The Protein Z-Dependent Protease Inhibitor Is a Serpin†

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ABSTRACT: In the presence of phospholipid vesicles and calcium ions, protein Z (PZ) serves as a cofactor for the inhibition of coagulation factor Xa by a plasma protein called PZ-dependent protease inhibitor (ZPI). To further characterize ZPI, its cDNA has been isolated and cloned from a human liver cDNA library. The ZPI cDNA is 2.44 kb in length and has a relatively long 5′ region (466 nt) that contains six potential ATG translation start codons. ATG's 1−4 are followed by short open reading frames, whereas ATG_5 and ATG_6 are in an uninterrupted open reading frame that includes the encoded ZPI protein. In vitro experiments show that ATG_6 is sufficient for the expression of rZPI in cultured Chinese hamster ovary cells. Northern analysis suggests the liver is a major site of ZPI synthesis. The predicted 423 residue amino acid sequence of the mature ZPI protein is 25−35% homologous with members of the serpin superfamily of protease inhibitors and is 78% identical to the amino acid sequence predicted by a previously described cDNA isolated from rat liver, regeneration-associated serpin protein-1 (rasp-1). Thus, ZPI is likely the human homologue of rat rasp-1. Alignment of the amino acid sequence of ZPI with those of other serpins predicts that Y387 is the P_1 residue at the reactive center of the ZPI molecule. Consistent with this notion, rZPI(Y387A), an altered form of ZPI in which tyrosine 387 has been changed to alanine, lacks PZ-dependent factor Xa inhibitory activity.

Protein Z (PZ)[1] is an M_r 62 000, vitamin K-dependent plasma protein whose function is uncertain (*1*, *2*). Its structure is homologous to those of the coagulation zymogens factor VII, factor IX, factor X, and protein C, but in the C-terminal domain of PZ the typical "activation" site is absent and the His and Ser residues of canonical serine protease catalytic triad are lacking (*3*, *4*). Further, the rates of association and dissociation of PZ with phospholipid vesicles are significantly slower that those of the other vitamin K-dependent coagulation factors (*5*).

Recently a protein Z-dependent protease inhibitor (ZPI) was isolated from plasma (*6*). ZPI is an M_r 72 000 single-chain protein whose N-terminal amino acid sequence, LAPSPQSPEXXA (X = indeterminate), did not match or show significant homology with sequences accessible in publicly available protein or DNA databases. In systems using purified components, ZPI produces rapid inhibition of factor Xa (>95% within 1 min by coagulation assay) that requires the presence of PZ, calcium ions, and phospholipids. The inhibitory process appears to involve the formation of a stoichiometric complex of factor Xa−PZ−ZPI at the phospholipid surface (*6*), but a covalent interaction between factor Xa and ZPI is not detectable by SDS−polyacrylamide gel electrophoresis. To further characterize ZPI, its cDNA has been isolated, cloned, and sequenced. Based on the primary amino acid structure of ZPI predicted by the cDNA sequence and additional studies of altered forms of recombinant ZPI, ZPI is a member of the serpin superfamily of protease inhibitors and likely contains a tyrosine as the P_1 residue at its reactive center.

MATERIALS AND METHODS

Materials. Fresh frozen human plasma was purchased from the Regional Red Cross (St. Louis, MO). Multiple human tissue RNA blot and human adult liver cDNA library were from Clontech (Palo Alto, CA); human fetal liver cDNA library was from Strategene (La Jolla, CA); nitrocellulose membrane from Schleicher & Schuell, Inc. (Keene, NH); PVDF membrane from Micron Separations, Inc. (Westborough, MA); [α-^{32}P]dATP from NEN Life Scientific, Inc. (Boston, MA); and dNTPs from Pharmacia Biotech, Inc. (Piscataway, NJ). Taq DNA polymerase, DMEM, fetal calf serum, and LipofectAMINE were from Gibco BRL, Life Technologies (Gaithersburg, MD). G418 was purchased from Mediatech, Inc. (Herndon, VA). Chinese hamster ovary (CHO) cells were from the ATCC (Manassas, VA). ITS+3 media supplement, protease inhibitor cocktail, soybean trypsin inhibitor, aprotinin, and rabbit brain cephalin were from Sigma Chemical (St. Louis, MO). Factor X deficient plasma was from George King Biomedical, Inc. (Overland Park, KS). Prestained molecular weight standards for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were purchased from Bio-Rad (Hercules, CA). The chromogenic substrates S2238 (H-D-Phe-Pip-Arg-pNA), S2366 (pyroGlu-Pro-Arg-pNA), S2444 (pyroGlu-Gly-Arg-

