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Research Article

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Unique C1 Inhibitor Dysfunction in a Kindred without Angioedema

II. Identification of an Ala^{443}→Val Substitution and Functional Analysis of the Recombinant Mutant Protein

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Abstract

We have determined the cause of an unusual C1 inhibitor abnormality in a large kindred. We previously found that half of serum C1 inhibitor molecules in affected kindred members are normal. The other half complexed with C1s but showed little complex formation with C1r. These molecules also appeared to be relatively resistant to digestion by trypsin. Taken together, the findings suggested that members of this kindred are heterozygous for an unusual C1 inhibitor mutation. Sequencing of genomic DNA from the kindred revealed that thymine has replaced cytosine in the codon for Ala^{443} (P2 residue) in one C1 inhibitor allele, resulting in substitution with a Val residue. To test the effect of this substitution, a mutant C1 inhibitor containing Ala^{443}→Val was constructed by site-directed mutagenesis and expressed in COS-1 cells. Both the Ala^{443}→Val mutant and the wild-type C1 inhibitor complexed completely with C1s, kallikrein, and coagulation Factor XIIa after incubation at 37°C for 60 min. In contrast, the mutant inhibitor failed to complex completely with C1r under the same conditions. Time course analysis showed that the ability of the mutant to complex with C1s is also impaired: although it complexed completely in 60 min, the rate of complex formation during a 0–60-min incubation was decreased compared with wild-type C1 inhibitor. The mutant inhibitor also formed a complex with trypsin, a serine protease that cleaves, and is not inhibited by, wild-type C1 inhibitor. The Ala^{443}→Val mutation therefore converts C1 inhibitor from a substrate to an inhibitor of trypsin. These studies emphasize the role of the P2 residue in the determination of target protease specificity. (*J. Clin. Invest.* 1995. 95:1299–1305.)

Key words: complement • complement activation • complement-activating enzymes • complement inactivators • serine protease inhibitors

Introduction

We have described a large kindred with hereditary reduced levels of the fourth component of complement (C4) (1). C4 metabolic turnover studies failed to demonstrate hypercatabolism of C4 and appeared to be compatible with C4 hyposynthesis, even though C4 structural alleles are intact in affected kindred members (2). Recently, we found that after a 15-min incubation, ~ 50% of serum C1 inhibitor in kindred members with decreased C4 levels did not complex with and inhibit C1r (3). Despite this failure, C1 inhibitor function, measured as inhibition of C1s and as the ability to form an SDS-stable complex with C1s, was normal in affected kindred members' sera (1, 3). In addition, approximately half of the C1 inhibitor molecules in affected members' sera appeared to be relatively resistant to cleavage by trypsin between the reactive center P1 Arg^{444} and P1′ Thr^{445} residues. Resistance to trypsin cleavage has been associated with amino acid substitutions at P1, decreased inhibition of C1s, and development of type II hereditary angioedema (HAE).[1] In this kindred, however, no member has ever had angioedema (3).

Diminished C1r binding, preservation of C1s binding, and relative resistance to trypsin suggested an unusual C1 inhibitor mutation. In this report, we demonstrate that affected kindred members are heterozygous for a P2 Ala^{443}→Val substitution, as demonstrated by nucleotide sequence analysis of the PCR-amplified gene. By site-directed mutagenesis of C1 inhibitor cDNA and expression in COS-1 cells, we show that the observed substitution decreased complex formation with both C1r and C1s and that the mutant has acquired the ability to complex with trypsin.

Methods

Kindred with incomplete C4 deficiency and dysfunctional C1 inhibitor. The kindred has been described previously (1–4). Incomplete C4 deficiency and dysfunctional C1 inhibitor are uniformly associated in 11 members of a five-family kindred spanning three generations. The pattern of inheritance is autosomal dominant, and there is no HLA linkage. The proband had systemic lupus erythematosus, but no member has had angioedema. Serum C4 levels in affected members are < 10 mg/dl (< 33% of pooled normal human serum) and have not fluctuated. Serum C2 levels measured by hemolytic titration have always been normal (3).

Preparation of genomic DNA. High molecular weight genomic DNA was isolated as previously described (5) from anticoagulated whole blood drawn from kindred member II-12 (2).

Amplification of the exons of C1 inhibitor by PCR. Exon VIII was amplified and sequenced first, since it encodes amino acids of the reactive center region of C1 inhibitor (6). Primers of 38 nucleotides with a 5′ BamHI site were synthesized on a 380B synthesizer (Applied Biosystems, Foster City, CA). 30 bases of each primer were complementary to nontranslated or intron sequences flanking exon VIII. The

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1. *Abbreviation used in this paper:* HAE, hereditary angioedema.

