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Heparin Cofactor II: cDNA Sequence, Chromosome Localization, Restriction Fragment Length Polymorphism, and Expression in *Escherichia coli*†

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ABSTRACT: Heparin cofactor II (HCII) is an inhibitor of thrombin in plasma that is activated by dermatan sulfate or heparin. An apparently full-length cDNA for HCII was isolated from a human liver λgt11 cDNA library. The cDNA consisted of 2215 base pairs (bp), including an open-reading frame of 1525 bp, a stop codon, a 3′-noncoding region of 654 bp, and a poly(A) tail. The deduced amino acid sequence contained a signal peptide of 19 amino acid residues and a mature protein of 480 amino acids. The sequence of HCII demonstrated homology with antithrombin III and other members of the α_1-antitrypsin superfamily. Blot hybridization of an HCII probe to DNA isolated from sorted human chromosomes indicated that the HCII gene is located on chromosome 22. Twenty human leukocyte DNA samples were digested with *Eco*RI, *Pst*I, *Hin*dIII, *Kpn*I, or *Bam*HI, and Southern blots of the digests were probed with HCII cDNA fragments. A restriction fragment length polymorphism was identified with *Bam*HI. A slightly truncated form of the cDNA, coding for Met-Ala instead of the N-terminal 18 amino acids of mature HCII, was cloned into the vector pKK233-2 and expressed in *Escherichia coli*. The resultant protein of apparent molecular weight 54 000 was identified on an immunoblot with ^{125}I-labeled anti-HCII antibodies. The recombinant HCII formed a complex with ^{125}I-thrombin in a reaction that required the presence of heparin or dermatan sulfate.

Heparin cofactor II (HCII)[1] is a 65 600-dalton glycoprotein in human plasma that inhibits the coagulation protease thrombin by forming an equimolar complex that is stable during SDS-PAGE (Tollefsen et al., 1982). HCII differs from other plasma protease inhibitors in its protease specificity. Unlike antithrombin III (ATIII), which inhibits all of the serine proteases of the intrinsic coagulation cascade, HCII specifically inhibits thrombin (Parker & Tollefsen, 1985). HCII also reacts slowly with the chymotrypsin-like protease cathepsin G (Parker & Tollefsen, 1985) and with chymotrypsin itself (Church et al., 1985).

HCII and ATIII differ from other protease inhibitors because their activities are stimulated approximately 1000-fold by certain glycosaminoglycans. Dermatan sulfate, heparin, and heparan sulfate increase thrombin inhibition by HCII in a dose-dependent manner, while chondroitin 4-sulfate, chondroitin 6-sulfate, keratan sulfate, and hyaluronic acid have no effect (Tollefsen et al., 1983). In contrast, only heparin and heparan sulfate increase the activity of ATIII. Thus, HCII is the principal inhibitor of thrombin in the presence of dermatan sulfate. Binding of heparin or dermatan sulfate to HCII appears to be required for the increased inhibitory activity observed in the presence of these glycosaminoglycans.

Recently, two partial cDNA clones encoding HCII have been identified. One consists of a 2.1-kb fragment which extends two nucleotides 5′ to the codon for the amino-terminal glycine of plasma HCII and contains 638 bp of 3′-noncoding sequence (Ragg, 1986). In our laboratory, a 1.2-kb cDNA was identified that contains 501 bp coding for the C-terminal 167 amino acids of HCII and 657 bp of 3′-noncoding sequence followed by a poly(A) tail (Inhorn & Tollefsen, 1986). These two sequences agreed entirely through the coding sequence. In this paper, we report a cDNA sequence that establishes the primary structure of the precursor of human HCII and have

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[1] Abbreviations: HCII, heparin cofactor II; ATIII, antithrombin III; SSC, 0.015 M sodium citrate/0.15 M sodium chloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); kb, kilobase(s); EDTA, ethylenediaminetetraacetic acid.

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expressed active recombinant HCII in *Escherichia coli*. In addition, the chromosome containing the HCII gene and a restriction fragment length polymorphism of the gene have been identified.

Materials and Methods

Materials. Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs, Bethesda Research Labs, or Amersham. M13mp18 phage, deoxynucleotide triphosphates, and dideoxynucleotide triphosphates were purchased from New England Biolabs, and 7-deaza-2′-deoxyguanosine 5′-triphosphate was from American Bionetics. Deoxyadenosine [α-^{35}S]thiotriphosphate ([α-^{35}S]dATP), [γ-^{32}P]ATP, and [α-^{32}P]dCTP were products of Amersham. Oligonucleotides were synthesized by using an Applied Biosystems Model 380A synthesizer in the laboratory of Dr. Evan Sadler. Plasmid vectors pGEM-3blue and pKK233-2 were products of Promega Biotech and Pharmacia, respectively. Affinity-purified rabbit anti-HCII antibody was isolated as described previously (Jaffe et al., 1985). Iodination of both thrombin and anti-HCII antibody was by the chloramine T method previously described (Tollefsen & Blank, 1981).

Radiolabeling of DNA Probes. The 30-base synthetic oligodeoxynucleotide GGGAGCAAAGGCCCGCTGGATCAGCTAGAG, corresponding to the 5′ end of the cDNA reported by Ragg (1986) (nucleotides 86–115 in Figure 2), was labeled at the 5′ end using polynucleotide kinase [γ-^{32}P]ATP as described by Maniatis et al. (1982). The HCII cDNA fragments used in screening of the cDNA library and Southern blotting were purified by electrophoresis in low melting point agarose and labeled with [α-^{32}P]dCTP by the random priming method of Feinberg and Vogelstein (1984) to a specific activity of $>1.7 \times 10^8$ cpm/μg.

