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Dysfunctional C1 inhibitor Ta: Deletion of Lys-251 results in acquisition of an N-glycosylation site

(hereditary angioneurotic edema/mutations/serpins/complement component C1)

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ABSTRACT **Hereditary angioneurotic edema is inherited as an autosomal dominant disorder and is characterized by potentially life-threatening episodic angioedema. In type II hereditary angioneurotic edema, a dysfunctional C1 inhibitor molecule is present together with low levels of normal C1 inhibitor. About 70% of these dysfunctional proteins result from reactive center (Arg-444) mutations. We describe the deletion of nucleotides encoding Lys-251 (AAG) in C1 inhibitor Ta, the dysfunctional C1 inhibitor from a family with type II hereditary angioneurotic edema. DNA sequence analysis was derived from clones obtained through polymerase chain reaction amplification of blood monocyte C1 inhibitor mRNA. As expected, clones with both normal and abnormal sequence were isolated. The deletion was verified by protein sequence analysis. These data, together with biochemical analysis of the protein and cell-free translation studies, suggest that this deletion, by altering the normal amino acid sequence from Asn-Lys-Ile-Ser to Asn-Ile-Ser, creates a new glycosylation site. The additional carbohydrate accounts for the larger size on SDS/PAGE and very likely interferes with protein function.**

C1 inhibitor, a member of the serpin family of serine protease inhibitors, inactivates proteases through the formation of inhibitor–protease complexes (1). The reactive center of serpins mimics the substrate of the protease. Recognition of this reactive-center peptide bond results in complex formation between the amino acid residue amino-terminal to this bond (the P1 residue) and the active-site serine of the protease. Proteases inactivated by C1 inhibitor recognize the Arg-444 (P1) —Thr-445 (P'1) peptide bond. *In vitro*, C1 inhibitor inactivates the complement components C1r and C1s, kallikrein, plasmin, and coagulation factors XI and XII (2–6). C1 inhibitor is a single polypeptide chain composed of 478 amino acid residues. It is a highly glycosylated plasma protein, with 6 N-linked and 14 O-linked oligosaccharide units (7). The majority of these carbohydrate groups are within the amino-terminal 100 residues. The molecular weight of secreted C1 inhibitor is 105,000 with only 52,869 for the polypeptide core. The carbohydrate does not appear to be necessary for protease inhibitory activity (8–10). No glycosylation variants have been described.

Hereditary angioneurotic edema (HANE) is an autosomal dominant disorder characterized by episodic edema of skin and mucosal surfaces. Gastrointestinal involvement may result in severe abdominal pain, and respiratory-tract involvement may lead to airway obstruction. Decreased circulating levels of functional C1 inhibitor are found in affected individuals (11, 12). HANE may be divided into two groups: type I is characterized by a parallel decrease in C1 inhibitor activity and antigen, and type II by decreased C1 inhibitor activity and normal to increased antigenic levels (11–14). Patients with type II HANE thus express a normal C1 inhibitor protein together with a mutant dysfunctional molecule. Two-thirds to three-fourths of type II patients have single amino acid substitutions at the P1 reactive-center residue (15–17). Only one mutation that results from a single base substitution in the hinge region and not at the reactive center has been described (18).

Previous analysis revealed that C1 inhibitor Ta differed from normal C1 inhibitor in mobility on agarose gel electrophoresis (13) and in apparent size on SDS/PAGE (19). This slight increase in size (≈4 kDa) was localized to a cyanogen bromide peptide that is now known to extend from amino acid residue 146 to 288. Therefore, cDNA corresponding to this fragment was amplified from blood monocyte mRNA by the polymerase chain reaction (PCR). This revealed a 3-base-pair (AAG) deletion that results in the loss of Lys-251 and reapposes a sequence creating a new N-linked glycosylation site. The carbohydrate addition and/or the amino acid deletion results in altered conformation and function.