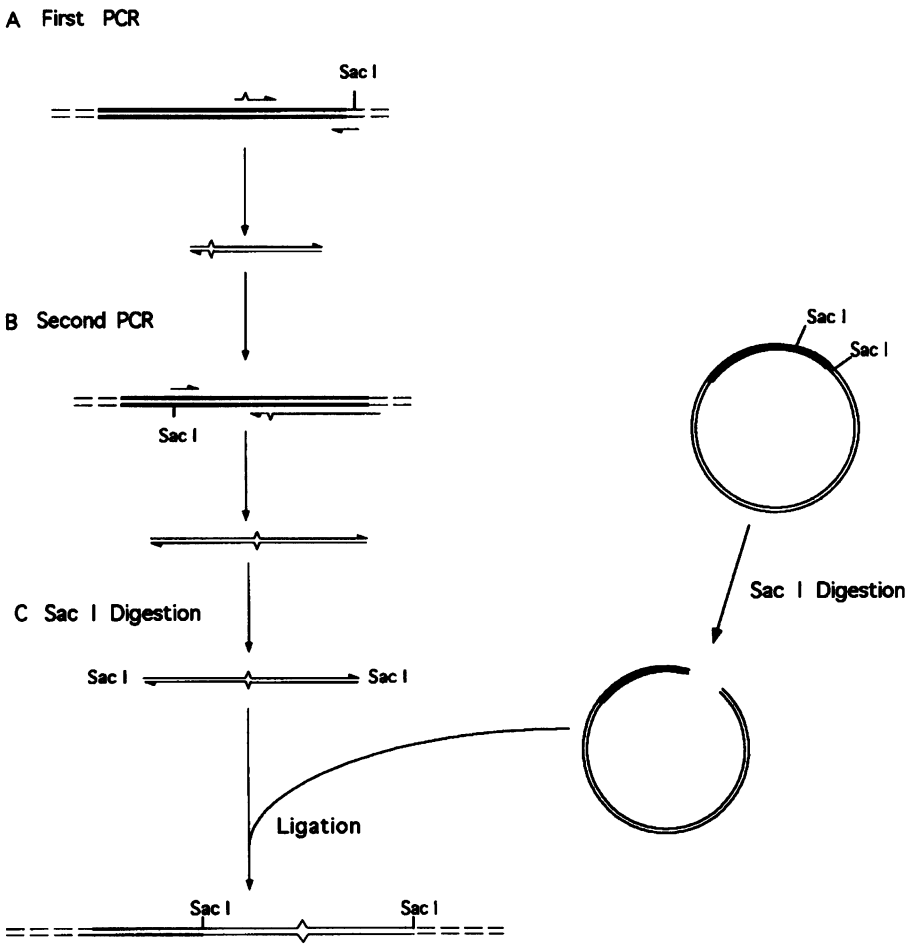

Figure 1. PCR-based site-directed mutagenesis. For simplicity, the template for the PCR, which is the full-length C1 inhibitor cDNA in pSVL, is shown in a linear format with only the immediate 5′ and 3′ segments of the vector included. (*A*) PCR using the mutagenic primer and a primer overlapping the SacI site within the vector. (*B*) PCR using the product of the first PCR and a second primer overlapping the SacI site within the C1 inhibitor cDNA. (*C*) digestion of the product of the second PCR with SacI followed by ligation of this fragment into the expression construct digested with SacI.

PCR amplification was done with 0.5 μg of genomic DNA, 25 pmol of each primers, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.0, 0.1% BSA, and 0.5 μl of *Thermus aquaticus* (Taq) polymerase (Perkin-Elmer Cetus instruments, Norwalk, CT) for 30 cycles (94°C for 1 min, 50°C for 2 min, and 72°C for 3 min). Amplified fragments were purified by isopropanol precipitation. The identical approach was used to amplify exons II–VII, except that the annealing temperature was modified as required to optimize annealing. Exon I was not amplified.