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[1] Abbreviations: PZ, protein Z; ZPI, protein Z-dependent protease inhibitor; t-PA, tissue-plasminogen activator; u-PA, urokinase-plasminogen activator; APC, activated protein C; rasp-1, rat regeneration-associated serpin-1; CHO cells, Chinese hamster ovary cells; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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pNA), S2390 (H-D-Val-Phe-Lys-pNA), S2222 [Bz-Ile-Glu-(γ-OR)-Gly-Arg-pNA], S2484 (pyroGlu-Pro-Val-pNA), and S2586 (MeO-Suc-Arg-Pro-Tyr-pNA) were from Pharmacia (Franklin, OH). Spectrazyme tPA (CH_3SO_2-D-HHT-Gly-Arg-pNA) was from American Diagnostica, Inc. (Greenwich, CT), and *N*-succinyl-Ala-Ala-Pro-Phe-pNA was from Sigma Chemical Company (St. Louis, MO).

Proteins. PZ and ZPI were purified from human plasma as previously described (*6*). A mouse monoclonal anti-ZPI antibody (MC4249.2) was produced using established and previously reported techniques (*7*). Thrombin, tissue-plasminogen activator (t-PA), urokinase-plasminogen activator (u-PA), and plasmin were purchased from American Diagnostica, Inc. Factor IXa and activated protein C (APC) were from Enzyme Research Laboratories (South Bend, IN); TPCK-treated bovine trypsin, TLCK-treated bovine chymotrypsin, leukocyte elastase, and cathepsin G were from Sigma; and factor VIIa was from Novo-Nordisk (Gentofte, Denmark). Meizothrombin was a gift from Michael Nesheim (Queen's University, Kingston, Ontario).

N-Terminal Amino Acid Sequencing of ZPI and Trypsin-Treated ZPI. Samples containing 20 μg of ZPI or 20 μg of ZPI that had been digested with trypsin (1:200 w/w) for 30 min at 22° C were reduced with 2-mercaptoethanol (5%), separated by 12% SDS−PAGE, and electro-transferred to a PVDF membrane. The membrane was stained for 10 min with 0.025% Coomassie Brilliant Blue R-250 in 10% methanol/7% acetic acid, washed with distilled water, and allowed to air-dry. Discernible protein bands of apparent molecular weight 72 000 for ZPI and 43 000 and 41 000 for trypsin-treated ZPI were cut from the membrane and sequenced by the Protein Chemistry Laboratory (Washington University, St. Louis, MO).

ZPI cDNA Cloning. The N-terminal amino acid sequence of the two tryptic peptides derived from ZPI was highly homologous to the amino acid sequence predicted by the previously reported cDNA for rat regeneration-associated serpin (rasp-1, GeneBank Accession No. 2143953)(see Results)(*8*). Nucleotide sequences derived from rasp-1 cDNA, 496−518 (ACCCAGGGTAGCTTTGCCTTCAT) and 805−825 (GTACATCATGGGCACCTTAAC), were used as the basis for 5′- and 3′-primers, respectively, in a PCR reaction to amplify a DNA fragment from a human fetal liver cDNA library (Strategene). The PCR product, ∼330 bp, was cloned into pGEM-T Easy (Promega, Madison,WI) and was found to be 80% homologous with rasp-1 cDNA by sequence analysis. Following radiolabeling with [α-^{32}P]dATP and random priming, the PCR product was used as a probe to screen approximately 2×10^6 plaque-forming units from a human liver cDNA library (Clontech). Hybridization was performed at 42 °C in 5 × SSPE, 5 × Denhardt's solution, 1% SDS, 50% formamide, and 100 μg/mL denatured salmon sperm. Filters were washed with 1 × SSC and 0.1% SDS solution at room temperature for 15 min and then washed 3 times with the same solution at 65 °C for 30 min. The twenty-one positive clones that remained after plaque purification contained cDNAs of four different lengths. A representative of the longest cDNA was sequenced in its entirety in both directions.

Northern Blot Analysis. ^{32}P-Labeled full-length ZPI cDNA was used as a probe for analysis of a human multiple tissue Northern blot membrane from Clontech (Palo Alto, CA) containing 2 μg of poly A+ RNA per sample. Hybridization was performed under the stringent conditions suggested by the manufacturer; autoradiography was allowed to proceed overnight.