METHODS

Patient. The type II HANE patient studied is a member of the family Ta, first reported in ref. 13. The function of C1 inhibitor isolated from members of this family (C1 inhibitor Ta) has been studied previously (20, 21), and the size of the protein and its cyanogen bromide peptides has been analyzed (19).

C1 Inhibitor Purification and Analysis. C1 inhibitor was isolated from normal and Ta plasma both by the method of Harrison (22) and by lectin affinity chromatography (23). SDS/PAGE (7.5% polyacrylamide) was performed according to Laemmli (24). Cleavage of isolated inhibitor at tryptophan residues with *o*-iodosobenzoic acid was performed as described by Mahoney *et al.* (25). The resulting peptides were desalted on a Bio-Gel P-2 (Bio-Rad) column (10-ml bed volume) and resolved by HPLC on an Aquapore RP300 column equilibrated in 0.1% trifluoroacetic acid and developed with a linear acetronitrile gradient to 70%. Automated Edman degradations were performed with an Applied Biosystems 477A pulsed liquid-phase protein sequencer. Deglycosylation with peptide:N-glycosidase F (Genzyme) and endoglycosidase F (Boehringer Mannheim) was performed as described (26–28).

PCR Amplification of Monocyte mRNA and Genomic DNA. Peripheral blood monocytes were isolated (29), and RNA was prepared by lysis with guanidinium thiocyanate and centrif-

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Abbreviations: HANE, hereditary angioneurotic edema; PCR, polymerase chain reaction; TBG, thyroxine-binding globulin.
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ugation through cesium chloride density gradients (30). First-strand cDNA was synthesized from 1 μg of total RNA by use of reverse transcriptase (Boehringer Mannheim). This first-strand cDNA was then used as template for PCR using 30-nucleotide primers (with 5′ *Bam*HI linkers) that corresponded to nucleotides 492–521 and 1032–1061 of the C1 inhibitor cDNA (7). The oligonucleotide primers were synthesized with an Applied Biosystems 380B DNA synthesizer. *Thermus aquaticus* (*Taq*) DNA polymerase was from Perkin–Elmer/Cetus; the cycle of denaturation (94°C, 1 min) and annealing/extension (72°C, 5 min) was repeated 30 times. Amplified DNA was subcloned into plasmid pUC18 and DNA sequence analysis was carried out by modification (DNA sequencing kit, United States Biochemical) of the dideoxy chain-termination method (31). For allele-specific hybridization, genomic PCR was performed with 30-nucleotide primers that corresponded to nucleotides 720–749 and 894–923 (both of which are within exon 5). The resulting amplified DNA was hybridized (after electrophoresis and Southern blotting) with allele-specific probes (nucleotides 842–861 and 840–863 with 851–853 deleted) at 37°C overnight. Blots were washed at room temperature with 2× SSC and at 50°C with 1× SSC (SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7).

Fibroblast Protein Synthesis and Cell-Free Translation. Fibroblast cell lines from normal individuals and from patient Ta were established as described (32). Cells were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. For tunicamycin treatment, the medium was aspirated, the cells were washed with Hanks' balanced salt solution, tunicamycin (5.0 μg/ml) was added in DMEM containing 1% bovine serum albumin, and the cultures were incubated overnight. These conditions resulted in some inhibition of protein synthesis, which occurred also with smaller quantities of tunicamycin and with incubation periods as short as 4 hr. The cells then were incubated with [^{35}S]methionine (250 μCi/ml; 1 μCi = 37 kBq) for 4 hr in the presence of the same quantity of tunicamycin. Supernatants were collected, cell lysates were prepared, and immunoprecipitations were performed as described (33). Total fibroblast RNA was prepared (30) for cell-free translation, which was performed using rabbit reticulocyte lysate (Promega) according to instructions provided by the manufacturer. The quantity of RNA used was 2.5 μg, and the reaction was allowed to proceed for 70 min. C1 inhibitor was immunoprecipitated as above and samples were subjected to SDS/PAGE. Gels were fixed, impregnated with En^{3}Hance, dried, and exposed at −70°C to Kodak XAR-5 film.