DNA sequencing. Amplified DNA fragments were sequenced by modifications of the dideoxynucleotide chain termination method (7). The 5′ oligonucleotide was 5′ end labeled with [^{32}P]ATP (New England Nuclear, Boston, MA) using T4 polynucleotide kinase (Boehringer Mannheim, GmbH, Mannheim, Germany). 1 pmol was added to a reaction mixture containing buffer, template, deoxynucleotide, and one dideoxynucleotide. The reaction was thermal cycled in the same fashion as the initial PCR. The mixtures were denatured in formamide and resolved on an 8 M urea/5% acrylamide (5% bis) gel in 0.09 M Tris-borate, 0.001 M EDTA buffer. The gel was dried and exposed to x-ray film (XAR-5, Eastman Kodak Co., Rochester, NY) overnight. Exons IV–VIII were also sequenced by automated laser fluorescence (Pharmacia/LKB Biotechnology, Piscataway, NJ), using a sequencing kit and protocol (Auto Read; Pharmacia/LKB Biotechnology).

Site-directed mutagenesis and expression in COS-1 cells. The full-length C1 inhibitor cDNA, subcloned into the pSVL expression vector (pC1-INH) (8) (kindly provided by Drs. Eric Eldering and Erik Hack, Amsterdam), was used as a template for PCR-based mutagenesis (Fig. 1). The first PCR was performed using the mutagenic primer (GCCATCTCTGTGGTCCGCACCCTGCTGGTC) that corresponds to nucleotides 16773–16802 with the C at position 16786 converted to a T (underlined). The other primer consisted of the complement of nucleotides 1523–1552 of the pSVL vector (GTATCTTATCATGTCTGGATCCGAGCTCC), which contains the only SacI site within the vector. The product of this reaction (180 bp) was used as a primer for the second PCR. The other primer for this second reaction consisted of nucleotides 8393–8422 of the C1 inhibitor gene (AGCAACAACAGTGACGCCAACTTGGAGCTC), which contains the only SacI site within the C1 inhibitor cDNA. The fragment generated from this PCR (810 bp) was purified by electrophoresis on low melting point agarose, digested with SacI, and exchanged for the equivalent fragment from pC1-INH. The presence of the mutation was confirmed by DNA sequence analysis of the resulting clone. Transfection of the wild-type and mutant expression clones into COS-1 cells was performed as previously described (9, 10). Transfected cells were cultured in the presence of [^{35}S]Met (Amersham, Arlington Heights, IL).

Thermal denaturation profiles. Native serine protease inhibitors (serpins), like C1 inhibitor, typically denature when incubated at 50–60°C. Prior cleavage of the polypeptide at or near the reactive center, however, leads to a structural rearrangement that stabilizes serpin molecules and prevents denaturation at ≥50°C. The thermal stability of recombinant C1 inhibitor was determined essentially as described by Stein et al. (11). COS-1 cell culture supernatants (100 μl) containing recombinant inhibitors were incubated with and without trypsin at 40, 50, 60, 70, and 80°C for 120 min and then were subjected to centrifugation at 14,000 *g* for 30 min. Quantitation of residual antigen in the supernatant was performed using an ELISA as described by Aulak et al. (12).

Complex formation between recombinant C1 inhibitor and target proteases. COS-1 cell culture supernates (40–100 μl) containing ~ 20 ng of recombinant normal C1 inhibitor or mutant C1 inhibitor (Ala^{443}→Val) were incubated at 37°C for 1 h with C1s (8.4 μg), C1r (6.8 μg) (each kindly provided by Dr. David Bing, Center for Blood Research,

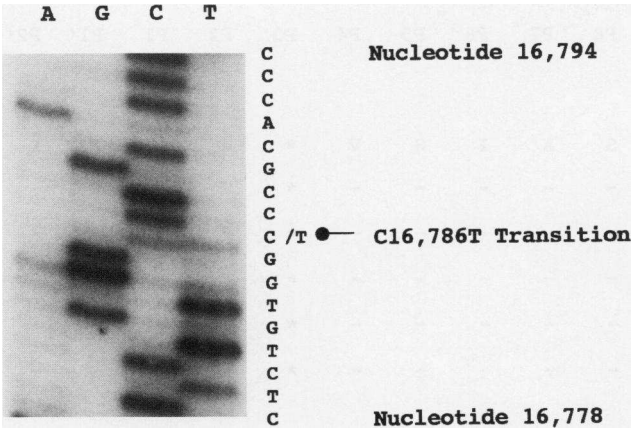

Figure 2. DNA sequence analysis of the C1 inhibitor gene amplified by PCR. Exon VIII was amplified inclusive of the intron/exon junctions. The reaction products were precipitated with isopropyl alcohol and sequenced by unidirectional PCR in the presence of dideoxynucleotides. Because both normal and mutant alleles are present in the genomic DNA template, both alleles are present in the PCR product, which results in identification of both a C and a T at nucleotide 16786. From left to right, the lanes on the autoradiograph of the sequencing gel are A, G, C, T.