In Vitro Expression of Wild-Type and Altered Forms of Recombinant ZPI (rZPI). A 2.2 kb fragment of the ZPI cDNA was produced by treatment with *Sac*I and *Hin*dIII and inserted into the multiple cloning site of pBluescript KS II. This fragment contained part of the 5′-untranslated region, the entire open reading frame, and the 3′-untranslated region of the ZPI cDNA. A 2.3 kb fragment of pBluescript-ZPI was released by *Pvu*II treatment and inserted by blunt-end ligation into the *Eco*RV site of the expression vector pCMV (*9*), producing pCMV-ZPI(WT). This 2.3 kb DNA fragment contained the following: (1) ZPI cDNA beginning 120 bp upstream of ATG_6 and lacking the remainder of the 5′-untranslated ZPI cDNA including ATG_1−ATG_5; (2) the coding and 3′-untranslated regions of ZPI cDNA; and (3) ∼200 bp of pBluescript KS II DNA. In pCMV, expression is driven by the cytomegalovirus early promoter/enhancer.

PCR-based site-directed mutagenesis with pCMV-ZPI-(WT) as template was used to change the codon for Y387 (TAT) in ZPI to that for alanine (GCT) or arginine (CGT). Mutations were confirmed by sequencing the ZPI cDNA between *Nsi*I (nt 1544) and *Spe*I (nt 1944) restriction sites which are upstream and downstream of the mutation site. These fragments were then inserted in pCMV-ZPI(WT) at *Nsi*I−*Spe*I to produce pCMV-ZPI(Y387A) and pCMV-ZPI-(Y387R). pCMV-ZPI(WT), pCMV-ZPI(Y387A), and pCMV-ZPI(Y387R) were cotransfected with pSV2neo into CHO cells using LipofectAMINE (GIBCO BRL) according to the manufacturer's instructions. Cell clones resistant to G418 were picked at 3 weeks and expanded. Nontransfected CHO cells and stable CHO clones expressing rZPI(WT), rZPI-(Y387A), and rZPI(Y387R) were cultured in 5% CO_2 with DMEM and 10% fetal calf serum in six well culture plates (Costar; Corning, Inc., Corning, NY). After the cells reached confluence, the medium was removed, and the cells were washed 3 times with 5 mL of DMEM before 1 mL of serum-free medium consisting of DMEM with ITS+3 media supplement (insulin, transferrin, selenium; Sigma) was added to each well. After an additional 48 h of culture, the conditioned medium was collected, centrifuged (14000*g* for 30 s) to remove cell debris, and analyzed by Western blotting and ZPI functional assay. In some experiments, aprotinin (1 μg/mL) and soybean trypsin inhibitor (2.5 μg/mL were included in the serum-free medium and a 1:10 dilution of protease inhibitor cocktail (Sigma) containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)(100 mM), pepstatin A (1.5 mM), *trans*-expoxysucciniyl-L-leucylamido(4-guanidino)butane (E-64)(1.4 mM), bestatin (4 mM), leupeptin (2.2 mM), and aprotinin (80 μM) was added to the conditioned medium at the time of its collection.

ZPI Functional Assay. A two-stage factor Xa inhibition assay was used to measure ZPI functional activity as previously described (*6*). Twenty microliters of rabbit brain cephalin (75 μM), 20 μL of $CaCl_2$ (25 mM), 20 μL of PZ (200 nM), or 20 μL of HSA (0.1 M NaCl, 0.05 M Hepes, pH 7.4, with 1 mg/mL bovine serum albumin), 20 μL of the sample to be tested, and 20 μL of factor Xa (1 nM) were incubated in the sample cup of a fibrometer at 37 °C. After