RESULTS

Localization of the Mutation in C1 Inhibitor Ta. On SDS/PAGE, C1 inhibitor isolated from Ta plasma consists of two bands, one of which appears the same size as normal C1 inhibitor and the other ≈4 kDa larger (Fig. 1). The relative quantities of the two species vary in different preparations as a result of variations in their relative quantities in plasma at different times. C1 inhibitor Ta as prepared by the method of Harrison (22) consists of virtually only these two species. Both normal C1 inhibitor and C1 inhibitor Ta isolated by lectin affinity chromatography also contained a band with apparent molecular mass of ≈75 kDa. This contaminant, which very likely is a fragment of the inhibitor, is present in C1 inhibitor isolates from stored plasma or serum prepared by the method of Pilatte *et al.* (23).

Previous studies indicated that the abnormality in C1 inhibitor Ta was contained within a cyanogen bromide peptide that included amino acid residues 146–288 (19). Efforts to localize the abnormality therefore were focused on the mRNA sequence encoding this peptide. Based on the known

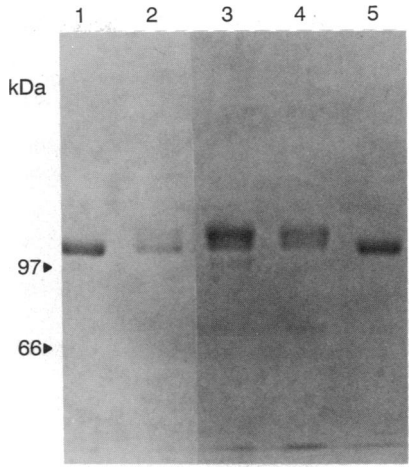

FIG. 1. SDS/PAGE of isolated normal C1 inhibitor and C1 inhibitor Ta. Lanes 1 and 5, normal C1 inhibitor (≈4 μg) isolated as in ref. 23; lane 2, C1 inhibitor Ta (2.5 μg) isolated by the same method; lanes 3 and 4, C1 inhibitor Ta (5 μg and 4 μg, respectively) isolated as in ref. 24.

mRNA sequence (7), oligonucleotide primers were selected to amplify the segment of mRNA corresponding to residues 131–320, thus encompassing all of this cyanogen bromide peptide. Peripheral blood monocyte mRNA was amplified using PCR. Clones from the resulting 570-base-pair amplified DNA corresponding to the normal and abnormal alleles then were isolated.

Sequence analysis revealed deletion of nucleotides 852–854 (AAG), which correspond to Lys-251 (Fig. 2). The sequences for the normal and mutant alleles were identical with the exception of this single codon. Two other oligonucleotide primer pairs were used to amplify the 5′ and 3′ ends of the C1 inhibitor mRNA, in order to generate DNA fragments that overlapped the above amplified fragment. The sequences of all resulting clones were identical with the normal sequence. In particular, all sequences analyzed coded for arginine and threonine at positions 444 and 445.

The deletion also was detected at the level of the C1 inhibitor gene (*C1NH*) by allele-specific hybridization of PCR-amplified genomic DNA (Fig. 3). The oligonucleotide corresponding to the normal allele hybridized with both normal and Ta DNA, although the hybridization with Ta DNA appeared less than with normal (Fig. 3, lanes 2 and 3). The deletion probe hybridized with Ta DNA and showed minimal background hybridization with normal DNA (lanes 5 and 6).