Boston, MA), kallikrein (15 μg), activated Hageman factor (coagulation factor β XIIa; 3.5 μg) (Enzyme Research Laboratories, South Bend, IN), or trypsin (0.5–5.0 μg) (Sigma Chemical Co., St. Louis, MO). After incubation, PMSF was added to a final concentration of 1 mM, and Triton X-100 (0.5%), deoxycholic acid (0.25%), SDS (0.5%), and EDTA (5 mM) were added to each sample. The IgG fraction of rabbit anti-human C1 inhibitor (3 μl of 3.71 mg/ml; Calbiochem, La Jolla, CA) was then added and incubated at 4°C for 18 h. A suspension (6 μl) of fixed *Staphylococcus aureus* (IgSorb, The Enzyme Center, Boston, MA) was washed, added to each sample, and incubated at 4°C for 1 h. The samples were subjected to centrifugation, and the precipitates were suspended in 15 μl of nonreducing SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS) and subjected to electrophoresis on a 7.5% polyacrylamide gel. Gels were fixed, incubated in ENHANCE (New England Nuclear), dried, and exposed to XAR-5 film at −70°C.

Results

DNA sequencing. Manual (^{32}P) and automated sequencing revealed the presence of both a cytosine and a thymine at nucleotide 16786 (numbered according to reference 13) (Fig. 2). This indicated heterozygosity at the second base in the codon for Ala^{443} (GCC), converting it to a codon for Val (GTC). No other mutations were detected.[2] Ala^{443} is at the P2 position; comparison of the sequence of this region of the mutant with normal C1 inhibitor and with several other dysfunctional C1 inhibitor proteins is shown in Fig. 3.

Thermal denaturation profiles of wild-type and mutant recombinant C1 inhibitors. Thermal denaturation curves of the recombinant wild-type and the Ala^{443}→Val mutant inhibitors were virtually identical (Fig. 4). Both recombinant proteins were stable at ≥50°C after reactive center cleavage by trypsin, indicating that each had undergone the conformational rearrangement thought to be required for inhibitory activity.

Interaction of recombinant normal C1 inhibitor and mutant C1 inhibitor (Ala^{443}→Val) with target proteases. The electrophoretic mobilities of the recombinant wild-type and Ala^{443}→Val C1 inhibitors on SDS-PAGE were identical. The wild-type and mutant inhibitors each reacted completely with C1s after a 60-min incubation at 37°C (Fig. 5 *A*). Both inhibitors were present as the complex with C1s (~190 kD) or as the cleaved product (~97 kD) that results from complex destabilization. The relative proportions of complexed and cleaved inhibitors were the same for both recombinant proteins. Incubation with C1r, however, revealed a striking difference: all of the wild-type inhibitor reacted with C1r, but only ~50% of the Ala^{443}→Val mutant had formed a complex with C1r after a 60-min incubation (Fig. 5 *A*). This suggests that the P2 mutant reacts less efficiently with C1r than does the normal protein. Fig. 5 *B* shows that the reaction of the Ala^{443}→Val mutant with kallikrein and β XIIa was complete after 60 min, like its reaction with C1s. The reactions between the mutant inhibitor, kallikrein, and β XIIa were identical to those between the wild-type inhibitor and each protease.

Fig. 6 shows mixtures of the recombinant inhibitors and target proteases incubated over time (0–60 min). Fig. 6 *A* confirms that complex formation between C1r and the Ala^{443}→Val mutant is impaired, and Fig. 6 *B* shows that complex formation between C1s and the mutant is also impaired. The decreased rate was more apparent with C1r: complex formation did not reach completion over the 60-min incubation time, whereas the wild-type inhibitor was completely complexed within 10 min. With C1s, all of the recombinant P2 mutant had reacted between 5 and 10 min (Fig. 6 *B*); the wild-type inhibitor was completely complexed in < 0.5 min. It is therefore possible that the degree of decrease in reactivity with C1s is as great as with C1r. However, because the wild-type protein reacts more slowly with C1r than with C1s, the change in reactivity of the P2 mutant is much more obvious with C1r.