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                                                                ctggagtggggta  13
agaggcgaattatagacacaaggggctcctctgcaggaaggaggccaagggaaagaggcttgaaaggcttgatat  88
ttcacccaccaccactcactgccggagtaagcaggtctccccttcccagggctgaggggaggcagggatgtgtgc  163
tgtcccagggctgagaagtggcaggtgagctggtgattccttactgcccaggttctgtctaggaaggtgcgtcct  238
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gagcaaagactttacaaagaccttaggcgatatatgtccaggagcacccaggaattactgggctaccactgcaga  388
ctgcaggacaagctccaagaacaggaaggaagtcttgcagctgaagggaggcactccttggcctccgcagccgat  463
cac atg aag gtg gtg cca agt ctc ctg ctc tcc gtc ctc ctg gca cag gtg tgg ctg  520/-4
     M   K   V   V   P   S   L   L   L   S   V   L   L   A   Q   V   W   L
gta ccc ggc ttg gcc ccc agt cct cag tcg cca gag acc cca gcc cct cag aac cag  577/16
 V   P   G   L   A   P   S   P   Q   S   P   E   T   P   A   P   Q   N*  Q
acc agc agg gta gtg cag gct ccc aag gag gaa gag gaa gat gag cag gag gcc agc  634/35
 T   S   R   V   V   Q   A   P   K   E   E   E   E   D   E   Q   E   A   S
gag gag aag gcc agt gag gaa gag aaa gcc tgg ctg atg gcc agc agg cag cag ctt  691/54
 E   E   K   A   S   E   E   E   K   A   W   L   M   A   S   R   Q   Q   L
gcc aag gag act tca aac ttc gga ttc agc ctg ctg cga aag atc tcc atg agg cac  748/73
 A   K   E   T   S   N   F   G   F   S   L   L   R   K   I   S   M   R   H
gat ggc aac atg gtc ttc tct cca ttt ggc atg tcc ttg gcc atg aca ggc ttg atg  805/92
 D   G   N   M   V   F   S   P   F   G   M   S   L   A   M   T   G   L   M
ctg ggg gcc aca ggg ccg act gaa acc cag atc aag aga ggg ctc cac ttg cag gcc  862/111
 L   G   A   T   G   P   T   E   T   Q   I   K   R   G   L   H   L   Q   A
ctg aag ccc acc aag ccc ggg ctc ctg cct tcc ctc ttt aag gga ctc aga gag acc  919/130
 L   K   P   T   K   P   G   L   L   P   S   L   F   K   G   L   R   E   T
ctc tcc cgc aac ctg gaa ctg ggc ctc aca cag ggg agt ttt gcc ttc atc cac aag  976/149
 L   S   R   N   L   E   L   G   L   T   Q   G   S   F   A   F   I   H   K
gat ttt gat gtc aaa gag act ttc ttc aat tta tcc aag agg tat ttt gat aca gag  1033/168
 D   F   D   V   K   E   T   F   F   N*  L   S   K   R   Y   F   D   T   E
tgc gtg cct atg aat ttt cgc aat gcc tca cag gcc aaa agg ctc atg aat cat tac  1090/187
 C   V   P   M   N   F   R   N*  A   S   Q   A   K   R   L   M   N   H   Y
att aac aaa gag act cgg ggg aaa att ccc aaa ctg ttt gat gag att aat cct gaa  1147/206
 I   N   K   E   T   R   G   K   I   P   K   L   F   D   E   I   N   P   E
acc aaa tta att ctt gtg gat tac atc ttg ttc aaa ggg aaa tgg ttg acc cca ttt  1204/225
 T   K   L   I   L   V   D   Y   I   L   F   K   G   K   W   L   T   P   F
gac cct gtc ttc acc gaa gtc gac act ttc cac ctg gac aag tac aag acc att aag  1261/244
 D   P   V   F   T   E   V   D   T   F   H   L   D   K   Y   K   T   I   K
gtg ccc atg atg tac ggt gca ggc aag ttt gcc tcc acc ttt gac aag aat ttt cgt  1318/263
 V   P   M   M   Y   G   A   G   K   F   A   S   T   F   D   K   N   F   R
tgt cat gtc ctc aaa ctg ccc tac caa gga aat gcc acc atg ctg gtg gtc ctc atg  1375/282
 C   H   V   L   K   L   P   Y   Q   G   N*  A   T   M   L   V   V   L   M
gag aaa atg ggt gac cac ctc gcc ctt gaa gac tac ctg acc aca gac ttg gtg gag  1432/301
 E   K   M   G   D   H   L   A   L   E   D   Y   L   T   T   D   L   V   E
aca tgg ctc aga aac atg aaa acc aga aac atg gaa gtt ttc ttt ccg aag ttc aag  1489/320
 T   W   L   R   N   M   K   T   R   N   M   E   V   F   F   P   K   F   K
cta gat cag aag tat gag atg cat gag ctg ctt agg cag atg gga atc aga aga atc  1546/339
 L   D   Q   K   Y   E   M   H   E   L   L   R   Q   M   G   I   R   R   I
ttc tca ccc ttt gct gac ctt agt gaa ctc tca gct act gga aga aat ctc caa gta  1603/358
 F   S   P   F   A   D   L   S   E   L   S   A   T   G   R   N   L   Q   V
tcc agg gtt tta caa aga aca gtg att gaa gtt gat gaa agg ggc act gag gca gtg  1660/377
 S   R   V   L   Q   R   T   V   I   E   V   D   E   R   G   T   E   A   V
gca gga atc ttg tca gaa att act gct tat tcc atg cct cct gtc atc aaa gtg gac  1717/396
 A   G   I   L   S   E   I   T   A   Y   S   M   P   P   V   I   K   V   D
cgg cca ttt cat ttc atg atc tat gaa gaa acc tct gga atg ctt ctg ttt ctg ggc  1774/415
 R   P   F   H   F   M   I   Y   E   E   T   S   G   M   L   L   F   L   G
agg gtg gtg aat ccg act ctc cta taa ttcaggacacgcataagcacttcgcgtgtagtagatgct  1840/423
 R   V   V   N*  P   T   L   L  ***
gaatctgaggtatcaaacacacacaggataccagcaatggatggcaggggagagtgttccttttgttcttaacta  1915
gtttagggtgttctcaaataaatacagtagtccccacttatctgaggggggatacattcaaagacccccagcagat  1990
gcctgaaacggtggacagtgctgaaccttatatatatttttttcctacacatacatacctatgataaagtttaatt  2065
tataaattaggcacagtaagagattaacaataataacaacattaagtaaaatgagttacttgaatgcaagcactg  2140
caataccataacagtcaaactgattatagagaaggctactaagtgactcatgggcgaggagcatagacagtgtgg  2215
agacattgggcaaggggagaattcacatcctgggtgggacagagcaggacaatgcaagattccatcccactactc  2290
agaatggcatgctgcttaagacttttagattgtttatttctggaatttttcatttaatgtttttggaccatggtt  2365
gaccatggttaactgagactgcagaaagcaaaaccatggataagggaggactactacaaaagcattaaattgata  2440
catatttttttaaaaaaaaaaaaaaaa
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FIGURE 1: Nucleotide sequence and deduced amino acid sequence of human ZPI cDNA. The amino acid sequence is shown in single-letter code beneath the nucleotide sequence. Nucleotide/amino acid numbers are shown in the column at the right. Translation is depicted as starting at ATG_6 (nt 467). An alternative initiation codon, ATG_5 (nt 347), is underlined with dashes (see text). Amino acid sequences derived from purified plasma ZPI are underlined. N* denote potential sites of N-linked glycosylation, and the tyrosine residue at the putative P_1 site at the reactive center of ZPI is shown in boldface print. The polyadenylation signal is doubly underlined.

