M_r of 120,000, which is in reasonable agreement with the sum of the M_r values of I' and β -Factor XIIa (88,000 + 28,000 = 116,000) (Fig. 5, lane D). In

^{1.} Abbreviations used in this paper: I' and I'', C1-inhibitor fragments produced by cleavage of the native inhibitor with neutrophil elastase.

Table I. Binding of 4C3 and 3C7: Effect of Heat Treatment

	Treatment	4C3	3C7
C1-inhibitor	None	838±103	5,139±260
	70°C for 1 h	753±126	4,266±384
Mixture of active kallikrein and active C1-inhibitor	None 70°C for 1 h	3,442±266 3,213±333	4,575±623 4,047±598
Mixture of active kallikrein and inactive heat-treated (60°C for 1 h) C1-inhibitor	None	1,019±122	4,181±140

Specific binding of ¹²³I-labeled F(ab)₂ fragment of sheep anti-mouse Ig to MAbs 4C3 and 3C7. Results are in counts per minute, mean±SD of triplicate determinations.

contrast, the addition of β -Factor XIIa did not modify the electrophoretic characteristics of I" (Fig. 5, lane D). The binding of MAb 4C3 to I' and I" was then examined by solid-phase RIA. The site for 4C3 was absent in a protein mixture containing intact C1-inhibitor and I' (Fig. 6, *diamonds*), but it became detectable when I" was present in the system (Fig. 6, *circles*).

Discussion

Peptide epitopes include antigenic determinants formed by random coiled sequences of adjacent amino acid residues, as well as determinants created by the assembly of residues that can be located far apart in the primary sequence of the antigen (33, 34). The former are termed sequential or contiguous epitopes, whereas the latter are designated conformational or dis-



Figure 5. SDS-PAGE analysis (7.5% gel, nonreduced) of the cleavage of C1-inhibitor by neutrophil elastase. Lane A, C1-inhibitor (15 µg i.e., 0.14 nmol in 15 μ l); lane *B*, protein mixture formed by the incubation of C1-inhibitor (15 μ g i.e., 0.14 in 15 μ l) with β -Factor XIIa (5.2 μ g i.e., 0.19 nmol in 37 μ l); lane C, protein mixture formed by a 30-min incubation of C1-inhibitor (15 μ g i.e., 0.14 nmol in 15 μ l) with human neutrophil elastase $(0.014 \ \mu g \text{ i.e.}, 0.5 \text{ pmol in 5})$ μ l); lane D, C1-inhibitor (15 μ g i.e., 0.14 nmol in 15 μ l) was incubated with human neutrophil elastase (0.014 μ g i.e., 0.5 pmol in 5 μ l); after 30 min, elastase was inhibited by the addition of α 1-antitrypsin (0.6

 μ g i.e., 11.3 pmol in 3 μ l); 10 min later, 5.2 μ g (0.19 nmol in 37 μ l) of β -Factor XIIa was added, and the resulting protein mixture was further incubated for 30 min. All incubations were performed at 37°C. Coomassie Blue was employed for protein staining. Right margin numbers are $M_r \times 10^{-3}$.



Figure 6. Binding of MAb 4C3 to C1-inhibitor fragments obtained by cleavage with neutrophil elastase. Diamonds, C1-inhibitor was cleaved using protocol A (see Methods), and the resulting protein mixture contained uncleaved C1-inhibitor (M_r , 105,000) and fragment I' (M_r , 88,000); circles, C1-inhibitor was cleaved using protocol B (see Methods), which yielded an antigen solution containing fragment I" (M_r , 77,000) as well as other

peptides with M_r values < 77,000. The ordinate indicates specific binding of ¹²⁵I-labeled F(ab')2 fragment of sheep anti-mouse Ig to ascites fluid. Specific binding is equal to total binding minus nonspecific binding (binding to native C1-inhibitor). Results are expressed as means±SD (n = 3). Solid lines, least-square fit of the experimental points; dotted line, binding of 4C3 to the protein mixture resulting from the inactivation of C1s by C1-inhibitor. The binding of 4C3 to native C1-inhibitor (nonspecific binding, mean±SD) at a 1:10,000 dilution was 514±73 cpm.