Isolation of HCII cDNA Clones. A cDNA library prepared from human liver mRNA cloned into the *Eco*RI site of λgt11 was provided by Dr. Savio L. C. Woo. The library was propagated in the *E. coli* strain RY1088 and plated at a density of ~50 000 phage/plate (150-mm diameter). Phage DNA was then adsorbed to nitrocellulose filters, and the filters were incubated with 8×10^7 cpm of the 30-base oligodeoxynucleotide in a 10-mL solution of 6× SSC, 12 mM EDTA, and 2× Denhardt's solution at 50 °C for 16 h. After hybridization, the filters were washed with 6× SSC twice at 23 °C for 15 min followed by 2 h at 50 °C and were autoradiographed. Duplicate filters of subclones of positive plaques were rescreened with two probes. One filter was hybridized to the 30-base oligodeoxynucleotide as described above. The other was hybridized to HCII1.2 (Figure 1), the 622 bp *Eco*RI fragment isolated by Inhorn and Tollefsen (1986) which corresponds to bases 1025–1647 in Figure 2. Hybridization was carried out in 50 mL of 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 10% dextran sulfate overnight at 68 °C. The filters were washed 3 times at 68 °C with 0.2× SSC and autoradiographed. Subclones hybridizing to both probes were analyzed further. DNA from λ phage was purified by the method of Helms et al. (1985). The cDNA inserts were isolated by *Eco*RI digestion followed by agarose gel electrophoresis.

DNA Sequence Analysis. The cDNA inserts were subcloned into M13mp18 and sequenced by the dideoxy chain termination method of Sanger et al. (1977). Sequencing reactions were carried out with [α-^{35}S]dATP, and the reaction products were electrophoresed on 6% polyacrylamide buffer gradient gels (Biggin et al., 1983). Synthetic oligonucleotide primers were used to obtain overlapping cDNA sequences. Sequences containing poor guanine-cytosine resolution were determined by using 7-deaza-2′-deoxyguanosine 5′-triphosphate (Barr et al., 1986). Portions of the sequence were determined by cloning the λ cDNA insert into pGEM-3blue and sequencing the double-stranded DNA by the method of Wallace et al. (1981).

Restriction Mapping of Human Genomic DNA. Purified human leukocyte DNA was isolated from the peripheral blood or bone marrow of 20 patients and was provided by Dr. Timothy Ley. Samples of DNA (5 μg) in 200 μL of the appropriate buffers were digested at 37 °C for 2–12 h with 40–100 units of *Eco*RI, *Bam*HI, *Pst*I, *Hin*dIII, or *Kpn*I. A 10-μL aliquot was removed immediately after addition of the endonuclease and incubated for 2 h with 0.45 μg of λ DNA. The sample containing λ DNA was analyzed by agarose gel electrophoresis to check for complete digestion. The digested DNA samples were electrophoresed and transferred to cationized nylon hybridization membranes (Gene Screen Plus, Du Pont) according to the manfacturer's protocol. The membranes were preincubated in 10 mL of a 1% SDS/10% dextran sulfate solution for 15 min at 65 °C. The ^{32}P-labeled cDNA probes were then hybridized to the leukocyte DNA in a solution of 1 M NaCl, 1% SDS, and 10% dextran sulfate at 65 °C for 12–20 h. The filters were washed twice in 200 mL of 2× SSC/1% SDS at 65 °C for 30 min followed by two washes in 0.1× SSC at 23 °C for 30 min and autoradiographed.

Chromosome Localization. Chromosome sorting was performed by the technique of dual laser flow sorting of fluorescent stained human fibroblast chromosomes as outlined previously (van Dilla et al., 1986). The chromosome DNA was hybridized to ^{32}P-labeled HCII1.2 (Figure 1).

Expression of Recombinant HCII. The *Eco*RI fragment HCII7.2 (Figure 1) was cloned into the vector pGEM-3blue, and the orientation was determined by restriction mapping. The resultant plasmid, pGEM-HCII7.2, with the polylinker adjacent to the 3′ end of the coding strand, was digested with *Pst*I, and the insert was cloned in the expression vector pKK233-2 at the *Pst*I site downstream from the Ptrc promoter to yield the plasmid designated pKK-HCII. The *E. coli* strain NM-522 was transformed by the $CaCl_2$ method (Cohen et al., 1972), and colonies were selected by ampicillin resistance. Plasmid minipreps were prepared according to Davis et al. (1986) to identify colonies containing recombinant plasmids and to check for proper orientation of the insert. Colonies containing a plasmid with the 5′ end of the coding strand adjacent to the promoter were grown overnight in 10 mL of medium containing isopropyl β-D-thiogalactopyranoside. The cell pellets were washed twice and resuspended in 1 mL of phoshate-buffered saline (0.15 M NaCl/0.02 M sodium phosphate, pH 7.4). The cells were lysed by sonication with three 10-s bursts of 150 W from a Biosonik sonicator, and the suspension was clarified by centrifugation. The supernatant lysates were subjected to SDS–PAGE on 7.5% polyacrylamide gels in the presence of β-mercaptoethanol as described by Laemmli (1970), and HCII was identified by immunoblot analysis of the slab gels as described previously (Towbin et al., 1979). To assay for complex formation, the lysates were incubated with ^{125}I-thrombin in the presence or absence of heparin or dermatan sulfate for 5 min at 37 °C prior to electrophoresis. Complexes were identified by autoradiography as described (Tollefsen & Blank, 1981).