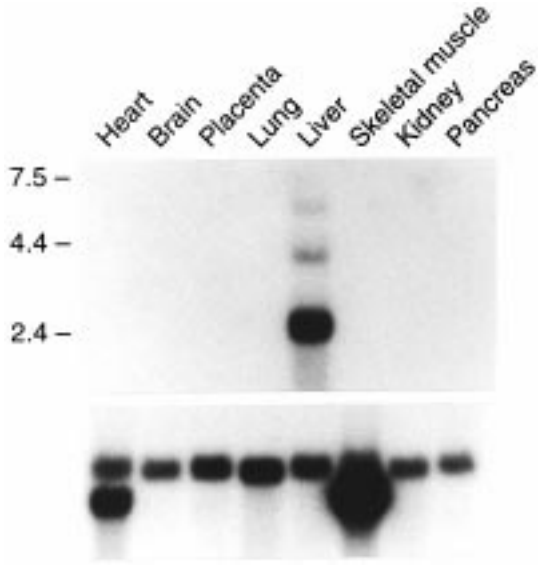

FIGURE 2: Northern analysis of multiple tissues for ZPI mRNA. (A) Northern blot nitrocellulose membrane containing 2 μg of poly A^+ mRNA from various human tissues in each lane was hybridized with a ^{32}P-labeled full-length ZPI cDNA probe (above) or a ^{32}P-labeled β-actin cDNA probe (below).

60 s, 50 μL of cephalin (75 μM), 50 μL of $CaCl_2$ (25 mM), and 50 μL of factor X-deficient plasma were added in succession, and the clotting time was measured. ZPI activity was determined by comparing the clotting time with a standard curve produced by using various concentrations of purified ZPI derived from plasma. One microgram of purified plasma ZPI was defined to possess 1000 milliunits (mU) of activity.