contiguous epitopes (34). The site for antibody 4C3 is of sequential nature because it withstands boiling. However, this site is expressed in a much weaker fashion by virgin C1-inhibitor than by C1-inhibitor that has reacted with plasma kallikrein (16). Thus, its emergence demonstrates that the conformation of the native serpin molecule has been modified. In the present study, we have examined the circumstances involved in the genesis of this modification. Our principal conclusion is that the expression of the epitope for 4C3 is probably a general consequence of proteolytic cleavage of the exposed loop of C1-inhibitor at or next to the reactive site peptide bond. This site is created when C1-inhibitor inactivates its target enzymes (Fig. 2), a reaction that is accompanied by cleavage of the serpin molecule at its reactive site, P1 residue Arg⁴⁴⁴ (35, 36). It is also created by the second cleavage of C1-inhibitor with neutrophil elastase (Fig. 6). Involvement of C1-inhibitor reactive center during this cleavage is established by the observation that the resulting species, fragment I", is an inactive molecule that cannot form a covalent complex with β -Factor XIIa (Fig. 5). The elastase of *Pseudomonas aerugi*nosa cleaves C1-inhibitor at the P3 residue Val⁴⁴² (35), and therefore, this residue is probably also the target for neutrophil elastase. Another mechanism could create the site for 4C3. The stability of α 1-antitrypsin or antithrombin is increased by proteolysis of the exposed loop of these serpins (7, 14, 15); a parallel observation has been made here with C1-inhibitor. Hence, the site for 4C3 may become accessible when the native stressed conformation of C1-inhibitor is converted into a more stable and relaxed state. However, the experiment with heatdenatured C1-inhibitor showed that increasing the molecular stability without proteolytic cleavage was not sufficient to expose the site for 4C3 (Table I).

Serpin-proteolytic enzyme complexes involving antithrombin, α 1-antitrypsin, and α 2-antiplasmin are rapidly cleared from the blood stream, while this is not the case for the respective native inhibitors (8–10). For example, the fractional metabolic rate of antithrombin-thrombin is 11 times higher

than the value determined for native antithrombin (8). Similar observations have been made comparing the clearance of α^2 antiplasmin-plasmin or α 2-antiplasmin-trypsin with the clearance of native antiplasmin (10). In addition, cold antithrombin-thrombin and α 1-antitrypsin-trypsin compete for the clearance of radioiodinated antithrombin-thrombin (9). Similarly, the complex between α 2-antiplasmin and plasmin (but not antitrypsin-trypsin) competes for the clearance of $^{125}I-\alpha 2$ antiplasmin-plasmin (10). Rapid metabolic clearance is also observed with complexes involving C1-inhibitor. Increased enzyme-dependent catabolism of this inhibitor is found in patients with hereditary angioedema (37, 38) or rheumatoid arthritis (39), who have activation of the proteolytic pathways dependent on C1-inhibitor. These results have led to the suggestion that receptor-mediated pathways are implicated in the clearance of serpin-serine protease complexes (8-10). A corollary for this proposition is that bimolecular enzyme-inhibitor complexes should possess a specific recognition site that allows for their cellular binding and uptake. Moreover, for the simplicity and the efficiency of such a clearance system, an identical site should be expressed not only by enzyme-inhibitor complexes, but also by serpin molecules that have been cleaved and inactivated by other mechanisms. The neoepitope identified by 4C3 has characteristics expected from such a site since it is undetectable on native C1-inhibitor, but is expressed on complexes formed between C1-inhibitor and its target enzymes, plasma kallikrein, C1s, or β -Factor XIIa, and cleaved and inactivated by neutrophil elastase on C1-inhibitor.

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