Confirmation of the Deletion of Lys-251 by Protein Sequence Analysis. Normal C1 inhibitor and C1 inhibitor Ta were cleaved at tryptophan residues with *o*-iodosobenzoic acid. The resulting HPLC-purified peptides were subjected to amino-terminal sequence analysis. The amino-terminal sequence of the peptide from the normal C1 inhibitor carboxyl-terminal to Trp-243 was exactly that predicted by the cDNA sequence (Table 1). The peptide isolated from C1 inhibitor Ta showed a single sequence identical with that of the normal from amino acid 244 to 250 (cycles 1–7), but two amino acids were identified at most subsequent cycles, beginning with residue 251 (cycle 8). At cycles 10 and 11, only one amino acid was detected; this very likely resulted from poor yields of the phenylthiohydantoin of arginine. At cycle 12, only leucine was detected, as would be predicted by the overlap of this residue in both peptides. One of the possible sequences in this mixture appeared identical with the normal, and the other skipped Lys-251 with succeeding residues previewing the normal sequence by one cycle.

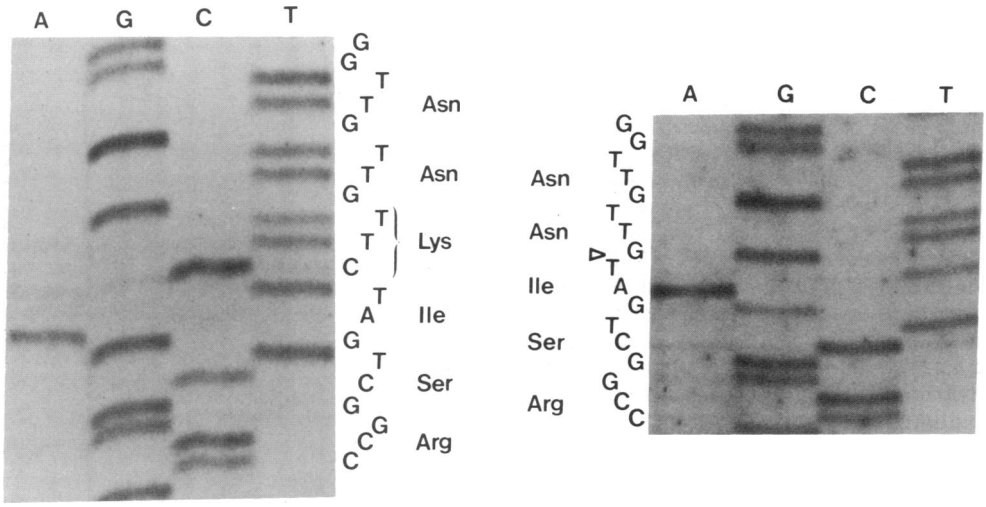

FIG. 2. Sequence analysis of the PCR-amplified Ta DNA from the normal and mutant alleles. The sequence of each is shown and corresponds in each case to the noncoding strand. The peptide sequence, therefore, is read from top to bottom. The deleted codon is indicated in the normal allele by the bracket and its position in the abnormal allele by the triangle.

Unique Glycosylation of C1 Inhibitor Ta. Deletion of Lys-251 alters the primary sequence from Asn-Lys-Ile-Thr to Asn-Ile-Thr, creating a potential new N-linked glycosylation site in C1 inhibitor Ta. C1 inhibitor Ta that was N-deglycosylated with endoglycosidase F or peptide:N-glycosidase F showed loss of the electrophoretic mobility differential on SDS/PAGE (Fig. 4). The two proteins shifted from apparent molecular masses of ≈105 and ≈109 kDa, respectively, to ≈80 kDa. When C1 inhibitor synthesized by normal and Ta fibroblasts in the absence or presence of tunicamycin (5 μg/ml) was analyzed, the intracellular product from Ta fibroblasts in the absence of tunicamycin consisted of two polypeptide bands, one slightly larger than the other, at ≈86 kDa (Fig. 5; the two bands are not well resolved and appear as one band that is broader than the band from normal fibroblasts). In the presence of tunicamycin, the intracellular normal C1 inhibitor and C1 inhibitor Ta appear to be single bands of ≈65 kDa. The secreted products in the presence of tunicamycin were also identical and had molecular masses of ≈80 kDa (data not shown). Cell-free translation confirmed this observation, showing the C1 inhibitor polypeptide from normal mRNA (Fig. 6, lane 3) and Ta mRNA (lane 4) to be of identical size on SDS/PAGE.