Western Blotting. SDS−polyacrylamide gel electrophoresis, the electro-transfer of proteins to nitrocellulose, and incubation of the blot with the monoclonal anti-ZPI antibody (MC4249.2) (10 μg/mL) were performed using previously described methods (*10*). Antibody binding to the blot was detected using horseradish peroxidase-labeled goat anti-mouse IgG antibodies (Sigma) and enhanced chemoluminescence (ECL) with Super Signal substrate (Pierce, Rockford, IL).

Protease Inhibition by ZPI. The proteases were incubated with or without ZPI at room temperature in the presence or absence of PZ, rabbit brain phospholipids (15 μM), and $CaCl_2$ (4 mM). After 10 min, the remaining protease activity was determined by coagulation or amidolytic assay. The tested proteases and their respective method of assay were as follows: thrombin (2.2 nM), S2238 (200 μM); meizothrombin (3.0 nM), S2238 (200 μM); factor VIIa (10 pM), coagulation assay (*11*); factor IXa (4.0 nM), coagulation assay (*12*); APC (7.1 nM), S2366 (250 μM); t-PA (14.7 nM), Spectrazyme tPA (250 μM); u-PA (18.9 nM), S2444 (250 μM); plasmin (12.0 nM), S2390 (150 μM); trypsin (1.7 nM), S2222 (100 μM); leukocyte elastase (33.9 nM), S2484 (1 mM); chymotrypsin (11.6 nM), S2586 (180 μM); and cathepsin G (125 nM), *N*-succinyl-Ala-Ala-Pro-Phe-pNA (1 mM). The concentrations of PZ and ZPI in each reaction were 40 nM and 35 nM, respectively, except for tests of leukocyte elastase and cathepsin G in which the PZ concentration was 160 nM and the ZPI concentration was 140 nM.

RESULTS

Isolation and Sequence of ZPI cDNA. In a search of publicly available protein and DNA databases, the N-terminal amino acid sequence of ZPI isolated from human plasma, LAPSPQSPEXXA (X = indeterminate), did not show significant sequence homology with previously reported gene products. However, the N-terminal sequences of peptides of 43 and 41 kDa produced by trypsin treatment of ZPI were the same, NLELGLTQGSFAFIHKDFDV, and showed 75% identity (16 of 20 residues) with an amino acid sequence predicted by the previously reported rat regeneration-associated serpin-1 (rasp-1) cDNA (*8*). Oligonucleotide primers based on rasp-1 cDNA sequence were used as PCR primers, and a human fetal liver cDNA library (Stratagene) was used as a template to produce a ~330 bp probe for the subsequent isolation of ZPI cDNA (see Materials and Methods). Twenty-one positive plaques containing inserts of four different sizes were isolated from a human liver cDNA library (Clontech). The nucleotide sequence and predicted amino acid sequence of the longest ZPI cDNA insert are shown in Figure 1. Restriction mapping and limited sequence analysis of clones representative of the three shorter ZPI cDNA insert sizes suggest they are 5′-truncated forms of the cDNA shown (data not shown).

The 5′ portion of the 2.44 kb ZPI cDNA contains six potential ATG translation start sites at nucleotides 156, 243, 250, 312, 347, and 467. The open reading frames following the first four ATG's encode 11, 22, 3, and 52 amino acids, respectively, before encountering stop codons. ATG_5 (underlined with dashes in Figure 1) is in the same reading frame as ATG_6, and translation initiation at ATG_5 would add the 40 amino acid sequence MSRSTQELLGYHCRLQDKLQEQEGSLAAEGRHSLASAADH to the encoded protein. Flanking nucleotides about the ATG codons, including ATG_5 and ATG_6, produce sequences that are not optimal for the initiation of translation (*13*). Nevertheless, ATG_6 is depicted as the initiator codon in Figure 1 because additional studies (see below) showed it was sufficient for ZPI expression. On Northern analysis of a human multiple tissue blot, ~2.4 kb of ZPI mRNA was strongly detected in liver, but undetectable in heart, lung, brain, spleen, testis, and kidney (Figure 2), suggesting that the liver is a major source of ZPI in vivo.