Results

Isolation and Characterization of cDNAs Coding for HCII. A full-length cDNA for HCII was isolated from a human liver

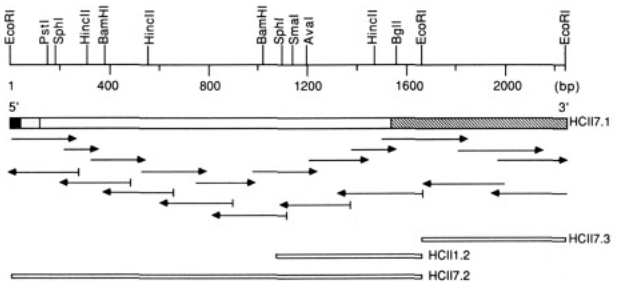

FIGURE 1: Partial restriction map of the cDNA for human HCII and the strategy used to sequence the cDNA inserts. HCII7.1, HCII7.2, and HCII7.3 were isolated from a partial *Eco*RI digest of a λgt11 clone. HCII1.2 is a 622 bp *Eco*RI fragment of the clone isolated by Inhorn and Tollefsen (1986). The solid bar indicates the 5′-untranslated region, the dotted bar the signal sequence, the open bar the sequence of plasma HCII, and the slashed bar the 3′-noncoding region. The extent and strand of sequencing are shown by the length and direction of each arrow. Sequences obtained from single-stranded (→) and double-stranded (⊢→) templates are indicated.

library in λgt11. Approximately 500 000 phage were screened with the 30-base synthetic oligodeoxynucleotide corresponding to the 5′ end of the cDNA reported by Ragg (1986). Twenty-two positive plaques were subcloned. These were rescreened with HCII1.2 containing the 3′ end of the HCII coding sequence (Inhorn & Tollefsen, 1986). Twenty of the 22 subclones hybridized to both probes and 8 of the strongest signals were chosen for further analysis. Partial digestion of one of the cDNA inserts with *Eco*RI resulted in three bands of approximately 2.2 kb (HCII7.1), 1.6 kb (HCII7.2), and 0.6 kb (HCII7.3) which were subcloned into M13mp18 and pGEM-3blue.

The nucleotide sequences were determined for both strands by the strategy shown in Figure 1. An open-reading frame of 1525 bp (positions 1–1525) was found. The first methionine codon occurs at positions 29–31, and the amino acid sequence deduced from the codons that follow is shown in Figure 2. A TAG termination codon occurs at positions 1526–1528 followed by 654 bp of noncoding sequence. The polyadenylation signal (AATAAA) begins at position 2158, 19 nucleotides upstream from the 33-nucleotide poly(A) tail.

Chromosome Localization of the HCII Gene. Sorted human chromosome DNA was blotted onto nitrocellulose filters and probed with ^{32}P-labeled HCII1.2. A strong hybridization signal was present with DNA isolated from chromosome 22 as shown in Figure 3.

Southern Blot Analysis of Human Genomic DNA. Twenty samples of human leukocyte DNA were digested with *Bam*HI, and Southern blots were hybridized with ^{32}P-labeled HCII1.2. Three distinct patterns of restriction fragments were observed as shown in Figure 4. Of the DNA samples, 3 yielded 1.3- and 2.6-kb fragments, 6 yielded 1.3- and 3.1-kb fragments, and 11 yielded 1.3-, 2.6-, and 3.1-kb fragments. This appears to represent a common intragenic restriction fragment length polymorphism. Hybridization of the *Bam*HI digests with the HCII7.3 probe yielded a single 1.3-kb band in all samples defining the 3′ end of the gene. Hybridization of the *Bam*HI digests with the HCII7.2 probe yield two invariant bands of 5.8 and 16.0 kb containing the 5′ end of the gene in addition to the 2.6/3.1-kb and 1.3-kb bands (Figure 4). Digestion with *Eco*RI, *Hin*dIII, *Pst*I, and *Kpn*I gave nonpolymorphic patterns (not shown).

Expression of Recombinant HCII in E. coli. The expression vector pKK-HCII was constructed as shown in Figure 5 (see Materials and Methods for details). The *Pst*I fragment of pGEM-HCII7.2 begins at the codon for Ala_{19} and extends through a portion of the 3′-noncoding region. Thus, the fragment lacks codons for the signal peptide and the N-terminal 18 amino acids of plasma HCII. The recombinant HCII gene contains two new codons at the 5′ end (Met-Ala-) derived from the fusion between plasmid and insert as shown in Figure 5.

E. coli transformed with the pKK-HCII plasmid produced a protein with an apparent mass of 54 000 daltons detected on an immunoblot probed with ^{125}I-labeled affinity-purified rabbit anti-HCII antibodies (Figure 6). No protein of this size was detected in the lysate of cells transformed with pKK233-2 lacking the cDNA insert. Purified plasma HCII migrated with an apparent mass of 72 000 daltons in the same gel (Figure 6). On the basis of the intensity of the immunoblots, approximately 5–10 ng of recombinant HCII was recovered from 40 μL of *E. coli* lysate. This represents ~0.01% of the total protein in the supernatant of the lysed bacteria based on A_{280} determinations.

To determine whether the recombinant HCII was active, we incubated cell lysates with ^{125}I-thrombin in the presence or absence of glycosaminoglycan and assayed for complex formation by SDS–PAGE and autoradiography. Purified plasma HCII formed a 96 000-dalton complex with ^{125}I-thrombin in the presence of heparin or dermatan sulfate as shown in Figure 7 (lanes 1–3). Similarly, lysates of *E. coli* transformed with pKK-HCII formed an 86 000-dalton complex with ^{125}I-thrombin in the presence of heparin or dermatan sulfate (lanes 4–6). This complex was not observed in lysates of *E. coli* transformed with pKK233-2 which lack the HCII cDNA insert. The 68 000-dalton band present in all of the lanes represented a radiolabeled contaminant in the ^{125}I-thrombin preparation. On the basis of intensities of the complexes in lanes 3 and 6, we estimate that approximately 2–3 ng of recombinant HCII in 30 μL of lysate was complexed to ^{125}I-thrombin. This result is in fair agreement with the total amount of HCII in the lysate determined by immunoblot analysis, suggesting that most of the recombinant HCII is active.