Interaction of the recombinant Ala^{443}→Val mutant with trypsin. Previous data suggested that the P2 mutant might be more resistant than the normal protein to digestion with trypsin (3). Therefore, the recombinant wild-type and mutant proteins (~20 ng) were incubated with trypsin (0.5–5.0 μg). At all concentrations of trypsin tested, the wild-type recombinant C1 inhibitor was efficiently cleaved to a lower molecular weight form (Fig. 7). The size of this fragment is consistent with cleavage at a site near the amino terminus and at another site immediately carboxy-terminal to the P1 Arg residue (3, 14, 20, 21). At the lowest concentration of trypsin (0.5 μg), the P2 mutant protein revealed an intermediate product, probably corresponding to a product with cleavage only at one of these two sites. More importantly, in this lane, a band clearly has appeared at a higher molecular mass (~120 kD) (Fig. 7), which is consistent with a complex formed by single molecules of trypsin and C1 inhibitor. The Ala^{443}→Val mutant therefore appears to have acquired the ability to complex with trypsin.

Discussion

Serological findings in this kindred suggest type II HAE. In type II HAE, one allele for C1 inhibitor encodes a normal

2. A completely reliable sequence for exon II could not be obtained for technical reasons with the primers and methods used for sequencing. A mutation could be present in this exon. However, since this exon encodes the signal peptide, it is unlikely that a dysfunctional inhibitor would result.

Reactive Center Residue	P15	P14	P13	P12	P11	P10	P9	P8	P7	P6	P5	P4	P3	P2	P1	P1′	P2′
C1 Inhibitor																	
Normal	G	V	E	A	A	A	A	S	A	I	S	V	*	A	R	T	L
Ala^{443} → Val	-	-	-	-	-	-	-	-	-	-	-	-	*	V	-	-	-
Arg^{444} → His (14)	-	-	-	-	-	-	-	-	-	-	-	-	*	-	H	-	-
Ala^{436} → Thr (9)	-	-	-	-	-	T	-	-	-	-	-	-	*	-	-	-	-
Ala^{434} → Glu (15)	-	-	-	E	-	-	-	-	-	-	-	-	*	-	-	-	-
Val^{432} → Glu (9)	-	E	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-
α_1-Antitrypsin	G	T	E	A	A	G	A	M	F	L	A	E	I	P	M	S	I

Figure 3. Comparison of normal C1 inhibitor, the Ala^{443}→Val mutant, and several other mutations in the reactive center loop of C1 inhibitor (14–19). The reactive center loop of α_1-antitrypsin is also shown. The asterisk at P3 indicates a deletion within the reactive center of normal C1 inhibitor compared with the sequence of α_1-antitrypsin and most other serpins. Several other substitutions at P1 have been reported (15–19) in addition to the His residue shown (14).

protein but the other encodes a dysfunctional protein. Diminished function of C1 inhibitor permits unregulated C1s-mediated activation of C4 and C2, as evidenced by decreased levels of these proteins in HAE patients' sera during and frequently between episodes of mucocutaneous edema. Diminished inhibition of C1s, kallikrein, and factor XIIa probably is all important in the pathogenesis of HAE, although the precise mechanism of edema formation is uncertain (22). Unlike kindred with HAE, however, C1 inhibitor dysfunction in this kindred does not lead to clinical symptoms of angioedema.

Previous work suggested that this kindred has an unusual mutation in C1 inhibitor that resulted in apparent selective failure to inhibit C1r (3). In the present study, PCR amplification and sequencing of genomic DNA from one affected kindred member revealed a single mutation: substitution of Val for Ala as the P2 residue in one of the two C1 inhibitor alleles (Fig. 2). Based on previous data and functional analysis of the P2 mutant, it is reasonable to conclude that all affected kindred members have the Ala^{443}→Val substitution. Studies of recombinant C1 inhibitor confirmed that this substitution decreases the mutant's ability to complex with C1r (Fig. 5 *A*). The recombinant mutant complexed normally with C1s, kallikrein, and β XIIa after a 60-min incubation (Fig. 5, *A* and *B*). Time course analysis revealed that its ability to complex with C1s is also diminished (Fig. 6). Previously, C1 inhibitor purified from affected kindred members, a 50/50 mixture of normal and mutant inhibitor, complexed completely with C1s in 10 min but not with C1r after 60 min, and C1 inhibitor activity, measured in