As depicted, the ZPI cDNA contains a 1335 bp open reading frame encoding a deduced protein of 444 amino acids. The predicted amino acid sequence has a typical 21 residue signal peptide that is followed by the N-terminal sequence of the purified ZPI protein. Five potential N-linked glycosylation sites are present. The nucleotide and predicted amino acid sequence of human ZPI are respectively 75% and 78% identical with those of rat rasp-1, suggesting that ZPI represents the human homologue of this rat protein. The ZPI amino acid sequence is also 25−35% homologous with other members of the serpin superfamily of protease inhibitors, including α_1-antitrypsin, antithrombin, heparin cofactor II, and protease nexin-1. The C-terminal region of ZPI shows the greatest similarity with the other members of the serpin superfamily, whereas the sequence of the N-terminal region of ZPI, which contains a very acidic domain (residues 26−43, Figure 1), does not show significant homology with these other serpins.

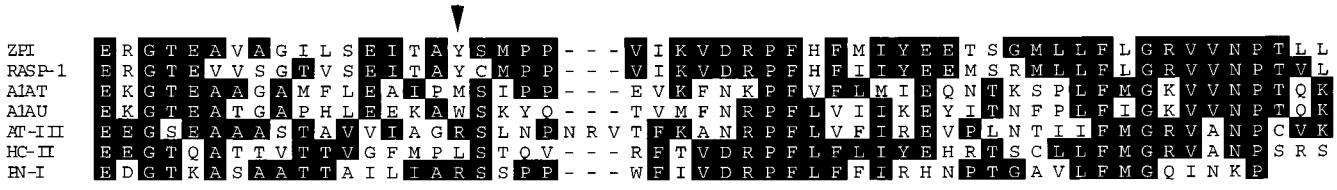

FIGURE 3: Alignment of the C-terminal amino acid sequences of ZPI and other serpins. Amino acid sequences of rat rasp-1 (RASP-1) and human α_1-antitrypsin (A1AT), antitrypsin-related sequence (A1AU), antithrombin (AT-III), heparin cofactor II (HC-II), and protease nexin 1 (PN-1) were extracted from the GenBank database (accession numbers 2143953, 1703025, 112891, 113936, 123055, and 121110, respectively). Identical amino acids are darkly shaded. The arrowhead indicates the column containing the P_1 residue at the reactive center of each serpin.

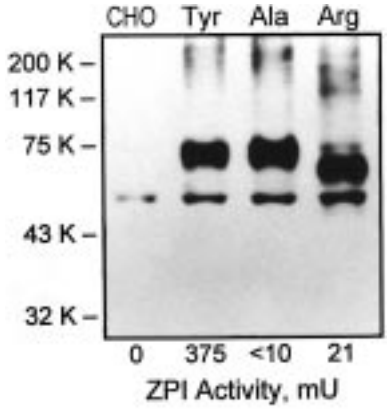

FIGURE 4: Western blot analysis of wild-type and altered forms of recombinant ZPI. Serum-free conditioned media (10 μL) from nontransfected CHO cells (CHO) and CHO cells expressing rZPI-(WT) (Tyr), rZPI(Y387A) (Ala), and rZPI(Y387R) (Arg) were analyzed by 12% SDS−PAGE and Western blotting using a mouse monoclonal anti-ZPI antibody. The migration of prestained molecular mass standards listed in kilodaltons is depicted on the left. Below the blot is listed the ZPI activity of each conditioned media (average of duplicate measurements). The protein band identified at ~54 000 Da is detected in the conditioned media of nontransfected CHO cells and appears to be unrelated to rZPI.

The C-terminal amino acid sequences of ZPI, rasp-1, and certain other serpins are shown in Figure 3. Based on this alignment, the putative P_1 residue at the reactive centers of human ZPI and rat rasp-1 is a tyrosine. Antitrypsin-related sequence (A1AU), an apparently nontranscribed DNA sequence highly homologous to that of antitrypsin and physically linked to the antitrypsin gene, also contains an aromatic residue (tryptophan) at the P_1 site (*14*, *15*). In common with many other serpins, the P_1' residue in ZPI is a serine, whereas the P_1' residue in rasp-1 is a cysteine.