DISCUSSION

Screening of a human liver cDNA library in λgt11 has resulted in isolation of a clone that encodes the amino acid sequence of the precursor of human HCII (Figure 2). The length of the HCII cDNA (2215 bp) agrees well with the length of the mRNA determined by Northern blot analysis (~2.3 kb; Ragg, 1986). A Met codon that could function as the initiator for protein synthesis is present at positions 29–31 of the cDNA. Since no stop codon occurs in the open-reading frame 5′ to the Met codon, a longer translation product cannot be ruled out. However, the putative initiator Met codon occurs in a nucleotide sequence (i.e., CCAAA*ATG*) that would efficiently promote initiation of translation (Kozak, 1986). The signal peptide (amino acid residues −19 to −1) contains a typical hydrophobic core (von Heijne, 1984). Cleavage of the signal peptide at the Gly_{-1}–Gly_1 bond would yield a protein with an N-terminal amino acid sequence that is identical with that of purified plasma HCII (Witt et al., 1983; Griffith et al., 1985).

Plasma HCII, as predicted by the cDNA, consists of 480 amino acid residues with the following composition: Ala, 22; Arg, 23; Asn, 32; Asp, 31; $^1/_2$-Cys, 3; Gln, 21; Glu, 28; Gly, 25; His, 13; Ile, 32; Leu, 50; Lys, 31; Met, 18; Phe, 29; Pro, 17; Ser, 29; Thr, 31; Trp, 4; Tyr, 12; and Val, 29. The molecular weight of the polypeptide is calculated to be 54 996. There are three potential Asn-linked glycosylation sites which occur at positions 30, 169, and 368 (Mononen & Karjalainen, 1984). Carbohydrate comprises ~10% of the circulating

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                          -19                                -11                                  -1  +1
                          Met Lys His Ser Leu Asn Ala Leu Leu Ile Phe Leu Ile Ile Thr Ser Ala Trp Gly Gly Ser Lys
CGAAACACAGAGCTTTAGCTCCGCCAAAATG AAA CAC TCA TTA AAC GCA CTT CTC ATT TTC CTC ATC ATA ACA TCT GCG TGG GGT GGG AGC AAA
1                                                  50
                        10                                 20                                  CHO
Gly Pro Leu Asp Gln Leu Glu Lys Gly Gly Glu Thr Ala Gln Ser Ala Asp Pro Gln Trp Glu Gln Leu Asn Asn Lys Asn Leu Ser
GGC CCG CTG GAT CAG CTA GAG AAA GGA GGG GAA ACT GCT CAG TCT GCA GAT CCC CAG TGG GAG CAG TTA AAT AAC AAA AAC CTG AGC
      100                                                 150
                            40                                 50                                  SO4
Met Pro Leu Leu Pro Ala Asp Phe His Lys Glu Asn Thr Val Thr Asn Asp Trp Ile Pro Glu Gly Glu Glu Asp Asp Asp Tyr Leu
ATG CCT CTT CTC CCT GCC GAC TTC CAC AAG GAA AAC ACC GTC ACC AAC GAC TGG ATT CCA GAG GGG GAG GAG GAC GAC GAC TAT CTG
                        200                                                 250
                                70          SO4                                 80                                  90
Asp Leu Glu Lys Ile Phe Ser Glu Asp Asp Asp Tyr Ile Asp Ile Val Asp Ser Leu Ser Val Ser Pro Thr Asp Ser Asp Val Ser
GAC CTG GAG AAG ATA TTC AGT GAA GAC GAC GAC TAC ATC GAC ATC GTC GAC AGT CTG TCA GTT TCC CCG ACA GAC TCT GAT GTG AGT
                                    300                                                                     350
                                100                                 110
Ala Gly Asn Ile Leu Gln Leu Phe His Gly Lys Ser Arg Ile Gln Arg Leu Asn Ile Leu Asn Ala Lys Phe Ala Phe Asn Leu Tyr
GCT GGG AAC ATC CTC CAG CTT TTT CAT GGC AAG AGC CGG ATC CAG CGT CTT AAC ATC CTC AAC GCC AAG TTC GCT TTC AAC CTC TAC
                                                400
120                                 130                                 140
Arg Val Leu Lys Asp Gln Val Asn Thr Phe Asp Asn Ile Phe Ile Ala Pro Val Gly Ile Ser Thr Ala Met Gly Met Ile Ser Leu
CGA GTG CTG AAA GAC CAG GTC AAC ACT TTC GAT AAC ATC TTC ATA GCA CCC GTT GGC ATT TCT ACT GCG ATG GGT ATG ATT TCC TTA
        450                                                 500
    150                                 160                                 CHO 170
Gly Leu Lys Gly Glu Thr His Glu Gln Val His Ser Ile Leu His Phe Lys Asp Phe Val Asn Ala Ser Ser Lys Tyr Glu Ile Thr
GGC CTG AAG GGA GAG ACC CAT GAA CAA GTG CAC TCG ATT TTG CAT TTT AAA GAC TTT GTT AAT GCC AGC AGC AAG TAT GAA ATC ACG
                        550                                                 600
        180                                 190                                 200
Thr Ile His Asn Leu Phe Arg Lys Leu Thr His Arg Leu Phe Arg Arg Asn Phe Gly Tyr Thr Leu Arg Ser Val Asn Asp Leu Tyr
ACC ATT CAT AAT CTC TTC CGT AAG CTG ACT CAT CGC CTC TTC AGG AGG AAT TTT GGG TAC ACA CTG CGG TCA GTC AAT GAC CTT TAT
                                650                                                                     700
            210                                 220                                 230
Ile Gln Lys Gln Phe Pro Ile Leu Leu Asp Phe Arg Thr Lys Val Arg Glu Tyr Tyr Phe Ala Glu Ala Gln Ile Ala Asp Phe Ser
ATC CAG AAG CAG TTT CCA ATC CTG CTT GAC TTC AGA ACT AAA GTA AGA GAG TAT TAC TTT GCT GAG GCC CAG ATA GCT GAC TTC TCA
                                                750
                240                                 250                                 260
Asp Pro Ala Phe Ile Ser Lys Thr Asn Asn His Ile Met Lys Leu Thr Lys Gly Leu Ile Lys Asp Ala Leu Glu Asn Ile Asp Pro
GAC CCT GCC TTC ATA TCA AAA ACC AAC AAC CAC ATC ATG AAG CTC ACC AAG GGC CTC ATA AAA GAT GCT CTG GAG AAT ATA GAC CCT
            800                                                 850
                    270                                 280                                 290
Ala Thr Gln Met Met Ile Leu Asn Cys Ile Tyr Phe Lys Gly Ser Trp Val Asn Lys Phe Pro Val Glu Met Thr His Asn His Asn
GCT ACC CAG ATG ATG ATT CTC AAC TGC ATC TAC TTC AAA GGA TCC TGG GTG AAT AAA TTC CCA GTG GAA ATG ACA CAC AAC CAC AAC
                                900                                                 950
                        300                                 310                                 320
Phe Arg Leu Asn Glu Arg Glu Val Val Lys Val Ser Met Met Gln Thr Lys Gly Asn Phe Leu Ala Ala Asn Asp Gln Glu Leu Asp
TTC CGG CTG AAT GAG AGA GAG GTA GTT AAG GTT TCC ATG ATG CAG ACC AAG GGG AAC TTC CTC GCA GCA AAT GAC CAG GAG CTG GAC
                                    1000
                            330                                 340                                 350
Cys Asp Ile Leu Gln Leu Glu Tyr Val Gly Gly Ile Ser Met Leu Ile Val Val Pro His Lys Met Ser Gly Met Lys Thr Leu Glu
TGC GAC ATC CTC CAG CTG GAA TAC GTG GGG GGC ATC AGC ATG CTA ATT GTG GTC CCA CAC AAG ATG TCT GGG ATG AAG ACC CTC GAA
                                                1100
                                360                                 CHO 370                                 380
Ala Gln Leu Thr Pro Arg Val Val Glu Arg Trp Gln Lys Ser Met Thr Asn Arg Thr Arg Glu Val Leu Leu Pro Lys Phe Lys Leu
GCG CAA CTG ACA CCC CGG GTG GTG GAG AGA TGG CAA AAA AGC ATG ACA AAC AGA ACT CGA GAA GTG CTT CTG CCG AAA TTC AAG CTG
                1150                                                1200
                                    390                                 400
Glu Lys Asn Tyr Asn Leu Val Glu Ser Leu Lys Leu Met Gly Ile Arg Met Leu Phe Asp Lys Asn Gly Asn Met Ala Gly Ile Ser
GAG AAG AAC TAC AAT CTA GTG GAG TCC CTG AAG TTG ATG GGG ATC AGG ATG CTG TTT GAC AAA AAT GGC AAC ATG GCA GGC ATC TCA
                                1250                                                            1300
410                                 420                                 430
Asp Gln Arg Ile Ala Ile Asp Leu Phe Lys His Gln Gly Thr Ile Thr Val Asn Glu Glu Gly Thr Gln Ala Thr Thr Val Thr Thr
GAC CAA AGG ATC GCC ATC GAC CTG TTC AAG CAC CAA GGC ACG ATC ACA GTG AAC GAG GAA GGC ACC CAA GCC ACC ACT GTG ACC ACG
                                                1350
    440                 ◊               450                                 460
Val Gly Phe Met Pro Leu Ser Thr Gln Val Arg Phe Thr Val Asp Arg Pro Phe Leu Phe Leu Ile Tyr Glu His Arg Thr Ser Cys
GTG GGG TTC ATG CCG CTG TCC ACC CAA GTC CGC TTC ACT GTC GAC CGC CCC TTT CTT TTC CTC ATC TAC GAG CAC CGC ACC AGC TGC
1400                                                            1450
        470                                 480
Leu Leu Phe Met Gly Arg Val Ala Asn Pro Ser Arg Ser  *
CTG CTC TTC ATG GGA AGA GTG GCC AAC CCC AGC AGG TCC TAGAGGTGGAGGTCTAGGTGTCTGAAGTGCCTTGGGGGCACCCTCATTTTGTTTCCATTCCAA
                1500                                                            1550