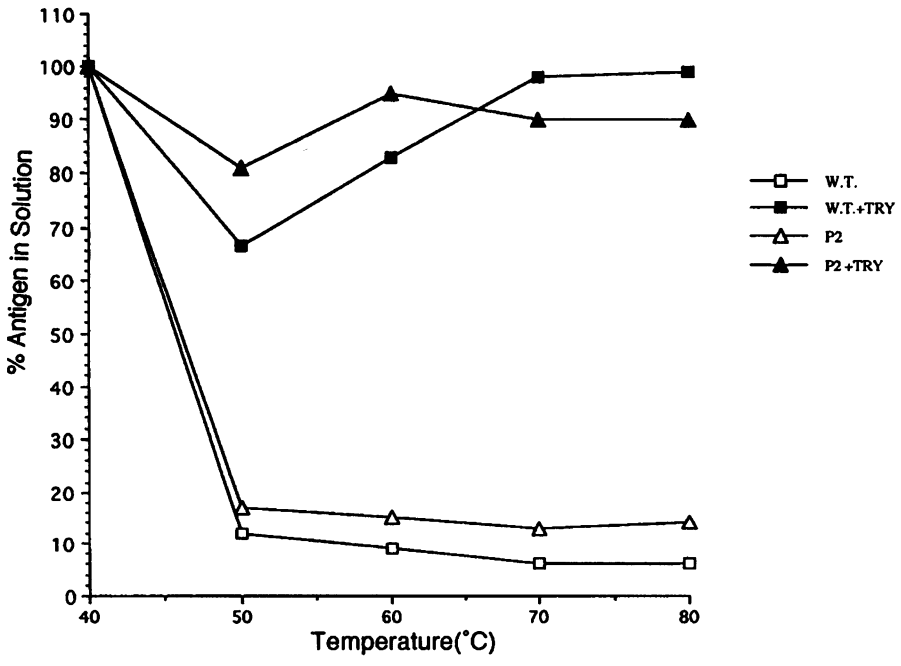

Figure 4. Thermal denaturation of wild type (*W.T.*) and P2 Ala→Val C1 inhibitor. Medium from transfected COS-1 cells was incubated alone or in the presence of trypsin, as described in Methods. Samples then were incubated at the indicated temperatures, and following centrifugation, residual protein was quantitated by ELISA.

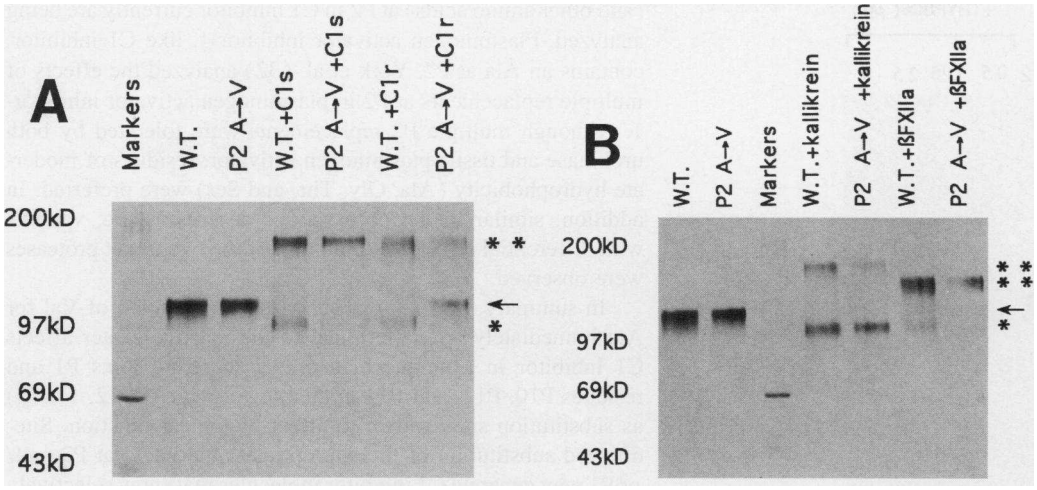

Figure 5. SDS–PAGE of recombinant wild-type (*W.T.*) and Ala^{443}→Val (*P2 A→V*) mutant C1 inhibitors after incubation with proteases. Each COS-1 cell supernatant (100 μl) containing [^{35}S]Met-labeled recombinant C1 inhibitor was incubated at 37°C for 1 h either alone or with the following proteases: (*A*) C1s (6.8 μg) and C1r (8.4 μg); (*B*) kallikrein (15 μg) and β XIIa (3.5 μg). Samples were immunoprecipitated with polyclonal antiserum to human C1 inhibitor and subjected to SDS-PAGE; autoradiography was performed as described in Methods. The position of the native unreacted C1 inhibitor is indicated by the arrow, that of the reactive center cleaved inhibitor is indicated by the asterisk, and the complex is indicated by the double asterisk.