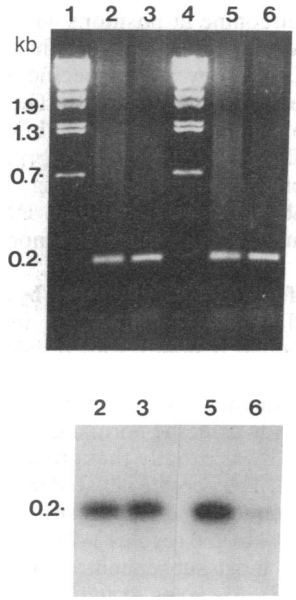

FIG. 3. Allele-specific hybridization of PCR-amplified genomic DNA. (*Upper*) Ethidium bromide-stained agarose gel of the 204-base-pair amplified DNA fragment. Lanes 1 and 4, size markers [given in kilobases (kb) at left]; lanes 2 and 5, Ta DNA; lanes 3 and 6, normal DNA. (*Lower*) Autoradiograph showing hybridization with the normal probe (lanes 2 and 3) and the deletion probe (lanes 5 and 6). The samples in each lane are as in *Upper*.

DISCUSSION

C1 inhibitor Ta is a mutant protein isolated from a patient with type II HANE. It does not contain a reactive-center substitution, which is the most common mutation type found in dysfunctional C1 inhibitor proteins (15–17). Rather, a 3-base-pair deletion (AAG), resulting in deletion of Lys-251, was revealed on analysis of the DNA sequence and was confirmed by the protein sequence. Consistent with the heterozygous nature of the defect, clones were also identified that did not have deletion of codon 251, and both sequences were detected on protein and DNA sequence analysis. Several other clones spanning the remainder of the coding sequence revealed no other abnormalities. Since full-length

Table 1. Amino acid sequence of the peptides isolated from normal and Ta C1 inhibitor after *o*-iodosobenzoic acid cleavage at Trp-243

Cycle	Normal (pmol)	Ta (pmol)
1	Val (292)	Val (87)
2	Ala (211)	Ala (68)
3	Lys (149)	Lys (46)
4	Asn (77)	Asn (32)
5	Thr (175)	Thr (52)
6	Asn (86)	Asn (33)
7	Asn (122)	Asn (18)
8	Lys (166)	Lys (17), Ile (34)
9	Ile (162)	Ile (35), Ser (21)
10	Ser (136)	Ser (10)
11	Arg (21)	Leu (50)
12	Leu (204)	Leu (77)
13	Leu (242)	Leu (59), Asp (5)
14	Asp (34)	Asp (4), Ser (15)
15	Ser (79)	Ser (18), Leu (46)
16	Leu (47)	Leu (47), Pro (12)
17	Pro (71)	Pro (17), Ser (16)

The yield(s) at each cycle, in picomoles, is shown in parentheses.

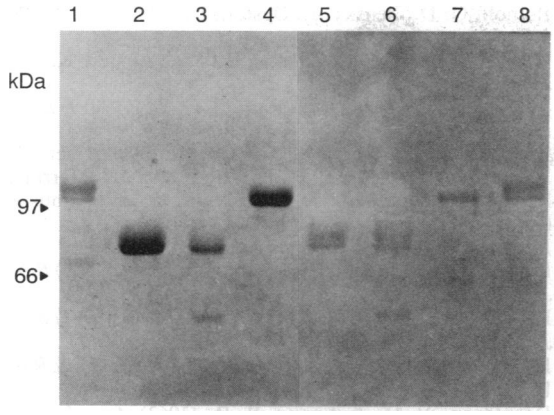

FIG. 4. Deglycosylation of normal and mutant C1 inhibitors with endoglycosidase F or peptide:N-glycosidase F. Lanes 4 and 7, normal C1 inhibitor; lane 2, normal inhibitor treated with endoglycosidase F; lane 5, normal inhibitor treated with peptide:N-glycosidase F; lanes 1 and 8, C1 inhibitor Ta; lane 3, C1 inhibitor Ta treated with endoglycosidase F; lane 6, C1 inhibitor Ta treated with peptide:N-glycosidase F.