CAACGAGAACAGAGATGTTCTGGCATCATTTACGTAGTTTACGCTACCAATCTGAATTCGAGGCCCATATGAGAGGAGCTTAGAAACGACCAAGAAGAGAGGCTTGTTGGAATCA
            1600                                                            1650                                1700

ATTCTGCACAATAGCCCATGCTGTAAGCTCATAGAAGTCACTGTAACTGTAGTGTGTCTGCTGTTACCTAGAGGGTCTCACCTCCCCACTCTTCACAGCAAACCTGAGCAGCGCG
                                                1750                                                            1800

TCCTAAGCACCTCCCGCTCCGGTGACCCCATCCTTGCACACCTGACTCTGTCACTCAAGCCTTTCTCCACCAGGCCCCTCATCTGAATACCAAGCACAGAAATGAGTGGTGTGAC
                                1850                                                            1900

TAATTCCTTACCTCTCCCAAGGAGGGTACACAACTAGCACCATTCTTGATGTCCAGGGAAGAAGCCACCTCAAGACATATGAGGGGTGCCCTGGGCTAATGTTAGGGCTTAATTT
                1950                                                            2000

TCTCAAAGCCTGACCTTTCAAATCCATGATGAATGCCATCAGTCCCTCCTGCTGTTGCCTCCCTGTGACCTGGAGGACAGTGTGTGCCATGTCTCCCATACTAGAGATAAATAAA
 2050                                                            2100                                2150

TGTAGCCACATTTACTGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
                                2200            2215
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FIGURE 2: Nucleotide sequence and translated amino acid sequence of HCII7.1. The potential Asn-linked glycosylation sites are indicated by CHO, and the sites of tyrosine sulfation are indicated by SO4. The reactive site is indicated by (◊) and the stop codon by an asterisk. The polyadenylation signal AATAAA is underlined.