affected members' sera as inhibition of C1s-mediated cleavage of a synthetic substrate, was increased compared with normal controls (3). Measurement of activity required incubation of the test serum and C1s for 20 min before substrate was added. This 20-min incubation time could account for *normal* serum C1 inhibitor activity, since the recombinant mutant reacted completely with C1s in ≤5 min (Fig. 6 *B*). However, it does not explain *increased* inhibition of C1s by C1 inhibitor in affected kindred members' sera (3). Although it seems unlikely, it may be possible that formation of the enzyme–inhibitor complex is enhanced in whole serum, even though the rate at which the recombinant mutant and C1s in isolation form SDS-stable complexes is somewhat diminished. Clarification of this question must await detailed kinetic studies.

Other C1 inhibitor mutants have demonstrated variable loss of activity against target proteases, but all of these mutants were associated with development of HAE, and none showed as selective a loss of activity as did the mutant in this kindred (23, 24). The best evidence that this mutation affects only target protease specificity and not the inhibitory mechanism is that, although it has lost activity against C1r, it has acquired inhibitory activity against trypsin (Fig. 7). The acquisition of a completely new inhibitory activity by alteration of any residue other than P1 is extremely unusual, if not unprecedented, and emphasizes the role of non–P1-reactive center loop residues in determination of inhibitory specificity. The best known example of the gain of inhibitory function against a nontarget protease is α_1-antitrypsin Pittsburgh, in which the P1 Met is replaced with an Arg (25). This converts the mutant to an efficient inhibitor of thrombin, plasma kallikrein, and factor XIIa (26, 27). The further substitution of the P2 Pro in α_1-antitrypsin Pittsburgh with an Ala (matching the P2 of C1 inhibitor) enhances its inhibition of kallikrein and confers the ability to inhibit C1s (albeit relatively weakly) (28). The C1 inhibitor P2 Ala→Val

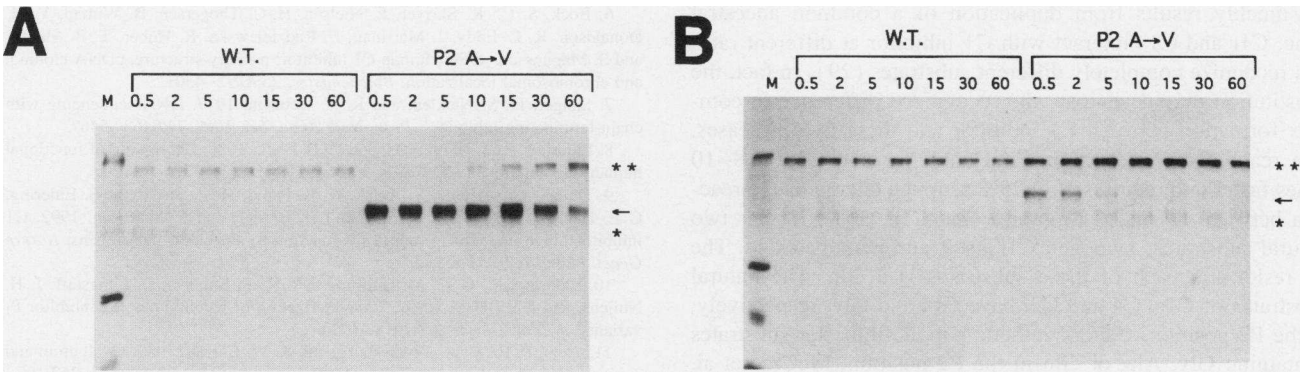

Figure 6. Kinetics of complex formation between proteases and the recombinant inhibitors. COS-1 cell supernatants containing wild-type C1 inhibitor (*W.T.*) (40 μl) or the Ala^{443}→Val mutant (*P2 A→V*) (100 μl) were incubated with C1r (*A*) or C1s (*B*) for 0.5, 2, 5, 10, 15, 30, and 60 min. Samples were immunoprecipitated and subjected to electrophoresis and autoradiography, as described in Methods. The position of the native unreacted C1 inhibitor is indicated by the arrow, that of the reactive center cleaved inhibitor is indicated by the asterisk, and the complex is indicated by the double asterisk. Molecular weight markers are as in Fig. 5.