Expression of Recombinant ZPI. To confirm that the protein encoded by the isolated cDNA possesses ZPI activity and to determine the importance of Y387 to ZPI function, rZPI(WT) and two altered forms of ZPI, rZPI(Y387A) and rZPI(Y387R), were expressed in Chinese hamster ovary (CHO) cells. Western blot analysis of the respective serum-free conditioned medias showed that the wild-type and altered forms of rZPI were present at similar concentrations (Figure 4). However, while rZPI(WT) and rZPI(Y387A) migrated with the same apparent molecular mass as plasma-derived ZPI (72 000 Da), the bulk of rZPI(Y387R) migrated with a molecular mass of 68 000 Da. Attempts to reduce the apparent proteolytic degradation of rZPI(Y387R) by including aprotinin and soybean trypsin inhibitor in the serum-free culture media and adding a protease inhibitor cocktail to the collected conditioned media were unsuccessful (see Materials and Methods). In a two-stage assay of PZ-dependent factor Xa inhibition, the serum-free conditioned media containing rZPI(WT) possessed substantial ZPI activity (375 milliunits/mL), whereas conditioned media containing rZPI(Y387A) lacked activity (<10 milliunits/mL) and conditioned media containing rZPI(Y387R) had markedly reduced activity (21 milliunits/mL) (Figure 4).

Spectrum of Protease Inhibition by ZPI. In the presence of PZ (40 nM), rabbit brain phospholipids (15 μM), and $CaCl_2$ (4 mM), the half-life of factor Xa (5 nM) coagulant activity is <10 s with the addition of ZPI (10 nM) (*6*). In contrast, under the conditions tested (see Materials and Methods), ZPI does not produce significant inhibition of thrombin, meizothrombin, factor VIIa, factor IXa, APC, t-PA, u-PA, plasmin, trypsin, or leukocyte elastase in the presence or absence of PZ, phospholipids, and calcium ions. Moreover, although it contains a putative tyrosine residue at its reactive center, ZPI does not inhibit chymotrypsin or cathepsin G.

DISCUSSION

Based on oligonucleotide and amino acid sequence homology, ZPI appears to be the human counterpart of rat rasp-1. Rasp-1 was initially identified as a gene whose transcription is increased 3−4-fold following subtotal hepatectomy in rats (*8*). However, rasp-1 expression is increased to a similar extent in sham-operated rats, suggesting that rasp-1 may be involved in the acute phase response. The rasp-1 gene product circulates in rat plasma with a reported molecular mass of ~50 000 Da, whereas the molecular mass of plasma ZPI is ~72 000 Da (*6*, *8*). This apparent difference in the molecular size between the rat and human gene products could be related to the extent of glycosylation. Constitutive expression of both rasp-1 (*8*) and ZPI genes is high in the liver and not detectable in brain, heart, lung, kidney, spleen, and testes by Northern analysis.

The ZPI cDNA is 2.44 kb in length and consistent with the smallest hybridizing species of ~2.4 kb noted in liver on Northern analysis (Figure 2). Hybridizing bands of greater size likely represent incompletely processed forms of ZPI mRNA. The 5′ region of the ZPI cDNA is relatively long (466 bp) and contains several potential ATG translation start codons. Four of these putative start codons are followed by termination codons, but the fifth ATG at nucleotide 347 is in-frame with the ATG at nucleotide 467 that we have tentatively designated as the authentic start codon. All these potential ATG initiation start sites are flanked by less than ideal nucleotide sequences (*13*). The long 5′-untranslated region, the presence of multiple upstream AUG codons that encode small open reading frames, and the lack of an optimal initiation sequence could all serve to suppress ZPI mRNA translation (*13*, *16*, *17*). Whether this is true, and whether

an alternative form of ZPI is produced through translation initiation at the fifth AUG (nt 347), will require direct testing.

ZPI has 25−35% overall homology with other members of the serpin superfamily, and its primary structure contains 40 of the 51 residues previously designated as essential for serpin tertiary structure (*18*). These conserved residues reside in the apolar core and the spine of serpin molecules. Amino acid alignment of ZPI and rasp-1 with other serpins suggests that the P_1 residue at their reactive centers is a tyrosine, which would set them apart from other serpins. To confirm the role of Y387 in the inhibition of factor Xa by ZPI, altered forms of ZPI in which this residue was changed to an alanine or arginine were evaluated. rZPI(Y387A) was stable under the tissue culture conditions required for its expression and lacked PZ-dependent anti-factor Xa activity. In contrast to rZPI(WT), rZPI(Y387R) was apparently proteolytically degraded during the production of conditioned media despite the use of multiple protease inhibitors. The proteolytic event reduces the mass of ZPI by ∼4000 Da, consistent with cleavage occurring following R387, but the enzyme(s) responsible for this proteolysis is (are) not known. In sum, the studies with rZPI(Y387A) and rZPI(Y387R) suggest that Y387 is critical for PZ-dependent factor Xa inhibition and are consistent with the notion that Y387 is the P_1 residue at the reactive center of ZPI.

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