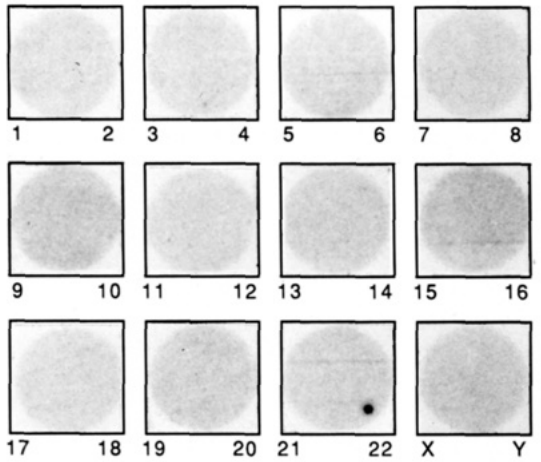

FIGURE 3: Chromosome localization of the gene for HCII. Sorted human chromosomes 1–22, X, and Y were blotted onto nitrocellulose and probed with HCII1.2. A hybridization signal can be seen with DNA from chromosome 22.

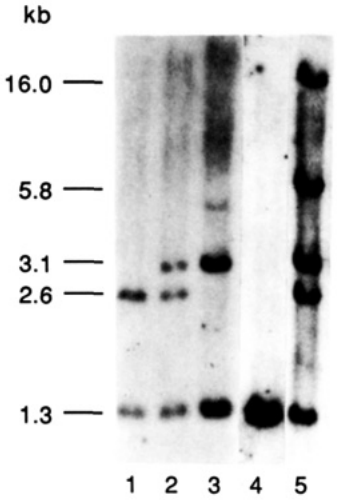

FIGURE 4: Southern blots of human leukocyte DNA digested with *Bam*HI. Lanes 1–3 are samples from three different individuals probed with HCII1.2. Lanes 1 and 3 are presumed to represent homozygotes with a polymorphic band of either 2.6 or 3.1 kb. Lane 2 is presumed to represent the heterozygote bearing both alleles. Lanes 4 and 5 are samples from a single individual probed with HCII7.3 (lane 4) or HCII7.2 (lane 5).

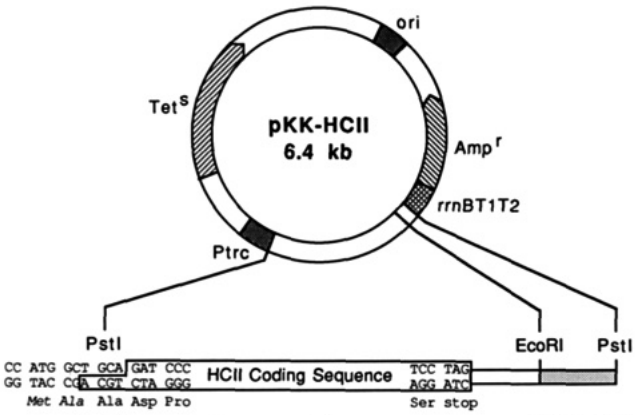

FIGURE 5: Schematic diagram of the expression vector pKK-HCII. The *Pst*I fragment of pGEM-HCII7.2 was cloned into the *Pst*I site of pKK233-2 as described under Materials and Methods. The positions of the promoter (Ptrc) and the transcription termination element (rrnBT1T2) are indicated. The nucleotides and codons at the junction of pKK233-2 and the 5′ end of the insert are shown.

protein (Tollefsen et al., 1982), yielding a calculated molecular weight of 61 110 for the glycoprotein. This is in good

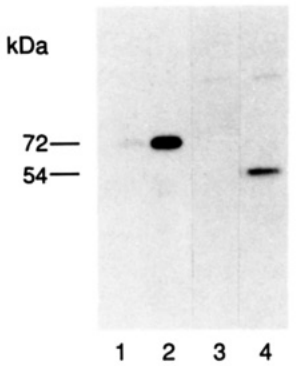

FIGURE 6: Immunoblot analysis of recombinant HCII. *E. coli* lysates were electrophoresed on 7.5% SDS–polyacrylamide gels and probed with ^{125}I-labeled anti-HCII antibodies. Samples containing 1 or 100 ng of purified HCII from human plasma are shown in lanes 1 and 2. The apparent mass of plasma HCII is 72 000 daltons. Lane 3 contains 40 μL of the lysate from *E. coli* transformed with the control plasmid pKK233-2 and demonstrates a faint 92 000-dalton band that is considered nonspecific. Lane 4 contains 40 μL of the lysate from *E. coli* transformed with pKK-HCII and demonstrates a 54 000-dalton band not present in the control (lane 3).

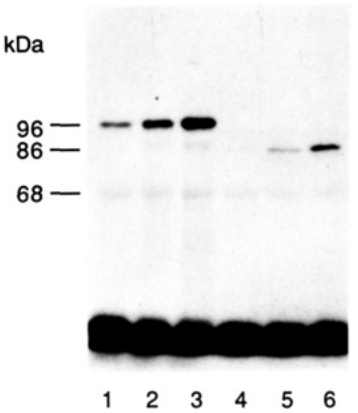

FIGURE 7: Complex formation between ^{125}I-thrombin and recombinant HCII. Ten nanograms of purified plasma HCII and 35 ng (20 000 cpm) of ^{125}I-thrombin were incubated for 5 min at 37 °C in 25 μL of phosphate-buffered saline without glycosaminoglycan (lane 1), in the presence of 40 μg/mL heparin (lane 2), or in the presence of 100 μg/mL dermatan sulfate (lane 3). The samples were then subjected to SDS–PAGE and autoradiographed. The 96 000-dalton band represents the ^{125}I-thrombin–HCII complex. Thirty-five microliters of lysate from *E. coli* transformed with pKK-HCII was incubated with 20 000 cpm of ^{125}I-thrombin without glycosaminoglycan (lane 4), in the presence of 40 μg/mL heparin (lane 5), or in the presence of 100 μg/mL dermatan sulfate (lane 6). The 86 000-dalton band represents the complex between ^{125}I-thrombin and recombinant HCII. The 68 000-dalton band present in lanes 4–6, but not the 86 000-dalton band, was also present in control lysates of *E. coli* transformed with pKK233-2. The radioactivity at the bottom of each lane represents uncomplexed ^{125}I-thrombin.

agreement with the molecular weight 65 600 (Tollefsen et al., 1982) and 62 100 (Tran et al., 1986) derived from sedimentation equilibrium analysis of plasma HCII.