clones were not generated, it cannot be stated unequivocally that both alleles have been sequenced completely. However, it is highly likely that the mutation defined here is the functionally relevant defect in C1 inhibitor Ta. This amino acid deletion results in alteration of the sequence Asn-Lys-Ile-Thr to Asn-Ile-Thr, thus creating a potential new N-linked glycosylation site. The data indicate that this new site is in fact glycosylated. C1 inhibitor Ta appears to be ≈4 kDa larger than normal C1 inhibitor on SDS/PAGE (ref. 19 and Fig. 1). Deglycosylation with endoglycosidase F or with peptide:N-glycosidase F reduced the normal and Ta mutant inhibitors to the same size (Fig. 4). In addition, cell-free translation using mRNA from Ta fibroblasts revealed a single gene product, identical in size to that produced by normal mRNA (Fig. 6). Finally, C1 inhibitor synthesized by Ta fibroblasts gave two bands, at 105 and 109 kDa, but a product the same size as normal in the presence of tunicamycin (Fig. 5). Although unexpected, it appears that the intracellular product (both normal and Ta) in the absence of tunicamycin is not completely O-glycosylated (86 kDa as compared with 105 kDa for the secreted protein). In the presence of tunicamycin, the intracellular C1 inhibitor is similar in size to the cell-free translation product (60–65 kDa), while the secreted protein is larger (80 kDa). This difference must therefore represent O-glycosylation. In any case, tunicamycin abolishes the size difference between the normal and mutant inhibitors. Protein sequence analysis of the relevant *o*-iodosobenzoic acid-derived peptide from the C1 inhibitor Ta preparation shows that the yield of asparagine from cycle 6 to cycle 7 decreased by approximately half. This also is consistent with glycosylation of this second asparagine residue in the mutant peptide.

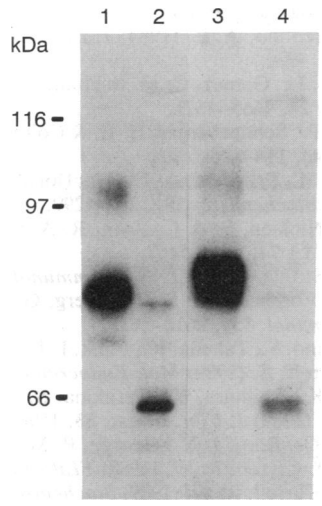

FIG. 5. The effect of tunicamycin on synthesis of normal and Ta C1 inhibitor, shown by SDS/PAGE of immunoprecipitates of [^{35}S]methionine-labeled lysates of normal fibroblasts (lanes 1 and 2) and Ta fibroblasts (lanes 3 and 4) in the absence of (lanes 1 and 3) or presence (lanes 2 and 4) of tunicamycin at 5 μg/ml. The fibroblasts incubated without tunicamycin (lanes 1 and 3) contained some fully glycosylated nonsecreted C1 inhibitor.

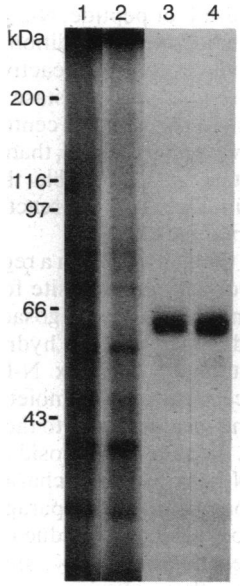

FIG. 6. Cell-free translation. Lanes 1 and 2, total *in vitro* translation products from mRNA isolated from normal and Ta fibroblasts, respectively. Lanes 3 and 4, products immunoprecipitated with anti-C1 inhibitor after translation of normal and Ta mRNA, respectively.