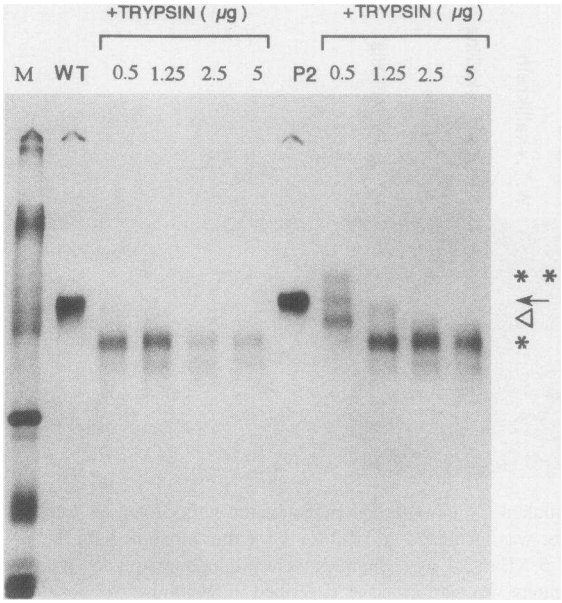

Figure 7. SDS–PAGE of recombinant C1 inhibitors with trypsin. COS-1 cell supernatants (100 μl) containing [^{35}S]Met-labeled recombinant wild-type (*W.T.*) or the Ala^{443}→Val mutant (*P2*) were incubated with trypsin (0.5, 1.25, 2.5, and 5 μg) at 37°C for 60 min. Samples were immunoprecipitated and subjected to electrophoresis and autoradiography, as described in Methods. The position of the native unreacted C1 inhibitor is indicated by the arrow, that of the putative bimolecular complex of trypsin and the P2 Ala→Val mutant is indicated by the double asterisk, that of the C1 inhibitor cleaved both at P1–P1′ and at the site near the amino terminus is indicated by the asterisk, and that of the intermediate product cleaved only at one site is indicated by the arrowhead. Molecular weight markers are as in Fig. 5.

mutant, however, is of further interest because wild-type C1 inhibitor is a trypsin substrate that is efficiently cleaved at P1–P1′ with catalytic quantities of trypsin (14). Further detailed analysis of the interaction of this and other C1 inhibitor P2 mutants with trypsin may help define the structural features that convert a given serpin from a substrate to an inhibitor of a specific protease.

Val for Ala is a relatively conservative substitution. Although Val is larger than Ala, nonpolarity and hydrophobicity at P2 are maintained. Despite their sequence homology, which presumably results from duplication of a common ancestral gene, C1r and C1s interact with C1 inhibitor at different rates and recognize completely different substrates (29). In fact, the substitution may accentuate the recognized difference in complex formation between C1 inhibitor and these two proteases. The reaction between normal C1 inhibitor and C1s is 8–10 times faster and requires only 25% as much energy as the reaction between normal C1 inhibitor and C1r (30). C1r has two natural substrates: zymogen C1r itself and zymogen C1s. The P2 residue in each of these substrates is a Gln. The natural substrates of C1s, C4 and C2, have Gln and Gly, respectively, at the P2 position. Using synthetic peptide thioester substrates containing Gly, Ala, or Gln in the P2 position, McCrae et al. (31) showed that C1r was less reactive and exhibited a higher substrate specificity than C1s. C1r was most reactive with substrates containing a Gly in the P2 position, whereas C1s preferred Ala or Gly. C1s was shown to hydrolyze a substrate with Val in the P2 position (31). The effects of substitution of Gly (and other amino acids) at P2 in C1 inhibitor currently are being analyzed. Plasminogen activator inhibitor-1, like C1 inhibitor, contains an Ala at P2. York et al. (32) analyzed the effects of multiple replacements at P2 in plasminogen activator inhibitor-1. Although multiple P2 replacements were tolerated by both urokinase and tissue plasminogen activator, residues of moderate hydrophobicity (Ala, Gly, Thr, and Ser) were preferred. In addition, similar to the observations described here, variants with differential effects on inhibition of the two target proteases were observed.

In summary, our findings show that substitution of Val for Ala immediately amino-terminal to the reactive center affects C1 inhibitor in a unique manner. P2 therefore joins P1 and residues P10, P12, and P14 in the hinge region (9, 12, 14, 15) as substitution sites known to affect biological function. Site-directed substitution of the appropriate amino acid at P2 and/or P1 may generate C1 inhibitor molecules that show selectively decreased or increased inhibition of target proteases, or that have acquired the ability to inhibit nontarget proteases.

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