Two tyrosine residues at positions 60 and 73 have been shown to be sulfated in a human hepatoma-derived cell line, and the sulfated form of HCII complexes with thrombin in a heparin-dependent fashion (Hortin et al., 1986a). The sequences flanking the sulfated tyrosine residues have a high content of acidic amino acids and represent a short internal repeat (Glu-Asp-Asp-Asp-Tyr-X-Asp, in which X is either Ile or Leu) within HCII. Several plasma proteins with similar acidic domains, including fibrinogen, fibronectin, and the C4 component of complement, undergo tyrosine sulfation (Hortin et al., 1986b). The only other serine protease inhibitor known to undergo tyrosine sulfation is α_2-antiplasmin, in which

FIGURE 8: Amino acid sequence alignment of HCII and ATIII. Alignment was performed by using the ALIGN computer program (Dayhoff et al., 1983). Asterisks denote residues that have been implicated in heparin binding to ATIII (see text). The N-terminus (Asn_{48}) of the partially degraded HCII characterized by Griffith et al. (1985) and the signal peptides are indicated.

sulfation occurs near the C-terminal end of the protein (Hortin et al., 1987).

The reactive site of HCII has been identified by isolation and sequence analysis of the peptide released from the inhibitor (corresponding to amino acids 445–480, Figure 2) when it reacts with thrombin or chymotrypsin (Griffith et al., 1985; Church et al., 1985). Both thrombin and chymotrypsin cleave the Leu_{444}–Ser_{445} bond of HCII and appear to form an ester linkage between the active-site serine hydroxyl group of the protease and the α-carbonyl group of Leu_{444}. The presence of leucine at the P1 position[2] is consistent with the ability of chymotrypsin and cathepsin G to react with HCII. The presence of Pro_{443} in the P2 position may enable HCII to react efficiently with thrombin, since proline is frequently found in the P2 position of other thrombin substrates (Inhorn & Tollefsen, 1986).

Three nucleotide differences were found when HCII7.1 was compared to the partial cDNA reported by Ragg (1986). One difference occurs at position 1608 in the 3′-untranslated region and has been described previously (Inhorn & Tollefsen, 1986). Two additional differences occur in the coding region: (1) C instead of T at position 532, resulting in a Gly codon in each case; (2) G instead of A at position 738, coding for Arg instead of Lys. These may represent genetic polymorphisms.

An additional polymorphism was identified by restriction mapping of human leukocyte DNA. *Bam*HI digests probed with an HCII cDNA fragment yielded three distinct autoradiographic patterns in which the 2.6- and 3.1-kb bands probably represent different alleles of the HCII gene (Figure 4). These alleles were found in both the homozygous and the heterozygous state. The frequencies of these alleles in the general population have not been determined.

The HCII gene occurs on chromosome 22 as determined by hybridization of the cDNA to sorted human chromosome DNA. Other serine protease inhibitor genes have been localized to chromsome 1 (ATIII; Bock et al., 1985), chromosome 14 (α_1-antitrypsin and α_1-antichymotrypsin; Rabin et al., 1986), and chromosome 11 (C1 inhibitor; Bock et al., 1986).

HCII is one of several homologous proteins that comprise the α_1-antitrypsin (Hunt & Dayhoff, 1980) or "serpin" (Carrell & Boswell, 1986) superfamily. HCII and ATIII differ from other protease inhibitors of the superfamily in their ability to become activated by binding to glycosaminoglycans. Binding of heparin to ATIII and HCII has been postulated to occur in regions of the protein rich in basic amino acid residues. For example, lysine residues in both ATIII and HCII may be involved in heparin binding, as chemical modification of these residues leads to a decrease in the heparin stimulatory effect (Rosenberg & Damus, 1973; Church & Griffith, 1984). One such region includes Arg_{47}, which has been found to be mutated in three kindreds whose ATIII molecules bind heparin with decreased affinity (Koide et al., 1984; Owen et al., 1987; Duchange et al., 1987). Furthermore, chemical modification of Trp_{49} decreases the affinity of ATIII for heparin (Blackburn et al., 1984). HCII is very similar to ATIII in this region. Beginning with Arg_{103} of HCII (corresponding to Arg_{47} of ATIII), 21 of the following 47 residues are identical (Figure 8). However, HCII lacks a tryptophan residue at position 105 (corresponding to Trp_{49} of ATIII), which might explain the fact that chemical modification of tryptophan residues in HCII has no effect on heparin binding (Church et al., 1986).

A recent report indicates that Lys_{125} of ATIII is protected from chemical modification in the presence of heparin (Peterson et al., 1987), implying that this residue is directly involved in heparin binding. HCII is also quite similar to ATIII in the vicinity of this residue. In the region from Ile_{179} to Arg_{193} of HCII, 8 of 15 residues are identical with those found in ATIII, including Lys_{185} (corresponding to Lys_{125} of ATIII), Arg_{189}, and Arg_{192}; in addition, Arg_{193} of HCII corresponds to a lysine residue in ATIII.

An α-helical domain of ATIII can be denatured with 0.7

[2] P1 designates the amino acid forming the new C-terminus after proteolytic cleavage and is preceded by P2, P3, etc. P1′ denotes the N-terminal amino acid following the cleavage site.