There are three other recent reports of mutations resulting in creation of N-linked glycosylation sites in abnormal proteins associated with human disease: thyroxine-binding globulin (TBG) Gary (34), fibrinogen Asahi (35), and antithrombin III Rouen (36). The mutations in these proteins result in single amino acid substitutions that create the N-glycosylation consensus sequence Asn-Xaa-Ser/Thr. One of the three (TBG Gary) has been shown to result from a single base change (34). The C1 inhibitor Ta mutation differs in that deletion of a codon leads to an amino acid deletion that creates the new glycosylation site. TBG is also a serpin family member, but thyroxin binding is not associated with the reactive-center region. TBG has no known protease inhibitory activity. The new oligosaccharide unit in TBG either interferes directly with thyroxin binding or alters the tertiary structure of the molecule. Fibrinogen Asahi has a defect in fibrin polymerization and crosslinking; presumably this is a result of interference in contact between the abnormally glycosylated D domains in the mutant fibrinogen (35). The mutation in antithrombin III Rouen, another serpin, similarly results in decreased heparin binding (36). The mechanism by which the additional oligosaccharide unit in C1 inhibitor Ta interferes with function remains unclear. The most straightforward mechanism would be steric hindrance of the reactive center so that protease is denied access to the P1-P'1 peptide bond. If this were the case, since glycosylation is not required for C1 inhibitor function (8–10), it might be possible to regenerate C1s inhibitory activity by deglycosylation of C1 inhibitor Ta. However, C1 inhibitor Ta deglycosylated with

either endoglycosidase F or peptide:N-glycosidase remained inactive (data not shown). In addition, the fact that C1 inhibitor Ta is cleaved within the reactive-center region by both trypsin and *Pseudomonas* elastase (unpublished data) indicates that access to the reactive center is not completely blocked. C1s, however, is larger than either trypsin or *Pseudomonas* elastase; it is possible therefore that these proteases could gain access to the reactive center whereas C1s access would be blocked.

It is also possible that Lys-251 is in a region of the molecule that provides a secondary contact site for C1s. Then either the deletion itself and/or the new oligosaccharide unit would alter this site. Endoglycosidase F hydrolyzes many high-mannose and biantennary complex N-linked oligosaccharides at the di-*N*-acetylchitobiosyl moiety, thus leaving one *N*-acetylglucosamine residue linked to the asparagine residue in the protein (26). Peptide:N-glycosidase F, on the other hand, hydrolyzes N-linked oligosaccharides at the glycosylamine junction, converting the asparagine residue at the glycosylation site to an aspartic residue (28). Thus, deglycosylation may not regenerate activity, since the presence of aspartic acid rather than asparagine, or of *N*-acetylglucosamine together with deletion of lysine, by virtue of charge changes, could alter any potential secondary protease contact site.

A final possible explanation for the abnormality in function is that either the deletion itself or the abnormal glycosylation interferes with the normal folding pathway for C1 inhibitor. The final conformation of the protein thus may be altered to an extent that normal three-dimensional structure cannot be regenerated by removal of the abnormal oligosaccharide unit.

The location of the deletion and the resulting new glycosylation site is compatible with any of these potential mechanisms. C1 inhibitor almost certainly has a three-dimensional structure very similar to that of α1-antitrypsin (37). By comparison with the known crystal structure of α_1-antitrypsin, the mutation described here is at the beginning of an unusual conserved segment that forms a series of bulges made up of alternating polar and apolar residues corresponding to Arg-254 through Leu-259. Lys-251 is present in most serpins and in α_1-antitrypsin is hydrogen-bonded to the threonine residue corresponding to Thr-248. Thus, deletion of Lys-251 together with glycosylation of Asn-250 may significantly alter the secondary structure in this region of the molecule. Further analysis of the precise mechanism leading to alteration of function of C1 inhibitor Ta will be aided by functional analysis of expressed normal C1 inhibitor and C1 inhibitor Ta with different mutations created in this region of the molecule.

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