M guanidinium chloride, resulting in loss of heparin binding activity (Villanueva, 1984). This α-helical domain has been postulated to include the lysine residues at positions 290, 294, and 297 based on secondary structure predictions. In the region of HCII (Lys_{343}–Val_{358}) that corresponds to the putative unstable helical segment of ATIII, 7 of 16 residues are identical, but only 1 basic amino acid is conserved. Although the sequence comparisons noted above would be consistent with the hypothesis that the glycosaminoglycan binding site(s) of HCII and ATIII lie(s) in the N-terminal portion of the molecule, further experiments are required to identify the residues that are directly involved in glycosaminoglycan binding.

A slightly truncated form of the cDNA HCII7.2 was cloned into the *E. coli* expression vector pKK233-2. The N-terminus of the recombinant protein should differ from native HCII by substitution of Met-Ala- for the amino acids in positions −19 through +18 of the protein as shown in Figure 5. A protein with an apparent mass of 54 000 daltons was identified on immunoblots with monospecific anti-HCII antibodies. The lower apparent molecular mass of recombinant HCII compared to the native glycoprotein is probably due to the absence of glycosylation and the truncated N-terminus. Nevertheless, the recombinant protein maintained its ability to complex with thrombin in a manner that depended on the presence of either heparin or dermatan sulfate. These results suggest that glycosylation and tyrosine sulfation, neither of which has been reported to occur in *E. coli*, are not required for activity. A partially degraded form of plasma HCII lacking the N-terminal 47 amino acid residues also maintained glycosaminoglycan-dependent thrombin inactivation (Griffith et al., 1985), suggesting that these amino acids are not necessary for inhibitor function. Expression of site-specific mutants of the HCII cDNA may allow identification of residues important for protease and glycosaminoglycan binding.

The physiological function of HCII remains undetermined. The primary role of HCII may be to control thrombin activity in extravascular tissues, since cultured fibroblasts and smooth muscle cells, but not endothelial cells, accelerate thrombin inactivation by HCII (McGuire & Tollefsen, 1987). A definitive role in hemostasis has not been demonstrated, although two kindreds have been described in whom thrombosis was associated with decreased HCII concentration in plasma (Sie et al., 1985; Tran et al., 1985). The genetic polymorphisms described in this report may allow determination of whether the decreased HCII levels observed in these and other patients result from abnormalities in the HCII gene.

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Registry No. DNA (human liver clone HC117.1 heparin cofactor II messenger RNA complementary), 111958-48-6; heparin cofactor II (human liver clone HC117.1 precursor protein moiety reduced), 111958-49-7; heparin cofactor II (human liver clone HC117.1 protein moiety reduced), 111958-50-0.

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Intersubunit Cross-Linking by *cis*-Dichlorodiammineplatinum(II) Stabilizes an α_2-Macroglobulin "Nascent" State: Evidence That Thiol Ester Bond Cleavage Correlates with Receptor Recognition Site Exposure†

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ABSTRACT: Treatment of human α_2-macroglobulin (α_2M) with proteinase results in cleavage of the α_2M subunits and subsequently in a conformational change in the inhibitor. This change irreversibly traps the proteinase and is accompanied by the generation of four thiol groups as well as exposure of receptor recognition sites. *cis*-Dichlorodiammineplatinum(II) (*cis*-DDP) causes extensive intersubunit cross-linking of α_2M. Incubation of α_2M or *cis*-DDP-treated α_2M with trypsin results in complete subunit cleavage; however, trypsin treatment of *cis*-DDP-α_2M does not result in a conformational change as determined by nondenaturing polyacrylamide gel electrophoresis (PAGE), receptor recognition site exposure, or appearance of thiol groups from the inhibitor. These results are in marked contrast to previous studies which demonstrated that incubation of *cis*-DDP-treated α_2M with CH_3NH_2 resulted in thiol ester bond cleavage and receptor recognition site exposure. *cis*-DDP-treated α_2M bound only 0.13 mol of ^{125}I-trypsin/mol of *cis*-DDP-α_2M. Incubation of trypsin-treated *cis*-DDP-α_2M with diethyldithiocarbamate (DDC), a potent chelator of platinum compounds, results in the removal of the intersubunit cross-links and completion of the α_2M conformational change as determined by nondenaturing PAGE. Complete receptor recognition site exposure and the appearance of 3.3 thiol groups/mol of α_2M also occur following this treatment. These results demonstrate that cross-linking of α_2M by *cis*-DDP prevents a conformational change in the inhibitor which is necessary for thiol ester bond activation and cleavage. Removal of intersubunit cross-links by incubation with DDC allows the completion of this conformational change. These studies also indicate that there is a strong correlation between thiol ester bond cleavage and exposure of receptor recognition sites on α_2M. It is further suggested that incubation of *cis*-DDP-treated α_2M with trypsin results in a "primed" form of α_2M which is very similar to "nascent" α_2M originally described by Sottrup-Jensen et al. [Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 128*, 127–132]. Incubation of trypsin-treated *cis*-DDP-α_2M with DDC results in the generation of a form of α_2M with complete subunit cleavage and complete thiol ester bond cleavage but essentially no proteinase binding.

Human α_2-macroglobulin (α_2M)[1] is a plasma proteinase inhibitor composed of four identical 180-kDa subunits that inhibits proteinases from all four classes (Swenson & Howard, 1979; Sottrup-Jensen et al., 1983a; Barrett & Starkey, 1973). The mechanism of proteinase inhibition by α_2M is unique in that cleavage of α_2M at a sequence of residues termed the "bait region", located near the middle of each α_2M subunit, leads to a conformational change in the inhibitor which results in the "trapping" of the proteinase (Barrett & Starkey, 1973; Harpel, 1973). Proteolysis of α_2M at the bait region also leads

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[1] Abbreviations: α_2M, α_2-macroglobulin; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); *cis*-DDP-α_2M, α_2-macroglobulin treated with *cis*-dichlorodiammineplatinum(II); PAGE, polyacrylamide gel electrophoresis; BPTI, bovine pancreatic trypsin inhibitor; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

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